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Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

35ème COLLOQUE SCIENTIFIQUE

35th SCIENTIFIC MEETING

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Dr. Poul Hyttel

Special Celebration

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Murcia, Spain, 13th and 14th September 2019

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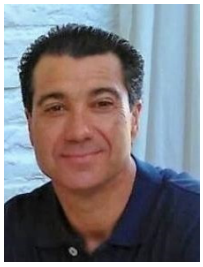

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Dr. Poul Hyttel

A.E.T.E. Medalist 2019

Dr. Poul Hyttel

A.E.T.E. Medalist 2019

Commendation of Dr. Poul Hyttel for AETE Pioneer Award 2019

Poul Hyttel was born September 19, 1954, in Noerresundby, Denmark to Bent and Inger Lis Hyttel. Poul's sister, Lene Hyttel, was born September 14, 1957 and in 1958 the family moved to Skagen, which is the northernmost town of Jutland, the mainland of Denmark. Skagen is situated on a peninsula that extends far out in to the sea, it is a special place where the waters from the seas of Skagerak and Kattegat meet and it has been famous for centuries, especially by Artists, for it's beautiful surrounding coastal scenery and evening light. Perhaps in another life, Poul would have lived among the Skagen Artist's colony; his artistic interpretations of oocyte and embryo molecular biology have enlivened many of his research papers, review chapters and presentations. Luckily for us, in addition to his artistic talent, Poul's passion and curiosity in animals was ignited by growing up on this narrow sand peninsula, where he adorned the family home with all kinds of animals, ranging from tropical finches to turtles. It was here that Poul developed the desire to study veterinary medicine with a focus on wildlife. Consequently, in 1973 at the age of 18, Poul moved to Copenhagen to study Veterinary Medicine at the Royal Veterinary and Agricultural University (RVAU), graduating as a DVM in 1979. During the course of the veterinary curriculum he became fascinated by embryology and reproduction, and after serving as a Dog Patrol Officer in the army, Poul embarked on his long, distinguished research career in Reproductive Biology. Under the mentorship of Professor Torben Greve, the 2004 recipient of the AETE Pioneer Award, Poul studied Repeat Breeding and Early Embryology in Cattle, for his PhD Thesis; he received his PhD in 1982. While carrying out the experiments for his PhD project, Poul developed his interest in electron microscopy, which he initially applied to bovine blastocysts. This passion and curiosity for ultra fine morphological detail was extended further and Poul conducted groundbreaking detailed ultrastructural analysis of oocyte maturation, fertilization and embryonic development in cattle, leading to his

degree as Doctor of Veterinary Sciences (DVSc.), in 1988, from RVAU. This was a very special time for Poul, his beloved son Mads was born in 1985 and together with Henrik Callesen, Poul was a member Torben Greve's research team behind the birth of the first IVF calf in Europe, in 1987 (Greve *et al.*, 1989a).

In 1990 Poul was appointed Professor of Veterinary Anatomy at the Department of Anatomy and Physiology at RVAU. In his new position he continued the cell biological studies on oocyte development, fertilization and initial embryogenesis and collaborating with scientists from around the world, extended these studies to many species, including horse, pig, sheep, fox, mouse, tiger and man. He was also one of the first scientists to try to understand the intrinsic differences between in vivo and in vitro derived embryos, using electron microscopy to identify the differences at the cellular level (Hyttel et al., 1988; 1989b). I first came to know of Poul and Torben through the publications arising from Poul's DVSc. research programme. I had been working on the kinetics of fertilization in cattle using only Orcein staining, it was quite a revelation to come across Poul's papers which revealed the ultrastructure of oocyte maturation and fertilization in cattle in exquisite detail. I was determined to learn from this man and in 1993, I had the good fortune of securing an EU Marie Curie International Fellowship which enabled me to move from Ireland to Denmark, to undertake my studies for my PhD as a student of Poul and Torben at RVAU. I joined the ranks of a series of PhD students who under Poul's guidance and supervision, spent many many hours at the transmission electron microscope in the basement of the old Anatomy Building. It was a wonderful time to belong to such a dynamic research group; projects on many aspects of sperm, oocyte and embryo development were ongoing across horses, pigs and cattle and we all rolled up our sleeves and helped one another. Torben and Poul were always open to collaboration with international scientists and they came from the USA, Africa, Europe and Asia on research sabbaticals or a passing visits. The research environment in the Reproduction Group at RVAU at that time, together with Poul's infectious curiosity and attention to detail, inspired me and many of their students to continue working in the field of Reproductive Biology. It also nutured many lifelong friendships.

Like many institutions in Europe, RVAU has been renamed several times, it is now titled the Faculty of Health and Medical Sciences within the University of Copenhagen, but Poul remains Professor of Anatomy, in the now, Department of Veterinary and Animal Sciences (DVAS). Professor Poul Hyttel is an internationally recognized scientist in the field of biomedicine, animal and human stem cells and early embryonic development. His

research profile exemplifies the principle of 'One Health': Poul's current research activities centre around pluripotent animal and human stem cells and in vitro embryo production. In 2015, Poul founded the transnational Stem Cell Centre of Excellence in Neurology, BrainStem, supported by Innovation Fund Denmark. Here advanced stem cell technologies such as human induced pluripotent stem cells (iPSCs) and gene editing are used for modelling neurodegeneration (e.g. Alzheimer's and Parkinson's disease). The iPSC activities have been reflected back towards veterinary medicine, where Poul leads a project investigating the condition, canine cognitive dysfunction, using canine iPSCs. However, Poul has never drifted too far from his bovine embryology roots; his research came full circle with the recent funding of the project 'EliteOva', by Innovation Fund Denmark, the aim of which is to optimize emerging technologies for in vitro embryo production and genomic selection of embryos in Danish cattle breeding.

To-date, Prof. Poul Hyttel has authored more than 280 refereed international articles, he has been the principal supervisor of more than 40 PhD-students and postdoctoal fellows and has attracted more than €18 million funding from the EU, NIH and Danish Funding Instruments for research projects. Prof. Hyttel has received numerous honours and esteemed leadership positions in our field. Most notably, Prof Poul Hyttel was President of the International Embryo Transfer Society in 1997, in 2011 he was awarded Doctor Honoris Causa at University of Antwerp, Belgium, in 2015 he was knighted 1st Class Order for his services to Denmark and in 2018 he was awarded Doctor Honoris Causa at Estonian University of Life Sciences, Tartu, Estonia.

Poul is married to Inge and another, very important title 'farfar' was bestowed on Poul in 2018, when Poul and Inge became first time grandparents to their grandson and granddaughter.

Kære Poul, tak for alt, tillykke med hvad du har opnået og de varmeste ønsker for fortsat lykke.

Trudee Fair M.Agr.Sci., PhD.
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Oocytes, embryos and pluripotent stem cells from a biomedical perspective

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Abstract

The veterinary and animal science professions are rapidly developing and their inherent and historical connection to agriculture is challenged by more biomedical and medical directions of research. While some consider this development as a risk of losing identity, it may also be seen as an opportunity for developing further and more sophisticated competences that may ultimately feed back to veterinary and animal science in a synergistic way. The present review describes how agriculture-related studies on bovine *in vitro* embryo production through studies of putative bovine and porcine embryonic stem cells led the way to more sophisticated studies of human induced pluripotent stem cells (iPSCs) using e.g. gene editing for modeling of neurodegeneration in man. However, instead of being a blind diversion from veterinary and animal science into medicine, these advanced studies of human iPSC-derived neurons build a set of competences that allowed us, in a more competent way, to focus on novel aspects of more veterinary and agricultural relevance in the form of porcine and canine iPSCs. These types of animal stem cells are of biomedical importance for modeling of iPSC-based therapy in man, but in particular the canine iPSCs are also important for understanding and modeling canine diseases, as e.g. canine cognitive dysfunction, for the benefit and therapy of dogs.

Keywords: embryonic stem cells, induced pluripotent stem cells, *in vitro* fertilization, Alzheimer's disease, dementia.

Introduction

The veterinary and animal science professions are rapidly developing in a shifting scientific environment. Worldwide institutional reorganizations towards larger entities result in absorption of veterinary and animal science faculties into broader entities with a focus on life, biomedical and medical sciences. While this development has a range of advantages creating novel scientifically rewarding collaborative landscapes it also challenges the conventional identity of the veterinary and animal science professions and their inherent and historical connection to agriculture. Consequently, the focus of veterinary and animal sciences has been extended with a major biomedical and even medical dimension; a development which is also sparked by a shift in funding opportunities with biomedicine and medicine having higher leverage than agriculture. Some consider the gradual increase in biomedical and medical focus, at the expense of agricultural attention, a risk. On the other hand, this development gives more room for investigating the complex area of "One Health" and may also give veterinarians and animal scientists access to new sets of competences, that may, in a synergistic and constructive way, feedback to more core classical veterinary and animal science.

It is fair to say that the biomedical and medical trend in science cannot be rejected and should be contemplated as an opportunity for contemporary development of the veterinary and animal science professions. It is the focus of this review to present a scientific development where research in assisted reproductive technologies (ARTs) and embryonic stem cells (ESCs) in the large domestic species has given opportunities for establishing a stem cell center of excellence in neurology focusing on human induced pluripotent stem cell (iPSC)-models for neurodegeneration and, finally, how the competences gained through these medical activities allowed for investigations of porcine and canine iPSCs feeding positively back to veterinary and animal science (Fig.1).

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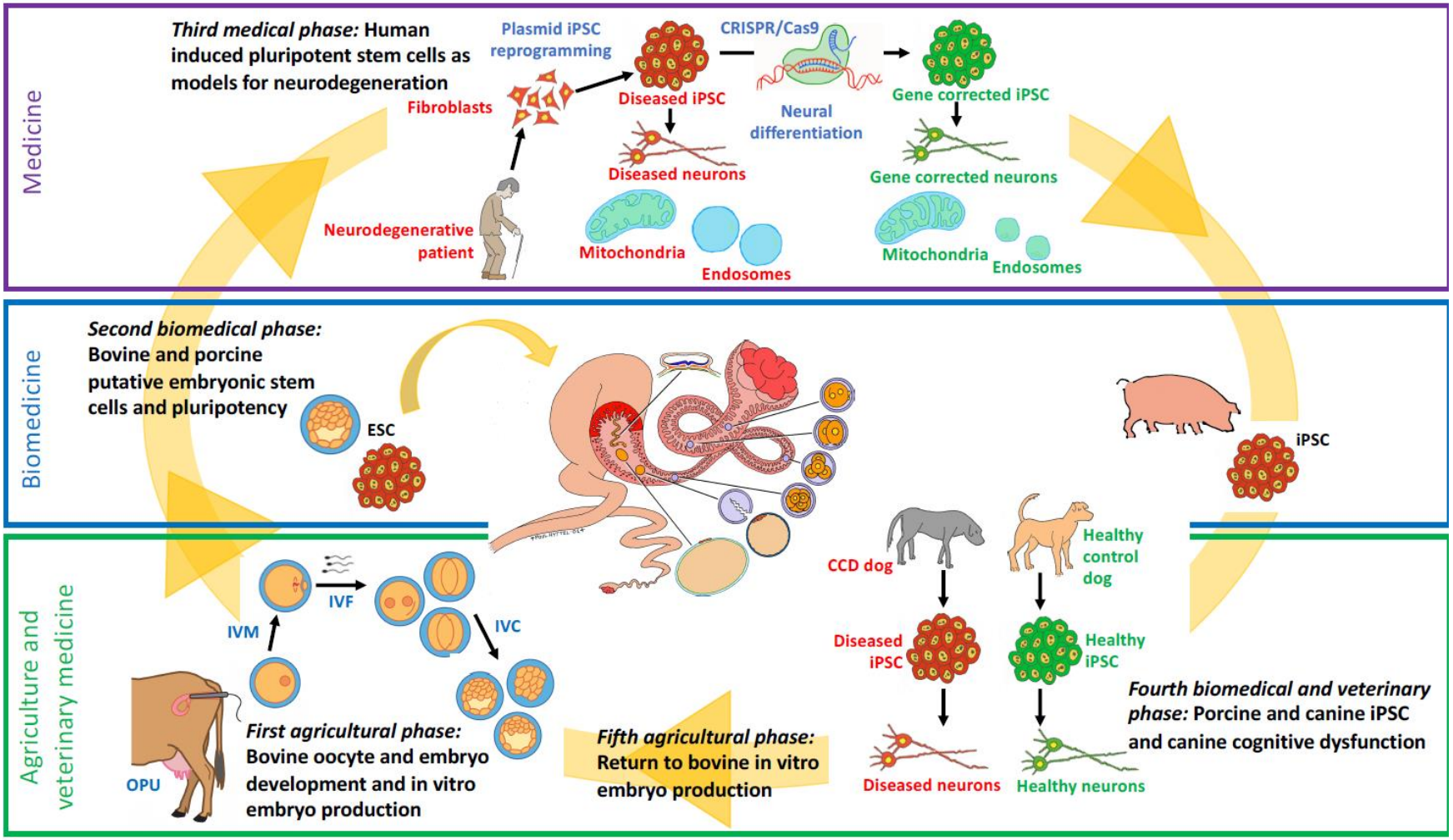

Figure. 1. The progression of research activities moving from agricultural and veterinary medicine through biomedicine and medicine and feeding back to agricultural and veterinary medicine. OPU: Ultrasound-guided ovum pickup; IVM: *In vitro* maturation; IVF: *In vitro* fertilization; IVC: *In vitro* culture.

First agricultural phase: Bovine oocyte and embryo development and *in vitro* embryo production

In vitro embryo production in cattle

Over the past 40 years there has been a gradual agricultural implementation of novel ARTs in large animal husbandry with particular focus on cattle (Greve and Callesen, 2005; Lonergan, 2007). Major components of this development have been the development and refinement of the multiple ovulation and embryo transfer (MOET), including cryopreservation of blastocysts, and of *in vitro* fertilization (IVF) culminating with the birth of the first IVF calf in 1981 (Brackett *et al*., 1982). Whereas contemporary *in vitro* production (IVP) of embryos in cattle includes *in vitro* oocyte maturation, *in vitro* fertilization and *in vitro* culture of the resultant embryos to the blastocyst stage, Brackett and colleagues flushed *in vivo* matured oocytes from the oviducts, performed IVF and transferred a 4-cell stage back to the oviduct. In 1987, the first European IVF calf, now resulting from *in vitro* oocyte maturation, was born in Copenhagen (Xu *et al*., 1987) where we invested great efforts in fundamental investigations of oocyte maturation and fertilization (Hyttel *et al*., 1986a; Hyttel *et al*., 1986b; Hyttel *et al*., 1988a; Hyttel *et al*., 1988b).

In parallel, the quest for defining optimal culture conditions allowing for the development of bovine zygotes to blastocysts took place including focus on coculture systems (Edwards *et al*., 1997), media composition (Holm *et al*., 1999) as well as the physical design of the culture platforms (Vajta *et al*., 2008; Smith *et al*., 2012). These efforts all became extremely relevant in the light of the astonishing adverse effects of improper *in vitro* culture conditions that were reported in 1997, and which coined the term Large Offspring Syndrome (LOS) (Kruip and den Daas, 1997). The risk of LOS caused severe drawbacks for the technologies, and in Denmark the practical implementation of *in vitro* embryo production in cattle breeding was abandoned mainly for this reason. Refined serum-free culture conditions, based on BSA supplementation, have now been developed allowing for improved fetal development and calving (George *et al*., 2008), and in 2013 an entire serum-free ready-to-use media suite for all the steps, maturation, fertilization and culture, was made commercially available by IVF Bioscience, UK, combining synthetic serum replacements and BSA. Finally, the combination of IVP and ultrasound-guided ovum pickup (OPU) has allowed for more sophisticated practical implementation of IVP in cattle breeding, and the year of 2018 became a turning point as the numbers of transferred bovine IVP embryos for the first time officially exceeded that of their *in vivo*-derived counterparts. According to the numbers collected by the IETS, almost 1.5 million (1,487,343) bovine embryos were produced by MOET or IVP worldwide in 2017 and two thirds (almost 1 million) were derived by IVP (Viana, 2018).

In order to pave the way for successful IVP of bovine embryos, we undertook a series of fundamental studies of oocyte development, fertilization and initial embryonic development in cattle, which are summarized in the following. Hence, we have characterized oocyte development and maturation, fertilization and initial embryonic development in cattle extensively by transmission electron microscopy (TEM).

The basic ultrastructure of the oocyte is generated during its growth phase in the primordial to the tertiary follicle. When the tertiary follicles in a cohort reach a diameter of about 3-5 mm in cattle, one dominant follicle is selected, and the structure of the oocyte in this particular follicle is modified during a process that may be referred to as capacitation or pre-maturation. The estrous cycle in cattle generally comprises 2 or 3 follicular waves, and the dominant follicle of the last wave becomes ovulatory. In the ovulatory follicle the oocyte undergoes a final maturation during an approximately 24 hour period between the peak of the LH-surge and ovulation.

Oocyte growth in cattle

During the growth of the bovine oocyte, the inside zona pellucida diameter of the gamete increases from less than 30 μm in the quiescent primordial follicle to more than 120 μm in the tertiary follicle. We have carefully characterized the ultrastructure, transcriptional activity and developmental competence of bovine oocytes in relation to the sequential stages of follicular development (Fair *et al*., 1996; Fair *et al*., 1997a; Fair *et al*., 1997b).

In the quiescent **primordial follicle** gap and intermediate junctions are present between adjacent granulosa cells, whereas exclusively intermediate junctions are seen between the granulosa cells and the oocyte. The transcriptionally quiescent nucleus of the oocyte, i.e. the germinal vesicle, occupies a central or slightly off center position and the organelles are concentrated in the perinuclear region. The **primary follicle** occasionally exhibits small portions of zona pellucida substance between the cuboidal granulosa cells and the oocyte. The continued zona-formation in the **secondary follicle** is associated with the embedding of granulosa cell processes and erect oocyte microvilli into the zona pellucida, and gap junctions are established between the granulosa cell processes and the oocyte. The oocyte nucleoli develop into a fibrillo-granular appearance and transcription is initiated. The oocyte in the **small tertiary follicle** up to about 1 mm in diameter exhibits a complete zona pellucida traversed by numerous cumulus cell projections forming gap and intermediate junctions to the oocyte. Clusters of cortical granules are numerous. The oocyte nucleoli are typical fibrillo-granular and transcription abundant. In the **larger tertiary follicles** the oocyte ultrastructure may be classified according to the inside zona pellucida diameter of the cell. In oocytes <100 μm the particular hooded mitochondria, unique to ruminants, are observed for the first time. Oocytes from 100 to 110 μm in diameter typically display formation of a perivitelline space, the process of which is associated with the release of the previously embedded microvilli from the

zona pellucida. The oocyte nucleus is displaced towards the periphery as are Golgi complexes and mitochondria, amongst which the hooded form becomes more numerous. The fibrillar centers of the nucleoli have typically migrated towards the nucleolar periphery and transcription is decreased. Oocytes from 110 to 120 µm typically present a well-developed perivitelline space and a peripherally located nucleus. The process of nucleolar inactivation has proceeded leaving the nucleolus to consist of a spherical nucleolar remnant with a fibrillar center attached. At a diameter of 120 µm, the oocyte has completed the growth phase and achieved the ultrastructure characterizing the fully developed gamete.

Interestingly, the oocyte achieves the competence to complete meiotic maturation to metaphase II *in vitro* at a diameter of about 110 µm coinciding with the de-activation of its transcriptional machinery, indicating that the necessary compartment of proteins and mRNAs has been formed at this stage of development.

Oocyte capacitation or pre-maturation in cattle

Further, we have carefully mapped the ultrastructural development of bovine oocytes in the dominant vs. the subordinate follicles (Assey *et al*., 1994a). With the growth of the dominant follicle, the ultrastructure of the fully grown oocyte is modified during its so-called capacitation or pre-maturation. During the days approaching the regression of the corpus luteum, i.e. the final period of the luteal phase, the cortical granule clusters are dislocated to more superficial locations and some granules migrate to solitary positions along the oolemma. During the period between luteolysis and the LH-surge individual cumulus cells exhibit elongation and some of the cumulus cell process endings are retracted to a more superficial location on the surface of the oolemma. Also, the oocyte nuclear envelope becomes undulating, especially in the regions facing the zona pellucida, and the nucleolar remnant displays vacuolization. Both of these phenomena are presumably related to the subsequent breakdown of the oocyte nucleus, i.e. germinal vesicle breakdown (GVBD). There are indications that the competence of the oocyte to produce blastocysts *in vitro* increases with completion of capacitation or pre-maturation in the dominant follicle (Hendriksen *et al*., 2000). Superovulation with exogenous gonadotropins may have an adverse effect on this process as indicated by a lack of at least the vacuolization of the nucleolar remnant (Assey *et al*., 1994b).

Oocyte maturation in cattle

The maturation of the oocyte, which in cattle occurs during the approximately 24 hour period from the LH-peak to ovulation, comprises the progression of meiosis from the diplotene stage of prophase I to metaphase II accompanied by a series of ultrastructural and molecular changes in the ooplasm. The ultrastructural changes have been described in detail in relation to the time of the LH-peak in unstimulated (Kruip *et al*., 1983) as well as gonadotropin stimulated cattle (Hyttel *et al*., 1986a). The breakdown of the oocyte nucleus (GVBD) occurs 9 to 12 hours after the LH-peak when the nuclear envelope becomes extremely undulating, the chromatin condenses, the nucleolar remnant is dissolved and there is a gradual decoupling of the cumulus cell endings from the oocyte (Hyttel, 1987). At about 15 and 20 hours after the LH-peak most oocytes have reached metaphase I and II, respectively, and the first polar body is abstricted. During the last hours of maturation, lipid droplets and mitochondria attain a more central location in the ooplasm and the cortical granules migrate to solitary positions along the oolemma. The peripheral migration of the cortical granules appears to be compromised to a certain degree during oocyte maturation *in vitro* (Hyttel *et al*., 1986b).

Growing and dominant follicles are capable of maintaining oocyte meiosis arrested at the diplotene stage of prophase I. However, numerous subordinate tertiary follicles undergo atresia. Interestingly, such atretic follicles may lose the ability to retain the oocyte in meiotic arrest. Hence, oocytes in atretic follicles may display different stages of meiotic maturation; even reaching metaphase II (Assey *et al*., 1994a). Through the described phases of growth, capacitation and maturation, the oocyte has now acquired the ultrastructural architecture for sustaining fertilization and initial embryonic development.

Fertilization and development of the zygote in cattle

The ultrastructure of bovine fertilization has precisely been described in relation to the estimated time of ovulation as determined by timing of the LH-peak in gonadotropin stimulated cows (Hyttel, *et al*., 1988a), and bovine *in vitro* fertilization have added to this understanding (Hyttel *et al*., 1988b; Hyttel *et al*., 1988c).

Upon acrosome reaction and penetration of the zona pellucida, the oocyte microvilli contact the equatorial segment of the sperm head where fusion between the two gametes initially occurs resulting in oocyte activation and cortical granule exocytosis establishing the block against polyspermic fertilization. With a correct Greek term, gamete fusion is termed syngamy; a term that erroneously is also widely used for the apposition of the pronuclei (see later). Within the first 2-3 hours after ovulation, the paternal chromatin is denuded from its membrane coverings and decondensed. In parallel, the maternal chromatin is advancing through anaphase and telophase II forming the second polar body. Pronucleus formation is initiated with smooth endoplasmic reticulum (SER) moving towards both the paternal and maternal chromatin to form nuclear envelope. About 4 hours after ovulation, the two sets of chromatin are completely surrounded by nuclear envelopes. The midpiece of the sperm tail remain spatially associated with the paternal pronucleus. Subsequently, the pronuclei swell to their characteristic spherical appearance accompanied by chromatin decondensation, and about 10 hours post ovulation most

zygotes exhibit spherical pronuclei (Laurincik *et al*., 1998). Along with this process, so-called nucleolus precursor bodies, very similar to the oocyte nucleolar remnant, which later act as enucleation sites for nucleolus formation, are formed in the pronuclei (Laurincik *et al*., 1996). The precursor bodies are not active in rRNA transcription and ribosome formation. The two pronuclei migrate to a close apposition, and about 14 hours after ovulation most zygotes exhibit apposed pronuclei. The S-phase of the first post-fertilization cell cycle takes place 12-19 hours after ovulation (Laurincik *et al*., 1994). Upon pronuclear apposition, pronounced undulations of the nuclear envelopes of the pronuclei are seen in the apposed regions probably preparing for breakdown of the envelopes, which is seen at about 24 hours after ovulation. This process is often referred to as synkaryosis, but it should be emphasized that the two pronuclei do not fuse, but undergo dissolution of the nuclear envelopes similar to the one seen at the breakdown of the oocyte nucleus (GVBD) at resumption of oocyte meiosis. Immediately after synkaryosis, karyokinesis and cytokinesis proceed resulting in the formation of two daughter nuclei enclosed in each their blastomere.

Pre-hatching embryonic development in cattle

Along with the initial cleavages, the embryonic genome is gradually activated during the so-called maternal-embryonic transition. Thus, a low rate of transcription of the embryonic genome has been detected as early as during the 1st, i.e. the zygote (Hay-Schmidt *et al*., 2001), and 2nd post-fertilization cell cycles (Hyttel *et al*., 1996; Viuff *et al*., 1996), and during the 4th cell cycle a major transcriptional activation occurs (Camous *et al*., 1986).

A number of other researchers have contributed to the understanding of the general embryonic ultrastructure based on either *in vivo* or *in vitro* developed embryos (Mohr and Trounson, 1981; Camous *et al*., 1986; Betteridge and Fléchon, 1988; King *et al*., 1988; Kopecný *et al*., 1989; Abe *et al*., 1999; Laurincik *et al*., 2000; Laurincik *et al*., 2003).

Early during the second cell cycle, i.e. the 2-cell stage, nucleolus precursor bodies resembling those described for the pronuclei are established in the nuclei. Hence, functional nucleoli are lacking and protein synthesis must be based on the ribosome pool inherited from the oocyte. Early during the third and fourth cell cycle, i.e. the tentative 4- and 8-cell stages, respectively, nucleolus precursor bodies resembling those from the previous cell cycles are again established. During the fourth cell cycle, however, the nucleolus precursor bodies develop into fibrillo-granular nucleoli displaying the typical components of actively ribosome-synthesizing nucleoli: Fibrillar centers, dense fibrillar component and granular component. The development of the nucleoli is a prerequisite for continued embryonic development and is a sensitive marker for the normality of this process. Abundant activation of embryonic transcription during the fourth cell cycle allows for the first cell differentiation and lineage commitments. External cells become connected by tight junctions while internal cells are only connected by focal membrane contacts. Mitochondria of the hooded form, which were established back during the development of the oocyte in the early tertiary follicle, become fewer, and elongated types with transverse cristae become more numerous.

The competences gained by our studies of oocyte maturation, fertilization and initial embryonic development in cattle allowed us to move into the stem cell area for creating novel potentials in agriculture and biomedicine.

Second biomedical phase: Bovine and porcine putative embryonic stem cells and pluripotency

Embryonic stem cells

Mouse embryonic stem cells (ESCs) were derived in 1981 (Evans and Kaufman, 1981; Martin, 1981) and paved the way for production of genetically modified mice (Thomas and Capecchi, 1987). Along with this development, an interest emerged in investigating the potentials for genomic modifications of the large domestic species for production, health, environmental and biomedical purposes.

Further studies of murine ESCs revealed that there are distinct states of pluripotency (naïve and primed) that differ both morphologically and functionally (De Los Angeles *et al*., 2012). Naïve murine pluripotent stem cells are derived from the inner cell mass (ICM) or early epiblast cells, proliferate in culture as packed dome-like colonies, are maintained in the undifferentiated state by LIF and BMP4 signaling, readily contribute to germline transmitting chimeric embryos, maintain two active X chromosomes (in female cells) and are relatively resistant to differentiation into primordial germ cells (PGCs) and extra-embryonic lineages (Kuijk *et al*., 2011). In contrast, primed pluripotent stem cells are derived from the epiblast of post-hatching murine blastocysts, are termed epiblast stem cells (EpiSCs), are molecularly and epigenetically different from murine ESCs (Brons *et al*., 2007; Tesar *et al*., 2007), have a more flattened colony morphology, depend on bFGF or TGFa/activin signaling, exhibit a limited ability to contribute to chimeras and have undergone X-chromosome inactivation (Brons *et al*., 2007). Human ESCs were first derived in 1998 (Thomson *et al*., 1998) and, surprisingly, they exhibit characteristics more like those of primed murine EpiSCs than their naïve murine ESC counterparts (Thomson *et al*., 1998).

The potentials of murine ESCs for the generation of transgenic mice sparked an interest in deriving ESCs in the large domestic species including activities in our laboratories focusing on cattle and pig. We and many others attempted to derive bovine ESCs (for review, see Ezashi *et al*., 2016) from different developmental stages from 2-cell embryos (Mitalipova *et al*., 2001) up to Day 12 hatched blastocysts (Gjørret and Maddox-Hyttel, 2005). However, even though

ESC-like cell lines were established and some of them could be cultured for extended periods of time, their characterization, especially with respect to functional contribution to chimeras, remained obscure. At present, it must be concluded that none of the derived cell lines have been capable of contributing to germline transmitting chimeras (Iwasaki *et al*., 2000) and , thus, can not be classified as *bona fide* ESCs. A very recent breakthrough indicates that a combination of FGF2 and an inhibitor of the canonical Wnt-signaling pathway may be the key to maintain bovine ESCs (Bogliotti *et al*., 2018).

Similar activities materialized in the pig where we and many others attempted to establish porcine ESCs (for review, see Telugu *et al*., 2010; Ezashi *et al*., 2016). However, even though a single report on a porcine ESC-derived chimera is found (Chen *et al*., 1999), none of the derived cell lines were capable of contributing to germline transmitting chimeras. Interestingly, cells from the inner cell mass from Day 6 to 7 porcine blastocysts are capable of contributing to such germline transmitting chimeras (Anderson *et al*., 1994; Onishi *et al*., 1994; Nagashima *et al*., 2004), and are, by this criterion, pluripotent and hereby a potential source of ESCs. Clearly, however, such is pluripotent cells loose this potential when cultured for even a short period of time. More recent data, where porcine ESCs again have been demonstrated to give rise to chimeric contribution, indicate that a novel medium including a combination of bFGF and LIF may represent a breakthrough although follow up with respect to germline transmission is warranted (Xue *et al*., 2016).

ICM and epiblast differentiation in the pig

In order to explain our lack of success in deriving bovine and porcine ESC, we undertook a set of fundamental studies of the porcine ICM and epiblast which clearly demonstrated that ungulate ICM and epiblast development and pluripotency show distinct differences as compared with its murine counterpart. A dynamic change in gene expression is the driving force for the first cell differentiation, i.e. the segregation of the compacting blastomeres into the ICM and trophectoderm. In the mouse, the ICM develops a stable regulatory circuit, in which the transcription factors Nanog (Chambers *et al*., 2003; Mitsui *et al*., 2003), OCT4 (Nichols *et al*., 1998; Schöler *et al*., 1990), SOX2 (Avilion *et al*., 2003), and SAL4 (Elling *et al*., 2006; Zhang *et al*., 2006) promote pluripotency and suppress differentiation. In contrast, in the trophectoderm-destined cells, the transcription factors CDX2 and EOMES are upregulated together with ELF5 and TEAD4, which are transcription factors acting upstream of CDX2 to mediate trophectoderm differentiation (Ng *et al*., 2008; Nishioka *et al*., 2008; Yagi *et al*., 2007). On the other hand, expression of the trophectoderm-associated transcription factors, CDX2, TEAD4, and ELF5, are repressed in the ICM by the regulatory circuit of Nanog, SOX2, and OCT4 (Ralston and Rossant, 2005). In the pig, the expression of CDX2 during preimplantation development appears conserved as compared with the mouse (Kuijk *et al*., 2007). OCT4 is, on the other hand, expressed in both the ICM and trophectoderm as opposed to the mouse (Keefer *et al*., 2007; Kuijk *et al*., 2008), and Nanog expression has not been observed in the porcine ICM (Hall *et al*., 2009). Hence, there are marked species differences with respect to the molecular background for ICM and trophectoderm specification.

The embryo hatches from the zona pellucida by Days 7 to 8, and in parallel the OCT4 expression, which was earlier present in both the ICM and the trophectoderm, becomes confined exclusively to the ICM (Vejlsted *et al*., 2006), whereas expression of Nanog is still lacking (Wolf *et al*., 2011) as opposed to the mouse. At the time of hatching, the ICM separates into two distinct cell populations. Hence, the most "ventral" cell layer towards the blastocyst cavity flattens and, finally, delaminates forming the hypoblast, whereas the "dorsal" cell population establishes the epiblast. The hypoblast subsequently extends along the inside of the trophectoderm forming a complete inner epithelial lining. The polar trophectoderm covering the epiblast (Rauber's layer) becomes very thin around Day 9 of gestation and gradually disintegrates exposing the epiblast to the uterine environment, which is very unlike the situation in the mouse, where the trophectoderm stays intact. Before the shedding of Rauber's layer, tight junctions are formed between the epiblast cells and the adjacent trophectoderm to maintain the epithelial sealing of the embryo despite the loss of the polar trophectoderm. Apparently, the porcine epiblast forms a small cavity, which finally opens dorsally followed by an "unfolding" of the complete epiblast upon the disintegration of Rauber's layer forming the embryonic disc (Hall *et al*., 2010). In parallel with the formation of the embryonic disc, the porcine epiblast starts to express not only OCT4, but also Nanog (Wolf *et al*., 2011b). At this stage of development, the first sign of anterior-posterior polarization develops in the embryonic disc: As mentioned earlier, the epiblast is underlaid by the hypoblast, and an area of increased cell density of closely apposed hypoblast cells develops. This area is approximately the same size as the embryonic disc, but it is dislocated about one third of its diameter anteriorly as compared with the epiblast of the embryonic disc (Hassoun *et al*., 2009; Wolf *et al*., 2011b). It is likely that this dense hypoblast region emits signals to the epiblast which suppress mesoderm-formation in the anterior epiblast regions. In this sense, the hypoblast may carry the blue-print for the specification of the epiblast.

During Days 11 to 12, the porcine embryonic disc develops into an oval shape, and a crescent-shaped accumulation of cells are found in the posterior region of the disc (Vejlsted *et al*., 2006). This crescent includes mesodermal progenitors which express the mesodermal markers, T (Brachyury) and Goosecoid (Blomberg *et al*., 2006; Wolf *et al*., 2011a), and apparently ingression of Brachyury-expressing extra-embryonic mesoderm is initiated from this crescent even before the "true" gastrulation starts with the appearance of the primitive streak (Wolf *et al*., 2011a), again, as opposed to the

mouse.

With the development of the embryonic disc, a very peculiar pattern of OCT4 and Nanog expression develops in the porcine epiblast: The majority of epiblast cells express OCT4, but small groups or islands of cells are OCT4 negative (Wolf *et al*., 2011b). The latter cells, on the other hand, express Nanog resulting in a mutually exclusive expression pattern. Subsequently, Nanog expression is lost in almost the entire epiblast, except for a few cell in the most posterior region of the embryonic disc, in which OCT4 is also expressed (Wolf *et al*., 2011b). The latter cells are believed to be the primordial germ cells (PGCs).

In conclusion, the efforts on establishing bovine and porcine ESCs have been plentiful but none of them resulted in *bona fide* ESC lines that were capable of giving rise to germ line transmitting chimeric embryos. Reasons for this lack of success are probably multifactorial (Ezashi *et al*., 2016). First of all, the initial embryonic development in cattle and pig differs significantly from that in the mouse: Bovine and porcine embryos have a more protracted development of the epiblast from the inner cell mass, in contrast to the mouse, the bovine and porcine epiblast penetrates the trophectoderm (Rauber's layer) and become exposed to the uterine environment and, finally, the bovine and porcine embryo adheres to the uterine epithelium instead of implanting through the epithelium as their murine counterpart. Second, the well-established markers of pluripotency are much less distinct and well-defined in bovine and porcine ESC-like cells than in their murine counterparts. Finally, the pluripotency states, i.e. naïve vs. primed, are not well recognized in bovine and porcine ESC-like cells. Hence, the culture conditions and needs for supplementation for maintenance of pluripotency are putative and in many studies both LIF and bFGF are used.

Importantly, in 1996 it was elegantly demonstrated that cloned sheep could be established by somatic cell nuclear transfer (SCNT) from a cultured cell line established from embryonic discs (Campbell *et al*., 1996). This breakthrough later led to the birth of Dolly (Wilmut *et al*., 1997), cloned from an adult mammary epithelial cell line, and to an alternative avenue for production of genetically modified large domestic species by SCNT utilizing genetically modified cell lines (Schnieke *et al*., 1997; McCreath *et al*., 2000). With this development, the practical importance of bovine and porcine ESCs became less evident as seen in an agricultural and biomedical perspective.

Third medical phase: Human induced pluripotent stem cells as models for neurodegeneration

Through our struggles towards establishing bovine and porcine ESCs, we developed a skill set that allowed us to embark on human iPSCs and the use of these fascinating cells for modelling neurodegeneration. Eminent funding opportunities prompted us to move from an agricultural focus into the medical arena.

Human iPSC reprogramming and mesenchymal-to-epithelial transition

In 2006, Takahashi and Yamanaka published their conceptual work on the establishment of murine iPSCs, where they elegantly narrowed down the need of reprogramming factors to the so-called Yamanaka factors: *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM), which were introduced by retroviral vectors (Takahashi and Yamanaka, 2006). Only one year later, two groups independently reported on the establishment of human iPSCs (Takahashi *et al*., 2007; Yu *et al*., 2007). Since these first publications a range of iPSC reprogramming technologies have been developed and refined with respect to both reprogramming factors (gene sequences, mRNA, miRNA, protein) and vectors (integrating and non-integrating viruses, minicircle vectors and episomal plasmids) in combination with different epigenetic modifiers (for review, see Malik and Rao, 2013). As the most novel approach, it has been demonstrated that the use of CRISPR transcriptional activators for prompting endogenous pluripotency gene expression can result in iPSC reprogramming (Weltner *et al*., 2018).

We have refined and characterized a non-integrative episomal plasmid-based human iPSC reprogramming strategy first published by Okita *et al.* (2011). Our reprogramming is based on the use of electroporation of fibroblasts with three plasmids encoding a short hairpin to TP53 (*shp53*) combined with human *OCT4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28*. We have clearly demonstrated that this strategy, including transient p53 suppression, increases reprogramming of human fibroblasts without affecting apoptosis and DNA damage (Rasmussen *et al*., 2014). Moreover, we have performed a detailed investigation of the gene expression and ultrastructural changes associated with the mesenchymal-to-epithelial transition (MET) that is a vital component of the iPSC reprogramming process (Høffding and Hyttel, 2015). We clearly demonstrated that the sequential acquisition of an epithelial epiblast-like ultrastructure was accompanied by a reorganization of actin and beta-catenin localization from the cytoplasm to the plasma membrane region as well as appearance of plasma membrane-associated E-cadherin and Occludin and of Nanog in the nucleus. In parallel, the mesenchymal marker vimentin disappeared. At the transcriptional level, the relative expression of the epithelial markers *CDH1*, *OCLN* and *EPCAM* was, accordingly, dramatically increased through MET. On the other hand, transcription of the mesenchymal markers *VIM*, *ZEB1* and *SLUG* appeared constant or slightly downregulated. The true downregulation was probably masked by the large number of non-reprogrammed fibroblasts in the samples. These studies clearly demonstrated that a well-orchestrated MET is a major component of iPSC reprogramming.

The investigations referred to above gave us a solid platform for iPSC-based disease modelling, which was materialized in the stem cell center of excellence in neurology, BrainStem.

Human iPSCs for modelling neurodegeneration

The iPSC technology gives access to an infinite source of pluripotent cells from an individual, offering great potentials for future disease modelling and cell therapy (Condic and Rao, 2010). *In vitro* disease modelling has become a major tool in the potential identification of novel disease phenotypes and drug targets as well as in drug screening. Worldwide, iPSCs are used for modelling a variety of disorders, but they are especially useful in research focusing on late progressive disorders such as neurodegenerative diseases, like frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Alzheimer's (AD) and Parkinson's disease (PD), where early symptomatic brain samples are impossible to obtain (Hargus *et al*., 2014; Hedges *et al*., 2016; Lee and Huang, 2017). The iPSC technology allows for creation of "micro-brains" in a dish and studies of the specific pathology and disease progression in an easily assessable and manipulated environment.

Previously, research in the underlying mechanisms of neurodegeneration, as e.g. AD, has been based on data from transgenic AD mice models, which are unfaithful in mimicking AD, or post-mortem AD brain tissue, which exclusively represents the terminal disease pathology. These shortcomings are a major setback for the development of novel therapeutics, which need to combat early disease progression. The iPSC technology, on the other hand, offers the opportunity to investigate early disease mechanisms in the relevant targets: The human neurons, astrocytes and microglia. An example of modelling of frontotemporal dementia (FTD) is presented in the following as it encompasses all components of stem cell biology and gene editing required for dissecting early disease mechanisms.

Frontotemporal dementia linked to chromosome 3 (FTD3) is a rare heterozygous early-onset form of frontotemporal dementia, which is caused by a point mutation in the gene encoding the charged multivesicular protein 2B (CHMP2B) located to the human chromosome 3. FTD3 is characterized as a behavioral variant of frontotemporal dementia mainly associated with initial mild personality changes and social inabilities and as the disease commences potential development of apathy and aggressive behavior (Seelaar *et al*., 2011). FTD3 slowly progresses from the age of onset around the late 50'ties with a mean duration of approximately 10 years, and is thus defined as an early-onset form of dementia (Isaacs *et al*., 2011; Rossor *et al*., 2010; Tang *et al*., 2012). The Danish version of FTD3 has spread in a large family and is caused by a single nucleotide mutation translated into shortened and altered C-terminus of the CHMP2B protein (Skibinski *et al*., 2005; Urwin *et al*., 2010; Zhang *et al*., 2017). CHMP2B is an important part of the endosomal sorting complex required for transport-III (ESCRT-III) and for a proper function of the intracellular endolysosomal pathway (Krasniak and Ahmad, 2016; Urwin *et al*., 2010; van der Zee *et al*., 2008). The mutation results in truncated CHMP2B unable to mediate the endosomal-lysosomal fusion and processing. Patient-derived iPSCs have proven to be very useful in identification of cellular and molecular FTD3 phenotypes and future studies utilizing such models will likely reveal potential therapeutic targets. The stepwise process of FTD3 disease modelling is presented in the following (Zhang *et al*., 2017).

Skin fibroblasts were harvested from the patients and reprogrammed into iPSCs as by means of the non-integrative episomal plasmid approach described above (Rasmussen *et al*., 2014). Before using the iPSC lines for experimentation, they were carefully characterized with respect to their expression of pluripotency markers, ability to differentiate into all three germ layers in-vitro, normality of karyotype and absence of episomal plasmids in their genome.

For disease modelling of neurons, the iPSCs were submitted to neural induction via a dual SMAD inhibition using the small molecules SB431542 and LDN193189, which inhibits the TGFβ and the BMP pathway, thus promoting ectodermal and neuronal differentiation, respectively (Zhang *et al*., 2017). The maturation of the neuronal progenitor cells into glutamatergic forebrain cortical neurons was initiated and maintained with growth factor supplements of BDNF, GDNF and the γ-secretase inhibitor DAPT (Zhang *et al*., 2017).

Until recently, reference iPSCs were derived from healthy age- and gender matched control individuals and used as a comparison to the iPSCs derived from the patients. With the introduction of clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing, it is now possible to create isogenic controls from the patient's own cells to use as a reference instead, eliminating obvious bias due to genomic variance (Poon *et al*., 2017). This so called CRISPR/Cas9 technology is derived from a natural adaptive immune defence mechanism in bacteria providing protection against DNA sequences invading from bacteriophages (Rath *et al*., 2015). Today, this microbial immune mechanism has been biotechnologically transformed into a versatile tool for genome editing. Hence, a single stranded guide-RNA sequence (sgRNA), designed to recognize a specific site in the genome, is combined with a Cas9 protein, capable of cleaving double stranded DNA. Once the target DNA is cut by Cas9 by a double stranded break, the cell repairs the break by either non-homologous-end-joining (NHEJ) or homology-directed repair (HDR) (Ran *et al*., 2013). NHEJ, which is by far the most common of the two, is a random default-prone mechanism where the DNA-recombinase repairs the break by adding random nucleotides until the two DNA strands once again are connected. NHEJ is likely to result in insertions or deletions (Indels), which often results in formation of a codon shift creating a premature stop codon. NHEJ can, however, be bypassed by HDR where an alternative, often single stranded, DNA template carrying a designed sequence with overhangs matching the DNA regions beside the cut is provided. This oligo will, in successful cases, function as a template for DNA-repair (Hsu *et al*., 2014; Ran *et al*., 2013; Yumlu *et al*., 2017).

In the case of FTD3, isogenic controls were created in three different patients (Zhang *et al*., 2017).

Based on the use of patient iPSC-derived neurons and their isogenic controls, we have clearly demonstrated specific disease phenotypes in the FTD3 neurons, all of which can be rescued by correction of the disease-causing mutation. These include mis-regulated expression of genes related to endosomes, mitochondria and iron homeostasis, which was verified by immunocytochemistry, electron microscopy and cellular assays demonstrating large neuronal accumulations of endosomes, lack of mitochondrial axonal distribution and cristae formation, reduction in mitochondrial respiration capacity and intracellular iron accumulation (Zhang *et al*., 2017). Further studies in disease modelling of FTD3 using iPSCs will potentially reveal additional novel disease phenotypes and therapeutic strategies.

The central nervous system holds a glial/neuron ratio of 1.48 (Friede and Van Houten, 1962; Sica *et al*., 2016), which emphasize the glial importance and points towards potential pathological implications of glia in neurodegenerative disorders like FTD. Consequently, we applied an astrocyte differentiation protocol where growth factor supplements, mimicking *in vivo* embryonic astrogenesis, promoted the differentiation and maturation of the neuronal progenitor cells into astrocyte progenitors and further towards astrocytes expressing the astrocytic markers AQP4, S100β, SOX9 and GFAP (unpublished data). Our studies of FTD3-derived astrocytes and their isogenic controls clearly demonstrated that the FTD3 astrocytes displayed accumulation of autophagosomes and increased astrocyte reactivity with a subsequent toxic effect on neurons (unpublished data). Hence, not only neurons, but also the prominent glial compartment is affected by FTD3. Continued research on co-cultures between neurons and glial cells, including both astrocytes and microglia, will further aid unravelling the molecular mechanisms behind this autophagic imbalance and induced neurotoxicity.

Fourth biomedical and veterinary phase: Porcine and canine iPSCs and canine cognitive dysfunction

The competences gained from the human iPSC-based disease modeling allowed us to return our focus to studies of porcine and canine iPSCs of more biomedical, veterinary and, potentially, agricultural relevance.

Porcine iPSCs

Porcine iPSCs have attracted great attention due to the fact that pigs are excellent biomedical models where potentials, but also risks associated with iPSC-based therapy may be investigated. The use of this model enables long-term studies of, for example, cell or organ transplantation, and a multitude of genetically modified pigs are emerging as models for human diseases (Perleberg *et al.*, 2018). In addition to being used for modeling cell-based therapy, porcine iPSCs may also facilitate the generation of genetically modified pigs for use as preclinical models and, potentially in the future, production of animals with valuable traits through the use of chimeric or nuclear transfer technologies. For these reasons we set out to derive integration-free porcine iPSCs.

As alluded to earlier, *bona fide* porcine ESCs have not been generated (Gandolfi *et al*., 2012). The derivation of iPSCs, therefore, is of great importance, and at least 25 studies have already described putative porcine iPSC production (for review, see Pessôa *et al*., 2019). The production of porcine iPSCs until now has predominantly utilized integrative viral vectors carrying human or murine *OCT4*, *SOX2*, *KLM4* and *C-MYC*, including some variations such as *NANOG* and *LIN-28*. However, persistent expression of the integrated transgenes has been widely reported, as opposed to the mouse, and failure to inactivate the exogenous factors is considered a major flaw in the generation of *bona fide* porcine iPSCs (Ezashi *et al*., 2016).

Contribution of porcine iPSCs to live chimeric offspring and germline transmission has only been achieved by one group thus far (West *et al*., 2010; West *et al*., 2011). In this study, porcine mesenchymal stem were used for the iPSC reprogramming and this approach resulted in more than 85% of the live-born piglets being chimeras. Interestingly, this approach also allowed for germline transmission where 2 out of 43 next generation piglets were of iPSC-origin. One of these piglets was, however, stillborn and the other only lived to Day 3 indicating that underlying potential epigenetic aberrancies are incurred.

As for the putative porcine ESCs, the pluripotency state, i.e. naïve vs. primed, of the porcine iPSCs has remained elusive and unclarified. Interestingly, the porcine iPSCs giving rise to germline transmission were cultured in the presence of bFGF being typical for primed murine ESCs, which are not capable of giving rise to germline transmitting chimeras (West *et al*., 2010; West *et al*., 2011). Again, this underlines the lack of clarity regarding the pluripotency states in the pig.

We have particularly focused on the derivation of integration-free porcine iPSCs according to the protocol we optimized for human iPSC reprogramming (Rasmussen *et al.*, 2014). Porcine iPSCs were successfully generated by this methodology and cultured in the presence of bFGF as well as MEK/ERK (PD0325901) and GSK-3β (CHIR99021) inhibitors (Li *et al*., 2018). In order to assess the transgene status with respect to genomic integration or plasmid persistence in our iPSCs, PCR analysis on total DNA extractions, which included genomic DNA and episomal plasmid DNA, were performed. These revealed that at least two of the three episomal plasmids were still present in all lines examined at passage 10. However, at passage 20 the abundance of the two plasmids was significantly diminished in all iPSC lines with one plasmid being completely undetectable. This promoted us to select the a porcine iPSC line, which showed the weakest PCR products for the two plasmids, for single cell subcloning under the assumption that the cell line might show a

certain diversity with respect to plasmid integration or retention. Indeed, 6 out of 8 subclones were completely free of episomal vector DNA. We hereby succeeded in generating porcine iPSCs free of the reprogramming constructs. One of the most striking findings during this quest was that subcloning appears to be crucial in order to obtain integration- and episomal-free porcine iPSCs using the plasmid approach.

During our efforts in implementing the plasmid-based iPSC reprogramming in the pig, we discovered a small population of stage-specific embryonic antigen 1 positive (SSEA-1+) cells in Danish Landrace and Göttingen minipig embryonic fibroblasts, which were absent in their Yucatan counterparts (Li *et al*., 2017). Interestingly, reprogramming of the SSEA-1+ cells after cell sorting led to higher reprogramming efficiency. These SSEA-1+ cells exhibited expression of several genes that are characteristic of mesenchymal stem cells.

Canine iPSCs

Dogs are considered as very interesting models for human diseases; not only due to the over 200 hereditary canine diseases with equivalents in humans, but also due to the physiological similarities as well as equivalence in response to therapy (Starkey *et al*., 2005; Gilmore and Greer, 2015). Based on the competences gained from our human iPSC modelling of neurodegeneration, we extended our studies back to the veterinary field focusing on the dog. Recently, the neurobehavioral syndrome canine cognitive dysfunction (CCD), which shares many clinical and neuropathological similarities with human aging and early stages of AD, has been characterized in dogs, and it is increasingly evident that humans and dogs demonstrate commonalities in brain aging associated with cognitive dysfunction (Studzinski *et al*., 2005; Cotman and Head, 2008). The prevalence of CCD in dogs over 8 years of age has been estimated to 14.2-22.5 % (Azkona *et al*., 2009; Salvin *et al*., 2010). Hence, we set out to further characterize the CCD condition in iPSC-derived neurons from aged demented and control dogs, which will also allow the comparison of CCD with human AD at the cellular level. Such studies have several perspectives: The dog may in the future serve as a model for spontaneous AD in humans and from a veterinary point of view, novel treatment modalities of CCD may become available.

The first information on potential canine iPSCs was reported some years after Yamanaka's breakthrough (Takahashi and Yamanaka, 2006), and the quest for deriving fully reprogrammed and stable canine iPSCs is still ongoing (Shimada *et al*., 2010; Lee *et al*., 2011; Luo *et al*., 2011; Whitworth *et al*., 2012; Koh *et al*., 2013; Baird *et al*., 2015; Nishimura *et al*., 2017; Gonçalves *et al*., 2017; Chow *et al*., 2017; Tsukamoto *et al*., 2018). In the first studies on canine iPSCs, canine reprograming factors were utilized for reprogramming (Shimada *et al*., 2010). The presumptive iPSCs were positive for OCT4 and alkaline phosphatase and were capable of directed differentiation into representatives of all three germ layers. However, the cells were not extensively characterized. In the subsequent work, researchers used mostly human or mouse OSKM reprogramming factors, occasionally with addition of *LIN28* and *NANOG* (Whitworth *et al*., 2012), introduced using retroviral (Shimada *et al*., 2010; Koh *et al*., 2013; Baird *et al*., 2015) or lentiviral approaches (Lee *et al*., 2011; Luo *et al*., 2011; Whitworth *et al*., 2012; Nishimura *et al*., 2017; Gonçalves *et al*., 2017). Lastly, non-integrative Sendai virus have been attempted (Chow *et al*., 2017; Tsukamoto *et al*., 2018). Again, as earlier described for the pig, the silencing of the integrated transgenes in the canine iPSCs seems to represent a consistent problem and was only described in a few studies (Baird *et al*., 2015; Gonçalves *et al*., 2017). Regarding culture conditions and supplementation requirements, canine iPSCs seem to be dependent of both LIF and bFGF, with some exceptions (Whitworth *et al*., 2012; Nishimura *et al*., 2017; Gonçalves *et al*., 2017; Chow *et al*., 2017), as well as the cells are dependent on culture with feeder cells, except for a single report (Nishimura *et al*., 2017).

In general, the reports on putative canine iPSCs do not refer to the naïve vs. primed pluripotency state of the generated cells. However, based on the expression of pluripotency markers, one can speculate that most of the generated cell lines represent a primed status, characterized by expression of markers such as SSEA4, TRA-1-60 and TRA-1-80 (Lee *et al*., 2011; Luo *et al*., 2011; Whitworth *et al*., 2012; Baird *et al*., 2015; Nishimura *et al*., 2017; Chow *et al*., 2017). Nevertheless, canine iPSCs expressing naïve pluripotency markers, like SSEA1, have also been described (Koh *et al*., 2013; Tsukamoto *et al*., 2018). The expression of pluripotency markers, however, may differ between species making it difficult to draw firm conclusions on the state of pluripotency just based upon such markers. Overall, the potential canine iPSCs have been reported to show different combinations of classic pluripotency markers, such as OCT4, Nanog, SOX2, amongst others (for review, see Pessôa *et al*., in press), and some of these cells lines were also able to form teratomas (Lee *et al*., 2011; Whitworth *et al*., 2012; Koh *et al*., 2013; Gonçalves *et al*., 2017; Chow *et al*., 2017; Tsukamoto *et al*., 2018). Contribution of iPSCs to the development of chimeric embryos has, however, not been described so far.

All the previously cited reports deal with reprogramming of fibroblasts or adipose tissue cells from canine embryos, fetuses or younger adults, the oldest donors being 3-year-old beagles and a 6-year-old male standard poodle (Koh *et al*., 2013; Chow *et al*., 2017; respectively). Our studies of CCD focused on geriatric dogs, and it turned out that iPSC reprogramming of fibroblasts from such elderly dogs is a major challenge. Our efforts, however, have just started to pay off. We have attempted to reprogram adult fibroblasts to pluripotency using an excisable lentiviral vector containing human and/or murine OSKM (Sommer *et al*., 2009; Gonçalves *et al*., 2017). After a longer series of experiments, the first iPSCs colonies have now emerged around 14 days after transduction with human factors in skin fibroblasts of a 14-year and 9 month-old female west highland white terrier. So far, colonies obtained are flat, present high

nuclei to cytoplasm ratio, are tightly packed, present well defined edges, and are positively stained for alkaline phosphatase and hanog The potential iPSCs are dependent on both LIF and bFGF and are in the process of expansion for further characterization. Once these cell lines are well stablished and characterized, we hope they will provide valuable information for iPSC-based disease modeling and veterinary research.

Fifth agricultural phase: Return to bovine *in vitro* embryo production

This review began with *in vitro* production of bovine embryos. Even though great advances were made in this area in Denmark during the eighties and nineties, the practical implementation of the technologies failed due to concerns related to animal welfare and ethical considerations related to the OPU procedure and the risk of LOS. As alluded to earlier in the text, the refinement of media for bovine IVP has more or less eliminated the risk for LOS, and the OPU procedures have also become less harmful. These developments have led to a Danish reconsideration of the use of the technologies in cattle breeding and have given leverage to funding of the project EliteOva by Innovation Fund Denmark. EliteOva aims at implementing OPU and IVP combined with genomic selection of the embryos in commercial Danish Holstein dairy breeding.

It has been a great privilege to encompass a full circle of scientific progress from bovine oocytes and embryos through bovine and porcine stem cells into human stem cell-based disease modeling and back to animal stem cells and, finally, practical implementation of bovine oocyte and embryo technologies.

Conclusions

The veterinary and animal science professions are rapidly developing and their inherent and historical focus on agriculture has been extended with a major biomedical and even medical dimension. This biomedical and medical trend in science cannot be rejected and should be contemplated as an opportunity for contemporary development of the veterinary and animal science professions. With an open scientific mind it is possible to embark on such biomedical and medical adventures and gain new competences that can feed back to novel ideas and projects in the veterinary and animal science field. Hence, seek opportunistic scientific avenues and see the possibilities in gaining novel competences that will, in turn, benefit veterinary and animal science.

Author contributions

PH: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; LVFP: Conceptualization, Investigation, Writing – original draft, Writing – review & editing; JB-MS: Funding acquisition, Conceptualization, Investigation, Writing – review & editing; KSD: Conceptualization, Investigation, Writing – original draft, Writing – review & editing; KF: Funding acquisition, Conceptualization, Investigation, Writing – review & editing; VJH: Funding acquisition, Conceptualization, Investigation, Writing – review & editing; TF: Investigation, Writing – review & editing; RJA: Investigation, Writing – review & editing; JL: Funding acquisition, Investigation, Writing – review & editing; HC: Writing – review & editing; TG: Funding acquisition, Supervision, Writing – review & editing; LBS: Funding acquisition, Conceptualization, Investigation, Writing – review & editing.

Conflict of interest

The authors declare that they have no competing interests. LBS: Scientific Advisor for IVFBioscience.

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Commercial Embryo Transfer Activity in Europe 2018

Collated by Marja Mikkola

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Bovine *In vivo* embryo production

	Dairy				Beef				All		
Country	**Collections conv semen**	**Collections sexed semen**	**Embryos & ova**	**Transferable embryos**	**Collections conv semen**	**Collections sexed semen**	**Embryos & ova**	**Transferable embryos**	**Collections total**	**Transferable Embryos total**	**Embryos/ collection**
Austria	335	11	3 357	2 488	17	1	95	138	364	2 626	7,2
Belgium	121	8	813	543	875	0	5 656	4 198	1 004	4 741	4,7
Bosnia and Herzegovina	0	0	0	0	1	0	5	5	1	5	5,0
Croatia	0	0	0	0	0	0	0	0	0	0	
Denmark	716	0	6 179	4 161	54	0	593	392	770	4 553	5,9
Estonia	0	0	0	0	0	0	0	0	0	0	
Finland	431	55	4 007	2 714	3	0	24	4	489	2 718	5,6
France	4 497	1 053	50 792	29 275	1 242	94	13 825	7 494	6 886	36 769	5,3
Germany	3 166	0	31 472	21 100	464	0	4 654	2 499	3 630	23 599	6,5
Greece	0	0	0	0	0	0	0	0	0	0	
Hungary	7	11	124	116	36	6	385	255	60	371	6,2
Ireland	664	0	8 190	3 904	0	0	0	0	664	3 904	5,9
Israel	41	1	239	156	1	0	6	6	43	162	3,8
Italy	2 432	0	28 397	19 418	150	0	1 575	1 083	2 582	20 501	7,9
Latvia	11	0	52	43	0	0	0	0	11	43	3,9
Lithuania	0	0	0	0	0	0	0	0	0	0	
Macedonia	0	0	0	0	0	0	0	0	0	0	
Netherlands	2 735	16	20 847	15 675	0	0	0	0	2 751	15 675	5,7
Norway	87	0	888	586	11	0	155	54	98	640	6,5
Poland	131	138	2 193	1 493	0	0	0	0	269	1 493	5,6
Portugal	60	50	572	352	10	0	103	38	120	390	3,3
Romania	9	0	48	40	0	0	0	0	9	40	4,4
Russian Federation	109	442	5 205	2 682	1 530	4	13 143	11 416	2 085	14 098	6,8
Serbia	5	0	22	14	0	0	0	0	5	14	2,8
Slovakia	0	0	0	0	0	0	0	0	0	0	
Slovenia	12	2	52	26	0	0	0	0	14	26	1,9
Spain	102	374	4 030	2 626	272	15	1 382	2 002	763	4 628	6,1
Sweden	127	0	864	562	3	0	4	2	130	564	4,3
Switzerland	212	147	3 615	2 129	16	1	149	67	376	2 196	5,8
Turkey	0	0	0	0	0	0	0	0	0	0	
Ukraine	10	8	261	209	8	4	175	125	30	334	11,1
United Kingdom	116	15	970	709	112	0	216	572	243	1 281	5,3
Grand Total	**16 136**	**2 331**	**173 189**	**111 021**	**4 805**	**125**	**42 145**	**30 350**	**23 397**	**141 371**	**6,0**

Bovine *In vitro* embryo production (OPU-IVP)

	Dairy								Beef								All		
	Non-stimulated				Stimulated				Non-stimulated				Stimulated						
Country	OPU Conv semen	OPU Sexed semen	Oocytes	Embryos	OPU Conv semen	OPU Sexed semen	Oocytes	Embryos	OPU Conv semen	OPU Sexed semen	Oocytes	Embryos	OPU Conv semen	OPU Sexed semen	Oocytes	Embryos	Sessions	Oocytes	Embryos
Estonia	0	0	0	0	0	0	0	0	0	0	0	0	0	8	74	24	8	74	24
Finland	368	0	1 809	67	315	0	2 946	385	0	0	0	0	0	0	0	0	683	4 755	452
France	206	13	924	214	277	80	3 928	831	0	0	0	0	46	0	456	166	622	5 308	1 211
Germany	809	0	11 844	2 621	0	0	0	0	20	0	337	89	0	0	0	0	829	12 181	2 710
Italy	119	0	1 327	188	0	0	0	0	0	0	0	0	0	0	0	0	119	1 327	188
Netherlands	694	85	11 300	2 502	9 750	0	104 103	27 300	0	0	0	0	0	0	0	0	10 529	115 403	29 802
Poland	4	10	112	62	0	33	324	128	0	0	0	0	0	0	0	0	47	436	190
Romania	0	0	0	0	4	0	5	1	0	0	0	0	0	0	0	0	4	5	1
Russian Federation	0	831	4 621	936	0	0	0	0	0	300	2 461	723	0	0	0	0	1 131	7 082	1 659
Serbia	11	7	76	28	8	15	128	32	0	0	0	0	0	0	0	0	41	204	60
Spain	0	0	0	0	0	50	770	151	68	0	473	239	0	0	0	0	118	1 243	390
Switzerland	40	29	841	145	0	0	0	0	0	0	0	0	0	0	0	0	69	841	145
Grand Total	**2 251**	**975**	**32 854**	**6 763**	**10 354**	**178**	**112 204**	**28 828**	**88**	**300**	**3 271**	**1 051**	**46**	**8**	**530**	**190**	**14 200**	**148 859**	**36 832**

Bovine embryo transfer - *In vivo*

Country	Dairy breeds			Beef breeds			Non-separated breeds			Total transfers
	Fresh	Frozen	Frozen Foreign	Fresh	Frozen	Frozen foreign	Fresh	Frozen	Frozen foreign	
Austria	1 000	1 402	15	14	90	13	0	0	0	2 534
Belgium	111	451	578	614	2 295	16	0	0	0	4 065
Denmark	2 386	1 102	0	92	96	0	0	0	0	3 676
Finland	789	1 938	165	4	23	50	0	0	0	2 969
France	14 604	13 153	654	2 344	4 333	170	43	90	12	35 403
Germany	8 983	11 680	0	689	1 246	0	0	0	0	22 598
Greece	0	0	7	0	0	0	0	0	0	7
Hungary	60	58	0	29	129	0	0	0	0	276
Ireland	1 565	1 904	0	0	0	0	0	0	0	3 469
Israel	146	19	0	6	0	0	0	0	0	171
Italy	0	0	0	0	0	0	7 750	0	0	7 750
Netherlands	4 381	14 332	0	0	0	0	1 634	0	0	20 347
Norway	31	75	143	15	61	52	0	0	0	377
Poland	667	705	438	0	0	0	0	0	0	1 810
Portugal	87	423	0	14	23	17	0	0	0	564
Romania	23	17	0	0	0	46	0	0	0	86
Russian Federation	220	1 005	91	885	10 072	105	0	0	0	12 378
Serbia	5	0	7	0	0	0	0	0	0	12
Slovenia	24	2	0	0	0	4	0	0	0	30
Spain	1 130	1 005	110	570	404	4	0	0	0	3 223
Sweden	0	0	0	0	0	0	142	396	103	641
Switzerland	729	810	422	3	38	21	0	0	0	2 023
Ukraine	12	74	0	61	58	0	0	0	0	205
United Kingdom	51	45	4	81	309	349	340	327	42	1 548
Grand Total	**37 004**	**50 200**	**2 634**	**5 421**	**19 177**	**847**	**9 909**	**813**	**157**	**126 162**

Bovine embryo transfer - *In vitro*

Country	OPU				Abattoir		Total transfers IVP
	Fresh	Frozen	Frozen foreign	OPU Exports	Fresh	Frozen	
Belgium	0	0	1 315	0	0	0	1 315
Estonia	17	0	0	0	0	0	17
Finland	19	457	0	0	0	0	476
France	369	348	54	88	0	0	859
Germany	2 007	909	0	0	0	0	2 916
Hungary	0	0	105	0	0	0	105
Italy	126	15	0	0	0	0	141
Netherlands	11 124	10 354	0	0	0	0	21 478
Poland	23	17	0	0	0	0	40
Romania	0	1	0	0	12	3	16
Russian Federation	111	521	0	0	0	0	632
Serbia	12	37	0	0	0	0	49
Spain	59	158	0	0	49	282	548
Switzerland	0	14	81	0	0	0	95
United Kingdom	0	117	0	0	0	0	117
Grand Total	**13 867**	**12 948**	**1 555**	**88**	**61**	**285**	**28 804**

Embryo production and transfer in other species - *In vivo*

Country	Embryo collection		Embryo transfer		
	Collections	Viable embryos	Fresh embryos	Frozen domestic	Frozen foreign
SHEEP					
France	7	25	0	0	0
Hungary	5	39	0	0	45
Italy	12	58	58	0	0
Serbia	3	16	0	0	0
Spain	56	160	34	0	0
Sweden	20	67	0	67	365
United Kingdom	537	3 296	3 247	116	0
Total	**640**	**3 661**	**3 339**	**183**	**410**
GOAT					
Spain	25	201	0	0	0
United Kingdom	3	26	26	0	0
Total	**28**	**227**	**26**	**0**	**0**
HORSE					
France	1 315	736	818	0	0
Italy	309	211	0	0	0
Netherlands	281	157	160	0	0
Poland	5	4	2	0	0
Portugal	211	84	76	8	0
Russian Federation	26	18	8	3	0
Spain	170	93	91	0	0
Sweden	23	15	15	0	0
Switzerland	90	42	22	1	0
United Kingdom	65	35	43	0	0
Total	**2 495**	**1 395**	**1 235**	**0**	**0**
BUFFALO					
Romania	3	6	0	0	0

Embryo production and transfer in other species - *In vitro*

Country	Oocyte collection			Embryo transfer IVP			Embryo exports
	OPU	Oocytes	Embryos	Fresh embryos	Frozen domestic	Frozen foreign	
SHEEP							
Spain	39	909	457	303	0	0	0
GOAT							
Spain	6	1 710	158	108	50	0	0
HORSE							
Italy	1 062	12 605	1 431	65	352	0	858
Netherlands	456	5 933	664	38	20	273	0
Switzerland	68	191	12	0	4	0	0
Total	1 586	18 729	2 107	**0**	**0**	**0**	**0**
BUFFALO							
Italy	2	49	21	27	0	0	0

Bovine *In vitro* embryo production – abattoir

	Dairy			Beef		
Country	**Donors abattoir**	**Oocytes**	**Embryos**	**Donors abattoir**	**Oocytes**	**Embryos**
Greece	8	0	30	0	0	0
Italy	0	0	0	5	168	28
Netherlands	84	2 350	705	0	0	0
Norway	0	0	0	0	0	0
Poland	86	695	166	0	0	0
Portugal	0	0	0	1 144	5 088	0
Romania	4	160	48	0	0	0
Spain	8	51	26	63	1 178	436
Grand Total	**190**	**3 256**	**975**	**1 212**	**6 434**	**464**

Bovine embryo technologies – embryo genotyping

	Sexed embryos		Genotyped embryos	
Country	**In vivo**	**In vitro**	**In vivo**	**In vitro**
Finland	0	33	38	33
France	3 054	0	1 642	0
Germany	0	0	310	55
Netherlands	0	0	101	2 708
Grand Total	**3 054**	**33**	**2 086**	**2 796**

Bovine embryo exports

Country	**Dairy**	**Beef**	**Non-separated breeds**
Austria	40	9	0
Belgium	20	900	0
Denmark	36	0	0
France	637	351	0
Germany	81	0	0
Norway	18	38	0
Spain	50	0	0
Switzerland	61	0	0
United Kingdom	0	0	63
Grand Total	**943**	**1 298**	**63**

INVITED LECTURES

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Intrafollicular barriers and cellular interactions during ovarian follicle development

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Abstract

Follicles are composed of different interdependent cell types including oocytes, cumulus, granulosa, and theca cells. Follicular cells and oocytes exchange signaling molecules from the beginning of the development of the primordial follicles until the moment of ovulation. The follicular structure transforms during folliculogenesis; barriers form between the germ and the somatic follicular cells, and between the somatic follicular cells. As such, communication systems need to adapt to maintain the exchange of signaling molecules. Two critical barriers are established at different stages of development: the zona pellucida, separating the oocyte and the cumulus cells limiting the communication through specific connections, and the antrum, separating subpopulations of follicular cells. In both situations, communication is maintained either by the development of specialized connections as transzonal projections or by paracrine signaling and trafficking of extracellular vesicles through the follicular fluid. The bidirectional communication between the oocytes and the follicle cells is vital for driving folliculogenesis and oogenesis. These communication systems are associated with essential functions related to follicular development, oocyte competence, and embryonic quality. Here, we discuss the formation of the zona pellucida and antrum during folliculogenesis, and their importance in follicle and oocyte development. Moreover, this review discusses the current knowledge on the cellular mechanisms such as the movement of molecules via transzonal projections, and the exchange of extracellular vesicles by follicular cells to overcome these barriers to support female gamete development. Finally, we highlight the undiscovered aspects related to intrafollicular communication among the germ and somatic cells, and between the somatic follicular cells and give our perspective on manipulating the above-mentioned cellular communication to improve reproductive technologies.

Keywords: cellular communication, extracellular vesicles, granulosa cells, oocyte, ovarian follicle, transzonal projections

Introduction: Follicle development

The ovarian follicle development starts long before birth during the intra-uterine period (Russe, 1983). The primordial germ cells migrate to the genital ridge, colonize, and proliferate. After this highly proliferative period, a human female fetus has approximately 6-7 million germ cells around the 20th week of gestation, however a vast majority of these germ cells are lost and approximately 1 to 2 million oocytes remain viable at birth (Motta *et al*., 1997; Sun *et al*., 2017). In bovines, the maximum number of germ cells is around 2.5 million at about the 15th week of gestation (Erickson, 1966) and thirteen days after birth bovine germ cells number decrease approximately to 68 thousand. This dramatic loss of germ cells close after birth occurs in most female mammals (Paulini *et al*., 2014).

Once mitotic proliferation stops, these germ cells arrest at meiotic prophase I to form the germ cell nests (Buehr, 1997; Tilly, 2001; Sun *et al*., 2017). Close to birth, breakdown of the germ cell nests occurs with the formation of the primordial follicle. Two cell types characterize this primordial follicle: a primary oocyte surrounded by a single layer of pre-granulosa cells (Fortune, 1994; BrawTal and Yossefi, 1997; Eppig, 2001). The primordial follicle population in the ovary serves as a reservoir for developing follicles and oocytes throughout the female reproductive life (Zuckerman, 1951; Kerr *et al*., 2013). After puberty, groups of primordial follicles are periodically recruited to initiate folliculogenesis.

Although the precise mechanisms that regulate germline nest breakdown and primordial follicle formation are mostly unknown (Wang *et al*., 2017), several growth factors and hormones play essential roles in primordial follicle formation (Pepling, 2012), for example estradiol-17β (E2) and members of the transforming growth factor beta (TGF-β) superfamily (Knight and Glister, 2006; Wang and Roy, 2007; Chakraborty and Roy, 2017). The TGF-β family members are secreted by the oocyte and include bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), which act via autocrine and paracrine mechanisms, regulating follicle growth and differentiation, as well as granulosa and thecal cell function during follicular development (Dong *et al*., 1996; Eppig *et al*., 1997; Gilchrist *et al*., 2004; Sanfins *et al*., 2018). By secreting these members of TGF-β family the oocyte is the main responsible for activating primordial follicles (Eppig, 2001).

Ovarian follicle development is a continuous process that has two different phases: the preantral and antral. The first phase, preantral, is gonadotropin-independent and relies on local growth factors. As

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folliculogenesis progresses, the follicle becomes gonadotropin-responsive and develops until secondary follicles. The second phase, antral, is gonadotropin-dependent and is characterized by the presence of the tertiary follicles, which has the presence the antrum, a cavity filled with follicular fluid (Dvořák and Tesařík, 1980; Erickson and Shumichi, 2001). This entire process of growth and differentiation of the follicle is accompanied by the oocyte growth and acquisition of competence (El-Hayek and Clarke, 2015; Monniaux, 2016).

Stimulation by the locally secreted factors activates the primordial follicles initiating the preantral growth phase for development into a primary follicle. Factors responsible for primary follicle development are not fully known; however, it is known that granulosa cell-derived anti-Mullerian hormone and activins participate in the regulation of this process (reviewed by Matzuk *et al*., 2002). The primary follicles are characterized by the presence of an oocyte covered with a single layer of cuboidal granulosa cells. As the oocyte grows, the granulosa cells proliferate to envelop the surface of the expanding oocyte (vandenHurk *et al*., 1997).

Continuous granulosa cell proliferation results in multiple layers of cells surrounding the oocyte and the follicles are referred to as secondary follicles. At this stage, the formation of the theca cell layer starts, separated from the granulosa by a basement membrane (BrawTal and Yossefi, 1997). At the same time, oocytes undergo alterations as the formation of cortical granules in the cytoplasm (Fair *et al*., 1997) and the beginning of mRNA synthesis (McLaughlin *et al*., 2010). At this stage, the formation of the zona pellucida (ZP) around the oocyte starts, to form the first significant barrier between the oocyte and the somatic granulosa cells (BrawTal and Yossefi, 1997; Clarke, 2018) (Fig. 1A).

As the secondary follicle develops, more layers of granulosa cells form, and an antral cavity filled with follicular fluid develops between them. With the initiation of the antral phase of follicular growth, the follicle is now a tertiary follicle. During the transition of the secondary to tertiary follicle the second significant barrier between follicular cells is formed (Fig. 1B). Indeed, the antrum induces the differentiation of granulosa subpopulations, the original granulosa cells present in the outer wall of the follicle and that specialized granulosa cells, now cumulus cells, that directly surround the oocyte during further development. Mural granulosa cells and cumulus cells became exposed to opposing gradients of follicle-stimulated hormone (FSH) and oocyte-secreted factors (OSF) (Fortune, 1994; Eppig, 2001; Wigglesworth *et al*., 2015). In this phase of intense follicle growth, the oocyte slows down or even stops its growth, while stromal cells form two layers of cells external to the basement membrane, the internal and external theca cell layers (Fair *et al*., 1997; Hyttel *et al*., 1997; Guo *et al*., 2016).

In bovine, from the beginning of antral phase until a diameter of approximately 8 mm, follicle growth is stimulated by FSH secreted by the pituitary gland. Follicles develops by the rapid proliferation of granulosa and theca cells that contribute to the further enlargement of the antrum and the follicle itself. From a diameter of 8 mm onwards, the follicle develops mainly by the trophic stimulation of LH, and eventually, after the LH surge, will be termed preovulatory follicles (Eppig *et al*., 1997).

At the end of its growth, the dominant follicle reaches a plateau phase of non-exponential growth with fewer cell divisions and slower diameter increase (Girard *et al*., 2015). Following the preovulatory gonadotropin surge, follicular cells initiate morphological, endocrine, and biochemical changes associated with luteinization process (Smith *et al*., 1994; Revelli *et al*., 2009). In monovulatory species, only one follicle continues its growth to become an ovulatory follicle, while the remaining antral follicles regress and undergo atresia (Hennet and Combelles, 2012).

For the follicle formation and its steady growth during the whole folliculogenesis process, the bidirectional communication within the follicle environment is essential for the complete development of the follicle as well as the oocyte. The crosstalk between the oocyte and somatic follicular cells and between the somatic follicular cells occurs through the interactions mediated by paracrine signaling factors, by gap junctions and, as recently described, by extracellular vesicles. The paracrine signaling occurs through the secretion of factors from the oocyte or from the somatic cells. The gap junctions are structures formed by connexins that allow the transport of molecules of low molecular weight (<1 kDa) as ions, metabolites and amino acids between granulosa cells and cumulus cells, and between cumulus and oocyte cells. These junctions connect neighbor follicular cells or germ and somatic cell, at the bulk end of transzonal projections. The extracellular vesicles consist in a communication system mediated by vesicles secreted by cells. These vesicles, may have proteins, miRNAs and mRNAs as cargo, and are secrete and uptake by follicular cells (reviewed by Del Collado *et al*., 2018).

Hence, there are two physical barriers existing in the follicular environment, the ZP and the antrum. In both cases, cellular communication mechanism overcomes these barriers to maintain the exchange of messages via transzonal projections (TZPs) and extracellular vesicles (EVs; Fig. 2). These barriers, the communication mechanisms within, and the importance of such communication for the follicle and oocyte development are discussed in the following part of the review.

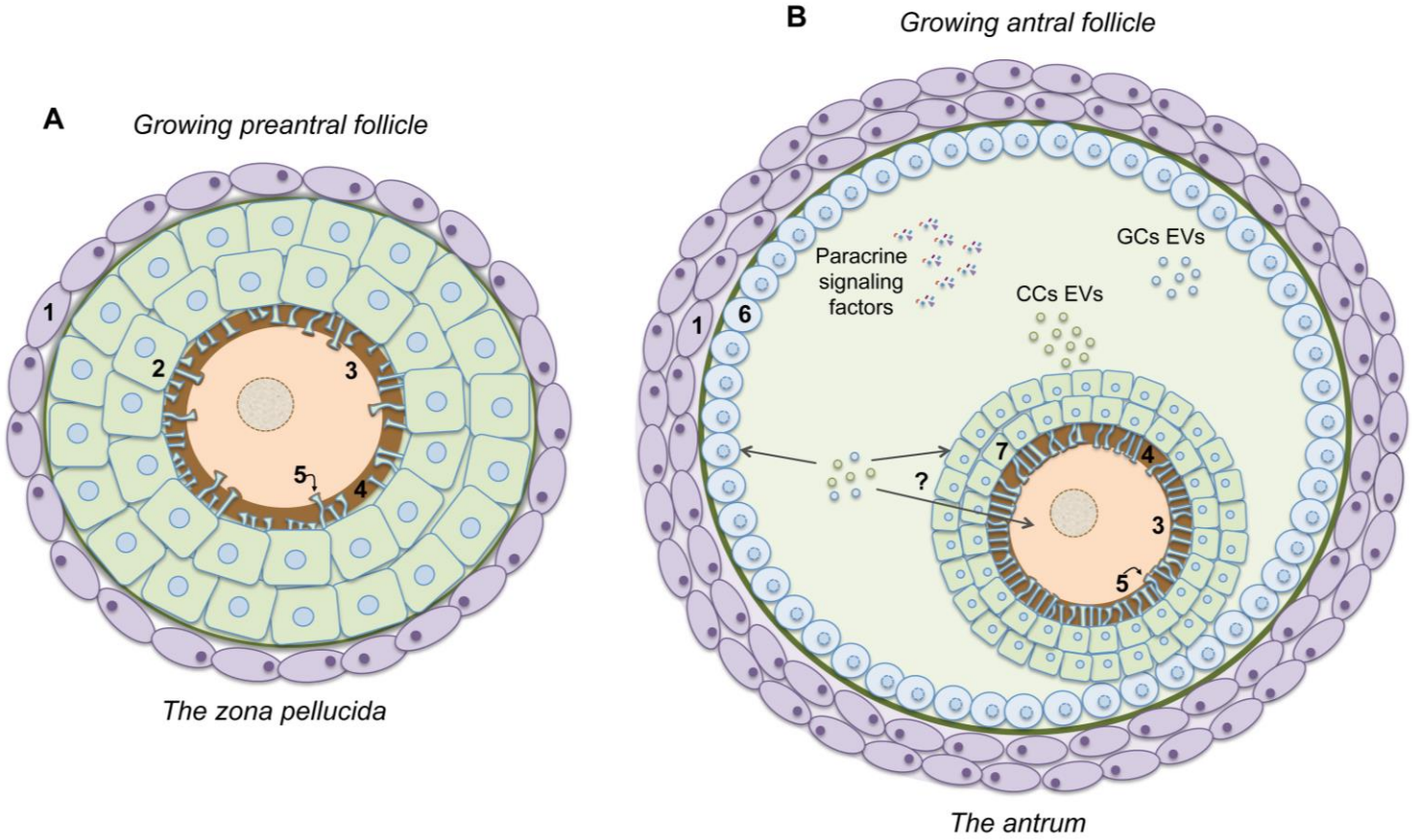

Figure 1. *Physical barriers to cell-to-cell communication in the ovarian follicle are established during folliculogenesis.* During preantral growth, zona pellucida, the first significant barrier between the oocyte and follicular cells, is formed. This barrier between the germ and somatic cells results from the deposition of glycoproteins by the oocyte. For continuous maintenance of a cytoplasmic bridge between germ and somatic cells, the oocyte stimulates the granulosa cells to generate specialized cytoplasmic filaments connecting both cells – the transzonal projections **(A)**. In antral growing follicles, a second significant barrier among follicular cells is formed – the antrum. Bilateral communication is maintained by paracrine signaling and extracellular vesicle traffic. Paracrine signaling of oocyte-secreted factors and transactivation of the EGF receptor by LH signaling drives follicle development and ovulation. Extracellular vesicles are secreted into the follicular fluid and are taken up by different cells types by a cargo delivery mechanism. The direct transfer of EVs-cargo from the follicular fluid to the oocyte remains elusive **(B)**. CCs – cumulus cells; EVs – extracellular vesicles; GCs – granulosa cells; Theca – theca cells.

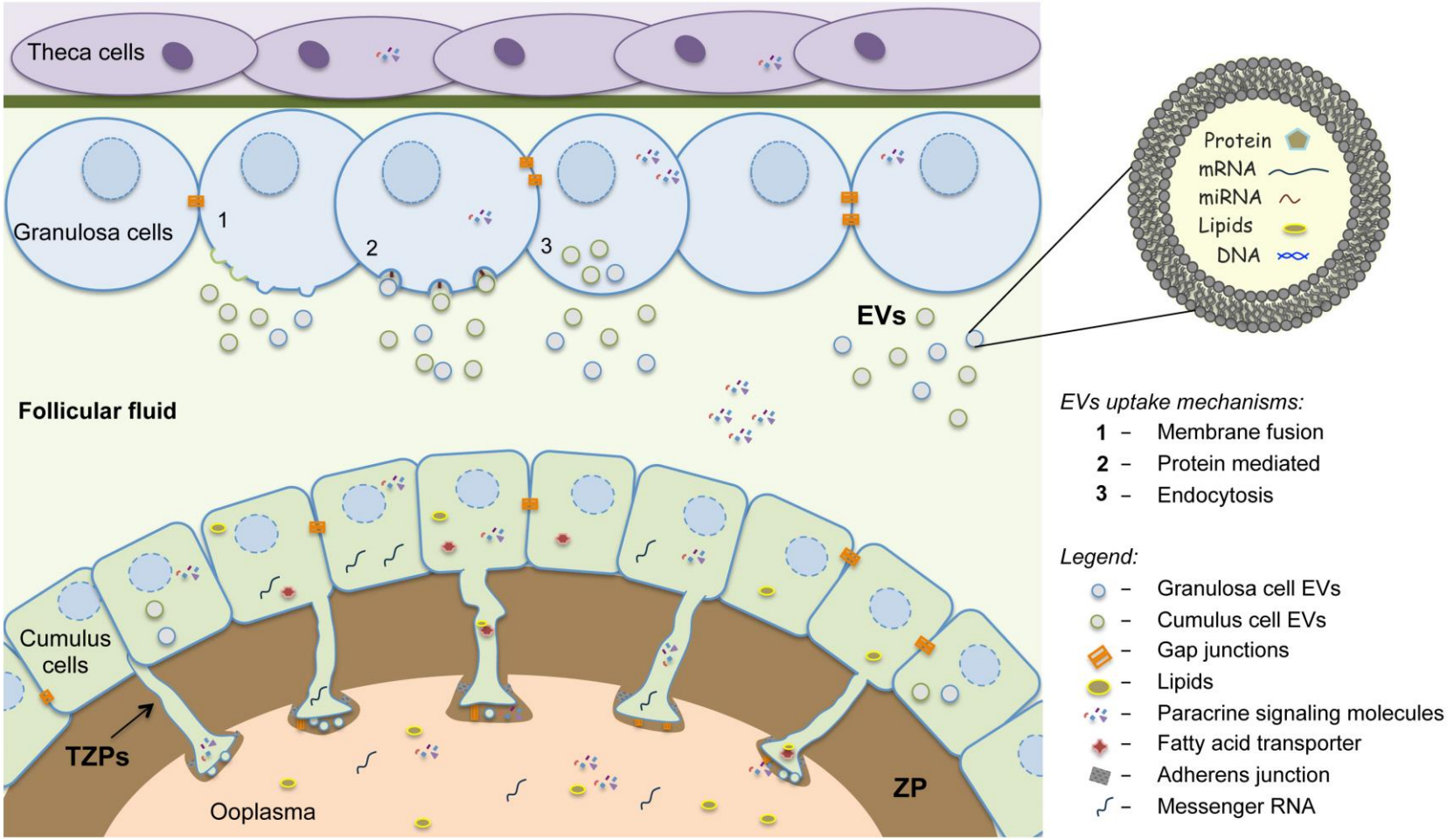

Figure 2. *Bidirectional communication within the ovarian follicle microenvironment.* The bidirectional crosstalk between cells that compose the follicle is associated with follicular development and acquisition of oocyte competence. Cellular crosstalk between germ-somatic cells and between somatic cells is mediated by the secretion of paracrine factors, by the communication through transzonal projections (TZPs) via gap junctions, and via extracellular vesicles (EVs) trafficking from the bulk end of TZPs to the oocyte, or trafficking into the follicular fluid. TZPs are specialized cytoplasmic projections that extend across zona pellucida (ZP) and allow the exchange of small molecules such as sugars, pyruvate, amino acids, and nucleotides, and large molecules such as mRNAs, lipids, and small organelles. Follicular fluid EVs are lipid bilayer vesicles loaded with proteins, mRNAs, microRNAs, lipids, and DNA and are taken up through endocytosis, protein recognition, and membrane fusion by distinct cell types within the ovarian follicle allowing communication with distant cells.

The first barrier

ZP formation and the germ-soma barrier

The ZP is a relatively thick extracellular coat that surrounds all mammalian oocytes. It is formed in the preantral phase of follicular development during the formation of secondary follicles, when the oocytes are arrested in the late diplotene stage and undergoing active growth (Wassarman and Litscher, 2012). This porous extracellular coat is formed by three or four glycoproteins depending on the species (Bleil and Wassarman, 1980; reviewed by Gupta, 2015). The ZP has essential functions during oogenesis, fertilization, and pre-implantation development (Wassarman and Litscher, 2012). For example, during oocyte development, the ZP integrity is important to maintain the communication between the oocyte and the cumulus cells (Wang *et al*., 2019). During fertilization the ZP play fundamental roles, as the block of non-specific fertilization, the block of polyspermia (Wassarman, 1999; Florman and Ducibella, 2006). And during early embryo development the ZP permit that cleavage stage embryos move freely along the oviduct and protect the growing embryo until implantation (Gupta *et al*., 2015) so that the ZP presence is necessary for normal early development in uterus (Modliński, 1970).

As an oocyte increases in diameter, its ZP increases in thickness (Wassarman and Litscher, 2013) and separate the oocytes and the surrounding cumulus cells. Depending on the species, the ZP ranges in thickness from less than 1 mm to more than 25 mm (Keefe *et al*., 1997). Despite this physical separation, the oocytes and cumulus cells maintain contact by the formation of TZPs (Albertini *et al*., 2001; Eppig, 2018). TZPs mainly originate from the cumulus cells in the layer immediately adjacent to the oocyte, but they are also shown to arise from cumulus cells positioned in oocyte more distant layers (Jaffe and Egbert, 2017). Since the number of TZPs present in the ZP of a developing oocyte is quite high, it is likely that each somatic cell surrounding the oocyte emits multiple projections towards the oocyte. Also, some projections extend from a single point of origin at the granulosa cell membrane and subsequently dividing into several TZPs towards the ooplasm (El-Hayek *et al*., 2018).

There are two hypotheses for the formation of TZPs: by "stretching" or "pushing." In the "stretching" model, the adhesion sites between the oocyte and granulosa cells are already present before ZP formation and remain during the ZP deposition process, to become stretched cytoplasmic filaments called TZPs. The second hypothesis, known as "pushing," proposes that the TZPs are elaborated from the granulosa cells after the deposition of the ZP and grow towards the oocyte where they establish contact with their plasma membrane (Clarke, 2017).

The growing oocyte induces somatic cells to generate the TZPs (El-Hayek *et al*., 2018). Factors secreted by the oocyte, such as GDF9 and FSH, correlate with the development of TZPs. Recent studies show that GDF9 produced by the oocyte acts via the SMAD signaling pathway to stimulate neighboring granulosa cells to generate TZP structures (El-Hayek *et al*., 2018). Besides that, recent functional studies verified that GDF9 maintains stable mRNAs that encode TZPs structural components (El-Hayek *et al*., 2018) and the absence of oocyte GDF9 leads to morphologically abnormal TZPs in mice (Dong *et al*., 1996; Carabatsos *et al*., 1998). FSH induces the retraction of TZPs, but the specific pathways by which this happens are still unclear (Combelles *et al*., 2004).

Oocyte and soma interactions through TZPs

The TZPs form concomitantly with the ZP and are specialized filopodia characterized as communication channels of approximately 2 um in diameter without the fusion of membranes (Macaulay *et al*., 2014). These channels originate from the cytoplasmic filaments of actin or tubulin, and their function depends on the composition. TZPs formed by tubulin filaments are related to cell adhesion while actin TZPs are involved in cell communication, and the latter are prevalent in oocyte ZP (Li and Albertini, 2013).

These projections allow communication between the oocyte and somatic cells. As the TZPs are free-ended structures, the exchange of small molecules occurs at the bulk end of the projections by gap junctions and intermediate junctions (zonula adherens-like junctions) that keep the cytoplasmic membranes of both cells in close contact (Hyttel *et al*., 1997; Albertini and Barrett, 2004). Additionally, at the bulk end of TZPs, a cleft is formed between the plasma membrane of the TZP and the oolemma. Extracellular vesicles were identified at this cleft (Macaulay *et al*., 2014) and are involved in potential mechanisms by which cargo transfer occurs from somatic cells to oocyte.

Some molecules, such as mRNAs, lipids, pyruvate and cGMP, are shown to be transported through TZPs, suggesting the importance of these communication mechanisms between the oocyte and the surrounding cumulus cells. TZPs have distinct roles, for example: i) in mRNA accumulation, as evidenced by passage of polyadenylated transcripts (Macaulay *et al*., 2014; Macaulay *et al*., 2016), ii) in metabolic and nutritional cooperation, due to continuous exchange of small molecule ions, cyclic nucleotides, and amino acids (Thomas *et al*., 2004; Lodde *et al*., 2013), iii) and providing energy substrates such as pyruvate, lactate (Scantland *et al*., 2014), and other metabolites. Recent results show that TZPs also have a role in lipid transport from cumulus cells to oocyte. The TZPs lipid transport was proved by co-localization of fatty acid binding protein 3 (FABP3), a protein responsible for carrying lipids, with TZPs within zona pellucida and by the increase of oocyte lipid droplets dependent on the presence of TZPs, indicating that TZPs might be involved in the oocyte lipid accumulation during maturation (del Collado *et al*., 2017).

The communication through TZPs have fundamental role in the oocyte meiosis control and oocyte maturation, since the transport of essential molecules as cAMP, is mediated by TZPs from somatic

cells to the oocyte (Eppig *et al*., 2005; Gilchrist *et al*., 2016). *In vitro* studies with bovine cumulus-oocyte complex revealed that the communication is maintained until the resumption of meiosis and onset of detachment within 9 h of maturation, and gradually decreases up to 22 h, when it eventually comes to a stop (Macaulay *et al*., 2014). A recent study pointed out that inclusion of the pre-IVM phase with a combination of cAMP modulators, resulted in maintenance of the density of TZPs after 20 h of *in vitro* maturation, resulting in improvement in the cumulus-oocyte communication leading to enhanced oocyte developmental competence (Soto-Heras *et al*., 2019). In aging females, the ability of somatic cells to respond to oocyte signals is reduced, resulting in lower formation of TZPs. Consequently, the reduced oocyte-somatic cell communication is the presumed cause for the reduced fertility in aged females (El-Hayek *et al*., 2018).

Interestingly, most of the time the TZPs are not in contact with the oocyte, they subdivide and form gap junctions between each other (Baena and Terasaki, 2019), a sign that these projections also have other essential functions such as communication between somatic cells. The TZPs are involved in essential processes for oocyte and consequently, embryo development. Studies investigating the transport mechanisms present in TZPs and how the *in vitro* environment influences these projections are still ongoing. This knowledge will probably be helpful to prevent lipid accumulation, aging consequences, and to improve *in vitro* oocyte maturation, with broad implications for animal and human assisted reproduction technologies.

The second barrier

Antrum formation and the cumulus-granulosa barrier

The antral follicles are characterized by the formation of a cavity filled with the follicular fluid. The follicular fluid originates from two sources, the bloodstream of thecal capillaries present in the ovary cortical region, and the components secreted by follicular cell layers, especially the granulosa cells and the fluid production, which intensifies with the enlargement of the follicles (Rodgers and Irving-Rodgers, 2010; Hennet and Combelles, 2012). The main hypothesis on follicular fluid formation suggests that an osmotic gradient is generated by granulosa cells production of hyaluronan and the chondroitin sulfate proteoglycan versican. This gradient generates influx of fluid derived from the thecal vasculature (Rodgers and Irving-Rodgers, 2010).

The follicular fluid contains a complex mixture of ions, proteins, metabolites, hormones, lipids, energy substrates, and reactive oxygen species (Leroy *et al*., 2004; Meeker *et al*., 2009; Ambekar *et al*., 2013). It serves as a source of regulatory molecules, such as gonadotrophins, steroids, growth factors, enzymes, proteoglycans, and lipoproteins (Revelli *et al*., 2009). This diverse array of molecules suggest that the follicular fluid is more than a reservoir and also supports intense metabolic activity, with substantial impact on follicular cells (Freitas *et al*., 2017) and oocyte. Roles of the follicular fluid were already reported, such as the participation in oocyte's acquisition of developmental competence (Fayezi *et al*., 2014; O'Gorman *et al*., 2013; Wallace *et al*., 2012) and in oocyte meiosis (Byskov *et al*., 1995; Mendoza *et al*., 2002). For example, hormone level in the follicular fluid, such as FSH (Suchanek *et al*., 1988), hCG (Ellsworth *et al*., 1984; Enien *et al*., 1998) and LH (Cha *et al*., 1986) have been reported to promote oocyte maturation and to increase chances of fertilization. The gonadotropins induce granulosa cells to secret hyaluronic acid (Mendoza *et al*., 2002) affecting oocyte development; they also act synergistically with estradiol (E2) enhancing cytoplasmatic maturation and controlling oocyte meiosis via cAMP secretion (Mendoza *et al*., 2002; Revelli *et al*., 2009). Another example is the fatty acids found in the follicular fluid that are incorporated by the oocyte and that have influence on oocyte maturation and quality. Excess of fatty acids were reported to negatively impact fertility outcomes (Shaaker *et al*., 2012).

The signaling mechanism for the formation of the antrum is not well understood; however, it was shown that FSH and type 1 insulin-like epidermal growth factors promote the formation of the antrum in cultured follicles *in vitro* (Gutierrez *et al*., 2000; Hillier, 2009). The growth of the antral follicles in bovines occurs in two distinct phases. The first is the slow phase where the follicles take approximately 30 days to advance from 0.3 mm in diameter to the stage of small antral follicles, which are about 3 mm in diameter. In this period of follicular growth, the oocyte reaches its final growth, approximately 110 µm in diameter, which relates to the acquisition of competence for development (Fair *et al*., 1997; Rodriguez and Farin, 2004). The second phase is the active phase when small follicles, approximately 3 mm in diameter, take from five to seven days to become dominant follicles, more than 8 mm in diameter. This phase is followed by a variable period of dominance, culminating in the development of the preovulatory follicle and ovulation (Bleach *et al*., 2001; Mihm and Bleach, 2003).

Given that antrum formation separates follicular cells and gametes, the need to maintain the communication between these cells is accomplished mainly through paracrine signaling. An example of paracrine signaling is the oocyte-secreted factors (GDF9 and BMP15) that interact with molecules such as FSH, IGF1 and androgens to promote mural granulosa proliferation and cumulus cells differentiation (Gilchrist *et al*., 2004).

Other examples of critical paracrine factors are found in preovulatory follicle. The signaling cascade triggered by the pre-ovulatory LH peak propagate through the ovulatory follicle via paracrine factors and stimulates the release of epidermal growth factor (EGF) ligands from the mural granulosa cells, that move across the follicular fluid to reach the cumulus cells. In these target cells induce changes in gene expression that will decrease the cGMP concentration in the cumulus cells

and consequently in the oocyte, resulting in cumulus cells expansion and meiosis resumption (Conti *et al*., 2012). The OSF also play vital roles regulating extracellular matrix stability, leading to ovulation (Gilchrist *et al*., 2004). Thus, this cascade is essential for the induction of gene expression required for follicle rupture, oocyte maturation and ovulation.

A novel communication system in the antrum

While paracrine signaling communication in follicular fluid have been described for many years, a novel communication mechanism, mediated by extracellular vesicles, has recently been described. Extracellular vesicles are phospholipid bilayer vesicles that transport biomolecules such as proteins, microRNAs, mRNAs, DNA, and lipids (Taylor and Gercel-Taylor, 2013; Di Pietro, 2016; Ávila *et al*., 2019). Their content varies and reflects the cell of origin (Akers *et al*., 2013). It was shown that the secretory cells actively select the number and the cargo of EVs depending on specific physiological and environmental conditions, such as diseases, nutritional status and stress (van Niel *et al*., 2018).

EVs are classified into microvesicles (MVs) and exosomes (Exos) according to their characteristics such as size, shape, membrane proteins, structural lipids, and their origin. The MVs are big with a diameter ranging from 500-1000 nm, have an irregular shape, and originate from the rupture of the cellular plasma membrane, which makes MVs a more heterogeneous population. On the other hand, the Exos have an approximate diameter of 50-100 nm (Crescitelli *et al*., 2013) and appear in electron microscopy as a cup-shaped form, depending on the preparation method. Importantly, EVs originate from the late endosomes, also called multivesicular bodies and are released into the extracellular space by fusion of the multivesicular body membrane with the plasma cell membrane (Taylor and Gercel-Taylor, 2013).

There are three modes of interaction between the EV and their target cells; i) the first is through direct interaction between membrane proteins of the EV with receptors on the target cell membrane, ii) The second by membrane cleavage of the EV proteins by proteases present in the extracellular space, and the release of products which act on the receptors of the target cell, and iii) the third by direct fusion of the EV membranes with the cell membrane, releasing the EV content in the cell and incorporating proteins and receptors into the cell membrane (Mathivanan *et al*., 2010).

EVs are present in several body fluids and were first described in the follicular fluid a few years ago (da Silveira *et al*., 2012). Follicular cells secrete these vesicles into the follicular environment (Andrade *et al*., 2017a) and the EVs are taken up by the granulosa and cumulus cells, in *in vivo* and *in vitro* systems (da Silveira *et al*., 2012; Di Pietro, 2016). Additionally, the oocyte surrounding cumulus cells could provide an entry point to deliver to oocyte the molecules that cannot pass through gap junctions, such as RNAs, miRNAs, proteins and lipids (Macaulay *et al*., 2014; Macaulay *et al*., 2016). Interestingly, *in vitro* studies showed that follicular fluid EVs alter transcript levels in oocytes (Dalanezi *et al*., 2017) and enhance oocyte competence to develop until the blastocyst stage (da Silveira *et al*., 2017).

The follicular fluid undergoes dynamic changes over late stages of folliculogenesis and its EVs content modify as consequence. As an example, the follicular fluid EVs from different size follicles have distinct concentrations and miRNA content (Navakanitworakul *et al*., 2016), and it has been described that they can differentially stimulate granulosa cells proliferation *in vitro* (Hung *et al*., 2017). Additionally, female age (Diez-Fraile *et al*., 2014; da Silveira *et al*., 2015a), and endocrine environment (da Silveira *et al*., 2015a) are important factors that can alter EVs content. Regarding female age, the miRNAs content of EVs from follicular fluid varies according to age. In old mares compare to young, a group of highly expressed miRNAs negatively modulates TGF-β, resulting in compromised maturation of oocytes (Da Silveira *et al*., 2015b). Also, the miR-23a, highly expressed in old mares (Da Silveira *et al*., 2015b), is correlated with human granulosa cells apoptosis pathway by inhibition of X-linked inhibitor of apoptosis protein (XIAP) and an increase in caspase 3 protein levels (Yang *et al*., 2012; Mobarak *et al*., 2019).

The bidirectional communication through EVs is associated with follicular development, oocyte growth, and quality, in humans and domestic animals (da Silveira *et al*., 2012; Sang *et al*., 2013; Sohel *et al*., 2013; Hung *et al*., 2015). During the maturation process, the EVs induce cumulus cell expansion and alter expression of genes related to the expansion process, when used as a supplement on cumulus-oocyte complexes (COCs) maturation medium (Hung *et al*., 2015). In another study, EVs stimulated granulosa cell proliferation by modulating Src, Pi3K/Akt and mitogen-activated protein kinase (MAPK) pathways, and interestingly, the EVs from small follicles were preferentially taken up by granulosa cells (Hung *et al*., 2017). Further, using EVs as supplements for embryo maturation in culture media, partially altered genes related to metabolism and development as well as miRNA and global DNA methylation and hydroxymethylation of bovine embryos produced *in vitro* (da Silveira *et al*., 2017). Another study observed a positive effect of follicular fluid EVs during *in vitro* maturation; they protected COCs from the harmful effects of heat shock stress (Rodrigues *et al*., 2019).

Although EVs carry different molecules, many studies show that the effect of EVs on cells are related to miRNAs and their regulatory effects, mainly because they are very stable and show resistance to degradation. The miRNAs in the follicular fluid EVs were associated with fertilization and embryo quality (Machtinger *et al*., 2017). Some studies, in humans and animals, have shown the role of miRNAs present in the follicular fluid, regulating follicular growth and development, cellular signaling, oocyte meiosis, and ovarian function (Martinez *et al*., 2018). EVs miRNAs modulate such important reproduction processes by the regulation of

pathways as insulin, epidermal growth factor receptor (ErbB), MAPK, Wnt signaling, TGF-β and PI3K-Akt signaling among others (da Silveira *et al*., 2012; Santonocito *et al*., 2014; Andrade *et al*., 2017b). Due to the importance of cellular communication in this environment and the immense potential of EVs, efforts are dedicated to better understand its functions and importance during oocyte maturation and early embryo development *in vitro*.

Conclusions and perspectives

Normal oocyte development depends on a finely regulated, constant, and reciprocal cell-to-cell communication between the follicle components. The crosstalk may occur by paracrine signaling and exchange of small molecules via gap-junctions; these are well-studied mechanisms. However, novel mechanisms of communication in the follicle microenvironment have been recently identified. These mechanisms allow the exchange of large molecules, such as nucleic acids, proteins, and lipids between follicular compartments, and are mediated by the trafficking of vesicles from the bulk-end of TZPs to the oolemma, or by the transit of EVs in the follicular fluid.

The importance of these novel communication mechanisms is exemplified by circumstances for which association with communication mediated by TZP or EVs has already been demonstrated, such as the accumulation of maternal transcripts in the oocyte, the acquisition of oocyte competence, and the decline in oocyte developmental potential associated with aging. Moreover, there is increasing evidence that assisted reproductive technologies disturbs the intrafollicular interactions, but their short and long-term effects are yet to be studied.

Studies in this field are limited, and in addition to the lack of knowledge about the effects of disrupting such communication mechanisms, there are plenty of unanswered questions in the subject area. Among other questions, it is unknown how the passage of molecules into the oocytes is regulated, and whether EVs in the follicular fluid can directly or indirectly modulate the oocyte. A thorough understanding of the biology of TZPs and EVs-mediated communication will potentiate the advancement of assisted reproductive technologies. These possibilities include modulation of the function of TZPs, regulation of EVs-cargo, and its use in *in vitro* culture conditions.

Author contributions

GMA: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing; MC: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing; FVM: Funding acquisition, Resources, Writing – review & editing; JCS: Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing; FP: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review& editing.

Conflict of interest

The authors declare that they have no competing interests.

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Reproductive physiology of the heat-stressed dairy cow: implications for fertility and assisted reproduction

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Abstract

Heat stress causes a large decline in pregnancy success per insemination during warm times of the year. Improvements in fertility are possible by exploiting knowledge about how heat stress affects the reproductive process. The oocyte can be damaged by heat stress at the earliest stages of folliculogenesis and remains sensitive to heat stress in the peri-ovulatory period. Changes in oocyte quality due to heat stress are the result of altered patterns of folliculogenesis and, possibly, direct effects of elevated body temperature on the oocyte. While adverse effects of elevated temperature on the oocyte have been observed *in vitro*, local cooling of the ovary and protective effects of follicular fluid may limit these actions *in vivo*. Heat stress can also compromise fertilization rate. The first seven days of embryonic development are very susceptible to disruption by heat stress. During these seven days, the embryo undergoes a rapid change in sensitivity to heat stress from being very sensitive (2- to 4-cell stage) to largely resistant (by the morulae stage). Direct actions of elevated temperature on the embryo are likely to be an important mechanism for reduction in embryonic survival caused by heat stress. An effective way to avoid effects of heat stress on the oocyte, fertilization, and early embryo is to bypass the effects through embryo transfer because embryos are typically transferred into females after acquisition of thermal resistance. There may be some opportunity to mitigate effects of heat stress by feeding antioxidants or regulating the endocrine environment of the cow but neither approach has been reduced to practice. The best long-term solution to the problem of heat stress may be to increase genetic resistance of cows to heat stress. Thermotolerance genes exist within dairy breeds and additional genes can be introgressed from other breeds by traditional means or gene editing.

Keywords: heat stress, lactating cow, reproduction, fertility, embryo, oocyte.

Introduction

The overall reproductive function of a herd of dairy cows is often estimated by calculating pregnancy rate, i.e., the product of estrus detection rate (how many cows in estrus are detected in estrus by farm personnel) and conception rate (a misnomer but a measure of how many cows that are inseminated are diagnosed as pregnant). A pregnancy rate of 100% would mean that every cow eligible to be pregnant in a 21-day period becomes pregnant in that time. By this measure, the reproductive function of the heat-stressed dairy cow can be very low indeed. Data in Figure 1 illustrate how heat stress can affect characteristics of estrous activity; only 19% of estrus periods were detected by farm workers in the summer in one study in Florida (Thatcher *et al*., 1986). Fertility after artificial insemination (AI) can also be low during heat stress. In a survey of dairy herds in Israel, less than 20% of inseminations resulted in pregnancies in the summer and pregnancy per AI (P/AI) in the worst herds (those with milk low production and a moderate amount of cooling) was only 3% (Fig. 2; Flamenbaum and Galon, 2010). In another study, P/AI at day 32 after insemination for lactating cows in Minais Gerais, Brazil was 17% when cows experienced two of more occurrences of a morning rectal temperature greater than 39.1°C at days -3, -2, 0 and 7 relative to timed AI *vs* 25% for cows with one occurrence and 37% for cows with no occurrence (Pereira *et al*., 2013).

Fortunately, the situation is not always so bleak. For example, P/AI at day 36 after insemination for multiparous cows in Oklahoma and Kansas during hot weather was 25-27% (Voelz *et al*., 2016). In a study in Florida, P/AI for multiparous cows in the summer on a free-stall dairy in Florida with fans and sprinkler was 32% (Zolini *et al*., 2019). Higher pregnancy rates during heat stress can be ascribed to superior cooling systems and implementation of timed AI programs. Not only do protocols for timed AI eliminate the need for estrus detection but, for some protocols, they can increase cow fertility (Carvalho *et al*., 2018).

Further improvements in fertility during the summer are possible by exploiting knowledge about how heat stress affects the reproductive process. Here, the impact of heat stress on follicular development, oocyte quality, fertilization, and embryonic development will be briefly outlined and the consequences of those changes for strategies to improve fertility will be discussed. Since AI using frozen semen can bypass effects on the bull, and timed AI bypasses effects on estrus behavior, the focus will be on biological processes important for establishing a high level of fertility. Keep in mind that there are effects of heat stress after establishment of pregnancy, most notably in late gestation when heat stress can affect milk yield of the cow after calving and the epigenetic program, growth and milk yield of the resulting calf (Dahl *et al*., 2017; Skiebiel *et al*., 2018). However, this important aspect of actions of heat stress on the pregnant cow is beyond the scope of the current review.

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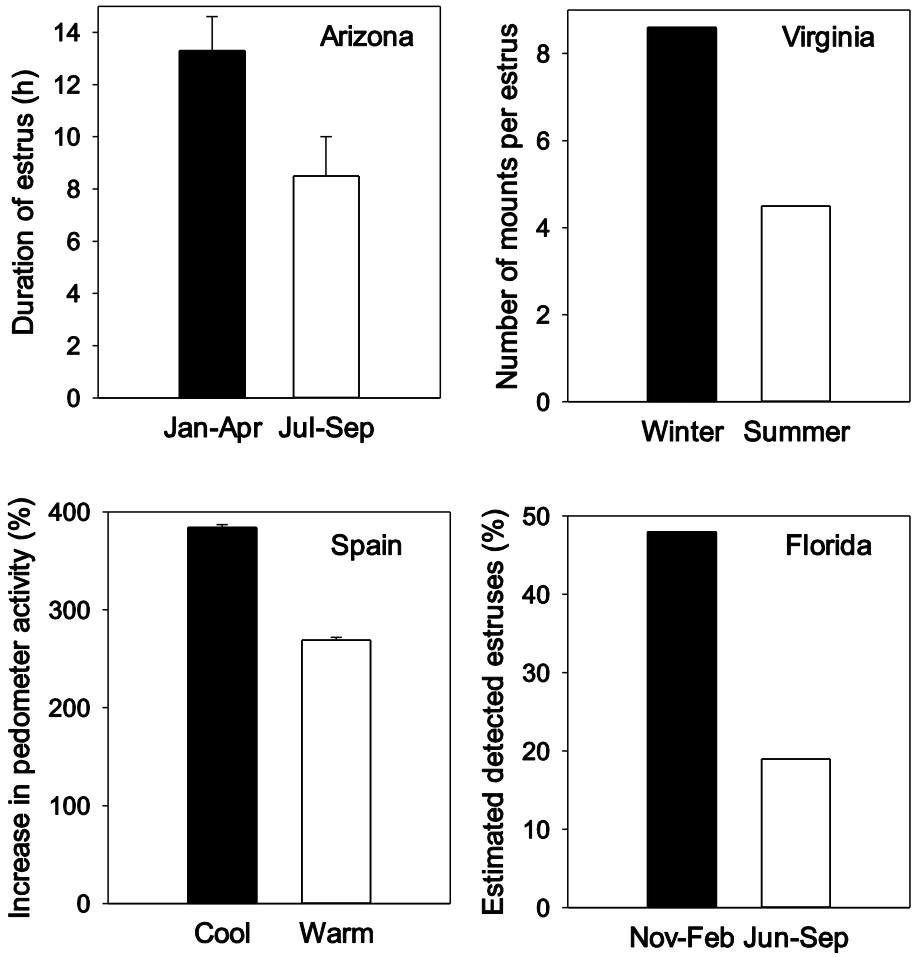

Figure 1. Seasonal variation in characteristics of estrus in lactating cows. Shown are data on duration of estrus in Arizona (Wolff and Monty, 1974), number of mounts per estrus in Virginia (Nebel *et al*., 1997), the increase in pedometer activity at estrus in Spain (López-Gatius *et al*., 2005a) and estimated percent of estrus periods detected by farm personnel in Florida (Thatcher *et al*., 1986). The figure is reproduced from Hansen (2017) with permission of the American Dairy Science Association.

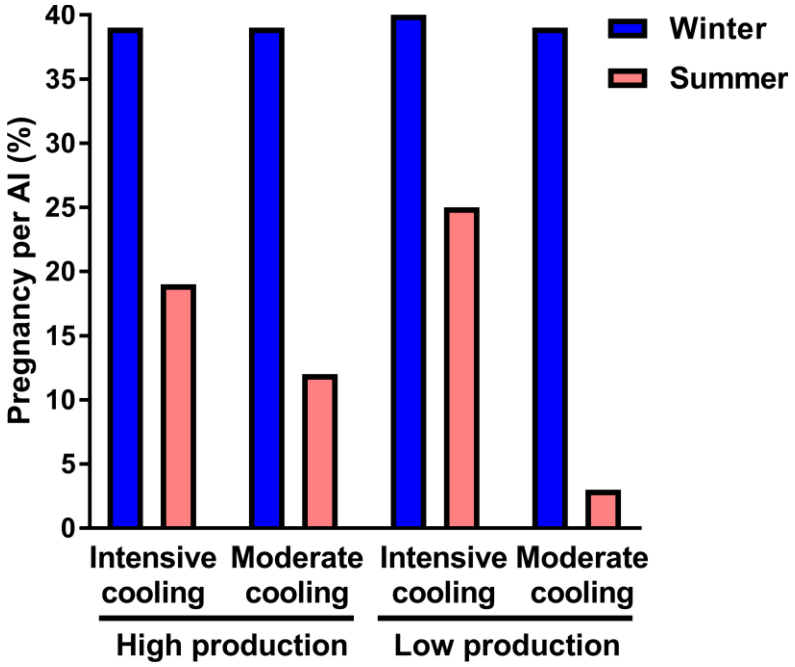

Figure 2. Pregnancies per artificial insemination of herds surveyed in Israel (Flamenbaum and Galon, 2010). Herds were classified based on the overall level of milk production (high *vs* low) and on the degree of cooling that cows receive (intensive *vs* moderate).

Reproductive Events Susceptible to Heat Stress

There have been attempts to reduce the impact of heat stress by cooling lactating cows for a limited period of time around ovulation, fertilization and early development but the improvement in fertility has been slight (Stott and Wiersma, 1976; Her *et al*., 1988; Ealy *et al*., 1994). This is because there is a broad window of time in which the reproductive process of the cow can be disrupted by heat stress. An experiment with Gir cattle indicates that heat stress can compromise development of the oocyte at the earliest stages of folliculogenesis. In particular, competence of oocytes to develop to the blastocyst stage after *in vitro* fertilization was reduced by heat stress occurring as early as 105 to 133 days before oocyte retrieval (Torres-Júnior *et al*., 2008). In an experiment with lactating Holsteins, secretion of androstenedione from cultured thecal cells and estradiol from cultured granulosa cells from medi,um-sized follicles was reduced by exposure of donor cows to heat stress 20 to 26 days earlier (Roth *et al*., 2001a). Additional evidence that heat stress affects the oocyte early in folliculogenesis are observations that oocyte competence for supporting embryonic development after *in vitro* activation was restored only gradually after the end of summer (see review by Hansen, 2013a; Roth, 2017). Moreover, treatments to increase follicular turnover, including multiple follicular aspirations (Roth *et al*., 2001b), follicle stimulating hormone (Roth *et al*., 2002) or somatotropin (Roth *et al*., 2002), hastened restoration of oocyte competence.

The oocyte remains sensitive to heat stress in the peri-ovulatory period. Treatment of superovulated cows with heat stress for 10 hours beginning at the onset of estrus reduced the percent of embryos recovered at day 7 after estrus that were classified as having normal morphology and increased the percent of embryos that were retarded with development (Putney *et al*., 1989a).

Heat stress can compromise fertilization rate (Sartori *et al*., 2002; Hackbart *et al*., 2010). For example, fertilization rate in lactating cows bred by AI was lowered from 88% in winter to 55% in summer (Sartori *et al*., 2002). Low fertilization rates could reflect damage to the oocyte, sperm deposited in the uterus, or disruption of the fertilization process itself. Effects on the sperm or fertilization process may be a more important cause than effects on the oocyte. Competence of the oocyte to be fertilized *in vitro* was not reduced in the summer although there was a reduction in development of cleaved embryos to the blastocyst stage (Ferreira *et al*., 2011). Further, indirect evidence for an effect of heat stress on the sperm is the observation of Girolando cows that insemination in the morning was associated with a slight but significant increase in P/AI (Rocha de Zouza *et al*., 2016).

The first 7 days of embryonic development are very susceptible to disruption by heat stress. Experimental treatment of superovulated cows with heat stress during this time reduced the development of embryos at day 7 (Putney *et al*, 1988a). Moreover, among single-ovulating lactating cows, there was a large reduction in the percent of recovered embryos classified as viable during periods of heat stress as compared to periods of no heat stress (Sartori *et al*., 2002). During these 7 days, the embryo undergoes a rapid change in sensitivity to heat stress. Exposure of superovulated cows to heat stress on day 1 after estrus reduced the percent of embryos that were blastocysts at day 8 (Ealy *et al*., 1993). However, heat stress at day 3, 5 or 7 had no effect on embryonic development. Thus, the resistance of the embryo to heat stress increases greatly in just a few days of development.

There is little known about sensitivity of the bovine embryo to heat stress after day 7. There is one report in beef cattle that heat stress from day 8 to 16 can reduce conceptus weight at day 16 (Biggers *et al*., 1987). However, the effectiveness of embryo transfer as a tool for improving fertility during heat stress (see discussion further in this paper) is indicative that embryo survival is not dependent to any large extent on the occurrence of maternal heat stress after day 7.

A proportion of cows initially diagnosed as pregnant around day 28-60 of pregnancy subsequently lose the pregnancy. There is some evidence that the frequency of this loss can be increased by heat stress (García-Ispierto *et al*., 2006; Santolaria *et al*., 2010; El-Tarabany and El-Tarabany, 2015). For example, pregnancy loss between days 34 to 45 of gestation and day 90 of gestation were 2% for cows in the cool season *vs* 12% for cows in the warm season (García-Ispierto *et al*., 2006). Attempts have been made to understand the crucial period in the reproductive process during which heat stress acts to increase late embryonic and fetal mortality by relating indices of heat stress at specific phases in the reproductive process to pregnancy loss. Such an approach is difficult to decipher because environmental conditions at one period are often highly correlated with environmental conditions at another period.

Physiological Causes of Effects of Heat Stress on the Oocyte and Embryo

Effects of heat stress are related to the inability of the affected cow to maintain its body temperature within the regulated range. As mentioned, P/AI is related to rectal temperature (Pereira *et al*., 2013). It has been estimated that fertility begins to decline when uterine temperature rises about 0.5°C above normal (Gwazdauskas *et al*., 1973). One reason why lactating cows are more susceptible to the negative effects of heat stress on fertility than heifers (Badinga *et al*., 1985) is because the metabolic heat production associated with lactation makes it more difficult for cows to regulate body temperature during heat stress than non-lactating heifers (Sartori *et al*., 2002). Effects of heat stress on the ovary, oviduct, uterus, and embryo could result from either physiological changes caused by heat stress or by the direct effects of elevated temperature on cells involved in reproduction.

Alterations in oocyte quality due to heat stress probably involve deviations in patterns of folliculogenesis. Follicular dominance is reduced in cows exposed to heat stress so that there is an increase in number of large follicles on the ovary, prolonged

period of dominance of the ovulatory follicle, increased circulating concentrations of follicle stimulating hormone (FSH) and reduced concentrations of estradiol-17β and inhibin (Wolfenson *et al*., 1995; Roth *et al*., 2000; Trout *et al*., 1998; Wilson *et al*., 1998). Heat stress can also dampen the preovulatory surge of luteinizing hormone and estradiol-17β (Gwazdauskas *et al*., 1981; Gilad *et al*., 1993; Armengol-Gelonch *et al*., 2017). Indeed, heat stress can increase the proportion of cows that fail to ovulate after administration of GnRH. Ovulation failure was 12% during the warm period *vs* 3% during the cool period (López-Gatius *et al*., 2005b). Use of more active analogs of gonadotropin releasing hormone (GnRH) can reduce the incidence of ovulation failure (García-Ispierto *et al*., 2019).

There are direct effects of elevated temperature (i.e., heat shock) on the competence of the oocyte undergoing maturation to develop into a blastocyst following fertilization or artificial activation (see Roth, 2017 for review). Possible local cooling of the ovary and protective effects of follicular fluid may limit these actions *in vivo*. Work by López-Gatius and Hunter (2017, 2019a,b) has revealed that the ovary experiences a cooler temperature than that measured in the rectum or on the surface of the uterus. Additionally, culture of maturing oocytes in a medium containing follicular fluid or follicular fluid exosomes reduced the negative effect of elevated temperature on oocyte competence for cleavage and blastocyst development after fertilization (Rodrigues *et al*., 2019). Direct effects of elevated temperature on the follicle may be important in some circumstances, however. Cows in which follicular temperature was lower than rectal temperature were more likely to ovulate and achieve pregnancy than cows in which the gradient between follicular and rectal temperature was low (López-Gatius and Hunter, 2019ab).

Direct actions of elevated temperature on the embryo are likely to be an important mechanism for reduction in embryonic survival caused by heat stress after ovulation. Indeed, the changes in embryonic resistance to maternal heat stress observed *in vivo* (Ealy *et al*., 1993) are also seen with effects of heat shock on cultured embryos. Exposure of the zygote and 2-cell embryo causes a large reduction in percent of embryos developing to the blastocyst stage (Edwards and Hansen, 1997; Sakatani *et al*., 2012; Ortega *et al*., 2016). Embryos at the 4- and 8-cell stage are also susceptible to heat shock but the magnitude of the deleterious effect is reduced as compared to that for the 2-cell embryo (Edwards and Hansen, 1997). Physiologically-relevant heat shock has little effect on development of morula-stage embryos (Edwards and Hansen, 1997; Eberhardt *et al*., 2009; Sakatani *et al*., 2012). Mechanisms responsible for acquisition of thermotolerance are not known but probably involve activation of the embryonic genome at the 8-cell stage (Graf *et al*., 2014) so that the full range of cellular adaptations to heat shock can be employed.

It is also possible that changes in circulating concentrations of steroid hormones induced by heat stress could alter the oviductal or uterine environment and thereby affect embryonic development. As stated previously, heat stress can reduce plasma concentrations of estradiol-17β (Gwazdauskas *et al*., 1981; Wolfenson *et al*., 1995; Wilson *et al*., 1998). Short-term exposure to heat stress either had no effect on plasma concentrations of progesterone (Roth *et al*., 2000) or caused an increase (Trout *et al*., 1998; Wilson *et al*., 1998). Long-term exposure to heat stress may lead to reduced progesterone concentrations, however, because luteal concentrations of the hormone during the luteal phase have been reported to be lower in summer than winter (Howell *et al*., 1994). Additionally, cooling cows during the summer increased circulating concentrations of progesterone (Wolfenson *et al*., 1988). Some effects of heat stress on peripheral blood concentrations of hormones could be the result of changes in water balance during heat stress and reduced hematocrit (Richards, 1985; Lamp *et al*., 2015).

Embryo Transfer: The Most Effective Mechanism for Maximizing Fertility During Heat Stress

One way to avoid consequences of heat stress on the oocyte, fertilization, and early embryo is to bypass its effects through implementation of an embryo transfer program. Embryos are typically transferred into females at day 7 after estrus. By that time, embryos have gained resistance to effects of heat stress. Embryo transfer can be coupled with ovulation synchronization programs to allow timed embryo transfer and avoid the need for estrus detection.

One way to demonstrate the effectiveness of embryo transfer for improving fertility during heat stress is to compare pregnancy outcomes for embryo transfer as compared to AI. As summarized in Figure 3, pregnancy rates during heat stress have been consistently higher for cows receiving an embryo than for cows submitted to AI. The only exception is when embryos were produced *in vitro* and cryopreserved before transfer. Thus, there was either no improvement in fertility as compared to AI when vitrified embryos produced *in vitro* were transferred (Drost *et al*., 1999; Fig. 3B) or the improvement was less than if fresh embryos were transferred (Stewart *et al*., 2011; Fig. 3C). These results are the consequence of poor cryopreservation of *in vitro* produced embryos (Hansen and Block, 2004).

Another way to demonstrate how embryo transfer reduces the impact of heat stress is to examine seasonal variation in pregnancy success after embryo transfer. Of eight studies in which seasonal variation in pregnancy rates were evaluated, there were only two cases where there was a large difference in pregnancy rate between hot and cool conditions including an experiment in Florida with fresh embryos produced *in vitro* (Block *et al*., 2007; Fig. 4B) and an experiment in South Dakota with vitrified embryos produced *in vitro* (Chebel *et al*., 2008; Fig. 4C). There was no difference between seasons for embryos produced by superovulation in the southwest United States (Putney *et al*., 1988b; Fig. 4A) or for fresh embryos produced *in vitro* in Florida (Loureiro *et al*., 2009; Fig. 4B) or South Dakota (Chebel *et al*., 2008; Fig. 4C). In the largest trials, there was a slight reduction in pregnancy per

embryo transfer in the hottest months (Ferraz *et al*., 2016; Vasconcelos *et al*., 2011; Baruselli *et al*., 2011) but the difference in pregnancy outcomes between the coolest and warmest times were only 3 to 4% (Fig. 4C, 4E and 4F). Seasonal variation of that magnitude is much less than what would be the case for AI.

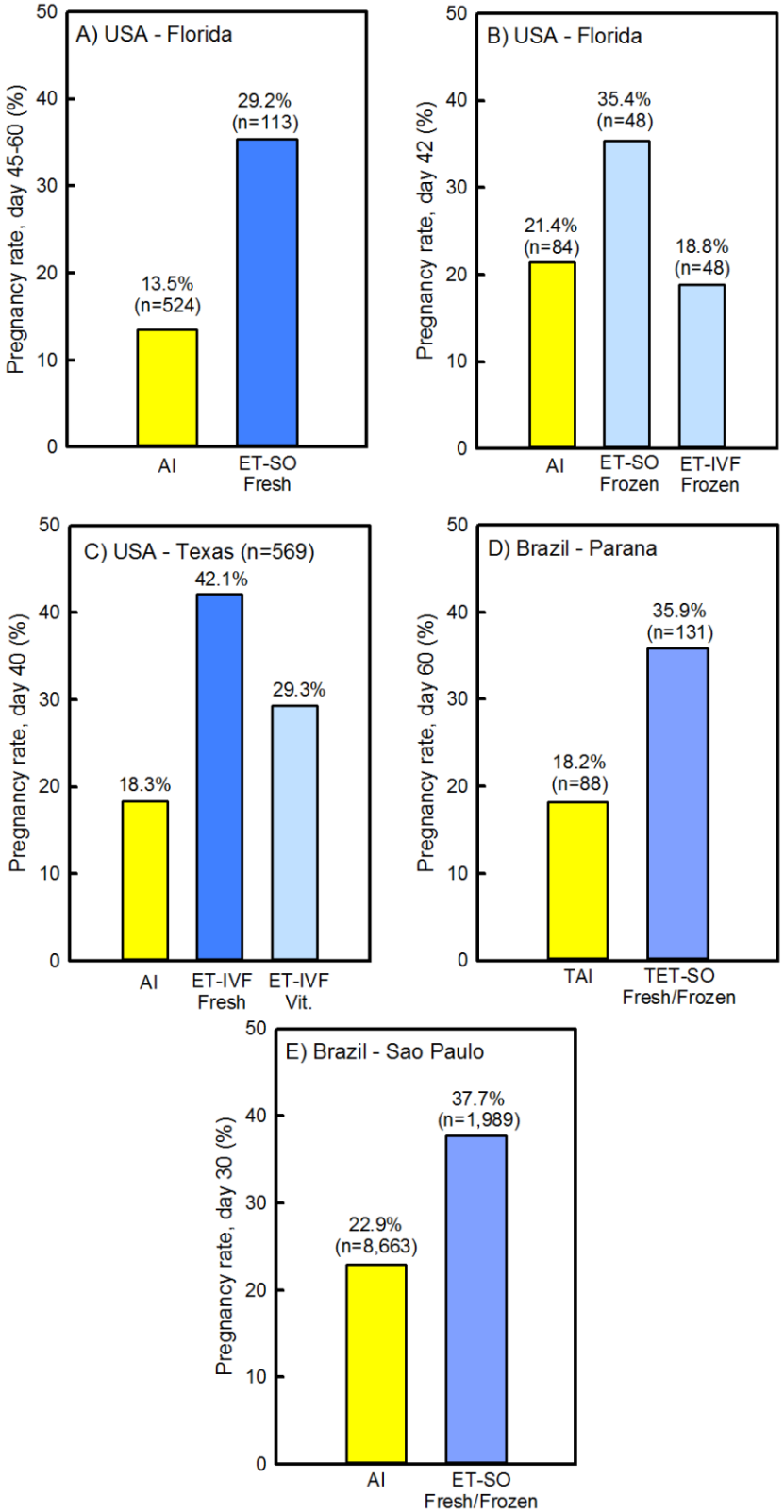

Figure 3. Comparisons of pregnancy success for artificial insemination *vs* embryo transfer in the summer. Data are from Putney *et al*. (1989b) (A), Drost *et al*. (1999) (B), Stewart *et al*. (2011) (C), Vasconcelos *et al*. (2011) (D) and Baruselli *et al*. (2011) (E). Abbreviations are as follows: AI, artificial insemination, ET, embryo transfer; IVF, *in vitro* fertilized; SO, superovulation; TAI, timed AI; TET, timed embryo transfer; Vit., vitrified. The figure is modified from a technical bulletin by Vetoquinol and is reproduced with permission.

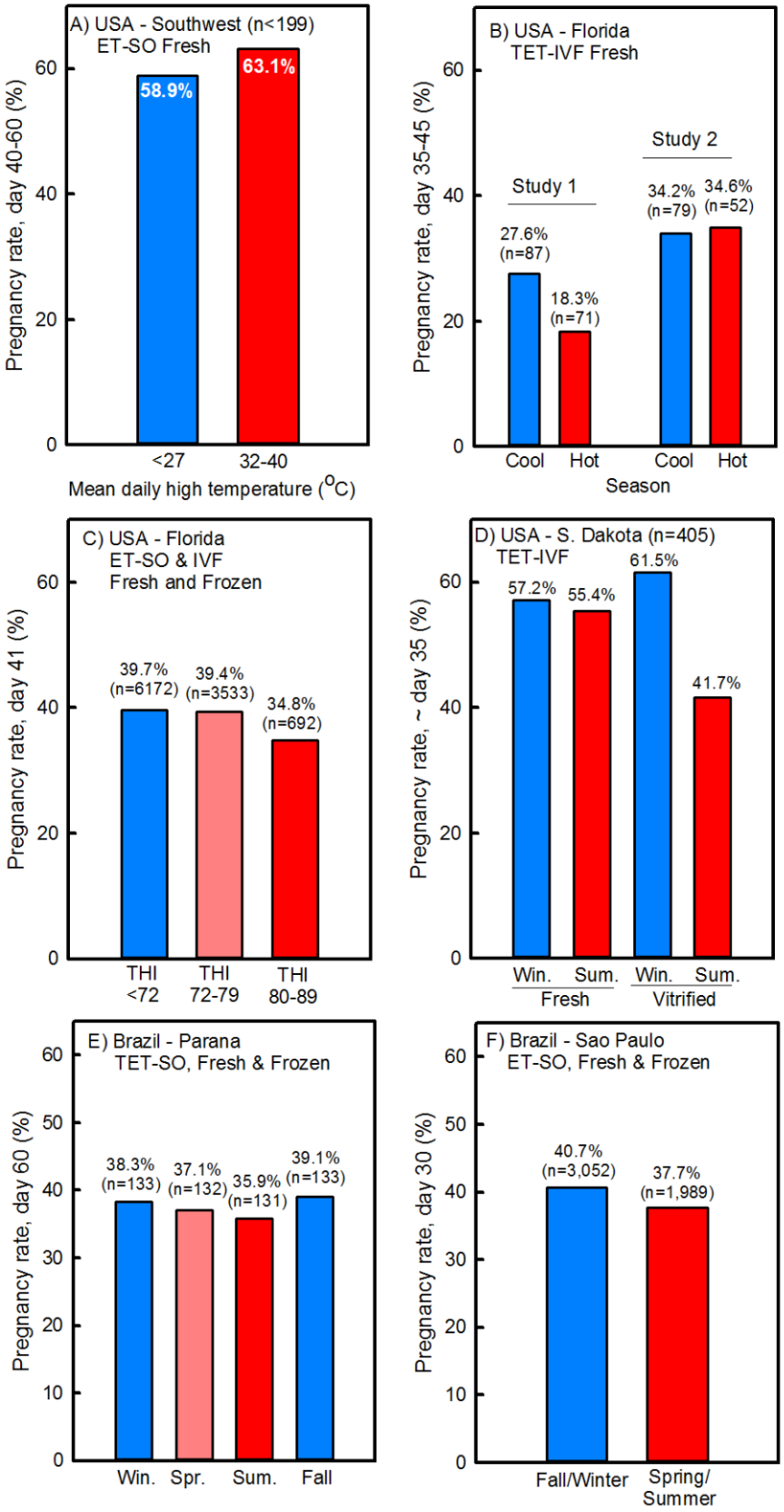

Figure 4. Comparisons of percent cows pregnant following embryo transfer in cool or hot weather. Data are from Putney *et al*. (1988b) (A), Block *et al*. (2007) and Loureiro *et al*. (2009) (B), Ferraz *et al*. (2016) (C), Chebel *et al*. (2008) (D), Vasconcelos *et al*. (2011) (E) and Baruselli *et al*. (2011) (F). Abbreviations are as follows: ET, embryo transfer; IVF, *in vitro* fertilized; S. Dakota, South Dakota; Spr., spring; SO, superovulation; Sum., summer; TET, timed embryo transfer; THI, temperature-humidity index; Win., winter. The figure is modified from a technical bulletin by Vetoquinol and is reproduced with permission.

Antioxidants

Exposure to elevated temperature can increase production of reaction oxygen species in oocytes (Nabenishi *et al*., 2012; Cavallari de Castro *et al*., 2019) and embryos (Sakatani *et al*., 2004; Sakatani *et al*., 2008; Ortega *et al*., 2016). *In vitro*, effects of heat shock on oocyte maturation can be reduced by provision of antioxidants (Lawrence *et al*., 2004; Nabenishi *et al*., 2012; Ispada *et al*., 2018; Cavallari de Castro *et al*., 2019). In the embryo, however, thermoprotective benefits of antioxidants *in vitro* have been inconsistent. There was no protective effect of dithiothreitol, glutathione, melatonin, taurine, or vitamin E (Ealy *et al*., 1995; Paula-Lopes *et al*., 2003a; de Castro *et al*., 2008; Ortega *et al*., 2016) but β-mercaptoethanol was protective (Sakatani *et al*., 2008). One interpretation of these data is that reactive oxygen species are a more important mediator of the embryotoxic actions of heat shock for the oocyte than the embryo.

Efforts to improve fertility of lactating cows exposed to heat stress by delivering antioxidants have generally not yielded positive effects (see review by Hansen, 2013b and Roth, 2017). There are two reports of beneficial effects of antioxidant administration, however. In the first, Aréchiga et al. (1998) found that a higher proportion of cows fed supplemental β-carotene from about day 15 after calving were pregnant at 90 d postpartum than control cows when the experiment was performed during the summer but not when the experiment was performed during the winter. Feeding supplemental β-carotene did not increase pregnancy per AI at first service so the effect on the proportion pregnant at 90 d reflects either alterations in fertility after first service or estrus detection. In the second report, administration of long-acting melatonin implants beginning at 220 d of gestation to cows during the summer shortened the interval to conception in the subsequent postpartum period and decreased the incidence of cows experiencing > 3 breedings per conception (García-Ispierto *et al*., 2013). The peak concentration of melatonin in the blood of cows receiving implants was low (i..e, 260-300 pM) and it might be that melatonin was acting as a hormone rather than as an antioxidant. Further studies are needed with both β-carotene and melatonin to evaluate efficacy of their administration for improving fertility during heat stress.

Hormonal Treatments

Much work continues on optimization of timed AI protocols in general and under the specific conditions of heat stress. Few studies have been performed to compare whether a specific improvement in a timed AI protocol works better for heat-stressed cows than cows not subjected to heat stress. One exception is for induction of ovulation. As already discussed, ovulation failure is more frequent during periods of heat stress and administration of more active analogs of GnRH can reduce the incidence of ovulation failure (García-Ispierto *et al*., 2019). In another study, Shabankareh *et al*. (2010) evaluated the summer-winter differences in P/AI at first service for cows bred at spontaneous estrus or following timed AI using either GnRH (Ovsynch) or estradiol cypionate (Heatsynch) to induce ovulation. There was no difference in P/AI (32, 30 and 30% for OvSynch, Heatsynch and spontaneous estrus) in the summer while P/AI in the winter was highest for spontaneous estrus (51%), intermediate in Ovsynch (40%) and lowest for Heatsynch (35%).

Several experiments have been conducted to evaluate effects of increasing circulating progesterone concentrations on fertility of heat-stressed cows. Results have been inconsistent and often dependent on the subset of cows treated. Administration of progesterone using a CIDR device from day 5 to 18 after insemination did not cause an overall increase in P/AI but there were positive effects of the treatment in cows with low body condition or postpartum uterine disorders (Friedman *et al*., 2012). In the study of Shabankareh *et al*. (2010), treatment with human chorionic gonadotropin (hCG) on day 5 after insemination increased P/AI in both summer (24 *vs* 38% for saline and hCG) and winter (35 *vs* 47%). Treatment with hCG at day 5 also increased pregnancy rate in cows during summer but the effect was seen only for primiparous cows (Zolini *et al*., 2019). Treatment with GnRH at AI or at both AI and day 12 of the estrous cycle increased P/AI in an experiment by López-Gatius *et al*. (2005c). In another research trial, there was no beneficial effect of treatment at day 0 on P/AI whereas treatment with GnRH at either day 5 or both day 0 and 5 increased P/AI but only for cows in third or greater lactation (Mendonça *et al*., 2017).

The idea that ovarian follicles can be compromised by heat stress at early stages of folliculogenesis has lead to the idea that fertility can be improved in the autumn by hastening the removal of damaged follicles from the ovary. Improved oocyte competence in the autumn, as measured by *in vitro* development to the blastocyst stage, has been achieved using several treatments to increase follicular turnover, including multiple follicular aspirations (Roth *et al*., 2001b) or treatment with FSH (Roth *et al*., 2002) or somatotropin (Roth *et al*., 2002). In addition, generation of three consecutive 9-day follicular waves by treatment with GnRH and prostaglandin $F_{2\alpha}$ has been reported to have some positive effects on fertility of lactating cows in the summer and autumn (Friedman *et al*., 2011). Treatment effects were seen for primiparous cows (37% *vs* 53% for control and treated cows) but not for multiparous cows (27 *vs* 29%).

Genetic Selection

Heritability estimates in Holsteins for body temperature during heat stress is 0.17 (Dikmen *et al*., 2012) and for the decline in milk yield during heat stress is 0.19 (Nguyen *et al*., 2016). Thus, it should be possible to reduce the impact of heat stress on reproduction by selecting genetically for thermoregulation. Data from Australia indicate that cows that are more thermotolerant genetically also have higher breeding values for fertility (Nguyen *et al*.,

2016). Unfortunately, they also have a lower genetic ability for milk yield so genetic strategies must be developed to allow selection for genes that confer superior thermotolerance without compromising milk yield.

One option is to introgress genes from thermotolerant breeds into dairy breeds using crossbreeding or gene editing. The prolactin receptor gene is one gene that has been mutated in a manner that leads to a slick hair phenotype characterized by a sleek, short hair coat and increased capacity for regulating body temperature (Dikmen *et al*., 2014). Arising in criollo breeds of cattle, several mutations in the gene exist that result in a truncated version of the protein to be synthesized (Porto-Neto *et al*., 2018). The gene has been introduced into Holsteins and is associated with reduced milk yield depression in the summer (Dikmen *et al*., 2014). Data from Puerto Rico indicate that slick-haired Holsteins are more fertile than Holsteins without the mutation (Ortiz-Colón *et al*., 2018).

There are also genetic effects on cellular resistance to elevated temperature. Embryos from *Bos indicus* breeds or the Romosinuano, a criollo breed, are more resistant to deleterious effects of heat shock on development of cultured embryos (Paula-Lopes *et al*., 2003b; Hernández-Cerón *et al*., 2004; Eberhardt *et al*., 2009; Silva *et al*., 2013). Fertility of cows in the summer was higher when inseminations were performed with Gyr semen than when Holstein semen was used (Pegorer *et al*., 2007). One gene that contains mutations that increases cellular resistance to heat shock is *HSPA1L*, as indicated by studies with lymphocytes (Basiricò *et al*., 2011) and embryos (Ortega *et al*., 2016).

Final Note

The decision as to which strategies to implement to reduce effects of heat stress on fertility is not a simple one. Embryo transfer, for example, while effective at minimizing the summer decline in fertility, is also expensive and may not be economically-effective unless the cost is constrained. In addition, getting cows pregnant in the summer can have long-term negative consequences for the resultant calf. Pinedo and De Vries (2017) have demonstrated that cows conceived in summer were older at first calving, had lower odds of surviving for a second calving, longer intervals from calving to first breeding and conception, and lower milk yield than cows conceived in winter. Thus, in some cases, non-uniform or seasonal calving may be the most profitable strategy. Genetic strategies that increase thermotolerance of the cow population are also desirable because, among other reasons, effects are permanent for that animal and extend to its offspring.

Author contributions

PJH: wrote and finalized the paper.

Conflicts of interest

The author declares no conflicts of interest.

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Inflammation: friend or foe of bovine reproduction?

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Abstract

Inflammation is not only the first line of defense of the organism but is also required in many reproductive processes such as ovulation, corpus luteum development, luteolysis, uterine clearance after insemination and post partum. Nevertheless, if excessive or persistent, inflammation can switch from a positive mechanism to a deleterious process, impairing oocyte quality and embryo development. Not only uterine but also non genital inflammatory sites can depreciate reproductive performances, with a carry over effect of 2 to 4 months. Since the metabolic challenges of the peripartum transition period make difficult for the cow to control inflammation, dairy cows are frequently in a pro-inflammatory stage, suggesting that inflammation, rather than infection, is a limiting factor of fertility in modern dairy cows. Within the first week after calving, cows have to mount an intense inflammatory response to the bacterial invasion of the uterine cavity with the challenge of being able to switch it off in no more than 5-6 weeks. The absence of neutrophils on endometrial smear is associated with the highest success rate at insemination. Since a fine tuning – rather than an absence - of inflammation is required along the reproductive cycle, anti-inflammatory drugs do not allow any improvement of pregnancy rate, except in the specific case of embryo transfer. Appropriate management of the transition period (especially nutritional) and in a long term perspective, genetic selection contribute to improve the aptitude of cows to controls the intensity of inflammatory process.

Keywords: inflammation; ovulation; post partum; cytokines; neutrophils.

Introduction

(Bacterial) infection has been long considered as an essential component of reproductive disorders, whereas (sterile) inflammation is nowadays identified as a major and frequent limiting factor of reproductive performances. In the medical approach, inflammation, hallmark of "–itis" diseases, is classically considered as a deleterious process, an unwanted response leading to immune dysfunction, diversion of nutrients from productive purposes, tissue damage, sepsis, organ failure and even death. Nevertheless, from a biological perspective, inflammation, involving chemokines and cytokines release, blood vessel dilation and immune cell infiltration, is the first line immune response of an organism facing a microbial infection or a tissue injury. Since the female genital tract is physiologically exposed to a range of tissue injuries (such as ovulation) and intrauterine bacterial challenges (after calving, at insemination/mating through sperm), inflammation also belongs to the physiology of reproduction. Moreover, some other reproductive processes, such as corpus luteum development and demise, or maternal recognition of pregnancy share some similarities with inflammatory events. The objective of this paper is to review the positive and negative relationships between inflammation and cow reproduction, to finally question the rationale of the use of anti-inflammatory drugs to improve reproductive performances. This review focuses on inflammation, trying to distinguish it from the effects of bacterial infections (including Lipopolysaccharide - LPS) and on the bovine female, despite inflammation is closely associated to many physiological and pathological aspects of reproduction in many other species, if not all (e.g. Freeman *et al*., 2013 in the bitch or Katila, 2012 in the mare).

The female genital tract is physiologically able to mount an inflammatory reaction

The female genital tract is naturally equipped to recognize pathogens and damages (Sheldon *et al*., 2018): some uterine, tubal and ovarian cells of the cow express receptors (Pattern recognition receptors, PRRs, sensors of 'danger') recognizing highly conserved microbial molecular signatures (MAMPs, Microbe-associated molecular patterns) or host-derived molecules indicative of cell injury (DNA fragments, mitochondrial content, but also free fatty acids and carbohydrates), referred to as DAMPs (for Damage-associated molecular patterns). Transmembrane toll-like receptors (TLRs) are probably the most classical PRRs and are expressed by bovine granulosa cells (Price and Sheldon, 2013), bovine oviductal epithelial cells, epithelial and stromal cells of the endometrium (Herath *et al*., 2009; Turner *et al*., 2014; Dadarwal *et al*., 2017; Danesh Mesgaran *et al*., 2018).

These different compartments are able to mount an early immune response: recognition of MAMPs or DAMPs by the genital cells initiate several signaling cascades (through NFκB or MAPkinase pathways for example), resulting in the expression of pro-inflammatory mediators (e.g. Tumor Necrosis Factor α - TNFα-, interleukin- IL 1 and 8),

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antimicrobial peptides and anti-apoptotic factors. Immune cells (mainly polymorphonuclear cells - PMN) are consequently attracted to the site of infection/injury, ensuring phagocytosis of invading microorganisms or cell fragments (Broom and Kogut 2018; Sheldon *et al*., 2019) together with the generation of *reactive oxygen species* (ROS) and the release of proteolytic enzymes. Pro-inflammatory cytokines also induce important microcirculatory events, at short (vasodilation) and long term (neoangiogenesis contributing to tissue healing).

Physiological inflammation in reproductive processes

Apart from playing a central role into innate immunity, inflammation is essential for successful cow reproduction since inflammatory (or inflammatory-like) processes are implicated in every step of fertility: in the cycle (ovulation, corpus luteum development, luteolysis), early pregnancy (maternal recognition of pregnancy) and later, in expulsion of fetal membranes and post partum uterine involution.

Ovulation

The ovulation exhibits many classical signs of local inflammation, with production of inflammatory mediators, locally increased blood flow, leukocyte infiltration, swelling, tissue digestion and ultimately tissue repair (Espey, 1980; Duffy *et al*., 2019). First responders to the LH surge are granulosa and theca cells, which produce chemokines and cytokines within hours after the LH surge. High concentrations of TNFα, IL1 and IL8 are found in follicular fluid at the preovulatory stage; consequently, not only the preovulatory follicle is invaded by high numbers of neutrophils and macrophages, but ovarian resident immune cells are activated (Jiemtaweeboon *et al*., 2011). Through proteolytic pathways, crucial within the ovulation process, these exogenous and endogenous cells regulate the reorganization of follicular stroma, the disruption of the granulosa basal lamina, and its invasion by vascular endothelial cells. LH-induced mediators also initiate cumulus expansion and cumulus-oocyte-complex detachment, together with extensive extracellular matrix remodeling and loss of the surface epithelium at the follicular apex. All these inflammatory phenomenons play a crucial role in the ovulatory process since treatment with antibodies directed against IL8 or neutrophils respectively suppress or decrease ovulation rate; administration of anti-proteases blocks ovulation; no blood flow increase is observed around large follicles that will finally fail to ovulate (Murdoch *et al*., 1997; Miyamoto *et al*., 2006).

Corpus luteum development

After ovulation, the remainder of the follicle undergoes intra-antral bleeding, colonization by a large variety of immune cells (mainly macrophages, neutrophils and eosinophils), secreting numerous cytokines (TNFα, interferon gamma, interleukins, prostaglandins) together with angiogenic factors. Follicular wall is rapidly remodeled, thanks to rapid angiogenesis and granulosa/thecal cells differentiation into luteal tissue, that finally fills the former follicular antral cavity. If ovulation can be assimilated to a specific physiological injury, corpus luteum (CL) development can be compared to a phase of tissue repair and organ healing.

Luteolysis

Not only CL formation but also lysis are inflammatory-like processes. Due to the short delay between prostaglandin F2α (PGF2α) secretion and the intraluteal immune reaction, luteolysis is even considered as an acute phenomenon (Shirasuna *et al*., 2012a). Leukocytes, especially eosinophils, macrophages and T lymphocytes, are recruited into the CL within the 5 first minutes after a PGF2α injection; as early as after 30 minutes, the expression of endothelial nitric oxide synthase is stimulated, accompanied by an increase in luteal blood flow and IL8 expression (Neuvians *et al*., 2004). Luteal blood flow increases within minutes in response to each peak of uterine PGF2α during spontaneous luteolysis in cattle (Miyamoto *et al*., 2005; Ginther and Beg, 2012). Interestingly, this "preluteolytic" blood flow increase is not observed in PGF2alpha refractory CL (Miyamoto *et al*., 2006). A little bit later, but as early as two hours, expression of pro-inflammatory cytokines (TNFα, IL1beta and interferon gamma) is increased and made responsible for apoptosis of luteal cells. CL regresses primarily through the loss of cells by apoptosis and apoptotic luteal cells are phagocytosed by macrophages. The large number of immune cells observed within the CL 6-24 hours after PGF2α are considered essential for a rapid demise of the CL tissue (Neuvians *et al*. 2004; Shirasuna *et al*., 2012b). As previously described, TNFα is also found involved into CL development: this dual effect may be due to a dose-effect, luteotropic at high doses or luteolytic at low doses, probably depending on the type of receptors activated (TNFRI or II) (Korzekwa *et al*., 2008). Four hours after PGF2α release, blood flow has felt back to the preluteolytic level and totally disappears after 24 hours (Miyamoto *et al*., 2005).

After insemination/mating: post-mating reaction

Spermatozoa, seminal plasma or extenders are recognized as "dangers" by the genital tract and can induce an inflammatory reaction though PRRs activation. Mating or artificial insemination (AI) are thus followed by a physiological influx of neutrophils into the uterine lumen which peaks between 1 and 12 hours after. This so called post mating reaction has been observed in the uterus, cervix and vagina but not into the oviduct (despite less well studied and probably more complex). Like bacteria, sperm are phagocyted by neutrophils either directly through cell-cell attachment or entrapped with neutrophil extracellular traps (NETs) which ensnare sperm and hinder their motility (Marey *et*

al., 2016). Rapid removal of sperm is thought to prevent acquired immune responses against sperm in dams since it is important for further embryo development that the female genital tract remains tolerant to paternal antigens (Katila 2012). In cattle, 60% of sperm are voided by 6 hours after AI and by 12-24 hours, only a few percent of sperm are left in the reproductive tract, the majority found within the vagina (Mitchell *et al*., 1985; Hawk, 1987). The duration of PMN infiltration is short, with a peak at less than 2 hours or at around 8-16 hours post AI or mating in cattle according to the different studies (reviewed by Katila, 2012): sperm and bacteria are rapidly eliminated, afterward the endometrium rapidly returns to a non-inflamed status, prepared to receive the embryo after its oviductal transit. If one can easily conceive than an excessive or persistent post mating response could decrease embryo survival rate, Kaufmann *et al*. (2009) suggested that the absence of post mating reaction in cows (no leukocytes intrauterine mobilization 4 hours after insemination) is associated with decreased pregnancy rates.

The situation is different in the oviduct whose epithelial cells face two opposite challenges: first, the protection against bacteria ascending from the uterus (and especially in oestrus, due to the opening of the cervical barrier and eventually insemination) and second, to favor fertilization and embryo development, whereas sperm and embryos are (semi) allogeneic to the maternal host. Interestingly, in presence of LH and estradiol, the oviduct generates a state of immunotolerance that ensures sperm survival until fertilization (Marey *et al*., 2016). Once sperm bound to oviductal epithelial cells, these cells are stimulated to secrete high levels of PGE2 that strongly suppress the PMN phagocytic activity to sperm and pro-inflammatory cytokines synthesis. Sperm binding thus favors the development of an anti-inflammatory immune environment and suppresses PMN sperm phagocytosis. More precisely, follicular fluid collected from pre-ovulatory follicle enhanced sperm phagocytosis by neutrophils in vitro whereas the oviductal fluid suppressed this activity. The oviductal environment seems thus to minimize the inflammatory effect of the follicular fluid released at the time of ovulation to allow sperm capacitation and fertilization (Yousef et al., 2019).

Placental expulsion

Placental maturation leading to fetal membranes expulsion also involves inflammatory mechanisms, mainly protease activity and leukocytes chemotaxis (Beagley *et al*., 2010). During the third trimester of pregnancy, fetal major histocompatibility complex (MHC) Class 1 molecules begin to be expressed by placental cells and initiate a maternal response (the fetus being an allograft) (Davies *et al*., 2000). Leukocytes are recruited through the placenta via several chemoattracting cytokines (TNFα, IL2 and IL 8) and phagocyte placental cells (Heuwieser and Grunert, 1987; Kimura *et al*, 2002). In addition, Matrix MetalloProteinase and collagenase activities increase in the maternal and the fetal part of the placenta (Maj and Kankhofer, 1997; Beagley *et al*., 2010). Both inflammatory components (leukocytes and enzymes) contribute to the loosening and subsequently the detachment of the villi. Importance of efficient inflammatory processes into placental expulsion in the cow is well demonstrated by the overexpression of anti-inflammatory associated genes and decreased expression of promoters of proteolytic activity in case of spontaneous placental retention (Nelli *et al*., 2019) even if not systematically reported (Walter and Boos, 2001).

Post partum uterine involution

Following the delivery of the calf, the uterine lumen, fulfilled with cellular and tissular debris, from placental and maternal origin, is physiologically colonized by bacteria (Sheldon *et al*., 2006). Both damages and bacterial invasion elicit a massive immediate cellular influx, whose intensity affects reproductive performances. Cows able to mount an early inflammatory response with more than 35-40% of neutrophils on endometrial smears 7 days after calving have shorter intervals from calving to pregnancy (Gilbert and Santos 2016; Cheong *et al*., 2017). This may be attributable to an early clearance of the uterine cavity from inflammatory stimuli. Inflammation is thus beneficial for the animal in the very early times after calving. However, it is important to distinguish local intrauterine cell mobilization - associated with a higher probability of ovulation from the first dominant follicle - and systemic inflammation, evaluated through haptoglobin concentration, conversely associated with a decreased ovulation rate (Cheong *et al*., 2017).

Excessive or persistent uterine inflammation

Once the initial danger of post-partum microbial invasion is contained, it is important that inflammation is resolved, otherwise chronic inflammation persists to the detriment of tissue function. Optimal reproductive performances thus require that the animal is able to mount a rapid, acute inflammatory response to control in a short term delay the microbial invasion. In a second step, after pathogen clearance, it is of equal importance that the animal is able to control the inflammation itself, to extinct it through a timely transition to an anti-inflammatory state, favorable to tissue repair processes. Rapid, targeted, effective and quick resolution are the hallmarks of a desired inflammatory response (Broom and Kogut 2018). Considering uterine health, after the intense PMN mobilization of the first week after calving, optimal reproductive performances are obtained if the percentage of PMN on endometrial smears falls below 5% between 21 and 35 days after calving, reaching a nadir (0-1%) around 45 days after calving and being maintained at this almost null level until the time of insemination (Deguillaume, 2010; Drillich *et al*., 2012; Bogado Pascottini *et al*., 2016; Machado Pfeifer *et al*., 2018; Fig. 1).

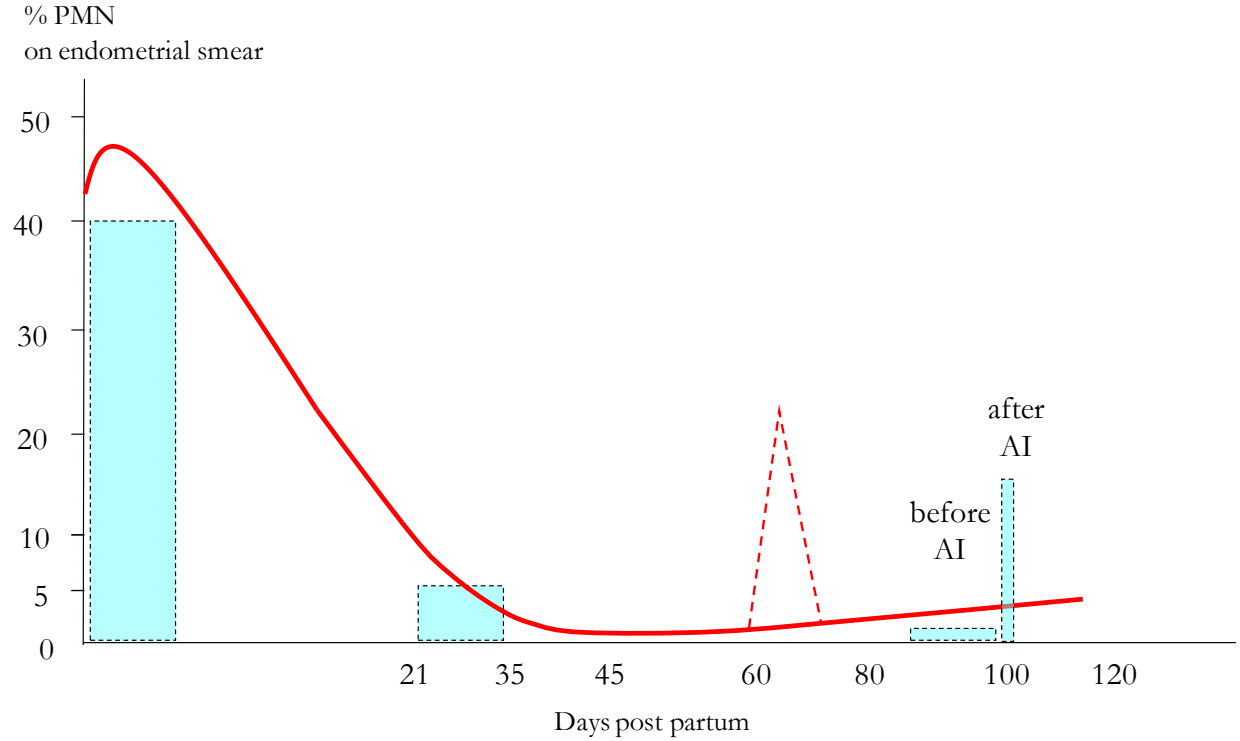

Figure 1. Intensity of endometrial inflammation from calving to insemination (% neutrophils on endometrial smear). All the thresholds indicated were determined based on a significant decrease of pregnancy rate. After an early intense mobilization of neutrophils after calving (>40%), inflammation is down regulated, becoming null around 40-45 days after calving and remaining null at the time of insemination. Between the nadir and the time of insemination, inflammation can be reactivated (interrupted lines). During a few hours after insemination, inflammation is transiently reactivated (post mating response).

But this fine tuning of uterine inflammation (massive during the first week after calving, rapidly controlled and finally extinct at the end of the first month and transiently reactivated during a few hours after insemination) is a difficult exercise for dairy cows, due to the delicate metabolic context of the post partum period (LeBlanc, 2014). Inflammation control is not just a passive extinction but rather requires the activation of anti-inflammatory pathways (including for example lipoxins and resolvins, Sheldon *et al*., 2017). Genital health relies on a fragile equilibrium between pro- and anti-inflammatory systems, difficult to maintain in dairy cows: the persistence of uterine inflammation at the time of insemination is a frequent situation (28 to 57% of Holstein cows according to the different studies). From three weeks before and until three weeks after calving (transition period), dairy cows are facing a negative energy balance (with production of non esterified fatty acids), oxidative stress (ROS production), together with digestive acidosis and social stress (Fig. 2), all situations that put the cow in a pro-inflammatory situation. Moreover, a vicious circle installs due to the huge energy expenditure associated to the inflammatory phenomenon itself. Dairy cows use more than 1 kg glucose in the first 12 hours after an LPS challenge (Kvidera *et al*., 2017), an expenditure corresponding to about 100 kcal/kg $BW^{0.75}$ (calculation from Gilbert, 2019), i.e. almost equivalent to maintenance. The depletion of the key cellular nutrients (such as glucose) reduces inflammatory responses, compromising the ability of animals to respond sufficiently to pathogens, resulting in the persistence of infections and chronic inflammation.

The tendency to an overactivity of pro-inflammatory systems and the instability of inflammation control in post partum dairy cows are pictured in endometrial smears follow-up: even when cows solved their uterine inflammation at 40-45 days post partum (0% PMN), transient episodes of reactivation of the uterine inflammation (up to 40% PMN) were observed after 60 days post partum (unpublished data). This explains why cows diagnosed as free from endometritis around 30 days post partum can be found with purulent uterine content at the time of insemination, probably due to a disruption of the equilibrium between pro- and anti-inflammatory systems.

To date, the unability of cows to down regulate inflammation is probably one important limiting factor of modern dairy cows fertility, due to the frequency of excessive uterine inflammation at the time of insemination, and its dramatic impact on insemination success rate (around 15 points decrease). As developed by Sheldon *et al.* (2019), uterine health is rather dependent on the endometrial tolerance to pathogens (ability to limit the disease severity induced by pathogens) than on its resistance (ability to limit the pathogen development).

Deleterious effects of inflammation on reproduction

Excessive or persistent inflammation has deleterious impact on fertility. But this applies not only to uterine inflammation, but also to extragenital inflammation. Due to cytokine release into the general circulation, ovaries, uterus and embryos may be somewhat "contaminated" by distant inflammatory sites, such as mastitis, podal inflammation, digestive inflammation consecutive to acidosis, all highly prevalent in dairy cows. Inflammatory diseases affect many steps of the reproductive process: GnRH and LH synthesis, folliculogenesis, follicular steroidogenesis, oocyte quality, ovulation, estrus expression, corpus luteum quality and lifespan, fertilization, embryo development and survival (Ribeiro and Carvalho, 2017; Fig. 3).

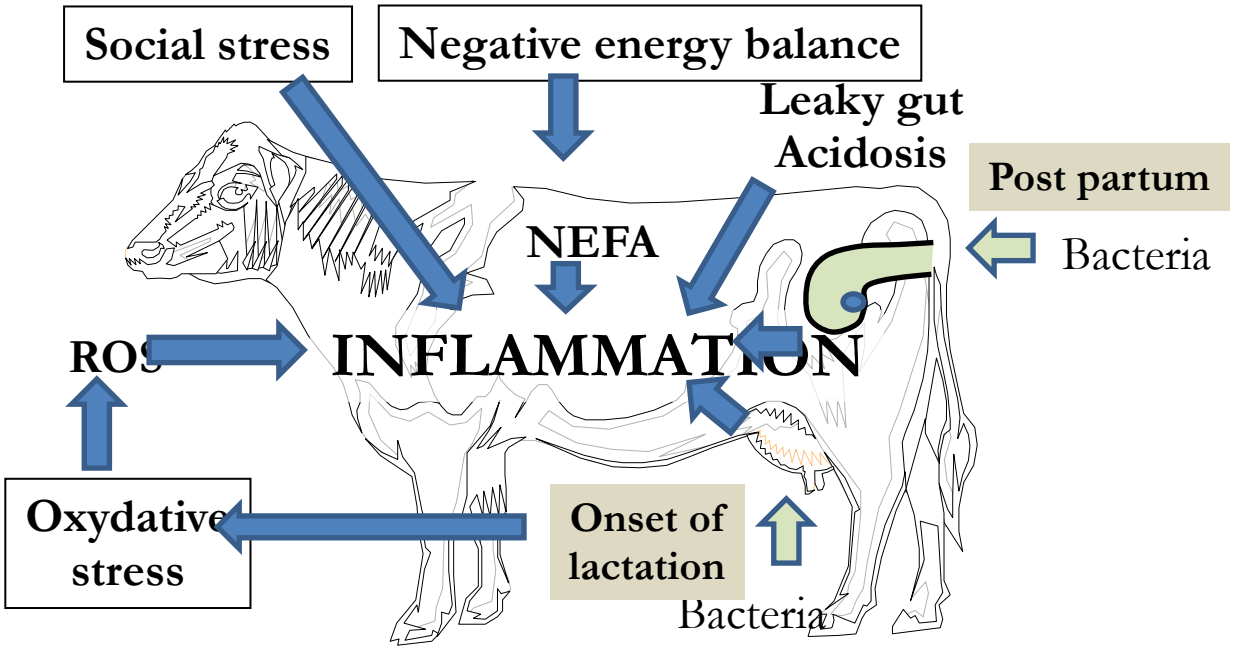

Figure 2. Determinants of the pro-inflammatory status during the post partum period of dairy cows. NEFA: Non Esterified Fatty Acids.

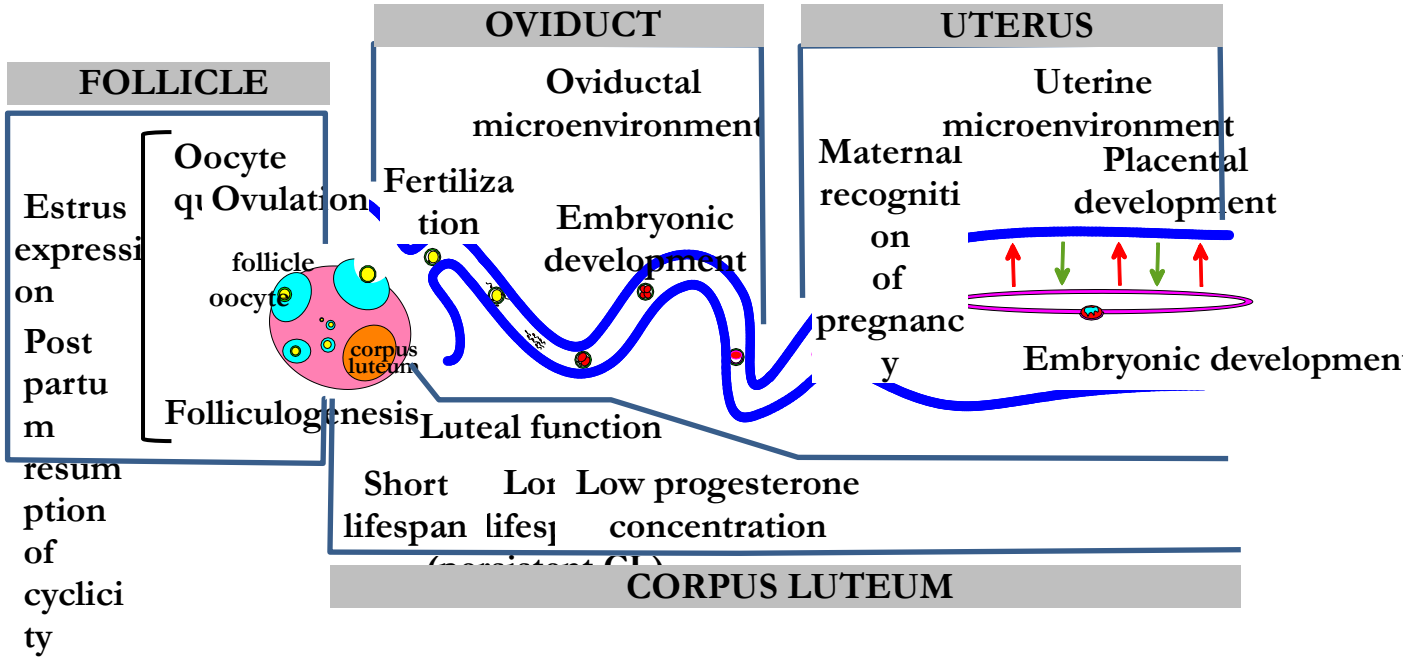

Figure 3. Steps of the reproductive process sensitive to inflammation.

Ovarian reserve

In humans and mouse, chronic inflammation is made responsible for destruction and/or premature activation of primordial follicles, leading to a decrease of the ovarian reserve, and thus Premature Ovarian Failure (phenomenon so called "inflamm-aging") (Huang *et al*., 2019). In the bovine, considering the post partum period as a prolonged period of inflammation with excessive oxidative stress and fatty acids release, Gilbert (2019) estimated plausible that inflammatory damages could be inflicted on developing oocytes and the resting oocyte pool, resulting in chronically diminished fertility (Sheldon *et al*., 2017).

Anovulation – Follicular cyst

Several anovulatory situations are associated with an increased expression of pro-inflammatory cytokines in the granulosa (IL1α, IL6 and TNF α) in humans (Luteinized unruptured follicle syndrom; Polycystic ovary syndrom) and in the cow (ovulation failure and follicular persistence, follicular cyst; Baravalle *et al*., 2015; Stassi *et al*., 2017).

Oocyte competence

Inflammation mediates changes in follicular fluid that diminish the ability of the oocyte to complete meiosis, undergo fertilization and support development of a conceptus. By the activation of granulosa PRRs, steroidogenesis and the interaction between oocyte and cumulus can be impaired (Herath *et al*., 2007). Inflammatory mediators have been also described to result into aberrant spindle formation and meiosis abnormalities (Bromfield and Sheldon, 2011; Banerjee *et al*., 2012).

Luteal insufficiency

Since inflammation affects granulosa and thecal cell function (before ovulation) and luteal cells (after ovulation), it is associated with inadequate function of the CL and insufficient circulating concentrations of progesterone, one of the major causes of infertility of modern cows (Diskin *et al*., 2011; Ribeiro *et al*., 2016).

Embryo/placental development

Inflammation may affect embryo survival both by its deleterious effect on oocyte quality and CL function but also by providing an inadequate uterine microenvironment and through direct effect of cytokines on embryonic/placental cells. The direct influence of inflammation *per se* on embryo has been elegantly demonstrated by Hill and Gilbert (2008) who induced a non infectious endometrial inflammation; after culture into the conditioned uterine medium, blastocyst cell number was decreased, affecting trophectoderm but not inner cell mass. Other authors observed consistently impaired elongation and decreased interferon tau secretion. Inflammation thus interferes with maternal recognition of pregnancy and later, if pregnancy is maintained, decreases placental weight from Day 42 of gestation (Lucy *et al*., 2016; Ribeiro *et al*., 2016). Interestingly, maternal inflammatory diseases even caused inflammation-like changes in the transcriptome of conceptus cells (Ribeiro *et al*., 2016).

Inflammation is thus involved into many reproductive diseases, namely abnormalities in ovarian resumption of cyclicity (delayed ovulation, short luteal phases, persistent corpus luteum), metritis/endometritis and repeat breeder syndrome.

Carry over effects of inflammation

The variety of targets sensitive to inflammation (oocyte, embryo, placenta) explains that inflammation affects reproductive performances at various distances from insemination. For example, mastitis negatively impacts on reproductive performances whatever it occurred before the first AI (even during the first month after calving), between first AI and conception or after conception, with a period at higher risk extending from 3 weeks before AI until 30 days after (Loeffler *et al*., 1999; Perrin *et al*., 2007; Lavon *et al*., 2011; Albaaj *et al*., 2017). Same observation was made with long lasting consequences of metritis on ovarian function, long after the resolution of the disease (Piersanti *et al*., 2019). This delayed effect of inflammation is reminiscent of what is known as the "Britt hypothesis" explaining the carry-over effect of negative energy balance on fertility (Britt, 1992). The carryover effect of inflammatory diseases on reproduction is attributable to the impact on oocyte quality together with an durably modified uterine environment. In case of uterine disease, inflammation can persist during several months as inflammatory lymphocytic foci within the endometrial wall, even during pregnancy (Lucy *et al*., 2016). The uterus may also be long-lasting impaired secondary to altered steroid synthesis. When previously diseased cows (retained fetal membranes, metritis, mastitis, lameness, and respiratory and digestive problems) are used as embryo recipients, establishment of diagnosed pregnancy is reduced and pregnancy loss rate is increased relative to that of previously healthy cows. The effect of inflammation on reproduction extends long beyond the resolution of the disease, until 4 months later (Ribeiro *et al*., 2016).

Transgenerational (epigenetic) effects of maternal inflammation are also suspected but with controversial observations. For Ribeiro and Carvalho (2017), female calves born from multidiseased cows have significantly lower incidence of mortality and morbidity before their first calving. Conversely, Ling *et al*. (2018) described that calves born to cows with a higher serum haptoglobin concentration (acute phase protein) during late gestation showed a lower TNFα plasma concentration after challenge, suggesting a compromised immune response to microbials.

Suppression of inflammation: NSAID and reproduction

Since inflammation (rather than infection) is now recognized as the limiting factor of reproductive performances and in the context of the reduction of the use of antibiotics, the interest of non steroidal anti-inflammatory drugs (NSAID) has been evaluated. When used as additional treatment, NSAID allowed to limit the reproductive impact of mastitis (MacDougall *et al*., 2016). Their administration at the time of AI did not improve pregnancy rates (Heuwieser *et al*., 2011); administration before ovulation was deleterious due to an inhibition of the ovulation process and follicular cyst formation (Pugliesi *et al*., 2012). Conversely, administration at the time of embryo transfer showed an improvement of pregnancy rates (+10 to 25 points), especially when transfer was qualified as difficult (Aguiar *et al*., 2013) or after transfer of low quality embryos (Scenna *et al*., 2005). Administration at mid luteal phase targeting maternal recognition of pregnancy did not show any significant improvement of insemination success rate.

Conclusion: Inflammation is not to be suppressed but regulated

Inflammation is a dual process, together mandatory at numerous steps of the reproduction process and deleterious for reproductive performances if excessive or persistent. Optimisation of insemination success rate depends not on the suppression of inflammation but on its fine regulation. The cow has to be able to mount intense inflammatory episodes and, more difficult, to control and shut them down rapidly, what is made complex by metabolic challenges post partum. Better regulation of the inflammation can be obtained through an appropriate dietary management during the transition period, targeting energy balance, Dietary Anions-Cations Difference, and anti oxidant reserves (LeBlanc 2012). Immunomodulators rather than anti-inflammatory drugs are an elegant strategy (such as pegbovigrastim, long acting-analog of bovine granulocyte colony-stimulating factor; Ruiz *et al*., 2017; Heiser *et al*., 2018). The genetic option is also promising, with the selection of females with high immune regulatory competences (Thompson-Crispy *et al*., 2012; Silva Silveira *et al*., 2019; König and May, 2019).

Authors contribution

SCM: Conceptualization, Writing – original draft, Writing – review & editing. MSD: Conceptualization, Writing – review & editing.

Conflict of interest

The Authors declare no conflict of interest.

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Developments of reproductive management and biotechnology in the pig

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Abstract

This review aims to describe changes in production environment, management tools and technology to alleviate problems seen with the present hyperprolific sow model. Successful parturition in the pig includes the possibility to express adequate maternal behaviour, rapid expulsion of piglets, complete expulsion of placenta, elimination of uterine contamination and debris, neonatal activity and colostrum intake. We focus on management of large litters, including maternal behaviour, ease of parturition, colostrum production, piglet quality parameters and intermittent suckling. There are also some interesting developments in technology to assess colostrum and immune state of the piglet. These developments may be utilized to improve the success rate of reproductive management around farrowing, lactation and after weaning. We also discuss new insights in how to examine the health of the mammary gland, uterus and ovaries of hyperprolific sows. Finally, we assess the latest developments on breeding and technology of hyperprolific sows, including artificial insemination (AI), real-time ultrasound of the genital tract and embryo transfer (ET). We conclude that 1) for the sow to produce sufficient colostrum, both the behavioural and physiological needs of the sow need to be met before and after parturition. Furthermore, 2) new ultrasound and biopsy technology can be effectively applied for accurate diagnosis of inflammatory processes of the udder and uterus and timing of AI regarding ovulation to improve insemination efficiency. Finally, 3) developments in cryopreservation of germ cells and embryos appear promising but lack of valid oocyte collection techniques and nonsurgical ET techniques are a bottleneck to commercial ET. These latest developments in management of parturition and reproductive technology are necessary to cope with the increasing challenges associated with very large litter sizes.

Keywords: large litter, sow, piglet, management, biotechnology.

Introduction

The pig appears to be superior in its reproductive ability at least when compared to other domestic animal species. This ability is based on the extremely high rate of fertility. Over the past three decades, efficient breeding and management has almost doubled the litter size of the domestic European sow breeds (Oliviero, 2019). During the same period, the duration of farrowing (second stage, from the first to the last fetus expulsed) has extended remarkably and is now four to five times longer than in the early 1990s (Oliviero *et al*., 2019). This may have resulted in an increase in farrowing complications such as postpartum dysgalactia syndrome (PDS, Kaiser *et al*., 2018a, b) and retention of placenta and a decrease in subsequent fertility (Björkman *et al*., 2017c; 2018c). Along with this development, we have seen a constant downward trend in the birth weight of the piglets and a similar trend in colostrum intake, which are connected and are the most important risk factors for piglet mortality (Oliviero *et al*., 2019). In the other hand, we have seen a tremendous increase in efficiency of production, which has considerably improved farming economy and related industry in a highly positive way. However, this may have come, at least to some extent, at the expense of animal health and welfare.

A large litter may be challenging for the metabolism of the sow such that there may be difficulties in resumption of ovarian cyclicity after weaning, especially in young sows in certain European breeds (Oliviero *et al*., 2013; Peltoniemi *et al*., 2016; Björkman *et al*., 2018c; Oliviero *et al*., 2019). Therefore, there appear to be major challenges associated with increasing litter sizes that are evident at farrowing, lactation and after weaning, which are periods when the foundations of the subsequent pregnancy are laid (Algers and Uvnäs-Moberg, 2007; Martineau *et al*., 2012). This paper discusses some of the key applications of reproductive biotechnology for the modern hyperprolific sow and her numerous offspring (beyond 17 piglets in a "large litter"). The first focus is on management and technology-related innovations used to address the challenges that sows and piglets face in terms of the ambient parturition environment and development of immunity around parturition and lactation. These innovations include optimizing colostrum intake and evaluation of colostrum yield and quality produced by the sow. Among the newer management interventions, intermittent suckling is aimed at not only hastening the production cycle, but also more importantly to improve the resilience of piglets after weaning. Secondly, we review some novel approaches to examine ovarian, uterine and mammary gland function *in vivo*. These involve both sampling and diagnostic imaging

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techniques that have been recently either discovered or considerably developed. Finally, we provide an updateon the use of artificial insemination (AI), which has been successful regarding use of fresh semen since inception of this technique, and future prospects of embryo transfer (ET) in the pig.

Management of large litters

Developments in parturition management of hyperprolific sows

Prolonged farrowing increases the risks of piglet asphyxia during parturition and less vital piglets at birth (Herpin *et al*., 2001). Yun et al. (2014) demonstrated that providing space and abundant nest building material before farrowing tended to increase sow plasma oxytocin concentrations (25 *vs*. 18 pg / ml in sows with abundant nesting material *vs*. sows with crates, respectively). Abundant nesting material also increased piglet serum IgG and IgM concentrations during early lactation (15 *vs*. 10 mg / ml (IgG) and 0,9 *vs*. 0,7 mg/ml (IgM) of sows with abundant nesting material vs. with sows in crates, respectively; Yun *et al*., 2014). Allowing for the intrinsic nesting behaviour to occur can reduce farrowing duration and therefore allow for more vital piglets (Jensen, 1986; Islas-Fabila *et al*., 2018) and for greater colostrum intake due to a shorter time interval gap from the start of farrowing to first suckling (Manjarin *et al*., 2018). Uncomplicated farrowing also reduces pain and inflammation in the sow (Björkman *et al*., 2017c; Kaiser *et al*., 2018a). Allowing the sow to farrow free and providing a substrate (straw, sawdust, paper) 1 to 2 days before farrowing can support the physiological nest building behaviour of the sow. This can significantly reduce farrowing duration and stillbirth rate (Oliviero *et al*., 2008; Gu *et al*., 2011).

With increasing occurrence of large litters, providing the sow with a good basis to produce enough colostrum is fundamental. Loss of back fat in late gestation and consequently sows arriving at farrowing with inadequate body condition affect colostrum yield (Decaluwé *et al*., 2013). Therefore, it appears essential that sows improve their body condition gradually during the whole pregnancy, arriving to farrowing in good body condition (backfat of 17 ± 3 mm) to fulfil protein turnover and sufficient colostrum yield (Oliviero *et al*., 2010; Decaluwé *et al*., 2013). During late pregnancy, not only adequate energy intake but also feeding composition seems to be of key importance in supporting the physiology of farrowing and colostrum quality. Many studies reported that specific essential fatty acids (conjugated linolenic, pinolenic and oleic acids) supplemented in gestating and lactating diets can improve sow colostrum immunoglobulins, piglet performance, average daily gain and weaning weight (Bontempo *et al*., 2004; Corino *et al*., 2009; Yao *et al*., 2012; Hasan *et al*., 2018). The feeding timing during pregnancy and especially in relation to parturition also seems to be of relevance regarding the success of farrowing. Feyera et al. (2018) observed that if the time lapse between the last feeding occasion prior to onset of farrowing lapsed beyond 3 hours, there was a positive linear correlation for time lapse and farrowing duration (Feyera *et al*., 2018). Glucose metabolism was considered to be of highest relevance behind this finding. However, other factors such as feeding fibre (involving bacterial metabolism of the GI tract) were also suggested to support more successful, quicker process of farrowing (Feyera *et al*., 2018). In conclusion, a proper ambient environment regarding food, metabolism, enrichment and space around farrowing are of key importance for successful processes of farrowing and colostrum yield, intake and quality.

Improving colostrum intake

Increased competition for colostrum intake is a critical factor for neonate piglets. These piglets are born without the protection of maternal immunoglobulins, as the epitheliochorial nature of the porcine placenta does not permit transfer of such large molecular weight structures from maternal to foetal blood circulation. Neonate piglets must acquire maternal immunoglobulins from ingested colostrum for passive immune protection before they produce sufficient endogenous immunoglobulins at approximately 3 to 4 weeks of age (Rooke and Bland 2002; Oliviero, 2013). The concentration of IgG piglet plasma shortly after birth is positively correlated with survival. Dead piglets have lower serum IgG concentrations than their surviving fellow piglets, which indicates low colostrum intake (Vallet *et al*., 2013). At farrowing, the IgG levels in colostrum are approximately 60 to 80 mg/ml. Within 10 to 12 h later IgG levels are reduced by half (35 to 40 mg/ml) and after 24 h a 70% reduction occurs (10 to 16 mg/ml), which is no longer an adequate level (Devillers *et al*., 2011; Quesnel *et al*., 2011; Hasan *et al*., 2016). Therefore, in large litters with prolonged farrowing of more than 6 hours, the immunity and viability of piglets are compromised. Furthermore, hyperprolific sows give birth to more piglets with low birth weight and with signs of intrauterine growth restriction (IUGR). There is an inverse relationship between number of piglets born in a litter and piglet birth weight; large litters are also associated with high variation in piglet birth weight within the litter (Quesnel *et al*., 2008; Akdag *et al*., 2009; Beaulieu *et al*., 2010; Smit *et al*., 2013; Matheson *et al*., 2018). A greater number of piglets born than the available teats at the sow's udder, lower birth weight and greater birth weight variation increase piglet competition for colostrum intake (Declerck *et al*., 2017). Similarly, lower birth weight and long farrowing duration are associated with lower piglet viability at birth, which can delay the access to the udder (Hoy *et al*., 1994; Islas-Fabila *et al*., 2018). Therefore, all underprivileged piglets should be provided with additional support to acquire a sufficient amount of good quality colostrum (e.g., should be assisted in suckling). To provide the best passive immunity, the procedure of split and assisted suckling should be effectively operated within the first 6 hours from the beginning of parturition, when the colostrum immunoglobulin content is at the maximum (Devillers *et*

al., 2011; Quesnel *et al*., 2011; Hasan *et al*., 2016). As small piglets or those with IUGR have difficulties to suckle from large nipples, the smallest functioning nipples should be used when assisting suckling. In conclusion, due to decreasing birth weight and colostrum intake per piglet, colostrum management around farrowing is of key importance for survival of piglets.

Technology to assess colostrum quality and immune state of neonatal piglets

Both colostrum yield and IgG content vary greatly among sows (Foisnet *et al*., 2010). Factors that affect the total colostrum yield are attributed to environment-related factors as well as to sow and piglet characteristics (Devillers *et al*., 2007; Farmer and Quesnel, 2009; Quesnel, 2011). IgG concentration in maternal colostrum significantly affects the acquisition of passive immunity (Kielland *et al*., 2015) and therefore knowledge on IgG content of colostrum may be essential to determine the correct action to reduce piglet pre-weaning mortality. The major practical point in assessing colostrum IgG content at the farm level may be identifying the sows with low colostrum IgG levels. Those sows are a risk for a successful acquisition of passive immunity in the piglets. This is of great importance particularly when large litters are present and cross-fostering and split suckling are common management practices employed to maximize colostrum intake. Therefore, if the estimated colostrum IgG content appears to be insufficient, a farmer with this advance knowledge can pay additional attention to the relevant management practices. Hasan et al. (2016) have proposed the use of a Brix refractometer to estimate IgG content in sow colostrum. When used in non-sucrose-containing liquids, the Brix percentage approximates the total solids (TS) percentage (Quigley *et al*., 2013; Hasan *et al*., 2016). At the start of farrowing, immunoglobulins represent a significant portion of the TS (Klobasa *et al*., 1987) and IgG represents 80% of the immunoglobulins in sow colostrum (Porter, 1969; Curtis, 1970). Colostrum samples evaluated with a Brix refractometer are positively correlated with the IgG level measured with ELISA (Hasan *et al*., 2016). Therefore, the Brix refractometer can be an inexpensive, rapid and satisfactorily accurate method for estimating IgG concentration. Differentiation between good and poor IgG content of colostrum is possible by interpreting the results with the categories proposed in Table 1. Hasan et al. (2016) proposed this classification following the nature of the IgG physiological curve during the first 24 h post-partum, when IgG levels peak in the first 3 h and decrease rapidly until values of 10 mg/ml are reached 24 h post-partum (Quesnel *et al*., 2015; Hurley, 2015). Brix values of <20% were correlated with very low IgG levels (14.5 mg/ml), which are not expected during early colostrogenesis. In conclusion, the Brix refractometer is an acceptable method to assess colostrum IgG content at the herd level during the initial hours of parturition, when IgG levels are expected to peak.

Table 1. Brix value categories to estimate sow colostrum IgG content according to Hasan et al. (2016). This interpretation scale is valid if the sample is obtained within 0-3 hours from the start of farrowing using a Brix refractometer with a scale range 0-53% (adapted from Hasan *et al*., 2016).

Brix %	IgG estimation categories
< 20	Poor
20-24	Borderline[a]
25-29	Adequate
≥ 30	Very good

[a]The category "Borderline" should not always be considered to estimate a not adequate IgG content, especially if the found Brix values are on the highest range of this category (23-24%), on the contrary levels falling at the lowest range of this category (20-21%) can be considered not optimal. Taking another sample, after 1-2 h, can allow better interpretation of the results, to see if the development of the estimated IgG content is stable, increasing or decreasing from the initial value (Hasan *et al*., 2016). In conclusion, IgG can be considered as a reliable indicator of colostrum quality. Use of Brix refractometers provide an effective tool to assess colostrum quality, which is essential in the management of a large litter.

Intermittent suckling

Management strategies to support large litters are numerous. They include at least use of nurse sows (Schmitt *et al*., 2019a, b), split suckling (Donovan and Dritz, 2000), use of substitute milk and automated milk replacers (Difilippo *et al*., 2015) and general neonatal management (Kirkden *et al*., 2013). Among the strategies, intermittent suckling (Kemp and Soede, 2012) is especially interesting, since it may provide a useful tool Alternative reproductive management strategies as IS have a considerable impact on grouping dynamics and reproductive functions in the pig (Peltoniemi *et al*., to postpone weaning of piglets, which becomes relevant for the industry based on the decreasing trend in colostrum intake and birth weights of piglets (Oliviero *et al*., 2019). Therefore, applying an intermittent suckling (IS) protocol, which encourages sows to become pregnant in the middle of lactation, seems like an appealing alternative.

However, IS also involves resumption of reproductive function in the middle of lactation, which may become a further metabolic challenge for the sow. 2016). Sows are in anoestrus during lactation and maturation of follicles is bound to the process of weaning. It is only after weaning that follicles are

provided with circumstances for growth and ovulation. This process heralding ovulation stems mainly from the continuous lack of suckling stimulus on the udder, high intake of feed rich in energy and daily application of boar stimulus.

Ovulation in the middle of lactation can be induced by essentially the same means as used after weaning, specifically temporary, transient interruption of suckling stimulus, high feed intake and proper application of boar stimulus. Recent studies (see Kemp and Soede 2012 for a review) have demonstrated that intermittent suckling can induce lactation oestrus especially when IS starts around the normal weaning and is combined with adequate boar stimulation. Oestrus may be induced in up to 90% of the older sows (Gerritsen *et al*., 2008; Soede *et al*., 2012) and over 70% in first parity sows (Chen *et al*., 2017) within 6 days during lactation; farrowing rates and litter size are comparable to controls. Thus, success is dependent on parity as primiparous sows do not appear to respond as well as older sows and there seems to be differences in the response to the IS protocol and in the breed used. The success rate of IS also seems to depend on the management issues around IS (van Nieuwamerongen *et al*., 2014). These include a proper audio-visual isolation of sow and the piglets during IS. Furthermore, group management during boar stimulation around separation time is essential for IS success (Tabe 2; Hasan *et al*., 2019; van Nieuwamerongen *et al*., 2014). In conclusion, lactation oestrus has the potential advantage that the lactation period can be extended while sows are pregnant and this allows piglets to be more developed when eventually weaned. Piglets seem to respond well in terms of growth performance and resilience to the opportunity for extended, although interrupted, suckling possibilities (van Nieuwamerongen *et al*., 2014).

Table 2. Descriptive result of individual herd data for a successful intermittent suckling program. Data presented in mean ± SD. Data adapted from Hasan *et al*., 2019.

Type of production	Herd number					
	1	2	3	4	5	6
	Traditional	Traditional	Traditional	Traditional	Traditional	Intermittent suckling
Gestation length, days	115	115.6	116.2 ± 0.1	115	114.4 ± 0.1	115.2 ± 0.2
Farrowing duration, min	211.9 ± 10.7	200.6 ± 12.9	329.2 ± 24.2	261.7 ± 22.1	306.7 ± 27.4	287.8 ± 23.9
Litter size	16.1 ± 0.5	16.7 ± 0.6	14.6 ± 0.6	17.1 ± 0.6	16.5 ± 0.5	16.1 ± 0.5
Live-born piglets	15.3 ± 0.5	15.5 ± 0.5	13.1 ± 0.5	16.5 ± 0.6	14.9 ± 0.4	15.4 ± 0.5
Stillborn piglets	0.8 ± 0.1	1.1 ± 0.2	1.4 ± 0.2	0.6 ± 0.2	2.7 ± 0.5	0.6 ± 0.2
Birth interval, min	14.4 ± 0.9	13.7 ± 0.8	26.4 ± 2.7	16.6 ± 1.8	18.6 ± 1.2	-
Birth time, min	112.1 ± 3.1	100.3 ± 2.9	180.8 ± 7.9	142.2 ± 6.7	147.5 ± 4.1	-
Litter characteristics						
Piglet BW_B (live born), g	1445.7 ± 14.1	1275.0 ± 12.4	1413.6 ± 14.5	1220.48 ± 16.5	1279.2 ± 10.4	1446.1 ± 21.7
Piglet weight (weaning: ear tagged), g	6918.8 ± 105.8	6757.4 ± 106.3	7718.4 ± 161.2	5392.0 ± 149.2	7939.5 ± 55.28	6061.0 ± 135.5
ADG* (ear tagged), g	257.8 ± 4.3	246.1 ± 4.6	212.9 ± 5.0	224.0 ± 7.5	228.2 ± 1.7	246.3 ± 7.1
Piglet age (weaning) days	21.0 ± 0.03	21.6 ± 0.02	29.6 ± 0.09	18.1 ± 0.09	28.9 ± 0.03	19.4 ± 0.2
CY**, g	4658.5 ± 221.5	4009.4 ± 145.9	4132.2 ± 223.1	4336. 1 ± 268.4	4710.6 ± 129.4	3846.5 ± 367.3
CI***, g	332.0 ± 6.6	274. 3 ± 5.8	343.5 ± 7.2	270.9 ± 8.1	331.1 ± 4.5	262.5 ± 10.0

ADG* average daily gain, CY** colostrum yield, CI*** colostrum intake.

Management of hyperprolific sows after parturition

Mammary gland function

The most important disease of the mammary gland of the postpartum sow is generally considered to be mastitis as part of PDS (Farmer *et al*., 2019), although the role of mastitis as part of the complex in modern sow lines has recently been questioned (Kaiser *et al*., 2005), the sows that had PDS had more hyperechoic images in the ultrasonographic examination of their mammary glands than sows without PDS. Björkman et al. (2017a) made the same observation in sows suffering from severe udder oedema prior to parturition, which is considered a risk factor for subsequent mastitis. In this case report, ultrasound of the mammary glands showed thickened dermal and subdermal tissues, hyperechoic

2018a,b). This disease (PDS) is suggested to be associated with large litters as a connection between farrowing duration and PDS has been established (Tummaruk *et al*., 2013; Björkman *et al*., 2018c). Diagnosis of mastitis is based mainly on clinical signs, as has been reviewed by Gerjets and Kemper (2009).

Recently, other methods such as ultrasound examination and biopsy isolation have been tested for feasibility as diagnostic tools for udder diseases (Baer and Bilkei, 2005; Spiegel *et al*., 2017; Björkman *et al*., 2017a, 2018a, 2018b). In the study by Baer and Bilkei

lobuloalveolar tissue with enlarged blood vessels and severe shadowing (Fig. 1). Sows with severe udder oedema also had lower colostrum quality (Björkman *et al*., 2018a). Therefore, PDS must be prevented to ensure the immunity of newborn piglets.

The objective of the study by Spiegel et al. (2017) was to verify by comparative bacteriological examinations of milk samples and mammary gland biopsies whether a better assessment of bacteriological status is possible using biopsies. Diagnostic investigations based on bacteriological examination are complicated, as a similar bacterial content can be detected in milk samples from both healthy and diseased sows. Contamination during sample collection may be a possible reason. Spiegel et al. (2017) obtained biopsies after local anaesthesia using a 7-cm biopsy needle and revealed that biopsy samples of the mammary gland did not provide advantages for bacteriological diagnosis compared to milk sampling. Furthermore, Spiegel et al. (2017) observed complications such as abscess formation following biopsy. The same method was also tested by Björkman et al. (2018b) using an automatic needle with a 14-gauge diameter, 10-cm length and a 22-mm penetration depth. Biopsies were obtained from the lateral-caudal part of three different mammary glands. Before biopsy, glands were disinfected three times with a povidone-iodine solution but no local anaesthesia was used. Sows were monitored until weaning and no complications (such as abscess formation) were observed. There was also no effect of the biopsy before parturition on colostrum production (Han *et al*., 2018). Biopsies can thus be collected in a rapid and humane way. This method seems to be of minor value for diagnosis of mastitis but can be used to study mammary gland function for research purposes, especially for comparison of sows with low and high colostrum or milk yield. Ultrasound imaging of the mammary glands can provide an effective tool for diagnosis of inflammatory processes of the udder, such as PDS.

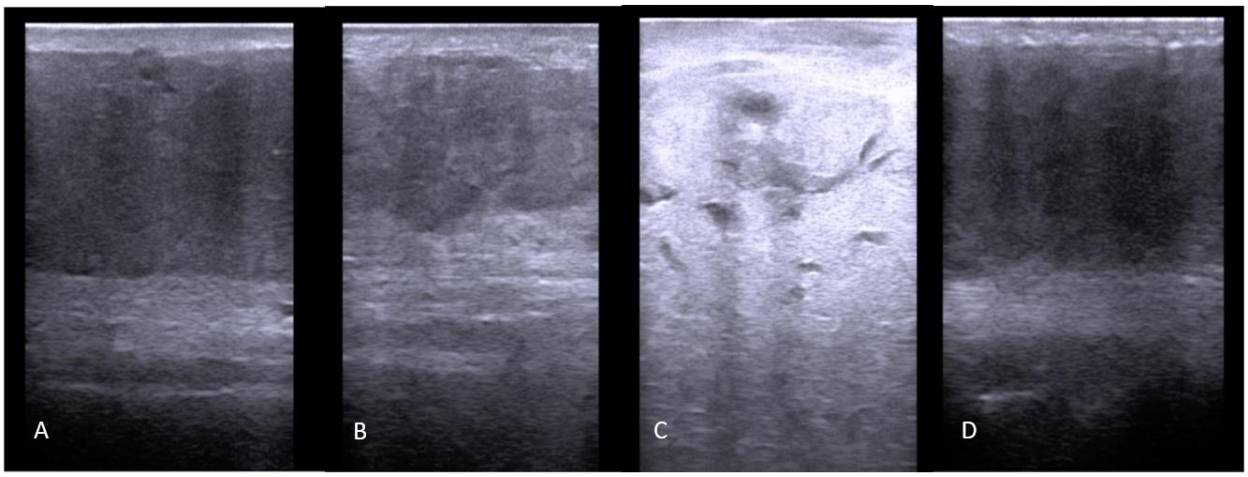

Figure 1. Ultrasound images of a mammary gland of a healthy sow (A) and from sows with from severe udder oedema (B-C). These images show thickened dermal and subdermal tissues (B, C), hyperechoic lobuloalveolar tissue (B, C) with enlarged blood vessels (C) and severe shadowing (D) (Björkman *et al*., 2017a, 2018a).

Uterine function

In recent years progress has been made in the use of ultrasonography to examine the non-gravid uterus. Timely and correct diagnosis of uterine disease, especially post-partum uterine disease, is essential to prevent subsequent subfertility (Kauffold and Wehrend, 2014). Oliviero et al. (2013) have shown that prolonged parturition can reduce subsequent fertility in the sow that may be associated with an increased incidence of post-partum uterine disease (Björkman *et al*., 2018c). In addition to prolonged parturition, obstetrical intervention, retained placenta and ≥2 stillborn piglets at birth have been shown to affect the incidence of post-partum endometritis (Björkman *et al*., 2018c). Ultrasonography is considered the best tool for diagnosis, not only for endometritis but also for cases in which placenta is retained (Björkman *et al*., 2017c). Examination of uterine structures currently utilizes the following three criteria: fluid echogenicity, echotexture and size (Kauffold and Althouse, 2007). Changes in echotexture reflect changes in endometrial oedema. Increased echotexture, unless attributed to circulating oestrogens originating from enlarged follicles, must be considered abnormal (Kauffold and Althouse, 2007). Furthermore, any fluid echogenicity, unless attributed to pregnancy, semen or oestrus, must be considered abnormal and indicative of an exudative inflammation of an acute or acute-chronic type (Kauffold and Althouse, 2007). Fluid echogenicity is often associated with uterine oedema and therefore increased echotexture and size of uterine cross-sections (Björkman *et al*., 2018c). In contrast, chronic endometritis, representing the most common type of uterine inflammation in pigs, cannot be definitively diagnosed by ultrasonography based on any of the criteria described above (Kauffold and Althouse, 2007). Therefore, it is essential to recognize acute endometritis in time. This can be achieved based on the criteria mentioned above. However, fluid echogenicity, uterine oedema and increased uterine size during the first few days after parturition are not unusual or abnormal

(Björkman *et al*., 2018c). Furthermore, when interpreting uterine size, the age and parity of the sow and the number of postpartum days must be considered. Björkman et al. (2018c) provide some reference values for the first postpartum week in Large White x Yorkshire sows.

Recently, the feasibility of transabdominal Doppler sonography (colour, power, pulse wave) to define uterine perfusion characteristics throughout the oestrous cycle in gilts (German Landrace x Pietrain) has been tested (Herlt, *et al*., 2018). These characteristics were perfused area, blood-flow velocity and intensity and resistance and pulsatility index. Colour Doppler sonography was the only feasible technique, as it was less affected by animal movements than power and pulse wave sonography. As determined by colour Doppler sonography, all five parameters determined showed specific patterns throughout the oestrous cycle. Perfused area and blood-flow velocity and intensity increased in proestrus, decreased in oestrus and remained low in midoestrus and most parts of dioestrus. The resistance and pulsatility index showed inversely paralleled patterns. Herlt et al. (2018) encourage the use of colour Doppler sonography for studying uterine capacity or uterus-related infertility, such as in cases of clinically unapparent endometritis. In conclusion, real-time ultrasound examination of the uterus is a fast, practical, efficient and accurate tool for diagnosis of acute inflammatory processes after parturition. Further developments in ultrasound technology, such as use of colour Doppler, may broaden the use of this technique beyond diagnosis of clinical disease of the uterus. In the future, it would be desirable to develop a uterine biopsy method for the sow for diagnosis of chronic uterine disease, like in the equine (Rua *et al*., 2018).

Ovarian function

Ovarian function postpartum can be monitored using ultrasonography. The use of B-mode ultrasound to determine follicular and corpus luteum size and the factors that affect the size of these structures have been reviewed (Soede *et al*., 2011; Langendijk, 2015; Soede and Kemp, 2015).

Recently, transabdominal colour Doppler sonography was used to assess ovarian blood flow characteristics during the course of the oestrus cycle in gilts (Stark, *et al*., 2019). These characteristics were perfused area, blood-flow velocity and intensity and resistance and pulsatility index. All parameters showed oestrous cycle-dependent patterns. Perfused area and blood-flow velocity were highest in diestrus, followed by proestrus, whereas the patterns of resistance and pulsatilty index were inversely proportional. Stark et al. (2019) concluded that ovarian blood flow was dependent on the stage of the oestrous cycle and was highest during the luteal phase and thus encouraged the use of colour Doppler ultrasonography to also investigate the reasons for ovary-based infertility, including corpus luteum insufficiency or seasonal effects on ovarian function.

Another technique that has recently been used is transvaginal ultrasound-guided biopsy of ovarian tissue. Björkman et al. (2017b) developed this method to obtain luteal tissue and to study corpus luteum function (Fig. 2). Biopsies were performed in four multiparous sows on days 9 and 15 of three consecutive oestrous cycles and the size and histological composition of the samples obtained were evaluated and the reproductive tract of the sows was monitored. Furthermore, biopsies were performed on 26 multiparous sows on days 10 and 13 after insemination and pregnancy rate, gestation length and subsequent litter size were evaluated. Altogether, tissue samples were obtained in 50% of the biopsy attempts. Sows from which one or more samples were obtained were older, heavier and had higher back fat compared to sows where no samples were obtained. No effects of the biopsies were observed on the cyclicity or reproductive organs of the sows or on subsequent corpus luteum diameter, pregnancy rate, gestation length and subsequent litter. The samples obtained had a diameter of 1 mm and contained heterogeneous tissue with various cell types. Björkman et al. (2017b) concluded that a transvaginal ultrasound-guided biopsy method for ovarian tissue can be used to study ovarian function. This method is relatively fast, minimally invasive and humane (Yun *et al*., 2017). Nevertheless, it should be noted that this method may not be used in young and small animals and tissue may be obtained in only half of the attempts. Furthermore, methods to select cells (e.g., laser microdissection) may be used to separate luteal from other ovarian cell types. In conclusion, advanced ultrasound techniques such as colour Doppler may be used to study ovarian dysfunction and seasonal infertility. A transvaginal ultrasound-guided biopsy of ovarian tissue has been developed for the pig and can be used for research purposes.

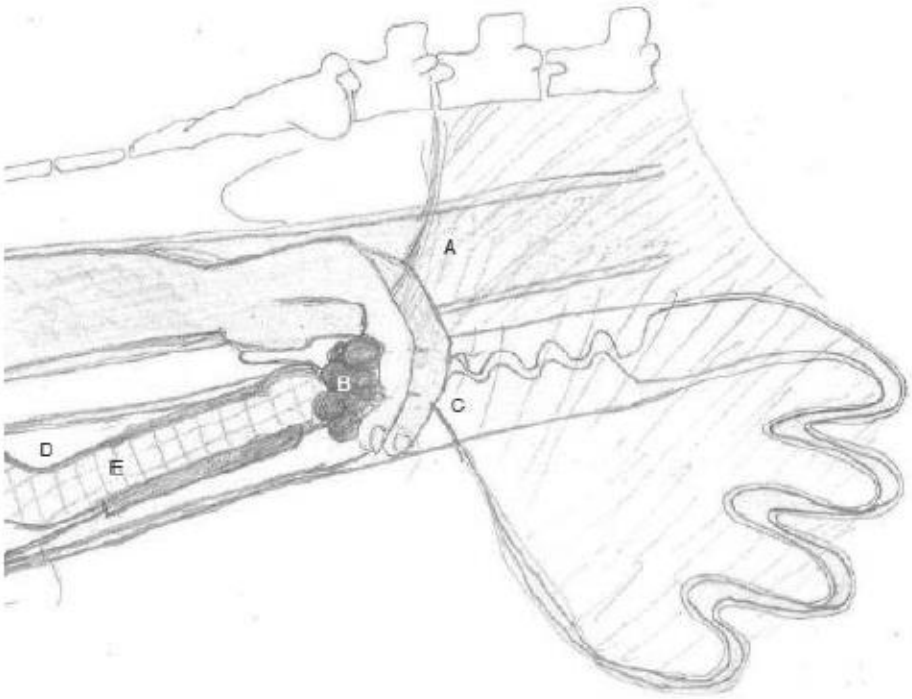

Figure 2. Illustration of the positioning of the transvaginal ultrasound-guided biopsy device. One hand is placed in the rectum (a) and the suspensory ligament of the ovary is palpated. After pulling on the ligament, the ovary is located and the proper ligament of the ovary held between the index and middle fingers, with the ovary on the palm side of the hand. The ovary (b) is pulled along the uterine cervix (c) into the peritoneal part of the pelvic cavity towards the cranial part of the vagina (d). With the other hand, the probe (e) is inserted into the vagina adjacent to the caudal part of the uterine cervix until the ovary becomes visible on the ultrasound screen (Björkman *et al*., 2017b).

Breeding hyperprolific sows

Artificial insemination

The pig is considered an intrauterine ejaculator (Senger, 2012). Therefore, deposition of semen in the uterus may be considered more of a physiological method than using the caudal portion of the cervix as the primary site of semen deposition. Generally speaking, intrauterine insemination (post-cervical, semen deposited into the uterine base) and deep intra-uterine (semen deposited into uterine horn) have been practiced to allow for a reduction of sperm number per dose, improved fertility or both (Watson and Behan, 2002; Martinez *et al*., 2002; Peltoniemi *et al*., 2009). A similar technique was developed to allow transcervical ET (Martinez *et al*., 2004). Results by Watson and Behan (2002) suggested that 2 or 3 billion spermatozoa/dose using intrauterine AI improve live-born litter size when compared with 1 billion spermatozoa/dose. However, it was subsequently shown that the number of spermatozoa may be reduced to 500 million spermatozoa/dose without detrimental effects on fertility (Martinez *et al*., 2006; Sumransap *et al*., 2007; Tummaruk *et al*., 2007; Roca *et al*., 2016; García-Vázquez *et al*., 2019). Post-cervical insemination seems to provide a number of advantages, such as a reduced sperm number requirement, less time required to perform insemination and faster genetic improvement (reviewed by García-Vázquez *et al*., 2019).

AI is used widely and globally by the industry. Despite these developments, some constraints such as cryopreservation of porcine semen prohibit efficient use of AI in international trade. Current research is focused on issues that affect AI such as freezing rates, cryoprotectants and storage (Yeste *et al*., 2016). Addition of antioxidants and the role of seminal plasma are being explored. As in other species like the horse, there seems to be large individual variation in semen freezability between boars (Yeste *et al*., 2016).

Timing of AI is another important factor in ensuring good fertility. Inseminating too early may not be successful, whilst if the sow is bred too late after ovulation, endometritis resulting in decreased litter size may be observed. Currently, two inseminations per oestrus is a commonly used practice to achieve a high pregnancy rate and large litter size. In a typical sow in oestrus, standing oestrus lasts for about 48 hours on average and ovulation occurs when two thirds of the standing oestrus has passed (Peltoniemi and Kemp, 2019). However, variation in the weaning to oestrus interval may affect the timing of AI. The later the sow enters oestrus after weaning, the sooner the optimal window for insemination (Roca *et al*., 2016). Ultrasound technology, in addition to a fixed-time AI after hormonal treatment protocol, may be used to pinpoint the optimal timing for AI in a specific herd, allowing for a good outcome after a single AI/oestrus (De Rensis and Kirkwood, 2016; Peltoniemi and Kemp, 2019). In conclusion, despite its wide use, application of AI in terms of dose deposition site within the uterus, cryopreservation of spermatozoa and timing towards a single AI are being further developed to advance the use of AI technology.

Use of ultrasound in the boar

Due to the increase in AI use in the pig breeding industry, there is interest in identifying males with suboptimal fertility to discard them or reduce their use (Pinho *et al*., 2018). This is especially important if a low number of spermatozoa per insemination dose is used and to meet the genetic potential of hyperprolific sows. In addition to proper mating management and insemination technique, high-quality semen from genetically superior sires is of high importance. Assessment of semen quality is one of the major evaluations for the selection of boars for breeding. For this reason, methods to assess the quality of semen before a boar starts reproductive life, or before using his semen for AI are required to predict their "fertility potential" (Pinho *et al*., 2018).

Therefore, studies in the past have focused on examining pre-pubertal and pubertal boars with ultrasound (Clark and Althouse, 2002; Clark *et al*., 2003; Ford and Wise, 2011; Kauffold *et al*., 2011; Pinho *et al*., 2018). The aims of these studies were to establish normal ultrasound parameters to identify subfertile boars and to establish correlations between these parameters and subsequent semen parameters. The first ultrasonographic evaluation of normal boar testes was performed more than 30 years ago. Cartee et al. (1986) compared the ultrasonographic appearance and testicle measurements with semen parameters in 14 Landrace boars but did not find any correlations. Nevertheless, they found significant differences in these parameters between 9-month-old and 15-month-old boars. Likewise, Clark et al. (2003) found an increased paired-testicular diameter in 18-month-old boars compared to 12-month-old boars. However, no correlation between paired-testicular diameter and the average total sperm number was established (Clark *et al*., 2003). Ford and Wise (2011) assessed pubertal development of boars derived from ultrasonographic determination of testicular diameter and length in 160 boars at 4, 5, 6, or 7 months of age. Boars were subsequently castrated and the weight of the testes, mean diameter of seminiferous tubules and percentage of the testis occupied by tubules were determined. At 4 and 5 months of age, although testicular diameter correlated positively with diameter of seminiferous tubules, this relationship was not significant at older ages.

Previously, Kauffold et al. (2011) conducted a study to describe the echogenicity pattern of the epididymis in boars using B-mode ultrasound together with grey-scale analysis. Ejaculate parameters were also determined for investigating the relationships between them and ultrasonographic findings. In the ultrasound images, all parts of the epididymis appeared homogeneous and regular in echotexture. However, while the echotexture of the caput and the corpus was normal, the cauda had a rather marbled echotexture (Kauffold *et al*., 2011). The echogenicity, expressed as the mean grey value, was different in comparison between the three segments of the epididymis (caput > corpus > cauda). The echotexture of the caput of the epididymis correlated slightly positively with the ejaculate volume and the total sperm count. Thus, ultrasound examination of the epididymis with analysis of caput echotexture provides information on semen parameters before semen collection.

Ultrasound examination of the accessory sex gland has also been successfully conducted and the appearance of each accessory sex gland has been described (Clark and Althouse, 2002) but no correlations with semen parameters have been made. It is unlikely that ultrasound examination of accessory sex glands can be implemented into practice. This method is quite challenging and dangerous as it requires rectalizing the boar. It is also not applicable in pubertal boars because of the anatomically small pelvic canal. This method may only be used in adult boars as a diagnostic tool in the work-up of subfertility. In conclusion, ultrasonographic determination of testicular diameter can be used to monitor testicular development during puberty but no correlations have been established with total sperm number in the ejaculate or with subsequent reproductive performance. It would be of particular interest to study whether ultrasound of the epididymis could also be used in prepubertal or pubertal boars to predict their future "fertility potential."

Embryo transfer in sows

Global need for foods and animals requires the development of strategies beyond traditional breeding to ensure offspring of high genetic quality and productivity while preserving genetic diversity. Demand for pork has been rising in recent decades due to changes in consumption patterns as incomes increase in developing countries with rapidly growing economies. Genetics from superior sows best meeting with breeding goals are sought internationally. The export of live animals is contentious due to animal welfare issues, biosecurity, economy and sustainability due to long transport times and crossing of borders. The challenges with AI regarding export of porcine genetics have been discussed earlier. Although sensitive to chilling and highly susceptible to intracellular ice formation, recent progress in oocyte and embryo cryopreservation is promising (Saragusty and Arav, 2011; Cuello *et al*., 2016; Nohalez *et al*., 2018). Porcine embryos have the potential to substantially accelerate genetic gain in pig populations and to facilitate international transport of genetics, while decreasing the carbon footprint due to reduced live animal transportation. New knowledge on ET in sows is therefore essential. ET in pigs was described for the first time in 1950 at the Pig Breeding Research Institute in Poltava, Ukraine (Kvasnitski, 1950). To our knowledge, no standardized and commercial ET service in sows exist. Today, porcine ET is carried out in private companies and institutes engaged in biomedical research (Petersen *et al*., 2008; Zheng *et al*., 2016). The main oocyte source for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) is ovaries mainly from prepuberal sows collected from the local slaughterhouse. Antral follicles are punctured in the laboratory for oocyte collection. Embryo collection after slaughter has the disadvantage of using donor sows only once.

Additionally, the stage of the oestrus cycle at slaughter or the reason for slaughter is commonly unknown. Therefore, when oocytes are recovered in this manner, they are heterogenous in terms of developmental competence (Bertoldo *et al*., 2012).

To establish a viable, commercial ET concept, oocyte and embryo retrieval should be feasible for trained veterinarians under field conditions. This suggests non-surgical oocyte or embryo retrieval. Recent reports by Björkman et al. (2017b) and Yun et al. (2017) are encouraging as transvaginal ultrasound-guided biopsies of the ovaries may not cause appreciable pain or distress in non-sedated sows. However, no successful non-surgical embryo collection has been reported in pigs, except for the studies of Hazeleger et al. (1989) and Kobayashi et al. (1989) that used surgical resection of uterine horns (Brüssow *et al*., 2000). The major reason for this restriction is the anatomy of the porcine genital tract.

Non-surgical ovum pick-up (OPU) has not gained significant importance in live sows. This is probably also due to anatomical challenges and the fact that sow ovaries must be placed near the cervix for proper visualization before transvaginal follicle puncture and oocyte isolation can be conducted. Rectal palpation of pig ovaries in can be challenging due to the long uterus horns and the limited length of the rectal mesentery (Okuyama *et al*., 2017). A recent report from Japan investigated transvaginal OPU and examined the effects of different aspiration vacuum pressures and the phases of oestrous cycle on oocyte recovery, the morphology of cumulus oocyte

complexes (COCs) and blastocyst formation in Berkshire pigs. The proportion of oocytes with several compact cumulus layers in 90 mmHg (27.2%) was significantly higher ($P < 0.01$) than in 120 mmHg (5.2%). The OPU technique enables repeated oocyte collection from highly valuable live pigs (Ikoma *et al*., 2014).

IVM, IVF and IVC have been extensively investigated in pigs taking known obstacles such as polyspermy into account (Romar *et al*., 2012; Yuan and Krisher 2012; Gil *et al*., 2017). IVM influences both nuclear and cytoplasmatic maturation of porcine oocytes and therefore pronuclear formation and cleavage (Laurincik *et al*., 1994). By modifying maturation media via addition of thiols and organic osmolytes, low incidents of male pronuclear formation after IVF can been counteracted (Funahashi and Day 1993). Polyspermic penetration of porcine oocytes range between 13% and 90% (Niwa, 1993). By simulating the oviductal environment, polyspermy is reduced and the final IVF increases the final efficiency by more than 48%. This was seemingly due to reduced sperm motility and lower capacitating status (Soriano-Úbeda *et al*., 2017). For IVC, NCSU23 containing taurine and hypotaurine promote the highest success rates in development from the single cell to blastocyst stage (Brüssow *et al*., 2000).

Table 3. *In vitro* and *in vivo*-related embryo transfer (ET) technologies in sows.

	Procedure	Need for research and development	References
I.	Selection of the indicated sows with superior fertility traits	Follicular fluid composition, seasonal infertility and follicle size effects on oocyte developmental competence and embryonic survival.	Peltoniemi *et al*., 1999; Bertoldo *et al*., 2013; Da Silva *et al*., 2018.
I.	Oocyte/ embryo retrieval from donor sows	Flushing equipment for sows, skill acquisition *in vivo*	Hazeleger *et al*., 1989; Kobayashi *et al*., 1989; Brüssow and Rátky 1996; Besenfelder *et al*., 1997
	Ovum pick-up (OPU) in donor sows	OPU device for sows, OPU technique optimization, skill acquisition on live animals	Brüssow *et al*., 1997; Antosik *et al*., 2007; Ikoma *et al*., 2014
II.	Gametes: *In vitro*	*In vitro* maturation (IVM), fertilization (IVF) and culture	Funahashi and Day 1993; Laurincik *et al*.,, 1994; Brüssow *et al*., 2000; Romar *et al*., 2012; Gil *et al*., 2017; Soriano-Úbeda *et al*., 2017
	Oocytes/ embryos: *In vitro*	Cryopreservation/ vitrification of embryos/ oocytes	Berthelot *et al*., 2000; Cuello *et al*., 2016; Nohalez *et al*., 2018
III.	Recipient sow synchronization	Hormonal synchronization protocol	Wilson *et al*., 1998; Martynenko *et al*., 2004; Brüssow *et al*., 2018
	ET on recipient sows	ET into the cranial portion of the corpus uteri	Webel *et al*.,, 1970; Galvin *et al*.,, 1994; Hazeleger and Kemp 1994; Li *et al*., 1996; Yonemura *et al*., 1996; Rátky *et al*., 2001; Martinez *et al*., 2004; Martinez *et al*., 2016

The selection of recipient gilts or sows will have a major impact on ET results (Brüssow *et al*., 2018). Recipient sows must be hormonally synchronous to the donors. Both the breed of the recipients and the recipient uterine environment can influence the ET results. Meishan pigs have been suggested as recipients due to their higher placental efficiency (Wilson *et al*., 1998), and post-ovulatory AI followed by ET could increase ET efficiency (Martynenko *et al*., 2004).

Embryos have mainly been transferred surgically into recipients, either into the oviducts or the cranial tip of the uterus. The need for surgical ET has certainly hampered the progress of bringing this method closer to implementation under field conditions. Multiple research groups have attempted to develop a nonsurgical ET procedure (Galvin *et al*., 1994; Hazeleger and Kemp 1994; Li *et al*., 1996; Yonemura *et al*., 1996; Martinez *et al*., 2004; Martinez *et al*., 2016).

Considerable research effort is still necessary before ET can be offered as a commercial breeding tool (Tab. 3). In conclusion, the prospects for ET in pigs have improved with recent developments in cryopreservation of oocytes and embryos. However, despite some recent developments, repeated collection of oocytes from live animals and the need for a surgical ET remain as bottlenecks for wider commercial use of ET in genetic improvement and international trade.

Conclusions

Management of the large litters of the present hyperprolific breeds involve a sufficient appreciation of the physiological and behavioural needs of the sow prior to and around farrowing. Meeting these needs improve the capacity of the sow to produce adequate colostrum, the quantity and quality of which can be managed and monitored by modern tools such as the Brix test. Feeding sows with higher levels of fibre and decreasing the time lapse between last feeding prior to onset of parturition provide new insights for the management of parturition. Use of intermittent suckling will hasten the production cycle while extending the lactation length of small piglets. This would make the process of weaning easier for piglets, but metabolically more demanding for the sow. Recent developments in real-time ultrasonography, together with ultrasound-guided biopsy techniques provide new and novel means to study inflammatory processes of the mammary gland, dysfunction of the uterus and the ovary, timing of AI and seasonal infertility. Advancements in cryopreservation of semen, oocytes and embryos appear encouraging in terms of establishing a foundation for further development of breeding, ET and trade of germ cells and embryos across borders. While litter size in domestic European pig breeds has doubled over the past two decades, duration of farrowing has extended four to five-fold. The birth weight of piglets and colostrum intake per piglet continue to decrease. These challenges of modern breeding need to be addressed in the future. We also urge more research into this area to resolve these emerging challenges of the hyperprolific sows lines.

Authors contributions

OP: Funding acquisition, conceptualization, writing - original draft, review & editing, commenting all phases; SB: Conceptualization, writing – original draft, review & editing, commenting all phases; MO-M: writing – original draft, review & editing, commenting all phases; CO: Conceptualization, writing – original draft, review & editing, commenting all phases.

Conflict of interest

There is no conflict of interest regarding any of the authors.

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SHORT COMMUNICATIONS

STUDENT COMPETITION

1. Generation of vascular deficient porcine embryos

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Keywords: organ generation, CRISPR/Cas9, porcine embryos

Organ transplantation is, in many cases, the only life-saving treatment for end-stage organ failure, but the main problem is the shortage of these organs. Currently, organs donations are insufficient to cover demand, so other alternatives are needed. Generation of human organs from pluripotent stem cells (PSCs) in animal recipients would provide an endless source of organs for clinical use. Blastocyst complementation is an extraordinarily promising approach to fulfil this unmet medical need. This technique allows the development of an organ/tissue that a genetically modified embryo is unable to form. It consists in the microinjection of PSCs into the cell type/organ-deficient preimplantational embryo and the completion of the embryonic development in the uterus of a foster mother. In this environment, the microinjected PSCs colonize the empty developmental niche and contribute entirely to its formation. Using blastocyst complementation strategy, a cell type/organ deficient pig embryo could be used to generate a humanized/human organ, owing to pigs are physiologically similar to humans as well as the size of their organs. Therefore, as first step, organ/tissue deficient pig embryos need to be generated. Because endothelial cells play a very relevant role in organ rejection upon xenotransplantation, we are generating vascular deficient pig embryos using CRISPR/Cas9 technology to erase ETV2 gene, since it is a master regulator of hematoendothelial lineages. For a preliminary study, we designed 5 different guides (Benchling, USA) against pig's ETV2 gene and were tested *in vitro* on pig's fibroblast. Genome editing was analyzed by Surveyor Mutation Kit (IDT, Spain) and 4 out of 5 guides showed cleavage capacity. Subsequently, the 4 selected guides were individually microinjected with Cas9 protein complex (100 ng/µl Cas9 protein and 50 ng/µl sgRNA) (IDT, Spain) 6h post fertilization into 1 cell-stage pig embryos. The embryos were cultured until blastocyst stage in a humidified atmosphere at 38.5ºC, 5% O_2 and 5% CO_2. Next, DNA amplification by PCR were performed before the deep sequencing analysis. Mutant embryos were obtained with the four microinjected guides, but only two of them achieved biallelic ETV2 disruption, although at low efficiency (Guide 1; 5% KO, Guide 2; 5% heterozygous, Guide 3; 5% KO and 5% Mosaic, Guide 4; 10% Mosaic and 5% heterozygous). Based on the foregoing, future experiments are required in order to optimize the generation of vascular deficient embryos. In the future, efficient ETV2 KO pig embryos could be used as recipient for human chimera-competent cells, that one may generate human vascularized organs. Moreover, this strategy can be expanding to any other organ in a deficient specific model embryo thus permitting the generation of fully humanized organs in a livestock animal and one day, resolve the organ shortage in the clinic.

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2. Vital tagging of potential naïve pluripotency in a mouse model

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Keywords: Pluripotency, Naive Cells, Endogenous retrovirus

Recent studies suggested that two distinct pluripotent states exist in mouse and human embryonic stem cells that are termed as naïve and primed. The naïve pluripotency constitutes a ground state with full differentiation potential, whereas the primed state has a restricted differentiation potential (Boroviak et al. *Nat. Cell Biol.* **16**, 516–528). The preimplantation epiblast represents a naïve pluripotent state, whereas the post-implantation epiblast reaches a primed state. Recently, it was shown that the expression of particular classes of endogenous retrovirus (ERV) mirror the naïve state in murine and human pluripotent cells (Wang et al. Nat Protoc. 11(2):327-46, 2016). We hypothesized that the naïve pluripotency could be tagged in vivo by a fluorescent reporter construct driven by the long terminal repeat (LTR) promoter of the human ERV7. To achieve this, the LTR7-GFP construct in a Sleeping Beauty (SB) transposon together with the SB transposase vector were co-injected into the cytoplasm of murine zygotes. The zygotes were transferred to surrogate animals, and subsequently the newborn mice were genotyped. Four founder transgenic animals were identified and used to establish stable lines. Expression of the LTR7-GFP was restricted to a subpopulation of the inner cell mass of late murine blastocysts. Additionally, expression of the reporter could be reactivated by reprogramming of fetal fibroblasts to induced pluripotent stem (iPS) cells. In somatic cells, no reporter expression was detected by fluorescence microscopy and RT-PCR. Thus the vital reporter labels a subpopulation of pluripotent cells in vivo and in vitro. Currently, additional experiments are ongoing to characterize this subpopulation in detail. This mouse model can be used to understand the development of pluripotency during early ontogenesis, and may provide insights into the regulation of pluripotency especially by temporal divergence in expression patterns of various endogenous retroviruses.

3. A proteomic approach to decipher embryo-maternal interactions in the oviduct

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K*eywords:* oviductin, mass spectrometry, morula

In vivo, the bovine embryo develops in contact with the oviductal fluid (OF) up to the 8-cell or morula stage. Oviduct proteins are known to be highly regulated across the estrous cycle. However, up to now, using immunohistochemistry, only few proteins, such as oviductin and osteopontin, have been identified as interacting with the developing embryo. The aim of this study was to use two complementary proteomic approaches: (i) bottom-up using nanoliquid chromatography coupled to tandem MS (nanoLC-MS/MS), and (ii) profiling by Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry (MS), to characterize new OF proteins interacting with the early bovine embryo. Pairs of bovine oviducts were collected at a local slaughterhouse and transported to the lab on ice. Only oviducts at the post-ovulatory stage (small hemorrhagic *corpus luteum* (CL)) and ipsilateral to the CL were used for OF collection by squeezing (n=22 cows). After 2 centrifugations (2000 g, 10 min then 12000 g, 10 min, 4°C), the OFs were pooled, aliquoted in small volumes and stored at −80°C. *In vitro* matured oocytes from slaughterhouse ovaries were fertilized with frozen semen. Zygotes were then cultured in SOF medium without proteins for 5 days. Pools of 25 embryos at the morula stage were incubated in 25 µL of OF (treated) or SOF (control group) for 6 h at 37°C then rinsed 3 times and stored at −80°C before MS analyses. For each proteomic approach, morulas from four replicates were analyzed. Bottom-up analyses were performed on pools of 25 embryos after protein extraction and trypsin digestion (n=4 pools/condition) and nanoLC-MS/MS (Tims-TOF, Bruker). Profiling analyses by MALDI-TOF (UltrafleXtreme, Bruker) in the 2-30 kDa mass range were performed on intact individual embryos (n=40 embryos/group). In parallel, the OF was analyzed both by nanoLC-MS/MS and MALDI-TOF MS. Proteins were considered as embryo-interacting proteins if they were detected in the OF and detected in treated but not in control embryos, or detected with significantly higher abundance in treated *vs.* control embryos (fold-change of mean normalized spectral counts > 2; p-value of t-tests < 0.05). By the bottom-up approach, a total of 561 proteins were identified, among which 21 OF interacting with embryos, including oviductin (OVGP1), galectin-3, transgelin-2, and several annexins (ANXA1, 2, 4). Among interacting proteins, seven had a signal peptide or were reported as secreted via non-classical secretory pathways. By the profiling approach on single embryos, a total of 221 masses were detected, among which five OF interacting with embryos. These masses were annotated as gluthatione S-transferase and several ribosomal proteins. In conclusion, high throughout proteomic methods were successfully used to identify embryo-interacting proteins originating from OF. Further analyses are requested to specify in which embryo compartments (zona pellucida, perivitelline space, blastomeres) these proteins are localized and which roles these interactions could play.

4. Proteomic analysis of ejaculated and epididymal sperm associated with freezability in Iberian ibex (Capra pyrenaica)

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Keywords: proteome, small ruminants, spermatozoa

The sperm proteome is known to affect cell cryoresistance and is reported to differ between epididymal and ejaculated sperm in small ruminants (C.J. Li *et al.*, Animal Reproduction Science, 173, 1–7, 2016; T. Pini *et al.*, Journal of Proteome Research, 15, 3700-11, 2016). However, studies aiming at identifying proteins involved on sperm freezing-tolerance are scarce. The aim of this study was to investigate the association between the freezing capacity and the proteome of ejaculated and epididymal sperm of the Iberian ibex. Ejaculates were collected from anesthetized animals by transrectal ultrasound-guided massage of the accessory sex glands combined with electroejaculation (n = 6), whereas epididymal samples were collected post-mortem by flushing (n = 6). After seminal/epididymal fluid removal, sperm cells were conventionally cryopreserved by slow freezing. Sperm quality parameters were assessed in fresh and frozen-thawed sperm to evaluate sperm freezability. Motility parameters were assessed by computer-assisted sperm analysis system and membrane and acrosome integrity were assessed by fluorescence microscopy. Tandem mass tag-labeled peptides were analyzed by high performance liquid chromatography coupled to a mass spectrometer (MS; Orbitrap Fusion Lumos) in three technical replicates. A false discovery rate of 1% was applied as protein identification threshold. The MS raw data were processed in Proteome Discoverer 2.2.0.388 and the statistical analysis was done using the moderated t-test of the R package limma. Epididymal sperm showed higher post-thaw total motility (57.46±8.58% *vs* 23.19±3.05%), progressive motility (37.70±6.38% *vs* 8.65±1.83%), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) than ejaculated sperm ($P<0.0001$). Post-thaw acrosome (89.50±0.56% *vs* 61.95±3.48%; $P<0.001$) and membrane integrity (57.33±7.26% *vs* 35.73±3.39%; $P<0.05$) were also higher in epididymal sperm. A total of 1660 proteins were quantified in both epididymal and ejaculated samples among which 310 proteins (18.7% of the total) were differentially expressed between both types of sperm when using a cut-off for significance (adjusted p-value <0.05) and fold-change (abs(log2 (fold-change)>1). Out of those proteins, 212 were significantly more abundant in epididymal sperm and 98 were more abundant in ejaculated sperm. Peroxiredoxin-4 (PRDX4) and superoxide dismutase [Cu-Zn] (SOD1) are proteins involved in cell protection against oxidative stress and were more abundant in epididymal than ejaculated sperm. Heat shock protein HSP 90-alpha (HSP90AA1) is a chaperone involved in structural maintenance and cell cycle control that was also more abundant in epididymal sperm. Besides updating the sperm proteome of small ruminants, this study revealed differences of cryoresistance between epididymal and ejaculated sperm of the Iberian ibex contributing to identification of candidate markers of sperm freezability.

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5. Culture under the physiological temperature registered along the reproductive tract of female pigs improves the blastocysts yield in vitro

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Keywords: temperature, assisted reproductive technology, pig

Despite temperature being one of the main external environmental factors that affects gene expression, thereby influencing the way an organism develops (Lobo, I. Nature Education vol. 1(1), p39, 2008), its oscillation pattern is barely taken into account in *in vitro* embryo production (IVP). Few studies obtaining direct measurements of temperature within the reproductive tract of the female pig have been reported and the procedures used in those previous studies have been surpassed today by the use of cutting-edge devices. While no reference temperature values have been published to date in the pig uterus, a temperature gradient within oviduct and ovary was described (Hunter et al., Reprod Biomed, vol. 24 (4), p. 377, 2012). We hypothesized that transferring physiological temperature conditions given in nature to IVP could help to reduce the prevalence of polyspermy after insemination in swine and improve the efficiency of that biotechnology in this species. Hence, we aimed to measure temperature within the isthmus, ampulla and uterus of sows (n=15) and use these values in IVP protocols. To this end, oviductal and uterine temperature was monitored adopting a laparo-endoscopic single-site surgery assisted approach along with a flexible and thin miniaturized probe previously used by López-Gatius and Hunter (López-Gatius and Hunter, Reprod Dom Anim, vol. 52(3), p. 366, 2017). The same temperature was retrieved in ampulla and isthmus (37.0 ± 0.5ºC) whereas a significantly higher value was found in uterus (38.7 ± 0.1ºC).This finding suggest that a lower temperature is required during IVF, while a higher temperature is needed during the embryo development. To test this assumption, *in vitro* matured oocytes were inseminated at two different temperature conditions: the routinely used value in pig IVP (38.5ºC) and a lower value (37ºC), recorded in the oviduct. At 18-20 hours post-insemination (hpi), putative zygotes were transferred to embryo culture medium and maintained at 38.5ºC in both groups. A sample of presumptive zygotes (n=218) was fixed and stained to assess the fertilization rates. At 180 hpi, development to blastocyst stage was evaluated. Data were analysed by one-way ANOVA. A P-value <0.05 was considered to denote statistical significance. Monospermy rate was significantly higher at 37ºC compared to 38.5ºC (65.0 ± 6.1% *vs.* 46.0 ± 6.1%), not being enough to improve the IVF yield. However, an increase in blastocyst yield when embryos were fertilized at 37ºC (39.0 ± 3.6%) was observed compared to those fertilized at 38.5ºC (24.0 ± 2.8%). Our study supports the recent data published by Hino and Yanagimachi in mice (Hino and Yanagimachi, Biol Reprod, in press, 2019) claiming that peristaltic movement within the oviduct and the continued ad-ovarian transport of oviductal fluid make a temperature gradient within the oviduct unlikely existent. In contrast, we found a temperature gradient between oviduct and uterus.

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TAI/FTET/AI

6. Comparison of INRA96 and Andromed as an extender for alpaca epididymal spermatozoa

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Breeding animals by artificial insemination rather than by natural mating has many advantages, for example, to prevent the spread of infectious disease and to allow males of superior genetic merit to produce offspring from a large number of females. However, the technique of artificial insemination is not well developed in alpacas for several reasons, one being the difficulty of working with the viscous ejaculate. Thus, it is difficult to develop protocols for semen handling, including choosing a semen extender. A first step in the development of such a protocol could be to use epididymal spermatozoa to test semen extenders. Two commercial semen extenders, Andromed (A; Minitüb; Tiefenbach, Germany) and INRA96 (I; IMV Technologies, L´Aigle, France), were chosen for this study. Neither of these extenders contains material of animal origin. Objective: to compare the two semen extenders for their suitability for alpaca epididymal spermatozoa. Materials and methods: scrotal contents were obtained from castration of males (n=10) for husbandry purposes. After removal from the animal, the organs were placed in a plastic bag containing phosphate buffered saline and were sent overnight to the laboratory at the Swedish University of Agricultural Sciences (SLU) in a Styrofoam box with a cold pack. This type of packaging is used to transport stallion semen and maintains the temperature at approximately 6 ºC overnight. The tunica vaginalis, connective tissues and blood vessels were removed; after isolating the cauda epididymis from the testis, it was placed in warm (37ºC) semen extender. From each animal, one cauda epididymis was placed in A and the other in I; several cuts were made in the epididymis to allow the contents to flow out. After incubation for 10 minutes at 37 °C, sperm motility was measured by computer assisted sperm analysis (CASA; SpermVision, Minitüb), membrane integrity (MI) was assessed after staining with SYBR14/propidium iodide (Live-Dead Sperm Viability KIT LIT L-7011; Invitrogen, Eugene, OR, USA), and acrosome status was determined by staining with FITC-conjugated peanut agglutinin (Sigma, St. Louis, USA). The CASA analysis was repeated incubation for 30 minutes. Means were compared by mixed model using the SAS® software (version 9.3); significance was set to $P \leq 0.05$. Results: LSMEAN (± SEM) after 10 minutes for A and I, respectively, were as follows: total motility 19±5% vs. 21±5% (not significant, NS), MI 58±9% vs. 56±9% (NS); intact acrosomes 65±7% vs 54±7% (NS). Total motility in A and I after 30 minutes was 29±4% and 35±4% (NS), respectively. Progressive motility in I after incubation for 30 minutes was 12±4% compared to 25±4% after 10 minutes ($p<0.05$). However, progressive motility in A was not different between the two time points (11±4% vs. 17±4%, respectively). Conclusion: viable epididymal spermatozoa could be obtained from the material even after overnight transport. There were no differences between the two extenders in the sperm parameters evaluated. Therefore, either extender could be used for alpaca spermatozoa and should be tested in an insemination trial.

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7. Influence of parturition number of the recipient on an embryo transfer programme in wool type ewes

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Keywords: ewes, ovulation rate, pregnancy

Embryo transfer is important for the multiplication and rapid propagation of sheep breeds of high genetic merit. The selection of the most appropriate genotypes of recipient ewes is essential to obtain high pregnancy rates. The objective of this study was to evaluate the fertility of two groups of ewes (nulliparous and multiparous) exposed to an embryo transfer program. The study was conducted from January to February of 2019 at the commercial sheep farm "Poza Rica", which is located in a temperate area named Singuilucan, in central Mexico. In total, 142 healthy and good body condition Hampshire ewes were used, from which 97 were nulliparous and 45 multiparous. The ewes were synchronized with intravaginal sponges containing 20 mg of micronized cronolone (Chrono Gest, Intervet, Netherlands), which were inserted for 12 days. On day 10, the ewes were injected intramuscularly with 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Kathadin rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined by laparoscopy. The recipients received an embryo of transferable quality (compact morula or blastocyst) within 3 h after its collection, coming from Dorper donor ewes using a laparoscope and standardized procedures. The embryos were kept in holding medium (Syngro, Vetoquinol, Canada) were transferred using a capillary glass tube in the ipsilateral horn to the ovary in which ovulation was recorded, and the presence of the best quality corpus luteum was determined based on its size. On day 35, pregnancy diagnosis was carried out using an ultrasound machine and a 3.5 MHz transabdominal probe (Aloka Prosound 2, Japan). The results of the incidence of estrus and pregnancy rate were analyzed as categorical variables with the GENMOD procedure, and ovulation rate with the GLM procedure, both of them available in SAS. All the ewes were detected in estrus in both treatments. The incidence of estrus was different ($p<0.05$) for ewes from nulliparous and multiparous at 24 h (62.9 and 55.5%), 30 h (32.9 and 45.4%), and 36 h (4.1 and 0%) after sponge removal. Ovulation rate was higher ($p<0.05$) in multiparous than nulliparous ewes (2.11 ± 0.12 vs 1.76 ± 0.08). Moreover, the pregnancy rate was lower ($p<0.01$) in nulliparous than multiparous ewes (29.9 vs 68.9%). In conclusion, under the conditions of the study, the results showed the feasibility of using multiparous Hampshire ewes as embryo recipients.

8. Effect of age of the recipient on an Embryo Transfer programme

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The reproductive technique of embryo transfer has been used to intensively reproduce high genetic merit animals of several species in different countries. The success of the technique relies on the control of every step to achieve high pregnancy rates. The objective of the study was to evaluate the fertility of two groups of ewes (nulliparous and multiparous) subjected to an embryo transfer program. The study was conducted from November to December of 2018 at the commercial sheep farm Rancho Poza Rica, which is situated in Singuilucan, Hidalgo, Mexico including a total of 46 ewes, from which 20 were nulliparous Katahdin ewes (T1) and 26 were multiparous Katahdin ewes (T2). The ewes were healthy, in good body condition (3.0) and were synchronized with intravaginal sponges containing 20 mg micronized cronolone (Chrono Gest, Intervet, Netherlands), inserted for 12 days. On day 10, the ewes were intramuscularly treated with 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Pelibuey rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined as a number of corpora lutea observed in ovaries during laparoscopy. The recipients received one transferable compact morula or blastocyst within 2 h after embryo recovery from a Charolais donor ewe using laparoscopy. The embryo recovered into holding medium (Syngro, Vetoquinol, Canada) was transferred using a capillary glass tube in the ipsilateral horn to the ovary in which ovulation was recorded or the presence of the best quality corpus luteum was observed, determined on the basis of its size. On day 45, pregnancy diagnosis was conducted using an ultrasound machine and a 3.5 MHz transabdominal probe (Draminski Animal profi 2, Poland). The results of incidence of estrus and pregnancy rate were analyzed as categorical variables with the Proc GENMOD function and ovulation rate with the procedure Proc GLM, both of them available in SAS. It was considered a $p \leq 0.05$ to establish significant differences between treatments. The general percentage of estrus was similar for ewes from T1 and T2 (76.9% vs 100%). The incidence of estrus was different ($p<0.05$) only for ewes from T1 and T2 at 24 h (50% vs 90%), but similar ($p>0.05$) at 30 h (19.2% vs 10.0%) and 36 h (7.7% vs 0%). Ovulation rate was higher in ewes from T2 compared to ewes from T1 (2.0 ± 0.22 vs 1.27 ± 0.13). Furthermore, 50% of the ewes were pregnant in both treatments. In conclusion, the results showed the feasibility of using Katahdin ewes as embryo recipients regardless of their age and parity.

9. Import of Belgian Blue embryos in tropical Indonesia: birth of first calves

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Keywords: Belgian blue cattle, Indonesia

The Belgian Blue breeding program has been started to improve local beef production in Indonesia. Belgian Blue cattle (BB) are characterized by a double-muscled phenotype caused by a deletion within the myostatin gene. Animals present less bone and fat, more muscle, as well as a higher muscle bone ratio than other breeds (Kolkman et al., Reprod Domest Anim 47, 365, 2012). Indonesia is Southeast Asia's biggest economy, and its population growth, rising incomes, and changing public tastes, caused the beef consumption in this country to increase over time (increase of 4.66% per year), while the growth of domestic beef production is only 3.20 % per year. As a result, beef import rose by 21.58% annually (Kusriatmi et al., Journal of the ISSAAS 20, 115-130, 2014). One of the efforts that the Indonesian government did was importing BB frozen semen and embryos from Belgium into Indonesia (Agung and Syahruddin, 16th AAAP Animal Science Congress 2, 10, 2014). Consequently, the first BB calves were born in South Asia following successful embryo transfer (ET). The result of BB born by ET is 94, with the total of pregnant cow is 138 from 588 of total pregnant checked by rectal palpation; while the result of BB born by artificial insemination (AI) is 168, with the total of pregnant cow is 278 from 545 of pregnant checked by the same method (Indonesian Animal Husbandry, 2019). From those result, we could find that the successful percentage of AI is higher than the ET application with 51% and 23% respectively. Furthermore, all of BB calves by AI was born by normal parturition, while the BB calves by ET was born by C-section. In order to follow up this program and to predict the future of BB in this tropical environment, we compared the birth weight of BB pure breed in Indonesia (by ET) with the crossbreeds (by AI with several Indonesian local cattle) with a total sample size of 105 calves. Furthermore, we also compare the birth weight of BB calves born by ET in Indonesia versus calves born in Belgium, with a total sample size of 115 new-born calves. The results indicated that the mean of BB pure breed birth weight in Indonesia 51,23 kg is higher rather than the crossbreed of BB with Friesian Holstein, Simmental, Limousine, Peranakan Ongole, Angus and Madura; with their mean of birth weight 44,80 kg; 43,5 kg; 36,14 kg; 29,59 kg; 46,6 kg; and 25,5 kg respectively. In addition, the result of birth weight of BB in Indonesia versus in Belgium showed that there are a significant different in their birth weight (α= 0,042 < 0,05), which the mean of BB birth weight in Belgium (52,39 kg) is higher rather than the mean of BB birth weight in Indonesia (52,00 kg). This significant difference might be related to the tropical condition in Indonesia. Based on (Brody S, Journal of Dairy Science 39, 6, 715-725, 1956), the environmental comfort zone for European cattle ranges between -1 and 15°C, while the temperature in Indonesia is on average 18°C-30°C (Indonesian Directorate of Animal Husbandry, 2019). In addition, the BB is assumed to be more susceptible to heat stress than most other breeds, owing to the reduced oxygen transport efficiency, caused by the relatively small volume of their heart and lungs in comparison with their body volume (Grobet et al., Mammalian Genome 9, 210, 1988).

OPU – IVF and ET

10. Effects of an oil covered culture system on bovine in vitro produced embryos

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Keywords: Embryo, Oil, Cattle

Embryos are usually produced in culture systems with an oil overlay, which conveys protection against the evaporation of water and microbial contamination. The oil can also release toxic substances and absorb essential components, such as hormones, which adversely affect the quality of the oocytes and the development of embryos in vitro. In addition, an oil overlay can be prohibitive when applying particular analysis such as concentration of lipophilic substances in medium, as steroids. The aim was to validate an oil-free bovine IVP system. This study compares bovine IVP with and without an oil overlay. Groups of 20 cumulus-oocyte-complexes (COC) collected from abattoir-derived ovaries were matured in tissue culture medium with BSA and eCG/hCG for 24 h with 5% CO2, fertilized in Fert-TALP for 19 h with 5% CO2 and cultured in SOFaa with 5% CO2 and 5% O2, all steps took place at 39°C. The quantity of medium in both groups (with and without an oil overlay) and throughout all stages of IVP was maintained at a volume of 100µl. The oil group was covered with 75µl paraffin oil (IVF Bioscience, Falmouth, UK). The maturation stage of oocytes was assessed using fluorescence staining (Hoechst 33342) after 24h of maturation. The developmental stage (number of blastocysts) were evaluated on day 8. The morphological quality of expanded day 8 blastocysts was determined by live-dead-staining (total cell number as well as ratio of live and dead cells). At least ten replicates were done. The statistical analysis was performed with 'R'. Evaluation of maturation and development rates were analysed using a binomial test. Data obtained from the live-dead-staining were analysed using a t-test. Oocytes matured in the absence of an oil overlay had significantly ($p < 0.05$) higher maturation rates (71.5 ± 6.8%) when compared against matured in medium with an oil overlay (60.2 ± 9.3%). The developmental rate was significantly higher after culture without oil overlay (without oil: 38.4 ± 14.8%, with oil: 33.5 ± 12.6%; $p < 0.05$). The total cell number and the live-dead-ratio was not significantly ($p > 0.05$) different (total cell number: without oil: 130.0 ± 30.2, with oil: 119.0 ± 30.0; live-dead-ratio: without oil: 20.5 ± 11.5, with oil: 19.0 ± 8.0). The osmolarity did not differed between both groups during the IVP. Currently, the medium is analysed with regard to steroid concentrations via radioimmunoassay. So far, based on the higher maturation and development rates, bovine oil-free IVP-systems can be suggested as an alternative to oil covered medium, especially for maturation.

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11. Individual serum-free and oil-based oocyte-to-embryo in vitro culture system is yielding high blastocyst rates and can be used as a basic system for individual follow-up

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Keywords: serum- free and individual culture system, embryo development, bovine

Bovine *in vitro* embryo production (IVP) is routinely performed by culturing oocytes in group at ratio 1:2 (25/50µL droplets). We have recently shown that individual culture of bovine embryos in SOF-medium supplemented with 0.4 % BSA and insulin, transferrin and selenium (SOF-ITS-BSA) is yielding day 8-blastocyst rates over 40 %. However, in order to get these high blastocyst rates, *in vitro* maturation and fertilization still have to be performed in group culture. Several groups have attempted to develop an *in vitro* maturation-fertilization-culture system allowing individual follow-up from oocyte until embryo. Different approaches such as attaching the oocytes to the bottom of the Petri dish with Cell-Tak®, using a mesh grid or culturing oocytes and embryos in the well-of-the-well system have been attempted. These systems work well but are technically often challenging. Here we describe a simple individual oocyte-to-embryo culture system which is yielding routinely over 30 % blastocyst rates. *In vitro* maturation, fertilization and culture were either performed in group or in individual culture. For group culture, sixty cumulus-oocyte complexes were aspirated from ovaries derived from cows slaughtered in a local abattoir and matured in 500 µL TCM199 supplemented with 20 ng/mL EGF for 22h. Next, mature oocytes were incubated in 500 µL IVF-TALP with 1 × 10^6 spermatozoa/mL for 20h and then denuded and cultured in groups of 25 presumed zygotes in 50 µL droplets of SOF-ITS-BSA under paraffin oil (7.5 mL) overlay. For individual culture, 3 dishes (60×15 mm) with 20 µL droplets under paraffin oil overlay were used, each droplet containing one cumulus-oocyte-complex for maturation and subsequent fertilization (in the same media as described for group culture), and after denudation, presumed zygotes were cultured individually in 20 µL droplets SOF-ITS –BSA under paraffin oil overlay until day 8. Each dish contained 17 droplets. Blastocysts were then subjected to differential staining. Blastocyst rates (5 replicates) were significantly lower for individual compared to group culture (32 % (79/244) versus 47 % (146/314)) (Independent sample t-test, SPSS 20; $P<0.05$), but higher than 30 % so still acceptable. Blastocyst quality was also significantly lower, with a lower total cell number (90 ± 1.31 vs. 118 ± 1.16) and higher apoptotic cell ratio (8.4 ± 0.25% vs. 5.2 ± 0.19%) for individual versus group culture respectively. This indicates that despite the high overall blastocyst rates, there is still room for improvement in the individual culture system. In conclusion, the serum-free and oil-containing individual culture system we describe here is yielding acceptable blastocyst rates and can as such be used as to investigate (1) how differences in initial oocyte quality can affect embryo outcome; and (2) how addition of specific biochemical factors to the single oocyte maturation medium can be used in order to improve oocyte maturation. We are now testing the addition of different components derived from bovine follicular fluid to maturation medium in order to evaluate their possible effect on individual oocyte maturation and further embryo development.

12. Nobiletin supplementation prior to EGA improves development and quality of bovine blastocysts in vitro

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One of the most important events in early embryo development is the maternal-to-embryonic transition when maternal transcripts and proteins are gradually degraded and the embryonic genome is activated (EGA). In bovine embryos, major EGA occurs at the eight- to 16-cell stage. At the same time, an increase in ROS levels during embryo culture in vitro induces oxidative stress leading to failed embryonic development and low quality of the blastocysts produced. Nobiletin is a polymethoxyflavone with antioxidant properties in different cell types. Therefore, we aimed to evaluate the effect of nobiletin supplementation to the culture medium of bovine embryos before major EGA on their development and quality. In vitro produced zygotes were cultured in four-well plates with 500 µl SOF+5% FCS (control), control with 5, 10 or 25 µM nobiletin (MedChemExpress, MCE, Sweden) (Nob5, Nob10 and Nob25 respectively) or control with 0.03% dimethyl sulfoxide (CDMSO vehicle for nobiletin dilution) from 18 to 54 hours post-insemination (hpi) at 38.5ºC, 5% CO_2, 5% O_2 and 90% N_2. For all groups, the speed of development was considered and embryos that reached ≥8-cells at 54 hpi were selected and cultured in control medium until Day 8. Cleavage rate (54 hpi) and blastocyst yield (D7-8) were evaluated, while quality of embryos were determined by assessing their total cell number, lipid content and mitochondrial activity (fluorescence intensity recorded in arbitrary units (a.u)). For this purpose, a representative number of D7 blastocysts (n=30/group/treatment) were fixed and stained with Hoëscht, Bodipy, and MitoTracker DeepRed, respectively. The images were obtained by confocal microscopy and analysed using Image J. Data obtained from 6 replicates were analysed using one-way ANOVA. No differences were found in cleavage rate while blastocyst yield at D8 was higher ($P<0.001$) for Nob5 (42.9±1.4%) and Nob10 (45.3±2.1%) compared to control (32.9±1.1%), CDMSO (32.6±1.4%) and Nob25 (34.2 ± 1.0%). For embryo quality evaluation, both controls and Nob groups with higher development were used. The number of intact cells per embryo was increased ($P<0.001$) in Nob5 (137.3±0.6) and Nob10 (126.7±0.8) compared to control (105.7±0.8) and CDMSO (106.4±0.8). The lipid content was significantly reduced ($P<0.001$) in Nob5 and Nob10 compared with both controls. For mitochondrial activity, fluorescence was significantly higher ($P<0.001$) in blastocysts from Nob5 and Nob10 compared with both controls. In conclusion, supplementation of nobiletin 5 or 10 µM/mL improves embryo development and the quality of blastocysts in terms of mitochondrial activity and cell numbers, while it reduces their lipid content.

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13. Effect of sperm selection using microfluidic sperm sorting chip on bovine embryonic development in vitro

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Keywords: IVF, microfluidic sperm sorting chip, cattle

The preparation of bovine sperm for in vitro fertilization (IVF) requires procedures such as a density-gradient centrifugation, which enables to select sperm cells with a higher rate of progressive motility and those that are morphologically intact. Microfluidic sorting devices have been demonstrated to effectively select motile human sperm without centrifugation. The aim of this study was to examine embryonic development after using a microfluidic sperm sorting chip for the selection of bovine sperm cells by the IVF procedure. Bovine ovaries were collected at slaughterhouse and placed and transported at 30°C in phosphate buffer solution until laboratory processing. In total 234 Cumulus-oocyte complexes (COCs) were obtained using the slicing method. The sperm samples were collected from a sire using an artificial vagina at artificial insemination centre. Sperm motility was assessed subjectively, and motile sperm (with more than 70% progressive motility) was prepared and used for IVF. After maturation, COCs (15-20 COCs in each drop) were fertilized with sperm cells that were selected after thawing at 30°C using either a standard density gradient (DG) protocol (SpermFilter®, GYNEMED GmbH & Co. KG, Lensahn, Germany) or a microfluidic sperm sorting (MSS) chip technique (Fertile Plus®, KOEK EU GmbH, Hannover, Germany). Motile sperm cells were added to the IVF drops to reach a final concentration of 1 × 106 cells/mL and were incubated with the COCs (19h, 5% CO2, 39°C). At the end of this co-incubation, presumptive zygotes were denuded using vortex, were washed and then placed into a synthetic oviduct fluid (SOF) under silicone oil. Cleavage and embryonic development rates (blastocyst formation) were recorded. For both treatment groups four replicates were performed. Statistical analysis was performed using Chi-square test with significance at $p < 0.05$. The MSS chip technique did not affect cleavage rates (MSS chip: 75.0% vs. DG: 71.2%, $p > 0.05$) and blastocyst rates at Day 7 (MSS chip: 18.1% vs. DG: 15.3%, $p > 0.05$). At Day 8, blastocysts rates were higher for oocytes, that were fertilized using MSS chip sorted sperms as compared to those selected by the DG technique (MSS chip: 33.6% vs. DG: 22.0%, $p = 0.048$). In conclusion, MSS chip sorted sperm may increase embryonic development rates and outcomes in routine IVF procedure. A larger number of sperm samples of different bulls will be studied in future studies to demonstrate the sperm quality and IVF outcomes after usage of MSS device.

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14. Estradiol benzoate and progesterone treatment in synchronization of embryo recipients and evaluation of the incidence of early pregnancy loss in the mare

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Keywords: Early pregnancy loss, embryo transfer, mare

Selection and management of recipient mares are the one of the most important factors affecting pregnancy in embryo transfer programs (Losino L & Alvarenga MA, Acta Sci Vet 2006; 25,34:39-49). It is well-documented that estrogen is responsible to activate progesterone receptors in the reproductive tract in preparation for pregnancy (Squires EL et al., Theriogenology 1999; 51:91-104). Furthermore, uterine edema is important for normal pregnancy in embryo transfer recipient mares (Losino L & Alvarenga MA; Acta Sci Vet. 2006; 25,34:39-49). The objective of this study was to compare early pregnancy loss (EPL) rates in acyclic mares treated with different hormones, at our embryo transfer center located in Tehran. A total of 20 acyclic recipients were examined by trans-rectal ultrasound scanning two times with 10 days interval and they did not have follicles more than 20 mm in each ovary. They showed no endometrial edema, having a typical homogeneous echotexture and grade 0 endometrial edema (subjective scoring system 0-5). On day 0, mares were randomly allocated to one of the following two treatments: 1) 5 mg/kg BW of estradiol benzoate i.m. (Vetastrol, Aburaihan, Tehran, Iran) for 5 days and 3 ml per 500 kg BW long acting altrenogest i.m. (Ready Serve, CEVA, Australia), twice with seven days interval; 2) long acting altrenogest (Ready Serve, CEVA, Australia) 3 ml per 500 kg BW i.m. twice with a 7 day interval. Endometrial edema and time of ovulation were determined once daily by trans-rectal ultrasound scanning. Seven days after ovulation, each mare received an embryo by transcervical transfer. Pregnancy diagnosis was performed on day 15 and 35 after ovulation using ultrasound scanning. Pregnancy rate and EPL rates were analyzed by Chi Squared, with differences considered significant at $p<0.05$. Pregnancy rate on day 15 after ovulation in group 1 was 80% and in group 2 60%. The grade of endometrial edema in group 1 was higher than in group 2 (>3 and <2, respectively). The EPL rate in group 2 was 50% and significantly higher than in group 1 (10%) up to 35 days of pregnancy. This study demonstrated that an estradiol treated acyclic mare seem to be suitable as a recipient with satisfactory incidence of EPL, as showed in other studies. The presence of moderate endometrial edema in acyclic mares was enough to indicate that the recipient was suitable for embryo transfer a couple of days later.

15. Melatonin increases the number of trophectoderm cells and total embryonic cells in in vitro-derived bovine blastocysts

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Keywords: Melatonin, inner cell mass, trophectoderm cells

It has been shown that *in vitro*-derived embryos have fewer trophectodermal (TE) cells than *in vivo*-derived embryos. The TE cells are important for attachment of the embryo to the uterine endometrium, the formation of the fetal placenta and ultimately pregnancy establishment. An aberrant allocation of inner cell mass (ICM) and TE cells could be related to insufficient placentation and thus embryonic/fetal losses. Excess of oxidative stress under *in vitro* conditions can alter many important reactions affecting the embryonic development. Recently attention has been directed towards melatonin as a non-expensive broad-spectrum antioxidant. In the present study, we investigated the effects of melatonin on allocation of ICM and TE cells in *in vitro*-derived bovine embryos. A total of 97 blastocysts (Day 8) produced *in vitro* in the presence or absence of two concentrations of melatonin (MT) [MT 0.01nanomolar (nM): n=25 and MT 1.0 nM, n=21], were differentially stained to determine the number of cells (ICM and TE cells). As melatonin has to be dissolved in ethanol a "sham" group containing ethanol (ETOH; n=27) and a standard control group (Control: n=24) were also included in the experimental setting. A modified differential staining technique was applied (Thouas et al., Reprod Biomed Online 3(1): 25-29, 2001). Cells were counted via fluorescence microscopy (470-490 nm excitation filter) (Olympus BX60F, Tokyo, Japan) at 400-fold magnifications. The chromatin in nuclei of TE cells and ICM cells was stained and visualized by red/pink or blue color, respectively. Data were statistically analyzed using the SAS/STAT® software (version 9.3) with the general linear model (PROC GLM). Significant differences were defined at $p<0.05$. The general mean for TE, ICM, and total embryonic cells were 88.9±2.6, 41.9±1.3 and 130.8±3.2; respectively. The number of TE cells was significantly higher ($p<0.05$) in MT 0.01 nM and MT 1.0 nM groups compared to the control and ETH groups (101.3±11.8 and 101.6±8.6 vs. 86.5±12.2 and 83.6±12.2, respectively). No differences ($p>0.05$) were observed in the number of TE cells in sham controls and controls, as well as between both melatonin concentrations. There were no differences ($p>0.05$) regarding the number of ICM cells between the different experimental groups (Control: 43.8±6.9; ETOH: 39.0±6.9; MT 0.01 nM: 42.0±4.9 and MT 1nM: 46.1±6.7). Supplementation of the media with melatonin at 1.0 nM and 0.01 nM increased ($p=0.05$) total cell number compared with control and ethanol groups (147.3±14.6 and 143.7±10.7 vs. 130.3±15.1 and 122.5±15.1, respectively). No differences ($p>0.05$) were found between the control and the ETOH group, neither between both concentrations of melatonin. In conclusion, these data indicate that the presence of melatonin in *in vitro* embryo production media increases the allocation of embryonic to the trophectoderm, as well the total number of embryonic cells. The physiological importance of this finding warrants further study and could have an important implication to reduce early embryo/fetal losses observed after *in vitro* embryo production.

16. Perfluorooctane sulfonic acid (PFOS) affects early embryonic development in a bovine in vitro model

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Keywords: Oocyte maturation, cattle, in vitro embryo production, PFOS

Perfluorooctane sulfonic acid (PFOS) is a chemical that has been widely used in products like food packaging, textile, impregnations and firefighting foams. It is now banned in many countries, including the EU, but still present in nature, animals and humans due to its persistent and bioaccumulating properties. The average reported human serum PFOS levels vary from 4-70nM (median 30 nM) in the literature (and higher in especially exposed groups). PFOS is endocrine disruptive and has toxic effects on reproduction in research animals although human data remains contradictory. The aim of this study was to examine the effect of environmentally relevant concentrations of PFOS on bovine early embryo development *in vitro* as a possible model for human early embryo development. This model was chosen as the process of maturation and fertilization in bovine oocytes shows more similarities to the human process compared to the murine. Abattoir derived oocytes were matured, fertilized and cultured in a bovine *in vitro* system. Oocytes were randomly divided into two treatment groups exposed to either 20 nM PFOS (P20) or 200 nM PFOS (P200) during *in vitro* maturation and to one control group (C) without PFOS. Cleavage rate as well as stage and grade of day 7 and 8 blastocysts were assessed. Further, neutral lipids were analyzed using HCS LipidTOXTM Green Neutral Lipid Stain (ThermoFisher Scientific, Waltham, USA) and nuclei were stained with Hoechst 33342 in paraformaldehyde-fixed day 8 blastocysts. Evaluations of the number of nuclei, total lipid volume, lipid volume of each blastocyst and lipid droplet size were performed using confocal microscopy. Mixed effect logistic regression was used to calculate the effect of treatment on the number of cleaved embryos and developed blastocysts. The effect of treatment on lipid droplets was performed using a linear mixed effect model with log-transformed values to assume normal distribution. From 13 batches with a total of 847 oocytes, 162 blastocysts were developed. Cleavage rate and cleavage rate above the 2-cell stage were significantly lower in the P200 group compared to the control group, although no significant difference could be seen on blastocyst development or grade on day 7 and 8 neither in P20 or P200. The blastocyst stage of development was significantly lower in the P200 group compared to the C group. In addition, in the P200 group PFOS had an effect on lipid droplet size in the early blastocyst stage where the lipid droplets were larger. The results from this experiment indicate that human relevant concentrations of PFOS impair bovine early embryo development. PFOS exposure delays development and affects the size of lipid droplets. These findings are in line with epidemiological studies linking PFOS exposure to lipid metabolism in adults. More studies are needed to elucidate the mechanisms and effects of PFOS on the early embryo development.

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17. Quality of mouse IVF blastocysts after addition of quercetin to the culture media at the morula stage.

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Keywords: IVF, culture-medium, blastocysts

Quercetin is a plant flavonol found in many fruits and wine, which has an important role as antioxidant in many pathological pathways associated with oxidative stress. The effects of the quercetin have been studied on the *in vitro* maturation, the competency of the oocyte and during the whole embryo development in different animal species. Nonetheless its effects have not been studied on mouse embryos obtained by IVF only during the stage from morula to blastocyst. The aim of this study was to examine the embryo development, cell death and cell number on mouse blastocysts obtained by IVF and cultured under conditions of normoxy, hypoxy and with different concentrations of quercetin. B6D2 strain female mice were hormonally stimulated to activate the recruiting of the follicles and to trigger the ovulation. Mature cumulus-oocyte complexes were obtained, used to perform IVF, cultured in KSOM and divided into 6 groups when they reached the morula stage (day 3): IVF_{KSOM}: embryos were cultured in KSOM until the blastocyst stage (control group); $IVF_{50\mu M}$, $IVF_{10\mu M}$,$IVF_{5\mu M}$ and$IVF_{1\mu M}$: morulae were cultured for 4 hours in KSOM media supplemented with 50 µg/ml, 10 µg/ml, 5 µg/ml or 1 µg/ml of quercetin, respectively, in an atmosphere of 5% CO_2, and transferred back to normal KSOM and cultured until the stage of blastocyst; $IVF_{3\%}$: morulae were cultured for 4 hours in KSOM in an atmosphere of 3% of O_2 (to resemble the uterus condition after the morula stage) and 5% of CO_2 until the stage of blastocyst. The blastocysts were used to study the embryo development (n=15 IVFs/group), the total number of cells (trophoblast cells and ICM by DAPI staining, n=30 blastocysts/group) and the cell death (studied by TUNEL assay, n=25 blastocysts/group). Our results showed that the mean of embryos that developed to blastocyst was 59.90% $\pm$ 25.83 for IVF_{KSOM}, 61.04% $\pm$ 25.9 for $IVF_{50\mu M}$, 72.14% $\pm$ 22.69 for$IVF_{10\mu M}$, 62.27% $\pm$ 29.59 for $IVF_{5\mu M}$,68.57% $\pm$ 20.55 for $IVF_{1\mu M}$ and 63.76% $\pm$ 26.86 for $IVF_{3\%}$ ($p>0.05$). The mean of the number of cells per blastocyst was 84.1 $\pm$ 7.82 for IVF_{KSOM}, 82.23 $\pm$ 11.33 for $IVF_{50\mu M}$, 89 $\pm$ 15.6 for $IVF_{10\mu M}$, 86.7 $\pm$ 9.5 for $IVF_{5\mu M}$, 87.56 $\pm$ 10.99 for $IVF_{1\mu M}$ and 88.2 $\pm$ 12.89 for $IVF_{3\%}$ ($p>0.05$). Results for the cell death showed that the mean of dead cells per blastocyst was 0.10 $\pm$ 0.27 for IVF_{KSOM}, 0.12 $\pm$ 0.33 for $IVF_{50\mu M}$, 0.04 $\pm$ 0.2 for $IVF_{10\mu M}$, 0.08 $\pm$ 0.27 for $IVF_{5\mu M}$, 0.08 $\pm$ 0.27 for $IVF_{1\mu M}$ and 0.30 $\pm$ 0.45 for $IVF_{3\%}$ ($p>0.05$). One-way ANOVA test was used for the statistical analysis and a $p<0.05$ was considered statistically significant. Based on the p values, there were no statistically significant differences between the groups in any assay. It is worth mentioning though that in embryos cultured with 10 µM of quercetin the number of cells per blastocyst was higher, the cell death was lower and a higher number of embryos reached the blastocyst stage. These results show that, even though the differences found were not statistically significant, the enrichment of the embryo culture media with 10 µM of quercetin at the stage of morula slightly improves the mouse blastocyst quality, hence showing potential to increase the implantation rates. Further studies are required though to verify this hypothesis.

18. Hormone levels differ between cow recipients carrying in vivo or in vitro-derived conceptus during early pregnancy

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Keywords: Embryo transfer, reproductive fluids, anti-mullerian hormone

Bovine embryo production is still far from optimal. In order to better mimic the natural environment, natural reproductive fluids (RF), collected from reproductive tracts, have been proposed as additives for embryo culture and results from RF-derived embryos after transfer (ET) have not shown adverse effect on pregnancy rate at day 30 when compared to a control (BSA) (Lopes, Animal Reproduction, v15, nr3, p550, 2018). However, pregnancy maintenance is dependent on several factors and hormones such as progesterone (P4), estrogen (E2), cortisol or, more recently studied, anti-Mullerian hormone (AMH), play an important role. P4 and E2 are protagonists during pregnancy and P4 has been related to early growth of conceptus in uterus (Shorten, J Dairy Sci, 101:736-751, 2018). Cortisol, on the other hand has been tagged as a meaningful participant in intrauterine regulatory system of early pregnancy in cattle (Majewska, J Rep Imuno, 93:82-93, 2012). AMH levels are known to influence the pregnancy maintenance, being low levels associated with pregnancy loss (Ribeiro, J Dairy Sci, 97:6888–6900, 2014). Therefore, we hypothesised that P4, E2, cortisol and AMH levels might have an influence on recipient's pregnancy outcomes. To test our hypothesis bovine IVP-blastocyts cultured with or without RF (RF and BSA groups) were transferred to synchronized recipients. An in vivo control was added by artificial insemination (AI) of recipients with frozen-thawed semen from the same bull used to produce IVP embryos. At day 30, pregnancy diagnosis was performed, embryos measured and blood samples from pregnant recipients were taken (Pregnancy/ET or AI: 12/54 from RF, 10/45 from BSA and 8/35 from AI group). Blood collected on the day of ET and 7 days post-AI was also analysed. Statistical analysis consisted of Pearson's correlation between variables (group, hormones, embryo size, day) followed by one-way (groups) or t-test (day) when significant correlation ($p<0.05$) was found. At day 7, P4 was significantly lower in AI vs. both RF/BSA. Cortisol had a tendency to be lower for RF group ($p=0,052$) but was not confirmed by post-hoc. AMH and E2 were not different between groups. At day 30, AMH and E2 were lower and higher, respectively, between AI & BSA, but RF had intermediate concentrations. P4 and cortisol were not different between groups. No correlation was found between embryo dimensions vs. hormone levels in any day nor group. P4 was, independently of the group, significantly lower at day 7 vs. day 30, whereas cortisol, E2 and AMH remained similar. In conclusion, in our study we observed that AMH and E2 levels at day 30 were significantly different between recipients holding AI vs. BSA embryos while recipients from RF group showed an intermediate value. P4 values, 7 days post-AI vs. day of ET were significantly lower for AI vs. ET recipients, but those differences disappeared by day 30. How the embryo influences hormonal levels remains to be further investigated and more analyses to the pregnant recipients as well as non-pregnant animals should be further addressed.

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19. Use of in-estrus heifer serum on in vitro culture of sheep embryos

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Keywords: embryo culture, supplement, blastocyst

Fetal bovine serum (FBS) is a universal supplement for embryo culture; however, alternatives have been sought to replace it. The study aimed to evaluate the estrus heifer serum (EHS) as a supplement of the culture medium, and to evaluate the differences between sera from donor Holstein heifers used individually or mixed in the development of ovine embryos to blastocyst stage cultured *in vitro*. A total of 1105 oocytes from ovaries of ewes obtained from a commercial slaughterhouse were used. The oocytes were *in vitro* matured (IVM) in TCM 199 (In vitro S.A., Mexico), supplemented with: 10% of FBS (Biowest, Mayimex, Mexico), 5 µg mL^{-1} FSH (Folltropin-V, Bioniche, Canada), 5 IU mL^{-1} hCG (Chorulon, Intervet, Netherlands) and 1 µg mL^{-1} 17-β estradiol (E8875, Sigma, Mexico). After 24 h of IVM at 5% CO_2 in air, at 38.5 °C and saturated humidity, the oocytes were fertilized (IVF) in commercial medium (In vitro S.A., Mexico) using fresh spermatozoa ($1x10^6$ mL^{-1}) from a Rideau Arcott ram of known fertility. The zygotes were cultured using Cleavage medium (Cook IVF, Brisbane, Australia) for 72 h until 16-cell stage. The rate of IVM, IVF and embryos in the 16-cell stage were registered (85.5, 67.0 and 65.4%, respectively). The embryos in the 16-cell stage (723) were randomly assigned to one of five treatments with Blastocyst culture medium (Cook IVF, Brisbane, Australia) plus 10% serum of different types: T1: FBS (control, n= 146); T2: EHS1 (n= 144); T3: EHS2 (n= 143); T4: EHS3 (n= 143) and T5: EHS mixture (T2, T3 and T4) (n= 147). The embryos were cultured for 96 h until blastocyst stage. The development and quality of the blastocysts were evaluated according to their morphology, while the diameter blastocyst was measured with a digital camera (AmScope, MU1803, China) using an inverted microscope (Nikon, Eclipse TS100, Japan). These variables were analyzed using the SAS program. The development data consider a comparison of binomial proportions with the construction of confidence intervals using GENMOD. Analysis of variance was used to analyze the diameter results with a classification criteria and fixed effects using GLM, while blastocyst quality was modeled according to a multinomial distribution and analyzed with GENMOD procedure. The percentage of blastocysts was similar between treatments (41.8, 40.3, 39.9, 50.4 and 43.5% for T1, T2, T3, T4 and T5, respectively, $p>0.05$). For blastocyst diameter, T4 and T5 were larger than T1 (238 *vs* 223 µm, p= 0.007, 234 *vs* 223 µm, p=0.04, respectively). Likewise, T4 was larger than T2 (238 *vs* 226 µm, p=0.03). No differences were observed among treatments for blastocyst quality ($p>0.05$). In conclusion, the estrus heifer serum used has similar effects as fetal bovine serum when the culture medium is supplemented at 72 h. Moreover, the individual use of serums may be better as a supplement than using a mixture under the conditions of this study.

20. The efficiency of collecting in vivo-developed porcine zygotes is not affected by 3-to- 5-days weaning-to-estrus interval

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Keywords: zygote, pig, embryo collection
The recently developed genome-editing (ZG-E) technology for pig zygotes, opening a new revolution in agriculture and biomedicine, depends of the efficient collection of large numbers of zygotes of the highest quality. Because IVP of zygotes in pigs still is inefficient, *in vivo*-collection remains as major source of zygotes. Little information is available on the efficiency of the collection procedures for in vivo-derived zygotes. Since the interval between pronuclear formation and the first division is very short in pigs, the collection of zygotes must be performed within a very narrow window. While the weaning is an efficient method to synchronize estrus and ovulation in sows, the weaning to estrus interval (WEI) can, due to its inverse relation with length of estrus and time of ovulation, interfere with ovulation and make it asynchronous. In addition, individual sows show variability in ovulation time, even after hCG treatment, which reduces the probability of obtaining zygotes during collection. This study compared the effects of three WEIs: 3d (N=57), 4d (N=131) or 5d (N=29) on the efficiency of zygote collection *in vivo*. The donors were super-ovulated with eCG (1,000 IU; i.m.) 24 h after weaning. Estrus was checked twice per day when allowing snout-to-snout contact of sows and a mature boar while applying manual backpressure. Sows in estrus at 48–72 h post-eCG were treated with hCG (750 IU; i.m.) at the onset of estrus. The donors were inseminated at 6 and 24 h after the onset of estrus and subjected to a laparotomy on Day 2 (Day 0: onset of estrus). After counting the number of corpora lutea, each oviduct was flushed with 20 mL of Tyrode's lactate-HEPES-polyvinyl alcohol medium. Collected structures were evaluated for morphology under a stereomicroscope and only those with a single cell and two visible polar bodies were considered as zygotes. Results were expressed as percentages or means±SD. Differences among groups were analyzed using Fisher's exact test or ANOVA as appropriate and were considered significant when $P<0.05$. A total of 217 out of 223 donors (97.3%) had embryos at collection. The mean ovulation rate (27.3±7.6 corpora lutea) and the mean number of structures (25.2±9.4) collected in these sows did not differ between groups. Of all recovered structures (N=5,468), 67.4%, 31.1% and 1.5% were zygotes, two-to-four cell embryos and oocytes-degenerated embryos, respectively. The different WEIs did neither affect the percentages of collected zygotes (range: 64.1% to 70.0%) nor the percentages of sows with zygotes at the collection (range: 70.2% to 73.3%). In conclusion, these results indicate that neither fertilization rates nor the number of zygotes collected at Day 2 of the cycle from superovulated sows were affected by a WEI of 3 to 5 days.
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21. Comparative study of growth parameters in piglets derived from embryos produced in vitro with or without reproductive fluids, and piglets derived from artificial insemination.

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Keywords: Growth, Reproductive Fluids, pig.

Different studies have suggested that the use of assisted reproductive technologies (ART) is associated to a higher incidence of low birth weight (Castillo et al., Hum Reprod doi:10.1093/humrep/dez025, 2019) as well as to alterations in the growth curve (Donjacour et al., Biol Reprod 90:80, 1–10, 2014). Recently, it has been described that porcine blastocysts produced *in vitro* with reproductive fluids (RF) in the culture medium show DNA methylation and gene expression patterns more similar to those produced in vivo than their counterparts produced without RF (Cánovas et al, eLife 1: e23670, 2017). However, it is unknown whether the presence of RF during fertilization and embryo development *in vitro* affects offspring growth-related parameters such as weight and length. The objective of this work was to compare growth parameters of piglets born after the transfer of embryos produced *in vitro* with RF (F-IVP, N=19) or without RF (C-IVP, N=29) added to the culture media with the same parameters in animals derived from artificial insemination (AI, N= 57). After birth, piglets were weighed at different days (0, 3, 9, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180) with a mobile scale and average weight daily gain (AWG) was calculated from total weight values (W) at every two consecutive days. All animals were measured with a zoo-metric tape from the frontal region of the cranium to the beginning of the rump (Cranium-rump length, CRL). All weight data and female (N=42) CRL data were analyzed by robust mixed ANOVA test while mixed ANOVA test was used for male CRL data (N=32) because Mauchly's test for sphericity came out significant but Greenhouse-Geisser (GGe) and Huyhn-Feldt (HFe)corrections were not valid. A total of 4 litters per group were studied, with the litter sizes differing between them (4.75±1.71, 7.25±2.06, 14.25±6.55, for F-IVP, C-IVP, and AI, respectively). Significant differences were reported most days studied for W, AWG and CRL measurements ($P \leq 0.05$) when they were compared by genders in AI piglets versus F-IVP and C-IVP groups, being these last two heaviest and longest than the first, although F-IVP showed intermediate values that could be related to a phenotype more similar to that obtained through AI. Similarly, significant differences were also observed between F-IVP and C-IVP for W, AWG, and CRL from day 9 to day 75. However, due to the low number of piglets under study and to the high differences in litter sizes between groups, further analyses are necessary to elucidate the influence of co-variables such as the mentioned litter size, with a possible strong influence in growth rates. Though preliminary, these are the first data in a large animal model, up to our knowledge, comparing growth parameters between ART-derived and AI derived offspring. In addition, they shed light on possible future phenotypic differences between ART-derived animals produces with or without RF.

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22. Differences in glucose tolerance between piglets born after in vitro fertilization/ embryo transfer and relatives born after artificial insemination

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Keywords: Glucose, Reproductive fluids, ART

Increasing evidence indicates a higher incidence of glucose metabolism abnormalities in children derived from Assisted Reproductive Technologies (ART) (Chen et al., Diabetes, 63:3189–3198,2014). Monophasic and biphasic patterns of blood glucose curves have been identified, biphasic curves being associated with better glucose tolerance and beta-cells function, increasing insulin sensitivity in humans (Bervoets et al., Horm Metab, 47:445–451, 2014). On the other hand, the addition of reproductive fluids (RF) to in vitro culture media used during ART has been proposed as a possible way to avoid ART-derived abnormalities in pigs (Cánovas et al., eLife 1: e23670, 2017). The aim of this study was to evaluate the response to oral glucose tolerance test in young growing pigs born from embryos produced in vitro with (F-IVP) or without RF (C-IVP), compared to animals born by artificial insemination (AI). Four litters of related animals per group were used. At 45 days of life, the two males and two females of highest and lowest weight per litter were selected for the study (N=14, N=15, and N=16 for F-IVP, C-IVP, and AI, respectively). After 18h overnight fast, water was withdrawn and 1h later, pigs drank 1.75 g/kg BW of glucose solution. Blood samples were collected from the auricular lateral vein before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after glucose intake. Blood glucose concentration (GC) was immediately determined by test strips with a glucometer (GlucoMenLX Plus+). One way ANOVA and Tukey post-hoc tests were applied ($P<0.05$). Pearson correlation coefficient was used to detect litter influence, resulting in a positive correlation between GC value and weight. A monophasic GC curve was observed in the three groups. GC steadily increased reaching a maximum at 45 min after glucose intake, thereafter, it decreased until basal values (range 74.13-78.67mg/dl). Significant differences between AI and F-IVP groups were observed at 15, 20 and 30 min, with F-IVP showing higher values. When the analyses were repeated splitting the animals by sex, males showed a monophasic curve with similar basal levels, and the glucose peak at 45 min in all groups (range 98.43-115,0mg/dl). Significant differences between AI and F-IVP groups were present at 20 min and between F-IVP vs.AI and C-IVP groups at 30 min. In contrast, females showed significant differences between AI and F-IVP groups before glucose intake and at 20 min, while F-IVP was different vs.AI and C-IVP at 15 min. In addition, females on average from F-IVP group presented a biphasic curve, with two peaks at 15 (range 83.50-128.67mg/dl) and 45 min (range 100.88-128.20mg/dl) compared with other groups. In conclusion, glucose tolerance in growing piglets is affected by sex and by the origin of the embryo, although all the basal and peak values are always into the physiological range.

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23. Effect of type of recipient on an embryo transfer programme in sheep

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Keywords: ewes, embryo transfer, pregnancy

The implementation of embryo transfer is important to help in the multiplication of high genetic merit animals, through the selection of the best males and females. The success of the technique depends on the possibility of achieving high levels of efficiency in each step to get high rates of pregnancy. The objective of this study was to evaluate the fertility of two groups of ewes (hair and wool types) exposed to an embryo transfer programme. The study was conducted from September to December of 2017 at the commercial sheep farm "Poza Rica", which is located in a temperate area named Singuilucan, in central Mexico. A total of 60 multiparous healthy and in good body condition (3.0) ewes were used, from which 30 were Suffolk x Hampshire (T1; wool type) and the other 30 were Katahdin x Pelibuey (T2; hair type). The ewes were synchronized with intravaginal sponges containing 20 mg of micronized cronolone (Chrono Gest, Intervet, Netherlands), which were inserted for 12 days. On day 10, the ewes received intramuscularly 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Katahdin rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined as the number of corpora lutea observed in ovaries during laparoscopy. The recipients received two embryos of transferable quality (compact morula or blastocyst) within 3 h after its collection, coming from Charolais donor ewes using a laparoscope and standardized procedures. The embryos recovered into holding medium (Syngro, Vetoquinol, Canada) were transferred using a Tom catheter in the ipsilateral horn to the ovary in which ovulation was recorded, and the presence of the best quality corpus luteum was determined based on its size. On day 60, pregnancy diagnosis was performed using an ultrasound and a 3.5 MHz transabdominal probe (Aloka Prosound 2, Japan). The results of the incidence of estrus and pregnancy rate were analyzed as categorical variables with the CATMOD procedure, and ovulation rate with the ANOVA procedure, both of them available in SAS. The total percentage of estrus was similar ($p>0.05$) between ewes of T1 and T2 (95 and 100%). The incidence of estrus was also similar ($p>0.05$) for ewes from T1 and T2 at 24 h (75 and 85%) and 30 h (25 and 15%). Ovulation rate for ewes of T2 was higher ($p<0.05$) than for ewes of T1 (2.26 ± 0.21 vs 1.80 ± 0.15). Also, pregnancy rate was lower ($p<0.10$) for ewes of T1 than for ewes of T2 (60 vs 80%). In conclusion, under the conditions of the study, the results showed the superiority of using hair type multiparous ewes as embryo recipients.

24. Prediction of pregnancy after transfer of bovine in vitro produced embryos based on recipients' blood plasma metabolomics

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Keywords: cattle, embryo recipients, metabolomics

In association with genomic selection schemes, embryo transfer (ET) of *in vitro* produced embryos (IVP) is steadily increasing worldwide in cattle, although calving rates remain lower than from *in vivo* embryos. Thus, identification of competent embryos and recipients able to reach pregnancy at term is a major objective in reproductive biotechnology. However, practitioners lack indicators to select suitable recipients, often leading to the exclusion of fertile animals. In that context, this study aimed to identify metabolite biomarkers in blood plasma of recipients belonging to several breeds (dairy, beef and crossbreed), that could predict pregnancy after ET of fresh or vitrified IVP embryos. Blood plasma of 130 recipients (67 Holstein for the training dataset; 63 for the validation dataset including 17 Holstein, 21 Asturiana de la Montaña and 25 crossbred) was collected at Day 0 (estrus) and Day 7 (4 to 6 hours prior to ET) and stored at -150º C until nuclear magnetic resonance (NMR) analysis. On Day 7, fresh (N=67; 34 for training and 33 for validation) or vitrified/warmed (N=63; 33 for training and 30 for validation) IVP embryos were transferred and pregnancy status was evaluated by trans-rectal ultrasound scanning at Day 40, 62 and at birth. NMR analysis led to absolute quantification of 36 metabolites. Average pregnancy rates were respectively 53.8 (50.7 for fresh and 57.1 for vitrified), 49.2 (44.8 for fresh and 54.0 for vitrified) and 43.8% (40.3 for fresh and 47.6 for vitrified) at Day 40, 62 and birth with no statistical differences between fresh and vitrified embryos. Data were examined for normality with Univariate procedure (SAS/STAT software). Thereafter, metabolites differentially expressed between pregnant and open recipients were identified by General Linear Model for each metabolite and each pregnancy checkpoint. Differences were considered significant at $p<0.05$ and FDR<0.05. Interestingly, putative biomarkers were only identified on Day 7 or by subtracting Day 0 and Day 7 (only for vitrified embryos) but not at Day 0. Biomarkers for fresh embryos were consistently identified on Day 40, Day 62 and birth, while vitrification led to a marked drop in biomarker abundance at birth. Overall classification accuracy was calculated to identify three types of biomarkers: 1) independent of breed and embryo type (2-Oxoglutaric acid; Ornithine); 2) specific for fresh embryos (L-Alanine, Ketoleucine, L-Threonine, 3-methyl-2-oxovalerate, Propionic acid); and 3) specific for vitrified embryos (L-Glycine, L-Glutamine, L-Methionine, L-Lysine). Metabolic enrichment analysis distinguished between recipients for fresh (enriched energy oxidative metabolism from fat in pregnant recipients) and vitrified embryos (low enrichment in lipid metabolism in pregnant recipients). Recipient selection by their pregnancy probability in a defined cycle seems to be possible using the biomarkers here identified for the first time. These findings may allow reliable recipient selection according to the cryopreservation status of the embryo, thus optimizing the efficiency of breeding programs.

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25. Short term temperature elevation during IVM affects embryo yield and alters gene expression pattern in oocytes, cumulus cells and blastocysts in cattle.

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Keywords: heat stress, embryo, cattle

Heat stress causes subfertility in cattle by inducing endocrine disruptions and deteriorating of oocyte and embryo quality. In this study we evaluated the effects of short lasting, moderate temperature elevation during IVM, on embryo yield, and on the expression of various genes. Abattoir derived oocytes were matured for 24 hours in TCM199 plus FCS and EGF at 39°C (controls n=549) or at 41°C from hour 2 to hour 8 of IVM (treated, n =867). Matured oocytes were fertilized by frozen/thawed swim-up separated sperm. Presumptive zygotes were denuded and cultured at 39°C in SOF supplemented with FCS for 9 days in microdroplets in groups of 25. In 8 replicates, cleavage and blastocyst formation rates were evaluated at 48 hours PI and on days 7,8,9 respectively. Cumulus cells, oocytes and blastocysts from 5 replicates were snap frozen for gene expression. qRT-PCR was used for analysis of expression pattern of genes related to metabolism, thermal and oxidative stress response, apoptosis, and placentation in oocytes (7 genes), cumulus cells (12 genes) and blastocysts (11genes). Three reference genes (YWHAZ, EEF1A1, UBA52) were used to normalize gene expression values per sample using their geometric mean and their suitability for normalization was checked with the geNorm program. Cleavage, embryo formation rates and gene expression between treated and control groups were tested by 2-tailed students t-test. Correlation analysis was performed by bi-clustering the samples according to their origin and their condition, which is an appropriate method for functionally heterogeneous data. Correlation and regression analysis were performed using gene expression data between groups, by the functions cor and rcorr implemented in R.

In treated group, cleavage and embryo formation rates were lower compared to controls (cleavage 86.7% vs 74.2%; blastocysts: day 7, 29.9% vs 19.7%, day 8, 34.2% vs 22.9% and day 9 35.9% vs 24.5%), in all cases $p<0.001$. Relative mRNA abundance of *HSPA1A, HSPB11, HSP90AA1, GPX1, GLUT1, PTGS2, GREM1, CPT1, G6PD, LDHA, CCNB1, MnSOD* in cumulus cells, *HSPA1A, HSPB11, HSP90AA1, G6PD, GPX1, CCNB1, MnSOD* in oocytes and *HSPA1A, HSP90AA1, DNMT3A, PTGS2, ACR1B1, PLAC8, GPX1, MnSOD, GLUT1, IGF2R, BAX* in day 7 blastocyst was measured by RT-PCR. No statistically significant difference was detected in any gene between treated and control groups. Heat treatment affected ($p<0.05$) the correlation of expression between *HSPB11 and G6PD, GPX1* and CCNB1 in oocytes. In cumulus cells HSP90AA1 was negatively correlated with *HSPA1A, LDHA* and *CCNB1,* while *CCNB1* was positively correlated with *HSPA1A, LDHA, GPX1* and *G6PD*. In blastocysts, heat treatment caused a negative correlation between *HSP90AA1, AKR1B1* and *PLAC8*. These results imply that exposure of oocytes to elevated temperature even for only 6 hours dramatically reduces the developmental competence of the oocytes, suppresses blastocyst yield and disrupts the coordinated pattern of a series of gene expression.

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26. Effect of antioxidant α-tocopherol on bovine oocyte's maturation

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Keywords: cattle, oocyte maturation, alpha-tocopherol

Vitamin E is an important natural antioxidant, and its most common and biologically active form is the α-tocopherol, being well known as a scavenger of free radicals in a hydrophobic milieu. As the chain-breaking of this antioxidant has not been reported to be present in mammalian spermatozoa, the present work was designed to evaluate the effect of the α-tocopherol on the maturation rate of bovine oocytes. For this purpose, 194 bovine ovaries divided by 12 replications, were collected at the slaughterhouse in the Terceira Island and transported to the laboratory. Follicles 2 to 8 mm in diameter were punctured, 779 cumulus-oocyte complexes (COCs) considered of quality 1 and 2 according to their morphological aspect, were assigned to maturation and randomly divided into 4 groups. Each oocyte group was maturated in a standard TCM 199 medium supplemented with fetal bovine serum, FSH/LH, Estradiol, Glutamine and Sodium pyruvate, added with different concentrations (0, 0.5, 1 and 2 mM) of α-tocopherol for 24 h at 38.5 °C, with 5% CO2 in the air and saturated humidity. Then, COC's, cumulus cells were removed, the oocytes were stained with aceto-orcein, observed under the phase contrast microscope, and the different nuclear phases were evaluated from prophase I to Metaphase II. *In vitro* maturation results are expressed as a percentage of oocytes reached the Metaphase II stage. Statistical differences among treatments were evaluated by the ANOVA test. α-Tocopherol at a concentration of 0.5mM increased significantly ($P<0.05$) the maturation rate, relative to the control group, 68.0% vs 60.8%, respectively. At a concentration of 1 and 2 mM, no significant differences were observed when compared to the results obtained in the control group, with maturation rates of 64.3 % and 60.6%, respectively. This study clearly suggests addition of α-tocopherol at a concentration of 0.5 mM increases the maturation rate of bovine oocytes, despite it did not reveal the mechanism by which the antioxidant acts to improve maturation results. Further studies on possible effects of different concentrations of this antioxidant on the developmental competence of *in vitro* produced embryos (IVM/IVF), and the viability of these embryos after transfer to recipient heifers on Day 7 post-estrus will be evaluated in our future research. Studies on vitamin E supplementation of bovine females will be also implemented.

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27. Influence of selected factors on the effectiveness of embryo transfer in cows.

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Keywords: embryo transfer, recipients, risk factors

The objective of the study was to identify risk factors associated with CR (conception rate) after ET(embryo transfer) in cows. It involved 952 cases of ET. Embryos were obtained from previously selected, hormonally synchronized (200 µg of cloprostenol) and superovulated (30 mg of pFSH in 8 decreasing intramuscular doses within 4 days and then 2 intramuscular injections of 500 µg cloprostenol) donors using a bloodless method (embryo flushing). Embryos' selection was carried out according to the evaluation of their morphology. Only embryos in stages of development 3-6 and quality 1-3 were used in the study (Bo 2013). Excellent quality embryos in the morula stage (n= 667) were intended for the direct transfer. Other embryos (n= 285) were destined for freezing and prepared for direct transfer after thawing. The group of embryo recipients consisted of 952 heifers of the Polish Holstein-Friesian breed with mean age 15.5 months and mean body condition score (BCS) 3.02. The heat was synchronized using 2 intramuscular injections of 500 µg cloprostenol given at 14-day intervals. Based on the ultrasound examination females having corpora lutea with diameter above 15 mm qualified for the ET. Embryos were placed in the recipients' uteruses between 6 and 8 days after synchronized or natural heat (757 vs. 195 heifers). The transfer procedure was performed with the use of Wörrlein gun (Goldenpick type) placed in the plastic sanitary case (Minitüb). Embryos were always inserted in the uterine horn ipsilateral to the ovary with the CL. After 2 months all recipients were clinically examined (transrectal palpation) for the pregnancy. It investigated the effect of season on ET, embryo quality or type (fresh vs. frozen), recipient's age and BCS, day of embryo introduction, depth of embryo insertion in the uterine horn, duration of the gun passage through the cervix, horn of the uterus (left vs. right), size and type of the corpus luteum (solid vs. cavitated) and type of treatment used before ET (hCG, flunixin meglumine (FM) and a combination of FM and hCG).To identify the determining factors of CR, the multivarious logit model was estimated using STATA software. The pregnancy was confirmed in 419 after fresh and 159 after frozen embryo transfer cases out of 952 of recipients (CR respectively: 62.8 and 55.8; total CR= 60.7%). The season as well as the embryo development stage were distinguished as statistically significant ($p < 0.05$). The best results (CR= 70%) were obtained in spring, the weakest in summer (CR= 50.2%). Most embryos were transferred at the stages "5" and "6" (n= 451, CR= 62.9). The more developed the transferred embryo was, the higher the CR in recipients. While embryos at the stage "4" led to pregnancy of 53% of the recipients (n=114), embryos at the stage of development "7" and "8" resulted in 66.7% and 83.3% of pregnancies respectively. However, embryos in these stages amounted only to 1.89% (n= 13). The significant ($p < 0.01$) impact of the 'condition' variable was also observed. The highest CR (67.6%) was noticed in recipients with 3.5 points (n= 157) and the lowest - 45.2% - in recipients with BCS higher than 4 (n= 42). The influence of other analyzed variables was not statistically significant.

Folliculogeneisis, Oogenesis, and Superovulation

28. Bisphenol S affects in vitro early developmental oocyte competence in ewe.

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Bisphenol A (BPA), an estrogenomimetic endocrine disruptor, causes deleterious effects on oocyte meiosis and maturation (Machtinger, R. Reprod Biomed Online. 29(4): p. 404. 2014). BPA was banned from food industry and replaced by structural analogs including Bisphenol S (BPS). Some studies on fish and rodent species, reported BPS effects on reproduction similar to BPA effects (Uzumcu, M. Reprod Toxicol. 23(3): p. 337. 2007; Giulivo, M. Environ Res. 151: p. 251. 2016; Ullah, H. Chemosphere. 152: p. 383. 2016). This study aims, therefore, to assess the acute effects of low doses of BPS during *in vitro* maturation on oocyte developmental competence in ewe, as bisphenol resistance was previously reported in rodent species. Cumulus-oocyte complexes (COC) were collected from ovine follicles > 2 mm (n=3789 ovaries). First, COC underwent *in vitro* maturation (IVM) for 24h (Paramio, M.T. Theriogenology. 86(1): p. 152. 2016), in untreated condition (control) or in presence of BPS (Sigma, Saint Quentin Fallavier) at low doses (1 µM, 10 µM) and at environmental doses (1 nM, 10 nM, 100 nM) (Liao, C. Environ Sci Technol. 46(12): p. 6860. 2012). Oocyte viability was assessed with Live dead® staining (Thermofischer, Illkirch, France) and fluorescence microscope observation (Zeiss, Munich, Germany) (n= 1159 oocytes). Nuclear oocyte maturation rate was evaluated by metaphase II oocyte count after chromatin Hoechst staining (Sigma, Saint Quentin Fallavier, France) (n= 978 oocytes). Then, matured COC were *in vitro* fertilized (IVF) and developed (IVD) during 7 days in SOF medium (Zhu, J. Int J Vet Sci Med. 6(Suppl): p. S15. 2018). Cleavage and blastocyst rates were determined on day 2 and on day 7 post-IVF respectively, by microscope observation (n= 2280 oocytes). Data were analyzed using logistic regression and generalized linear model (R package Rcmdr, R version 3.5.3).

Our results showed that, BPS 1 µM and 10 µM do not affect oocyte viability rate (98% [n = 245] and 97.2 % [n = 282] respectively) compared to control (99 %, n = 289). Metaphase II oocyte rate is decreased by 13 % with BPS 10 µM (76.6%, p = 0.0008) compared to control (88%). Among fertilized COC (about 300 per condition), the very low dose BPS 1 nM significantly increased cleavage rate by 28.4 % (70.1%) compared to control (54.6%, p= 0.0003). On the contrary, BPS 1 µM decreased by 12.7 % the cleavage rate (47.6%) compared to control (p = 0.004). Among cleaved embryos, BPS 10 nM and BPS 1 µM decreased blastocyst rate respectively by 34.8% (14.2%, p = 0.046) and by 42.6 % (12.5%, p= 0.017), compared to control (21,8%). Particularly, BPS 1µM significantly reduced blastocyst hatching rate by 65 % (3.3%, p = 0.032) compared to control (9.4%). BPS during *in vitro* maturation negatively affects ovine cleavage and blastocyst rates. Our data suggest BPS negatively influences early developmental oocyte competence. Further studies are needed to investigate the potential BPS effect on estrogen receptors transcripts and on signaling pathways.

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29. Global transcriptome alterations in porcine oocytes with different developmental competence

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Keywords: oocyte, transcriptome, follicle size

Although our knowledge regarding oocyte quality and development has improved significantly, molecular mechanisms regulating and determining oocyte developmental competence are still unclear. Therefore, the objective of this study was to identify and analyze the transcriptome profiles of porcine oocytes derived from different sized follicles and exhibited different developmental competence using RNA high throughput sequencing technology. Cumulus-oocyte complexes (COCs) of the same grades were aspirated from medium (MO; 3-6 mm) or small (SO; 1.5-1.9 mm) ovarian follicles and tested for developmental competence and chromatin configurations. After aspiration and removal of cumulus cells, oocytes were stained with Hoechst 33342 and chromatin configurations were assessed under a fluorescence microscope. COCs from the two groups were matured and cultured in vitro after parthenogenetic activation according to our previous protocol (Prochazka *et al*. 2011, *Reproduction* 141:425-435). After 144 h, the ability of embryos to reach the blastocyst stage was analyzed. For RNA sequencing, RNA libraries were constructed from both oocyte groups (three replicates each, n= 360) and then sequenced on an Illumina HiSeq4000. Raw expression data were normalized using the trimmed mean of M-values (TMM) normalization method. The differential expression analysis was done using the statistical Bioconductor software package EdgeR. Oocytes of MO group showed significantly higher blastocyst rate compared to the SO group (33.41±7.82 vs 15.51±3.44, respectively). MO group exhibited a significantly higher proportion of surrounded nucleolus chromatin configuration compared to the SO group which exhibited a higher percentage of the non-surrounded nucleolus configuration. Transcriptome analysis showed that 14,557 genes in total were commonly detected in both oocyte groups. A group of 930 genes was representing the top highly expressed genes (>5000 reads in each replicate) including genes related to cell cycle and oocyte meiosis and quality (*CCNB1, CCNB2, ESPL1, CPEB1, CUL1, CDC25B, CDC27, BMP15*, and *GDF9*). Differential expression (DE) analysis revealed 60 up- and 262 down-regulated genes (FDR< 0.05, FC≥ 1.5) in MO compared to SO group. *ACOD1, TNFSF11, and OAZ3* were among the top up-regulated genes, while *KCNJ14, IQCA1, CLDN15,* and *IGFBP2* were among the top down-regulated genes. Ontological classification of DE genes indicated that regulation of actin cytoskeleton, oxidative phosphorylation, and ECM-receptor interaction were among the significantly enriched pathways. In addition, biological processes related to cell growth and signaling, transcription, cytoskeleton, and extracellular matrix organization were among the highly enriched in DE genes. In conclusion, this study provides new insights into the transcriptome alterations of oocytes in relation to developmental competence.

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30. PGE2 concentration of the follicular fluid as a measure of heterogeneity of the response to hormonal stimulation of the bovine ovarian follicle

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Keywords: ovarian-stimulation, PGE2, bovine

The LH surge promotes prostaglandin E2 (PGE2) production within the preovulatory follicle. Oocyte microenvironment levels of PGE2 affect the developmental competence after fertilization. This study aimed to characterize the follicular fluid PGE2 enrichment during superovulation treatment. Six heifers (Holstein, 20.3 +/-0.85 months old) received FSH (Stimufol®, Reprobiol, Belgium): half dose, ie, 250 µg of porcine follitropin (pFSH), combined with 50 µg of porcine lutropin (pLH). At the rate of 8 injections, in degressive dose, spread over 4 days. PGF2α (Estrumate®, MSD Santé Animale, France) was injected at the same time as the 5th injection of Stimufol®.LH peak was assumed to occur between 35 and 40 hours after the PGF2α injection. Individual sampling of fluid from antral follicles was performed by ovum pick up 12 hours before PGF2α injection and 60 hours after PGF2α injection. This protocol was designed to allow the collection of fluids from ovarian follicle containing either a pre-matured or a matured oocyte. Each heifer was his own control as we took the "pre-matured" follicular fluid on a first ovary and the "matured" follicular fluid on the 2nd ovary, 3 days later. The punctures were repeated twice and were cross-checked for the next repetition to evaluate the impact of the ipsi or contralateral side of the corpus luteum (CL) on the follicular fluid composition. The volume of fluid was measured for each punctured follicle. The PGE2 concentration of the follicular fluid was measured by Elisa (Cayman Chemical) to determine the progress of terminal follicular differentiation. An average of 13 +/- 5.06 and 28 +/-13.9 follicles were punctured per session/heifer for respectively pre-matured (n=78) and matured (n=169) follicles. The mean collected volume differed between the two groups (pre-matured: 0.229+/-0.213 ml/follicle; matured: 0.575+/-0.379 ml/follicle; two samples t-test, pval<0.0001). No effect of the side of CL on fluid volume was detected (2-way Anova, p=0.397). The PGE2 concentration was determined in 25 pre-matured follicles and 127 matured follicles. The mean PGE2 concentration significantly differed between the two groups of follicular fluids (pre-matured: 7.2 +/-7.5 ng/ml; matured: 60.2 +/-58.6 ng/ml) No effect of the side of CL was detected (p=0.278). Surprisingly, there was no linear relationship between fluid volume and PGE2 concentration (adjusted R-squared: -0.0002, p-value=0.327). PGE2 concentrations were very spread out within the matured group. This important dispersion (Interquartile range=58.6 ng/ml) indicated that despite follicle growth in response to hormonal stimulation (FSH/LH) the ability of follicular granulosa and cumulus cells to synthesize PGE2 was imperfectly achieved. Only 48% of the follicular fluids in the mature group had higher PGE2 levels than those in the premature follicle group. In conclusion, despite the ability of the stimulation treatment to promote growth of many follicles, there was a great heterogeneity in terms of PGE2 synthesis. This alteration could represent defective signaling mechanisms that could impact the developmental competence of the oocyte.

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31. Effects of bisphenol S on ovine primary granulosa cells in vitro

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Keywords: Bisphenol S, Granulosa Cells, Steroidogenesis

Bisphenol A (BPA), a plasticizer used in food and drink packaging, medical devices and paper products (Giulivo M. *et al.*, Environmental Research, Vol. 151: 251-264, 2016), has been prohibited in food industry due to its deleterious endocrine effects on both male and female reproduction (Bloom M. S. *et al.*, Fertility and Sterility, Vol. 106: 857-863, 2016). Thus, BPA has been recently replaced by a structural analogue: bisphenol S (BPS). While its presence is exponentially increasing, BPS use is not regulated and its effects are still poorly understood, particularly on female reproduction. Several studies, especially in fish and rodents, already showed that BPS exhibits a similar impact to BPA in terms of both effects and intensity on the reproductive functions of these species, but this comparison of BPA vs BPS was not yet studied on granulosa cells (GCs) (Chen D. *et al.*, Environmental Science & Technology, Vol. 50: 5438-5453, 2016 ; Rochester J. R. *et al.*, Environmental Health Perspectives, Vol. 123: 643-650, 2015). GCs are essential for female reproductive function. They proliferate and secrete the hormones: progesterone and estradiol to allow the growth and maturation of the follicle and oocyte. The aim of this study is thus to investigate the *in vitro* effects of both BPS and BPA on ovine primary GCs. The ewe model was chosen as it is a relevant animal model for women reproduction. After follicle aspiration of approximately 1000 ovaries from local slaughterhouses, GCs were collected, purified and treated in complemented serum-free Mc Coy Medium, in absence (control) or presence of increasing concentration of BPS or BPA (1 nM, 10 nM, 100 nM, 1µM, 10 µM, 50 µM, 100 µM and 200 µM) for 48 hours. Progesterone and estradiol levels (12 and 5 independent cultures respectively) were measured by ELISA in the supernatant and normalized to the protein concentration of each well. Cell proliferation (13 independent cultures) was measured by ELISA assay after BrDU (BromoDesoxyUridine) incorporation. Data were analyzed using non-parametric permutational ANOVA and Tuckey post-hoc test. Our results showed that BPS did not affect cell proliferation, in contrast to BPA which significantly reduced cell proliferation at 50 µM ($p = 0{,}0007$) compared to the control. On the other hand, BPS significantly decreased progesterone secretion from 10 µM onwards (- 22 %; $p = 0.0038$) , whereas BPA lowered the level of progesterone only at 100 and 200 µM ($p < 0{,}0001$) compared to the control. BPS and BPA significantly increased estradiol secretion similarly from 10 µM onwards (+ 198 % $p = 0.0075$ vs. + 259 % $p < 0.0001$, respectively) compared to the control. These first results showed that BPS exhibits similar effects as BPA on steroidogenesis in ovine primary GCs, but not on cell proliferation. BPS even affected progesterone secretion at lower dose compared to BPA. Thus, BPS is probably not a safe alternative to BPA. Mechanisms disrupted by these molecules are currently studied in ovine primary GCs.

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32. Effect of LIF, IL-6 and IL-11 on microRNA expression of bovine cumulus cells and oocytes matured in vitro

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Keywords: Oocyte, microRNA, cytokines

Members of the interleukin (IL-6) family of cytokines including leukemia inhibitory factor (LIF), IL-6 and interleukin-11 (IL-11) are important for reproductive function, and the expression of specific cytokines is actually required for ovulation. They participate in follicular growth and development, oocyte maturation and implantation. IL-6 cytokines stimulate the intracellular Janus kinase/signal transducer, thereby activating JAK/STAT, MAP-kinase and PI(3)-kinase pathways. This alters downstream expression of genes and microRNAs (miRNAs) in oocytes and follicular cells, creating a microenvironment that improves oocyte quality and competency. However, the putative involvement of miRNAs in the JAK/STAT signal transduction pathway activated by members of the IL-6 family has not been fully elucidated. We, therefore, characterized the effects of LIF, IL-6 and IL-11 on miRNA expression in bovine cumulus-oocyte complexes matured *in vitro*. We assessed the expression of *miR-21*, *miR-155*, *miR-34c*and *miR-146a*, miRNAs previously implicated in oocyte maturation and cumulus expansion. Oocytes were distributed in 5 groups: GV (germinal vesicle), Control (matured in TCM199 + 10% FBS + FSH + LH + E2), LIF (TCM199 + 25 ng/mL LIF), IL-6 (TCM199 + 10 ng/mL IL-6), IL-11 (TCM199 + 5 ng/mL IL-11) and non-supplemented (TCM199). After 24h of IVM, cumulus cells were stripped from oocytes and both cumulus cells and oocytes were collected for miRNA extraction and qPCR analysis. The effects of treatment were analyzed by one-way ANOVA followed by a Sidak test ($p<0.05$). *MicroRNA-21*expression was significantly higher in cumulus cells from the control (FBS) and LIF groups and was higher in LIF-treated oocytes compared to TCM199 alone. IL-11 treatment increased *miR-146a*expression in oocytes while no significant differences were observed in the levels of *miR-146a*in cumulus cells. In cumulus cells, *miR-155*was significantly higher in controls, compared to oocytes, where no differences were observed between groups. The presence of cytokines during maturation had no effect on *miR-34c*expression in cumulus cells or oocytes in any group. miR-21 seems to be one of the most relevant miRNAs in oocyte function. It is the most abundant miRNA in cumulus cells in bovine. It is considered as an indicator of oocyte quality because it increases along oocyte maturation when the oocyte becomes competent for fertilization, and also for its anti-apoptotic role, as some of its target genes are related to apoptosis. miR-21 inhibition leads to an increase of active caspase 3 in granulosa cells, what results in an increased apoptosis. In conclusion, LIF addition to the maturation media may improve oocyte quality through increased expression of*miR-21*.It is relevant that LIF without serum and hormones could create a response in miR-21 similar to that in the controls. Further studies to evaluate the potential effects and mechanisms of action of LIF on bovine oocytes are warranted.

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Physiology of male reproduction and semen technology

33. Transcript abundance and antioxidant biomarker of buck semen cryopreserved with melatonin supplementation

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Keywords: Keywords: bucks, melatonin, motility, total antioxidant capacity, gene expression

This study was carried out to improve the freezability of buck semen using two different types of cryoprotectants with two doses of melatonin as antioxidant. Pooled samples from four sexually mature Egyptian Baladi Bucks were used in this experiment. Semen was diluted (1:8) of Tris-fructose-citric extender containing egg yolk. Either Glycerol or Dimethyl sulfoxide (DMSO) was used as cryoprotectant at a final concentration of 5 %. Extended semen was supplemented with different levels (10^{-6}mM and 10^{-9}mM) of melatonin (M5250, Sigma-Aldrich, St Louis, MO, USA) in addition to control group and cooled at 5 °C for 4h before deep-freezing at -196°C. Extended semen of all groups was adjusted to the same concentration and finally packed in 0.25 ml French straws (IMV).Computer assisted semen analysis (CASA) was used to evaluate semen after cryopreservation. Data was analyzed using the SAS GLM procedure (SAS, 2004) and applying the following model. Duncan's multiple range test was used to detect differences among means, the significance level was set at $P<0.05$. Quantitative real-time PCR data was analyzed using delta Ct method and values were reported as relative expression of target genes to the calibrator after normalization to reference gene (GAPDH). The progressive motility was higher ($p < 0.05$) in control sample extended with glycerol (71.6±2.3%) than that supplemented with DMSO (32.9±2.5%). The progressive motility was higher ($P<0.05$) in samples supplemented by low dose of melatonin (10^{-9} mM) compared with high dose (10^{-6}mM) in glycerol (74.4±2.4% and 64.4±2.5%, respectively) and in DMSO based extender (35.5 ±2.4% and 32.9 ±2.5%, respectively). The CASA parameters (VAP, VCL and VSL um/s) were significantly different in low melatonin dose from high melatonin dose in glycerol based extender being (57.4±1.1, 103.5±2.9 and 42.5±0.8) against (51.3±1.2, 91.8±3.0 and 37.7±0.8) respectively. The activity of total antioxidant capacity (TAC) was significantly greater in DMSO group supplemented with the low melatonin dose (0.49 mM/L ±0.09) than high melatonin dose (0.16 mM/L ±0.09) group. While, there was no significant differences in TAC between glycerol extender groups. Transcript abundance of genes enhancing mitochondrial activity CPT2, ATP5F1A and SOD2 was significantly ($p < 0.05$) increased in glycerol-based extender groups and this was more apparent in low melatonin dose compared with all other glycerol based extender groups. On contrast, gene regulating oxidative stress (NFE2L2) was up-regulated ($p < 0.05$) in groups cryopreserved with DMSO extender compared with those cryopreserved in glycerol based extender. It could be concluded that using glycerol based extender supplemented with low concentration of melatonin would be recommend for enhancing the fertilizing ability of buck semen.

34. Effects of increasing concentrations of LPS on In vitro ovine oocyte developmental competence

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Keywords: inflammation, lipopolysaccharide, oocyte developmental competence

Negative energy balance (NEB) during early lactation in dairy cows leads to an altered metabolic state that has major effects on animal reproduction. Feeding high concentrate diet, a common strategy for mitigation of NEB, enhance the risk of ruminal acidosis. Both ruminal acidosis and infectious diseases can enhance the concentration of lipopolysaccharide (LPS), an important bacterial component in circulation leading to disturbed reproductive performance. Nevertheless, some degree of LPS induced inflammation be beneficial through triggering antioxidant process to protect cell from oxidative stress. Although considerable number of researches investigated the effects of LPS on reproductive performance of dairy cows, the response of sheep to the increasing concentrations of environmental LPS is not defined. Ewes ovary were collected from slaughterhouse, sliced and the oocytes with more than three layers of cumulus cell and integrated cytoplasm were matured for 24 h under increasing concentrations of LPS (0, 0.01, 0.1, 1 and 10 µg/mL). In order to measure the intracellular glutathione (GSH) content, a number of matured oocytes were denuded and stained with cell tracker blue and then observed using an epifluorescence microscope and were analyzed by ImageJ software. A number of matured oocytes also were fertilized using frozen ram semen. Then, the rate of oocytes reached to the blastocyst stage were recorded at day 8 post-insemination. Data were analyzed with GLM procedure of R software. Our data showed that there was no difference ($P \geq 0.05$) between the groups in GSH content, although it was higher in medium with 10 µg/mL of LPS. Addition of LPS reduced the number of fertilized oocytes reached to blastocyst stage in a dose dependent manner (36.69, 34.21, 35.41, 16.66 and 14.28 % of oocytes reached to blastocyst stage, respectively for 0, 0.01, 0.1, 1 and 10 µg/ml of LPS; $P<0.05$). It has been shown that LPS induces the production of pre-inflammatory cytokines such as (Interleukin 6) IL-6 and (Interleukin-8) IL-8 from variety of cells. In mammals, transcription factors such as (nuclear factor-κB) NF-κB and IFN are activated after recognition of LPS by Toll like receptor (TLR-4). Moreover, bovine granulosa cells express TLR4 receptor complex and response to LPS through phosphorylation of TLR signaling components p38 and extracellular signal-regulated kinase and increase the IL-6 and IL8 transcripts. LPS was reported to affect intracellular redox status and increase apoptosis through enhancing pro-apoptotic factors. A group of antioxidant enzymes and non-enzymatic processes protects gametes and embryos against ROS damage during oocyte maturation and early stage of development. In this study, although the difference between groups in regards to GSH content was not significant but maybe higher concentration of glutathione in response to high level of LPS was a mechanism for confronting the inflammatory response created in those groups. In conclusion, our results demonstrate that LPS in 1 and 10 µg/mL concentrations may have detrimental effects on oocyte developmental competence in ovine.

35. CatSper inhibitor effect on porcine sperm in the presence of higher chemotactic activity of the follicular fluid

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Keywords: Boar spermatozoa, NNC effect, follicular fluid

Several components in follicular fluid (FF), in particular progesterone, have chemotactic capacity and depend on the entry of Ca2+ through membrane or CatSper channels in some species (Lishko P.V. et al., Nature, 471(7338):387–91 (2011)). On the other hand, P4 does not seem to be a clear CatSper agonist in porcine (Vicente-Carrillo A. et al., ReproBiol, 17 (1): 69-78 (2017)). The NNC 55-0396 inhibitory effect on CatSper in sperm has been demonstrated. The aim of this study was to investigate NNC effect in the presence or not of FF. The chemotaxis system used consists of two wells (A and B) connected by capillaries. Four wells (A) were filled with fresh sperm after washing in a discontinuous gradient of Percoll®, followed by TALP medium (20x106/mL diluted in 500 µL) from proved fertility boars (N=4) previously incubated or not with NNC alone, NNC and 0.25% of FF, while the opposite wells (B) were filled with TALP (control group) and TALP supplemented with 0.25% of FF. NNC (2 µM) without cytotoxic effects and 0.25% of FF were used. Two experiments were performed: Experiment I: the A and opposite B were filled with 1: Control group (TALP (A) – TALP (B)), 2: FF+ (TALP (A) – FF (B)), 3: NNC (TALP+NNC (A) – TALP (B)), 4: FF- (TALP+NNC (A) – FF (B)). Experiment II: the A and opposite B were filled with 1: Control group (TALP (A) – TALP (B)), 2: FF+ (TALP (A) – FF (B)), 3: NNC (TALP+NNC (A) – TALP (B)), 4: NNC+FF (TALP+NNC+FF (A) – FF (B)). In experiment I, treatment 3 and 4 were pre-incubated (10 min) with NNC before chemotaxis, likewise, for treatment 3 in experiment II. However, the treatment 4 in experiment II was pre-incubated (10 min) at the same time with NNC and 0.25% of FF before chemotaxis. After 20 min of chemotaxis, the sperm concentrations (%) from wells B were evaluated using the free statistical software, SAS University Edition (SAS, 2016). In experiment I, the highest percentage of attracted sperm was seen in FF+ (9.1%b) versus control group TALP (7.5%a), NNC group (5.6%a), and FF-group (6.8%a) ($p<0.05$). Similarly to experiment II: FF+ (5.7%b) versus control group (4.6%a), NNC group (3%c), and NNC+FF group (3.9%a) ($p<0.05$). These results may indicate the chemotactic effect of FF on boar spermatozoa. Moreover, NNC inhibited sperm chemotaxis even in the presence of higher chemotactic activity of FF, which suggests that at least the chemoattractant components in FF might act via CatSper. Further studies should be carried out to test this hypothesis.
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36. Factors affecting the sperm concentration assessment in commercial seminal doses in pigs

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Keywords: sperm assessment, quality control, sperm concentration
Artificial insemination is widely implemented worldwide with more than 90% of the sows inseminated with fresh semen. However, up to now, a quality control system for swine artificial insemination centers has not been internationally established. Evaluation of the sperm concentration in commercial seminal doses is a key point in the control of the dose's quality, since a clear relationship is established between total sperm number in the insemination dose and fertility outcome. The use of cell counting chambers is a cheap and simple methodology. Nevertheless, it tends to be less precise than other more sophisticated and expensive methodologies (Hansen, Theriogenology, 66, 2188. 2006). The aim of this study was the evaluation of different factors (dilution rate, pipetting repeatability, microscopy magnification, time of sample resting before evaluation and chamber area evaluated) that could modify the results in sperm concentration of commercial seminal doses, for further proposal of a scientific base a standardized protocol. Sperm concentration in 27 seminal doses was evaluated by one observer by dilution (1/10 or 1/20 rate) in saline solution (0.3% formaldehyde), pipetting by triplicate, disposed in a Neubauer chamber and observed after 1 or 5 minutes by contrast phase microscopy (x100, x200 or x400 magnification) and counting the number of spermatozoa present in 0.12 or 0.2 mm^2. Data were expressed as the mean ± SEM and analysed by ANOVA, considering the specific factors (dilution, pipetting, time, objective, area) as the main variable and sample as covariate. Bland-Altman analysis was applied to assess the degree of agreement, showing the bias (mean±SD). The pattern of relationship between difference and average was evaluated by lineal regression as quality of the agreement ($p<0.05$). Sperm concentration was not affected by dilution rate 1/10 or 1/20 (bias 1±5.42, p=0.74). No differences were found for concentration of samples by 3 pipetting procedures (p=0.81), between 1 or 5 minutes in the chamber before examination (p=0.73) and between counting areas of 0.12 vs. 0.20 mm^2 (p=0.69). However, the concentration measured using x10 objective was higher (41.56±2.54x10^6/ml) than using x20 (37.81±1.95, p=0.03) and x40 objectives (38.03±1.89, p=0.02). These differences were confirmed with significant regression for the difference x40-x10 (bias -3.53±9.14, p=0.01) and 20x-10x (bias -3.75±9.76, p=0.02). The overestimation with x10 objective could be related to difficulties to observe with precision the limits of the counting area or measuring as spermatozoa other different particles. These problems are minimized when higher magnification is used, although time consuming is higher with higher magnification. So, according to the obtained results, we propose a standard procedure with the selection of x20 contrast phase objective, dilution 1/20, 1 min sample resting and 0.2 mm^2 to optimize precision and time consuming.
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37. L-carnitine supplementation to UHT skimmed milk-based extender improves motility and membranes integrity of chilled ram sperm up to 96 h

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Keywords: L-carnitine, sperm, ram

The addition of new additives as L-carnitine (LC) to extenders could mitigate reactive oxygen species (ROS) production and improved motility and viability in chilled ram sperm as has been demonstrated in other species (e.g. human, bull, and mice). The aim of this work was to evaluate the antioxidant effect of LC on motility variables and integrity of plasma, acrosomal, and mitochondrial membranes of chilled (5 ºC) ram sperm up to 96 h. Twelve pools from 36 semen ejaculates were collected by artificial vagina from 12 Merino rams (3-9 years) in four sessions during non-reproductive season (June to August). Each pool was divided into 6 aliquots and then diluted at $200x10^6$ sperm/ml in UHT-based extender (skimmed milk-6% egg yolk) supplemented either 1mM (LC1), 2.5mM (LC2.5), 5mM (LC5), 7.5mM (LC7.5), and 10mM (LC10) of LC. A control group without LC was included in each pool. Sperm motility variables were assessed by CASA system (SCA®) and total sperm with intact plasma membrane / intact acrosome / intact mitochondrial membrane (IPIAIM,%) was assessed by triple fluorescence association test (PI/PNA-FITC/Mitotracker green) at 0, 48, and 96 h. The effects of LC concentration and cold-storage time were analyzed by one-way ANOVA and Bonferroni´s test ($p<0.05$). Overall, the results showed that kinetic variables and integrity of sperm membranes decreased ($p<0.05$) as cold-storage time increased in all groups. The results revealed a higher ($p<0.01$) sperm motility (SM, %) in all LC groups than control group at 48 h. However, at 96 h, both LC5 and LC10 groups showed a SM higher ($p<0.001$) than both LC7.5 and control group (87.9±2.2 and 88.0±1.8 vs 82.9±2.1 and 82.5±3.1, respectively). Progressive sperm motility (PSM, %) was higher with LC5 group than control group at 48 h (42.2±2.9 vs 36.7±1.8) and 96 h (35.7±3.4 vs 29.0±1.7). Surprisingly, straight line velocity (VSL, µm/s) was improved with all LC groups compared with control group at 0h ($p<0.01$), 48h ($p<0.001$) and 96 h ($p<0.001$). Moreover, at 96h VSL (µm/s) value was higher with LC7.5 group than all LC groups ($p<0.5$) and control ($p<0.001$) (LC7.5: 87.2±4.9 vs LC1: 75.1±4.5, LC2.5: 78.6.2±5.9, LC5: 79.4±5.0, LC10: 79.3±5.2, and control: 65.4±3.4). Likewise, IPIAIM percentage was higher ($p<0.001$) in all LC groups than control group during at 48 h and 96 h (LC1: 62.3±2.0, LC2.5: 66.3±1.7, LC5: 63.3±2.8, LC7.5: 66.5±2.4, and LC10: 66.3±1.9 vs control group: 49.2±2.9). These results revealed a kinetic-enhancer effect of LC supplementation to UHT skimmed milk-based extender, which might improve fertility following cervical insemination of sheep.

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38. Identification and localization of NADPH oxidase 5 in ram spermatozoa

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The aim of this study was to identify the presence of NADPH oxidase 5 (NOX5) in ram spermatozoa and to investigate if melatonin could modulate this enzyme during *in vitro* capacitation. Semen from nine *Rasa Aragonesa* rams was collected and pooled. Seminal plasma free spermatozoa were selected by a swim-up procedure (control sample). Spermatozoa were then incubated in TALP medium without (TALP sample) or with cAMP-elevating agents (cocktail sample, Ck) for 3 h at 39 °C and 5% CO_2. 1 µM melatonin was added to TALP and cocktail samples (TALP-Mel and Ck-Mel) (n=6). Capacitation status was evaluated by chlortetracycline (CTC) staining. Identification and distribution of NOX5 in ram spermatozoa was investigated by western-blot and indirect immunofluorescence (IIF) with the anti- rabbit NOX5 C-terminal antibody (ab191010, Abcam, Cambridge, UK). At least 200 spermatozoa were scored per sample in CTC and IIF assays. Differences between experimental groups in CTC staining and NOX5 immunolabeling were compared by means of chi-square test using GraphPad InStat software (Version 3.01). As expected, the inclusion of cAMP-elevating agents in the cocktail sample increased the capacitated-sperm pattern by CTC compared with TALP sample after *in vitro* capacitation ($p< 0.001$), whereas the presence of melatonin at 1 µM in both samples increased the non-capacitated-pattern relative to samples without hormone ($p<0.001$). Regarding the presence of NOX5 in ram spermatozoa, Western blot analyses revealed a band of 86 kDa compatible with that reported to NOX5 in human (Musset et al., The journal of biological chemistry, 287: 9376-9383,2012) and equine (Sabeur and Ball, Reproduction 134:263-270, 2007) spermatozoa. IIF revealed six differences immunotypes depending on the presence of NOX5 in the ram sperm: I: apical region II: acrosome, III: post-acrosome, IV: apical and post-acrosomal, V: acrosome and post-acrosome (all subtypes with midpiece labelling) and VI: labelling in the midpiece of the spermatozoa. In swim-up selected (control) ram spermatozoa, the predominant NOX5 immunotypes were I and II. After incubation in capacitating conditions, these immunotypes decreased in TALP samples and increased those III and V ($p< 0.001$) when compared to control. In cocktail samples, there was also an increase in the rate of spermatozoa with labelling only in the midpiece of the flagellum (type VI, $p<0.001$). However, the presence of melatonin in TALP medium (TALP-Mel) increased II subtype and in cocktail sample (Ck-Mel) increased V immnutype ($p< 0.001$), spermatozoa presented a NOX5 distribution very similar to that observed control and TALP samples respectively. In conclusion, these preliminary results reveal for the first time that NOX5 is present in ram spermatozoa, and that melatonin can prevent the NOX5 distribution changes associated with sperm capacitation.

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39. Effect of repeated ejaculates on seminal plasma composition and semen liquid storage in INRA180 ram

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Keywords: repeated ejaculate, semen storage, seminal plasma INRA 180 ram

In sheep, superior rams are used extensively for mating or as sperm donors for genetic improvement. Nevertheless, sperm production capacity and its storage are a major factor confining extensive use of rams over numerous ewes for a long period of time. The objective of this experiment is to evaluate the effect of repeated ejaculates on liquid storage sperm motility, and seminal plasma biochemical composition in INRA180 ram. Five INRA180 rams were collected weekly for 4 weeks at a rate of three ejaculates each 20 minutes. Concentration of total protein (g / l) (Prot) (Lowry et al.,1951. *J. Biol. Chem. 193, 265-275*), total lipid (g / l) (Lip) (Woodman and Price, 1972. *Clin. Chim. Acta., 38, 39-43*) and fructose concentration (g / l) (Fruc) (Mann, 1948, *J. Agric. Sci., 38, 323-331*) were evaluated. Immediately after collection and initial evaluation, the semen was extended in skim milk (SM) at 15°C to reach 0.8×10^9 spermatozoa/ml. Thereafter, the samples were evaluated at different storage times (0, 8, and 24 h). A CASA system was used to determine total (TM%) and progressive (PM%) motilities. All analyses were carried out using a statistical software program JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA). Variance analysis (one-way ANOVA) was performed. The statistical model included fixed effects of repeated ejaculates (first, second and third). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors for Prot, Lip and Fruc in seminal plasma, TM and PM in each storage duration (0, 8 and 24h) considering the significance level of $P < 0.05$. The results showed that, in seminal plasma, the total protein concentration was significantly higher in the first (25.30±0.22 g/l) and second ejaculates (25.17±0.29 g/l) compared to the third (24.36±0.23 g/l). The highest total lipid concentration was recorded in the first ejaculates (3.75±0.07 g/l) ($P < 0.05$), followed by the second ejaculates (3.63±0.08 g/l) ($P < 0.05$), while the third ones (3.46±0.09 g/l) recorded the lowest total lipid concentrations ($P < 0.05$). The fructose concentration was higher in the second ejaculates (5.49±0.16 g/l) ($P < 0.05$), followed by the first ejaculates (5.39±0.14 g/l) ($P < 0.05$), while the third ejaculates recorded the lowest fructose concentrations (4.96±0.17 g/l) ($P < 0.05$). Regarding semen liquid storage, the results indicated that the second ejaculate has significantly better sperm motility compared to the first and the third ones and this still true until 8 h of liquid storage. While at 24 h, the first ejaculate gives the best results ($P < 0.05$). To conclude, our results recommend the use of the second ejaculate for artificial insemination before 8 h of storage and the first ejaculate until 24 h of storage.

40. Effects of semen collection methods and equilibration times on post-thaw sperm kinematic parameters of Saanen bucks

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Keywords: electro-ejaculation, equilibration time, sperm kinematics

The success of AI depends on semen quality and female fertility. Buck semen is commonly collected by artificial vagina (AV). However, when bucks cannot be trained for semen collection or semen is collected to evaluate fertility of bucks before mating season, the electro-ejaculation (EE) method is usually utilized. However, differences on sperm characteristics between ejaculates collected by AV and EE have been reported. The equilibration process and type of extender are known to affect the quality of post-thaw sperm quality and this study hypothesize that semen collection methods also have confounding effects. The present study was conducted to investigate the effects of semen collection methods and equilibration times on sperm kinematic parameters of Saanen bucks. Eight bucks were divided into two equal groups (4 bucks) based on semen collection methods; AV or EE. In total, 12 ejaculates (collections) per buck were collected at weekly intervals. Freshly collected ejaculates were pooled per group and extended with clarified egg-yolk tris extender (CEY). Pooled semen samples were cooled to 4^{o}C within 2 h and equilibrated at 4^{o}C for 2, 4 and 6 h in separate aliquots. Thereafter, the samples were then frozen using standard procedure after completion of each equilibration time. Four straws of frozen semen per group per collection were thawed at 33°C for 30 sec and evaluated for post-thaw sperm motility and kinematic parameters using CASA system 24 h after freezing. Semen collected with AV had significantly ($p<0.001$) higher sperm curvilinear velocity (VCL: 122.21±1.23 µm/s), straight line velocity (VSL: 89.24±0.11 µm/s), linearity (LIN: 64.23±0.91 %), beat/cross-frequency (BCF: 7.21±0.02 Hz), total motility (98.12±0.34 %), rapid sperm (66.26±0.11 %) and progressive motility (77.51±1.12 %) compared to semen collected with EE method. Post-thaw sperm curvilinear velocity (VCL: 65.52±0.02 µm/s) were higher ($p<0.001$) for sperm equilibrated for 2 h in semen collected with AV methods compared to other equilibration times. Straight line velocity (VSL: 49.15±0.92 µm/s) was higher ($p<0.001$) for sperm equilibrated for 2 h in semen collected with AV methods. Average path velocity (VAP: 64.65±0.43 µm/s) was higher ($p<0.001$) for sperm equilibrated for 4 h in semen collected with AV. Linearity coefficient (LIN: 74.34±1.01 %) and straightness coefficient (STR: 77.89±0.45 %) in semen collected with AV were higher ($p<0.001$) for sperm equilibrated for 2 h compared to semen collected with EE and other equilibration times (4 and 6 h). Amplitude of lateral head displacement (ALH: 3.46±0.98; 3.39±0.02 µm) was higher ($p<0.001$) for sperm equilibrated for 4 h in semen collected with AV and 2 h equilibration in semen collected with EE. The AV method and equilibration times for 2 or 4 hours preserved sperm motility and kinematic parameters post-thaw.

41. Description of porcine spermatozoa-interacting proteins after contact with male and female reproductive fluids

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Keywords: porcine, biological fluids, sperm proteome

After ejaculation, sperm are deposited within the female genital tract by natural or artificial insemination, starting their journey towards the fertilization site. Along the way, sperm take contact with reproductive fluids, and only few of them reach the oocyte. The first fluid in which sperm are immersed during ejaculation is seminal plasma (SP), involved in aiding sperm transport and survival through the female genital tract by SP-proteins, improving their fertilizing ability (Bromfield, Animal, 104-109, 2018). Once deposited within the uterus, sperm contact with uterine fluid (UF), a dynamic female milieu that changes its proteome during the oestrus cycle (Soleilhavoup, Mol Cell Proteomics, 93-108, 2016). In sow, sperm-UF interaction is still unknown, but UF exerts a cytotoxic effect on sperm cells unprovided of SP (Kawano, Proc Natl Acad Sci, 4145-50, 2014). After crossing the uterus, selected sperm reach the oviduct, interacting with the oviductal fluid (OF), inducing sperm functional changes (Perez-Cerezales, Biol Reprod, 262-276, 2018). Since sperm interaction with fluids may change sperm proteome, the aim of this study was to identify proteins that adhere to ejaculated sperm (S) after contact with different reproductive fluids [SP, UF and OF (collected in slaughtered sows)] for a better understanding of sperm behavior during their journey previous to fertilization. The experimental groups used were: 1) S group (control): sperm without reproductive fluids; 2) SP group: S incubated with 20% SP; 3) UF group: S incubated with 20% UF (late follicular phase); 4) OF group: S with 20% OF (late follicular phase); 5) UF-SP group: S with 20% UF and 20% SP; 6) OF-SP group: S with 20% OF and 20% SP. All the groups were incubated for 180 min at 38°C, then centrifuged at 600 *g* for 5 min and the pellet was used for protein extraction, carefully performed to detect surface proteins. Sperm proteome was assessed by HPLC-MS/MS analysis. The total number of proteins identified was 88. Among these, 56 proteins were detected in S group and in UF, SP and UF-SP groups. 72 proteins were detected in S group and in OF, SP and OF-SP groups. This study has also allowed to identify a higher number of proteins in common between OF and OF-SP groups (29), than in UF group and UF-SP group (3 proteins in common). Furthermore, sperm incubated with UF-SP showed a lower number of proteins (17) than when incubated with SP (32) or UF (42). Instead, sperm showed 44 proteins when incubated with OF-SP, 32 proteins with SP and 42 proteins with OF. One of detected proteins, sperm acrosome membrane-associated protein 1, was expressed in all the groups except in OF-SP group. Moreover, sperm equatorial segment protein 1 was detected in all the groups except in UF-SP group. The combined use of SP with UF or OF suggests an interaction between these fluids that modify the sperm proteins probably caused by a steric hindrance. In conclusion, this study highlights how sperm proteome changes after interaction with different reproductive fluids, with a potential physiological impact during the *in vivo* fertilization process.

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42. Nuclear morphometrics and chromosome positioning in boar sperm

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Keywords: sperm, nucleus, morphometrics

The predicted increase in the global population and changes in dietary preferences have led to a rise in the demand for meat products. Artificial insemination is routinely used in commercial pig breeding, for which the use of high-quality semen samples during insemination is crucial. With an aim to reduce inter-operator variability and the laborious nature of manual semen analysis, we have developed a fast, unbiased software-based approach which allows comprehensive analysis of pig sperm nuclear morphometrics. Fresh ejaculated semen samples were identified as either fertile or sub-fertile using a combination of computer assisted sperm analysis (CASA) and manual assessment prior to use of samples in this study. Using CASA, 'normal morphology' was assigned to samples if less than 30% of the sample contained morphological defects such as bent tails, coiled tails, distal midpiece reflex (DMR), proximal droplets and distal droplets. Subjective manual assessment was used to score motility from 1 to 5, 1 being dead and 5 being excellent. Samples were categorised as fertile if more than 70% of the sample had 'normal morphology' and if at least 85% of the sample had a motility score of 4 or above. Those falling below these criteria were categorised as sub-fertile. Analysis of nuclear morphology from 50 fertile and 50 sub-fertile samples yielded measures from 11,534 and 11,326 nuclei respectively. Cluster analysis using measures of Area, Circularity, Variability, Bounding height and Bounding width by Ward linkage using squared Euclidean distance and standardised variables supported the existence of three clusters with different membership for fertile and sub-fertile sperm. Specifically, sperm heads from fertile animals were overrepresented in one cluster and underrepresented in another. The cluster in which sperm heads from fertile samples were overrepresented was characterised by a high mean nuclear area, which was a consequence of greater head width, and by low variability between sperm heads. We extended this analysis to determine if chromosome positioning in pig sperm also varies between fertile and sub-fertile samples. In a preliminary study, two fertile and two sub-fertile semen samples from Pietrain boars were analysed using fluorescence *in situ* hybridisation with locus-specific subtelomeric probes, and the position of pig chromosomes 10 and X were determined. This suggested that chromosomal position also differs between nuclei from fertile and sub-fertile samples. Based on this preliminary finding, we are currently extending this study to perform a complete analysis of nuclear organisation using a larger sample size of 20 samples and imaging more cells per sample. In conclusion, we show that there are morphological and chromosome positioning differences between sperm nuclei from fertile and sub-fertile samples. This approach therefore has the potential both to be used as a tool for sperm morphology assessment and as a way to investigate the causes of fertility differences.

43. Season affects refrigerated-stored semen doses from a commercial stud AI centre: A flow cytometry study of sperm physiology and chromatin status

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Keywords: spermatozoa, pig, seasonality

The pig industry rely on the production of semen doses in stud centers, which are distributed to the production farms. Advances in boar selection, extender formulation and storage allow for consistency on sperm quality and artificial insemination (AI) results. However, seasonality still affects semen quality (Porcine Health Manag 3:15, 2017). Our objective was to characterize the influence of the season in the doses produced in a modern center (NE Spain). We tested two hypotheses: Sperm quality was affected by the season, and this effect followed a yearly sinusoid pattern. Semen doses (40×10^6 ml^{-1}) were produced from 236 Pietrain boars in routine semen production (extender from Magapor, Zaragoza, Spain). The doses (436 from early 2017 to early 2019) were sent at 17 °C to the laboratory, being analysed by 48 h of storage. An aliquot was added to the staining solution at 10^6 ml^{-1} (BTS with Hoechst 33342, PNA-FITC, merocyanine 540, propidium iodide and Mitotracker deep red; ThermoFisher, Waltham, MA), for viability, acrosomal status, capacitation, and mitochondrial activity assessment (Theriogenology 80, 400–410, 2013). Another aliquot was submitted to ORT (osmotic resistance test, 15 min in 150 mOsm/kg BTS before staining). After 15 min at 37 °C, samples were run in a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). Sperm chromatin was assessed by SCSA (Sperm Chromatin Structure Assay; Methods Cell Sci 22:169–189, 2000), obtaining %DFI (DNA fragmentación) and %HDS (chromatin immaturity). Data were analyzed with the R statistical package, testing season effects with linear mixed-effect models (calendar season as fixed and male as grouping factors) and cosinor regression. We also tested the relationship between physiological and chromatin variables by Pearson correlations. Doses collected were 111 in spring, 96 in summer, 117 in fall and 112 in winter. Sperm quality was overall good (interquartile ranges, viability: 79.6, 89.6; intact acrosomes: 88.4, 93.2; viable-capacitated: 2.9, 5.5; viable-active mitochondria: 71.1, 97.2; ORT: 61.6, 87.1; %DFI: 0.4, 0.7; %HDS: 0.5, 1.6). We detected a season effect in viability, acrosomal integrity, mitochondrial activity, and %HDS ($P<0.001$), and in viable capacitated ($P=0.003$). Cosinor detected a yearly sinusoid pattern ($P<0.025$) (peak and low-point indicated) for: viability and mitochondrial activity (mid-spring/mid-fall); acrosomal integrity (early-fall/early-spring); capacitated (late fall/late spring); and %HDS (early summer/early winter). We also found significant correlations of %HDS with acrosomal integrity (-0.66, $P<0.001$) and mitochondrial activity (0.40, $P=0.048$). Overall, the effect size of the calendar season was small. However, its influence on the %HDS, being a chromatin structure parameter, merits study. Stud centers should take these results into account, since the season effect may increase in suboptimal situations, affecting the adherence to quality standards. Supported by RTI2018-095183-B-I00 (Ministry of Science, Innovation and Universities, Spain) and AGL2016-81890-REDT (MINECO, Spain). We thank EVB (Spain) and Lucía Tejerina for their collaboration in this study.

44. SLO1 channels are essential for acrosome reaction during in vitro capacitation of boar spermatozoa

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Keywords: sperm; capacitation; acrosome reaction; pig; SLO1 channels

The aim of the present study was to determine whether SLO1 channels, also known as big potassium (BK) or maxi K^+ channels, are involved in sperm capacitation and acrosome reaction in boar spermatozoa. With this purpose, we incubated semen samples from five boars in *in vitro* capacitation medium plus paxilline 100 nM (PAX), a specific blocker of SLO1 channels. Sperm samples were incubated in capacitation medium at 5% CO_2 and 38.5ºC for 240 min, with or without PAX. At 240 min, progesterone was added to control and PAX samples to induce the acrosome reaction. Samples were incubated for further 60 min (300 min). After 0, 60, 120, 180, 240, 250, 270 and 300 min of incubation, total and progressive motility were measured by Computer Assisted Sperm Analysis (CASA), and acrosome integrity, permeability of plasma membrane, and intracellular calcium levels measured by Fluo3 and Rho5 were determined by flow cytometry. After confirming that data distributed normality and variances were homogenous, a mixed model followed by post-hoc Sidak test was run. Total and progressive motility, as well as calcium levels measured by Fluo3, which preferentially stains calcium residing in the mid-piece, did not differ significantly between control and PAX samples at any incubation time ($P>0.05$). Despite permeability of plasma membrane and acrosome integrity being lower in PAX than in control samples after the addition of progesterone, these differences were not significant ($P>0.05$). In contrast, intracellular calcium levels measured by Rhod5, which has more affinity for calcium residing in the sperm head, and acrosome reacted spermatozoa were significantly ($P<0.05$) lower in PAX than in control samples after 250 and 300 min of incubation. We can thus conclude that, while SLO1 channels do not seem to play a key role for motility regulation of boar spermatozoa during capacitation and progesterone-induced acrosome exocytosis, they are essential for triggering the acrosome reaction. This involvement appears to be related with the modulation of calcium stores present in the sperm head.

45. In vitro assessment of sperm characteristics using semen from Norwegian Red bulls with high and low fertility

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Keywords: bull, non-return rate, sperm characteristics

The aim of this study was to investigate possible associations between *in vitro* parameters and fertility by assessment of several *in vitro* sperm characteristics and IVF using cryopreserved semen from Norwegian Red bulls of contrasting fertility. The bulls were characterized as low- or high-performing bulls based on non-return rate after 56 days (NR56) for an average of 1132 first AIs per bull, ranging from 47% to 79%. NR56 was calculated as LSmean for 507 bulls used in AI from 2013-2018, based on a General Linear Model (PROC GLM in SAS®) including the following parameters: bull, AI month and year, parity and double AI within 1-4 days. Totally 37 bulls with contrasting NR56 were selected for analyses, 19 bulls with NR56 LSmean ranging from 0.76 to 0.78 and 18 bulls ranging from 0.46 to 0.65. Cryopreserved semen doses were analysed for total sperm motility, progressive motility and hyperactivity by computer-assisted sperm analysis. Additionally, the ATP content was assessed using the CellTiter-Glo® Luminescence assay. Sperm chromatin, acrosome and plasma membrane integrity were analysed by flow cytometry using the Sperm Chromatin Structure Assay, Alexa 488 conjugated peanut agglutinin and propidium iodide, respectively. Furthermore, semen from selected bulls from the contrasting fertility groups were used for IVF. *In vitro* production of embryos were performed with media from IVF Bioscience using four well plates containing 500 µL of the respective media. Bovine ovaries were collected at a local slaughterhouse, transported to the laboratory and cumulus–oocyte complexes (COCs) were aspirated from follicles sized 3 to 15 mm in diameter. Groups of high quality COCs were matured for 22 h (6% CO_2, 38.8°C). Spermatozoa prepared at a concentration of 1 × 10^6 /ml were added to each group of oocytes followed by 18 h incubation (6% CO_2, 38.8°C). Cumulus cells were removed from the presumptive zygotes by vortexing prior to cultivation in a humidified atmosphere (7% O_2, 6% CO_2 and 87% N_2). At day 3 post-fertilization, the cleavage rate was evaluated. Further, the blastocyst rate at day 7 and day 8 was recorded. Cryopreserved semen from each bull was used in three replicate experiments including 180 oocytes per bull. Statistical analyses were performed by linear mixed models in Rstudio (v 1.1.463) using the *in vitro* sperm parameters and LSmean for NR56 as dependent and independent variables, respectively. In addition, bull, age and season at the time of semen collection were included in the model. Total motility, progressive motility and hyperactivity was positively associated with NR56 ($p<0.05$). Furthermore, sperm chromatin integrity, calculated as DNA fragmentation index and high DNA-stainable sperm, showed a negative association with NR56 ($p<0.05$). The ATP content and acrosome integrity were not associated with NR56. Preliminary results from IVF indicate that bulls of similar fertility (NR56) obtain different blastocyst yields *in vitro*. In conclusion, the results of the study showed that NR56 was associated with several *in vitro* sperm parameters. Prediction of fertility might be possible combining *in vitro* sperm analyses, where the use of IVF could provide valuable additional information.

Embryology, Developmental Biology, and Physiology of Reproduction

46. Embryo-induced alterations in the endometrial transcriptome of prepubertal bovine heifers

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Keywords: endometrium, prepubertal, calves

Advancing the age at which puberty is reached in replacement heifers is central to the financial and environmental sustainability of cattle production systems. Puberty onset is regulated by a complex network of biochemical processes and involves interaction between key metabolic, neuroendocrine and reproductive tissues. Most components that regulate the hypothalamic-pituitary-ovarian axis are in place before the occurrence of puberty. However, it is unclear if the prepubertal uterus is capable of responding to the presence of an embryo or conceptus. Thus, the objectives of this study were to determine the response of the endometrium of 5-month-old prepubertal heifers to i) Day (D) 7 blastocysts (Experiment 1), and ii) a D14 conceptus or 100 ng/ml of interferon tau (IFNT) (Experiment 2), and to compare this response to that of a postpubertal endometrium. Angus X Holstein-Friesian heifer calves (prepubertal group; n= 9) were euthanized at 21 weeks of age. Reproductive tracts were recovered to obtain endometrial explants. For Experiment 1, crossbred postpubertal beef heifers (n= 5) were synchronized and slaughtered on D7 of the cycle (D0 = expected ovulation) to obtain endometrial explants. Twenty D7 in vitro produced (IVP) blastocysts were placed on top of an explant from prepubertal (PreP-D7; n= 5) or postpubertal heifers (PostP-D7; n= 5), and co-cultured for 6 h. For Experiment 2, crossbred postpubertal beef heifers were synchronized and either used to generate D14 conceptuses following the transfer of IVP blastocysts on D7 (n= 9; 15 embryos/recipient) or were used to obtain D14 endometrial explants (n= 5). Conceptuses were recovered on D14 by post-mortem uterine flushing and placed individually on top of explants from prepubertal (PreP-D14; n= 4) or postpubertal heifers (PostP-D14; n= 5) and co-cultured for 6 h. In both experiments, endometrial explants were cultured with medium alone as a negative control (PreP-CTRL and PostP-CTRL; n= 4-5 /group). All explants were snap frozen for subsequent RNA-seq. Despite a large number of differentially expressed genes (DEG) between PreP-CTRL and PostP-CTRL on D7 (n= 6063), the response to D7 blastocysts was similar: 27 DEG between PreP-D7 and PreP-CTRL and 5 DEG between PostP-D7 and PostP-CTRL (all 5 also upregulated in the prepubertal endometrium). All D7 embryo-induced DEG were interferon-stimulated genes (ISG). Similarly, while a comparison between PreP-CTRL and PostP-CTRL on D14 revealed 3544 DEG, endometrial response to a D14 conceptus was similar: 42 DEG in PreP-D14 and 61 DEG in PostP-D14 (37 genes in common). All genes upregulated in PreP-D14 and 57 of the 61 DEG in PostP-D14 were ISG. Exposure to exogenous IFNT increased the expression of a similar number of genes (165 in PrepP-IFNT and 168 in PostP-IFNT, relative to the controls, 113 of which were shared). Of the 27 DEG induced in PreP endometrium by a D7 blastocyst, 26 were common with those induced by a D14 conceptus. In conclusion, prepubertal endometrium is capable of responding to D7 blastocysts, a D14 conceptus, and IFNT in a manner similar to that of postpubertal endometrium.

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47. Anti-cancer potential of pomegranate peel on human ovarian carcinoma cells OVCAR-3

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Keywords: pomegranate, cancer, ovary

Pomegranate (*Punica granatum*) is a unique and potent source of biologically active substances including flavonoids, anthocyanins, and especially ellagitannins and punicalagins with many beneficial properties. Furthermore, several scientific studies have focused on the bioactivity of pomegranate peel extracts, which possess remarkable antioxidant, antibacterial, anti-inflammatory and anti-cancer activities. In accordance with anti-cancer potential of pomegranate fruits, the aim of our study was to examine the *in vitro* effect of pomegranate peel extract at the different concentrations (0; 25; 50; 100; 200 µg/ml), in short-term application (for 24 h) on a human ovarian carcinoma cell model system (OVCAR-3). Analysis were focused on cell viability, production of reactive oxygen species (ROS), and expression of NAD-dependent deacetylase SIRT1 and histone γ-H2AX as a marker DNA double strand breaks. For this experiment, the ethanol extract from lyophilized pomegranate peel was prepared. Cells treated with ethanol in an amount corresponding to the highest used concentration of extract were used as positive controls (+Control) for the experiments. The metabolic activity was evaluated by AlamarBlue™ cell viability assay; the ROS production was quantified by chemiluminescence and the protein expression was detected by Western Blot analysis. Band intensity was quantified using Image Lab™ software (Bio-Rad, CA, US). Statistical significances were established by using One-way ANOVA along with Dunnett's test. All experiments were done in triplicate. The pomegranate peel extract significantly ($P \leq 0.001$) inhibited the viability of OVCAR-3 cells at all used concentrations in comparison to control. Moreover, ROS generation was significantly ($P \leq 0.01$) increased at all used concentrations of pomegranate peel extract in a dose-dependent manner. Interestingly, evaluation of the level of SIRT1 showed significant ($P \leq 0.05$) decrease in ovarian cancer cells OVCAR-3 in comparison to healthy cells human ovarian granulosa cells (HGL5). On the other hand, SIRT1 expression was significantly increase after pomegranate peel extract treatment at the concentrations 100 and 200 µg/ml in OVCAR-3 cells ($P \leq 0.05$). Additionally, all used concentrations of pomegranate peel extract led to significant ($P \leq 0.01$) γ-H2AX over-expression in ovarian cancer cells OVCAR-3 as a response to DNA damage. In conclusion, our data suggested that oxidative stress due to pomegranate-induced ROS production resulted in a decrease in the number of viable OVCAR-3 cells. The results show dose-dependent effect of pomegranate peel extract on human ovarian carcinoma cells OVCAR-3 and the potential role of pomegranate in the prevention or treatment of cancer by regulation of various signal pathways. Further studies are essential to understanding the therapeutic potential of pomegranate peel extract, however, it might serve to be a potential chemoprotective agent.

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48. Sexual dimorphism during early embryo development in the bovine: differential gene expression in relation with oxidative stress and culture conditions

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Keywords: bovine blastocyst, RT-qPCR, sex ratio

Female and male mammalian embryos differ from the onset of the embryonic genome. This is mainly due to differential gene expression related to sexual chromosomes. Indeed, large parts of X-linked genes are overexpressed in female embryos up to the inactivation of one X chromosome. Therefore, metabolism and adaptation to environmental conditions differ between sexes. Using the IVP bovine embryo as a model, we showed that culture conditions and induced oxidative stress differentially impact male and female embryos at the early blastocyst stage. Male embryos survived an oxidative stress induced by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) better in the presence of serum (FCS medium; sex ratio: 61 vs 46% in control embryos; $p<0.05$), while female embryos were more resistant than males in serum-free conditions (BSA-ITS medium; sex ratio: 44 vs 59% in control embryos; $p<0.05$). The quality of the surviving blastocysts, i.e. the apoptotic rates, also differed between sexes (Dallemagne et al., Theriogenology, 2018, 117, 49). In order to decipher the origin of those differences, the relative level of expression of several genes was evaluated by RT-qPCR on single blastocysts obtained in the same culture conditions. Briefly, bovine embryos were cultured in FCS or BSA-ITS medium. Oxidative stress was induced from D5 post-insemination (pi) using 0.1 or 1mM AAPH, respectively. At D7 pi, blastocysts were recovered and individually snap frozen in liquid nitrogen until RNA extraction. RNA extraction and reverse transcription were performed using the RNeasy Plus Micro kit from Qiagen and the iScript cDNA Synthesis kit from Bio-Rad, respectively. PCR was first performed on *DDX3Y* gene to sex the embryos. Then the samples were submitted to qPCR for 2 reference genes and 5 genes of interest (StepOnePlus, Applied Biosystem; 109 embryos; 3 replicates; 3 to 15 embryos per condition). The two reference genes, *YWHAZ* and *H2AFZ*, were selected for their stable expression whatever the condition (female or male, with or without oxidative stress, in FCS or BSA-ITS medium). Four X-linked genes (*AIFM1, XIAP, G6PD, HPRT*) and one autosomal gene (*BAX*) were selected based on the literature for their roles in the control of apoptosis or oxidative and their potential implication in the observed differences between the tested conditions. Statistical analysis was performed with the Standard Least Squares method (fixed effects: stress, sex, stress*sex; random effect: replication). All X-linked genes showed a higher expression in female embryos, whatever the culture medium ($p<0.01$; between 1.4 and 2.5 fold). AAPH treatment significantly decreased the expression of *XIAP* only in FCS containing medium (on average 0.78 fold), while it increased the expression of *BAX* (1.3 fold) and *HPRT* (1.3 fold) only in BSA-ITS medium ($p<0.01$). In this last medium, the impact of stress on *AIFM1* expression tended to depend on the sex of the embryo ($p=0.068$; females: on average 1.2 fold increase vs male 0.9 fold). In conclusion, the study confirms the higher expression of the tested X-linked genes in female embryos. The few differences observed between culture and stress conditions did not allow linking the expression of the studied genes to the sexual dimorphism observed for the developmental and apoptotic rates in the tested conditions.

49. Trolox during in vitro maturation of bovine oocytes can protect embryos from palmitic acid induced lipotoxicity during development: effects on mRNA transcript abundance

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Keywords: fatty acids, trolox, in vitro production of bovine embryos

Maternal metabolic disorders are associated with elevated concentrations of palmitic acid (PA), which is known to jeopardize bovine oocyte and embryo development and quality. Molecular analyses of PA exposed bovine oocytes and embryos point towards oxidative stress (OS) pathways. Previous research has shown that the detrimental effects of PA-exposure during oocyte IVM cannot be alleviated by antioxidant (AO) supplementation, e.g. Trolox (TR, water soluble VitE), during IVM or IVC. Exposing oocytes with TR during IVM protected subsequent embryo development under PA conditions (De Bie *et al*. 2018, AETE). In the present study, we examined the effects of TR on the quality of the produced blastocysts at the transcriptome level.

Bovine COCs were matured, fertilized and cultured in 2 different experiments (min 3 repeats each). In EXP1, COCs (n=1565) were exposed to pathophysiological follicular PA concentrations (150 µM, Sigma-Aldrich, BE), subsequent embryos were cultured under solvent control (ethanol) conditions (PA-SC). TR was added during IVM or IVC (100 µM, Thermo Fisher, BE; PATR-SC, PA-TR). In EXP2, COCs (n=1477) were matured under solvent control conditions, subsequent embryos were exposed to pathophysiological oviductal PA concentrations (230 µM; SC-PA). TR was added during IVM or IVC (100 µM; TR-PA, SC-PATR). In each experiment, a solvent control was included (SC-SC). Pools of min 10 day 8 blastocysts per treatment were examined for relative transcript abundance of genes (normalized to *H2AFZ* and *YWHAZ*) involved in OS (*CAT, GPX, SOD1, SOD2, PRDX1, PRDX3, NRF2*), mitochondrial function (*TFAM, HSPD1*), lipid metabolism (*PPARg*) and apoptosis (*BAX*) and analyzed by one-way ANOVA.A significant increase in *NRF2* and *TFAM* was found in blastocysts from PA exposed COCs (PA-SC) and embryos (SC-PA) compared with controls (SC-SC). Increased *NRF2* in blastocysts from PA exposed COCs (PA-SC) returned to control levels when TR was added during IVM or IVC (PATR-SC, PA-TR). In contrast, when embryos were exposed to elevated PA (SC-PA), adding TR during IVM or IVC (TR-PA, SC-PATR) was not able to alleviate elevated *NRF2* expression to control levels, suggesting activation of OS defense mechanisms. The addition of TR in each EXP significantly reduced *TFAM* gene expression to levels similar to controls (SC-SC), suggesting normalization of mitochondrial biogenesis. In EXP1, a significant increase in *CAT* was found in PA exposed oocytes (PA-SC) compared with their control counterparts. Adding TR during IVM or IVC (PATR-SC, PA-TR) significantly reduced blastocyst *CAT* expression to levels lower than controls. No significant PA-induced changes were found in the expression of other genes. In conclusion, the enhancement of the developmental capacity of PA-exposed bovine oocytes and embryos by TR is most promising when oocytes are protected by TR prior to the PA insult. Moreover, subsequent blastocysts appear to have control levels of expression of genes related to OS and mitochondrial function and increased expression of genes involved in OS relief.

50. Effect of Temopl on in vitro oocytes maturation in Egyptian Buffalo

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Keywords: Tempol, in vitro maturation, Buffalo oocytes

Oxidative stress is a major biological threat that negatively affect oocytes quality and subsequent maturity competence. Cellular antioxidant system, like superoxide dismutase (SOD), plays a substantial role for maintaining redox balance against the excessive accumulation of reactive oxygen species (ROS). Consequently, the antioxidants are frequently used in the in vitro culture system to promote the oocyte's maturation. Tempol is is a single chemical compound that facilitates hydrogen peroxide metabolism, scavenging ROS, and functionally similar to SOD. Therefore, the current study was conducted to study the effect of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) as an exogenous antioxidant on in vitro oocyte maturation rate of Egyptian buffalo. Cumulus-oocyte complexes (COCs) were recovered from animals slaughtered at local abattoir and grade A and B were used in this study. The maturation rate was assessed by nuclear status (oocytes reached metaphase II stage) and cumulus expansion of the oocytes as well as on the molecular level. Three different concentrations (0.5, 1 and 2 µM) of Tempol (Sigma-Aldrich, Cat no 176141,) were added to the buffalo oocyte maturation medium (Tissue Culture Medium, TCM-199 HEPES medium supplemented with 2% fetal bovine serum, 5µg/ml of FSH, 1µg/ml estradiol-17β, 0.15 mg/ml glutamine, 22µg/ml Na-pyruvate, 50µg/ml gentamycin). By the end of maturation period (22-24 h), COCs expansion rate and nuclear maturation rates were evaluated. After oocytes denudation, the oocytes from all experimental groups were stored at – 80°C for further genetic analysis. Two candidate genes regulating metabolic activity (CPT2) and antioxidant status (NFE2L2) were profiled using Real-Time PCR find out molecular action of Tempol on COCs during maturation. The GAPDH was used as a reference gene for relative expression quantification. The results revealed that 0.5 µM of Tempol (88.0±4.0 %) enhances buffalo COCs expansion rate comparing to control (75.7±3.0 %) and the other two concentrations of Tempol (1 and 2 µM were 79.9±4.1 and 68.1±4.2 %, respectively). While, the differences were significant ($p \leq 0.05$) between the higher and lower concentration of Tempol. Moreover, the metaphase II (indicator of maturation rate) oocytes were higher in 0.5 µM concentration (84.4 %) in comparison to 1 and 2 µM concentrations (68.9 and 76.7 %, respectively) and control group (75.8 %). For the molecular analyses, the transcriptional abundance of CPT2 showed a significant ($p \leq 0.05$) decline trend with the increase of Tempol concentration in the maturation medium being in addition significantly lower than control group. While, for NEF2L2, the expression level of NEF2L2 gene was comparable between the control group and the groups of oocytes matured with 0.5 and 1 µM of Tempol, However, 2 µM of Tempol concentration showed lower level of expression of NEF2L2 in comparison with all experimental groups. Moreover, the expression pattern of CPT2 and NEF2L2 genes strengthened the results of oocyte maturation rate in vitro. In conclusion, the 0.5 µM concentration of Tempol revealed potential significance for oocyte maturation competence as well as on the molecular level.

51. Embryo vitrification device has consequences at birth and adulthood

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Keywords: embryo, vitrification, phenotype

The development of assisted reproductive technologies (ART) over the past decades has provided tremendous advantages in livestock. Cryopreservation of reproductive cells is the second most used ART, which allows for long-term storage gametes/embryos by cooling them to subzero temperatures. This technique has become essential to enable the banking and the transport of embryos from high genetic value animals around the world. In this field a progressive replacement of slow freezing by vitrification methodologies has occurred. However, rather than a standardized method, an explosion of vitrification methods has been appeared over the last decade, using extremely variable vitrification media and more than 25 different vitrification devices. Moreover, it is increasingly common to find evidences that ART conditions can affect embryo development and, ultimately, the adult phenotype via epigenetic mechanisms that vary depending on the nature of the procedure (embryonic developmental plasticity). Therefore, using the rabbit as a model, the aim of this study was evaluate the effect over the growth performance of two vitrification devices, both based on the use of the minimum volume strategy but composed of different material (metal or plastic), which brings different cooling/warming rates. To assess this issue, 72-hours embryos (late morula/early blastocyst) were vitrified using metallic loops (n=102; ML) and Cryotop® (n=100; CP). Embryos were vitrified in a two-step addition procedure; equilibrium (10% EG + 10% DMSO + 10% Dextran) for 2 minutes and vitrification (20% EG + 20% DMSO + 10% Dextran) for 1 minutes. After thawing, embryos were transferred into the oviducts of 16 foster mothers. Birth rate (animals born / transferred embryos) was recorded and the pups were identified and weekly weighted until adulthood. Gompertz growth curve equation [$y= a \cdot EXP(-b \cdot EXP(-k \cdot t)]$ was used to determine the growth rate (k). Statistical analysis was performed through a general lineal model (considering $p<0.05$). The results showed that ML birth rate (44.1±4.67%) was lower compared to CP group (65.0±4.72%; $p<0.05$). However, CP animals were smaller (-9.6±2.69g; $p<0.05$) at birth, even after using the litter size as covariable. Finally, although growth rate was similar between both groups (average k value: 0.16±0.01), data suggest that differences at birth cannot be restored later in life. Therefore, CP animals showed lower body weight (-318.9±103.86g; $p<0.05$) at adulthood. In conclusion, the vitrification device does not seem to be trivial. Our results increase the studies that reports a significant effect of the vitrification device in the achievement of a successful vitrification. In addition, our findings provide evidences of an embryonic plasticity in response to the vitrification device, which modify the birth weight and the late growth performance. Therefore, we show for the first time to our knowledge that phenotype can change in response to a vitrification device.

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52. Immunohistochemical identification of CIRBP in bovine ovary and testicle

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Keywords: CIRBP, stress-induced protein, cattle

The cold-inducible RNA-binding protein (CIRBP) is a highly conserved stress-induced protein that helps cells to resist adverse environmental conditions via stabilizing specific mRNAs and facilitating their translation. CIRBP participates in anti-apoptotic and anti-senescent cytoprotective processes. In relation with gametes, CIRBP improves the developmental competence in vitrified-warmed yak oocytes and exerts a protective effect against spermatogenic injury caused by heat stress and cryptorchidism in mice. The purpose of this study was to identify the expression of CIRBP on different cell populations in adult bovine ovary and testicle. Tissues were obtained from healthy slaughtered animals (non-pregnant heifers and sexually matured males). Paraffin blocks containing tissue sections of ovary and testicle were processed for immunohistochemistry. Tissue sections were dewaxed, blocked for intrinsic peroxidase (15 min, 3% H_20_2 in methanol), and subjected to antigen retrieval (10 mM sodium citrate, pH 6.0, 30 min, 95°C). Nonspecific binding was blocked with 10% normal donkey serum (30 min). Tissues were incubated with two dilutions (1/250, 1/500) of two primary antibodies against CIRBP overnight at 4°C: monoclonal rabbit anti-CIRBP [EPR18783] (ab191885, Abcam) or polyclonal goat anti-CIRBP (ab106230, Abcam). Antibodies were detected with a commercial peroxidase kit and diaminobenzidine. Sections were lightly counterstained with hematoxylin. Some sections were processed for immunofluorescence and analyzed through confocal microscopy, using two secondary antibodies at 1/600: Alexa Fluor® 546 donkey anti-rabbit IgG or Alexa Fluor® 633 donkey anti-goat IgG (Invitrogen). Fluorescent sections were counterstained with DAPI. Sections stained on the absence of primary antibody (negative controls) demonstrated a lack of unspecific binding for the detection system. Both immunohistochemistry and immunofluorescent sections were analyzed with ImageJ Software. Two-Way ANOVA analysis was performed with GraphPad Prism Version 8.0.2. *Post hoc* comparisons were performed using the Tukey test. In ovary, CIRBP was present in follicular cells of primordial follicles and in the granulosa and theca cells of the subsequent follicular stages. Oocytes presented less intensity compared to follicular cells. Granulosa cells presented more intensity than theca cells. In testicle, CIRBP was present in Leydig, Sertoli, and spermatogenic cells but not in mature spermatozoa. Dilutions at 1/250 presented better-contrasted images in both primary antibodies. In conclusion, CIRBP was present in bovine male and female gonads as seen in other species. Slight differences can be found using monoclonal or polyclonal antibodies against CIRBP in both tissues. Despite CIRBP being known to play an important role in spermatogenesis, little is known about its role in folliculogenesis and developmental competence of oocytes. Further studies are needed on the function of CIRBP on bovine follicles, oocytes, and developing embryos.

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53. Metabolomic profiling of oviductal extracellular vesicles across the estrous cycle in cattle

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Keywords: Oviduct, extracellular vesicles, metabolomics

Extracellular vesicles (EVs) in oviductal secretions have been suggested to play major roles in the cross-talk between gametes/embryo and the oviduct. The aims of the present study were to determine the metabolomic profile of bovine oviductal EVs and to examine whether the metabolic content of oviductal EVs varies according to the stage of the estrous cycle and the side relative to ovulation.

Bovine oviducts were collected at a local slaughterhouse, transported on ice, and classified into 4 stages of the estrous cycle according to the ovarian and corpus luteum morphologies (n=34-54 cows/stage): post-ovulatory (Post-ov; Days 1-4 of estrous cycle, coinciding with the time of embryo presence in the oviduct), mid luteal (Mid-lut; Days 5-11), late luteal (Late-lut; Days 11-17) and pre-ovulatory (Pre-ov; Days 18-20, coincinding with the time of estrus). Additionally, follicular fluid was collected from the Pre-ov follicles to exclude animals with cystic follicles (intra-follicular concentrations of progesterone > 160 ng/ml and estradiol < 40 ng/ml). Oviductal fluids (OF) were collected from contra- and ipsi-lateral oviducts by squeezing. Then, OF was separated from cells and cell debris by centrifugation (10 min at 2,000 g then 15 min at 12,000 g). Oviductal EVs were isolated from pools of OF (8-19 cows per pool; 3-4 pools per stage and side; 397 ± 15 µl of OF per pool) by ultracentrifugation (90 min at 100,000 g twice) and resuspended in PBS. Finally, EV samples (with no trace of oviductal fluid) were assayed for protein concentration (12.9 ± 0.5 mg/ml per pool) and stored at – 80°C for metabolic analysis. Samples of EVs were analyzed by proton nuclear magnetic resonance spectroscopy (NMR) as previously described (Lamy et al. Reprod. Fertil. Dev. 2018). The concentration of each metabolite was normalized to 1 mg of protein. Normalized values were compared between stages of the cycle and sides relative to ovulation using two-way analysis of variance (ANOVA) followed by Tukey's tests with $P < 0.05$ considered significant. NMR identified 22 metabolites in oviductal EVs, from which 15 could be quantified. Among them, 5 were amino acids (alanine, glycine, isoleucine, methionine and valine) and 9 energy substrates including lactate, myoinositol, glucose-1-phosphate and maltose as the most abundant metabolites. With the exception of maltose, all metabolites identified in oviductal EVs were previously identified in the OF (Lamy et al. 2018). Except for maltose, the side relative to ovulation had no effect on metabolite concentrations. Interestingly, levels of methionine were significantly higher at Pre-ov compared to Late-lut ($P<0.05$). Furthermore, glucose-1-phosphate and maltose concentrations were greatly affected by the stage of the estrous cycle ($P<0.0001$), showing 10- to 40-fold higher levels at Mid-lut and Late-lut than at Pre-ov and Post-ov. The metabolites identified in the present study could be taken up by gametes/embryos via EVs and play key roles in gamete maturation, fertilization and/or embryo development.

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54. Parental contribution of splicing factors ZRSR1 and ZRSR2 in early embryo development

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Keywords: minor splicing, embryogenesis, zygotic gene activation

ZRSR1/2 have been implicated in 3' splice site recognition of U12 introns, a minor intron class (<0.4% of all introns) which is conserved across eukaryotic taxa with important roles in developmental processes. *Zrsr1* is a imprinting gene which is paternally expressed in mice, and *Zrsr2* is in the X-chromosome in all mammalian species analyzed. To determine the implications of minor splicing in early embryo development, CRISPR technology was used to produce *Zrsr1/2* mutant mice (*Zrsr1*^{mu} *and Zrsr2*^{mu}) that were viable with normal lifespan. We crossed homozygous *Zrsr2*^{mu} female with *Zrsr1*^{mu} male, being the double mutation (Zrsr1/2^{mu}) lethal, giving rise to embryos that stopped developing mainly between the 2- and 4-cells stages, just after zygotic gene activation (ZGA). This indicates that embryos need at least one normal *Zrsr1* allele from the father or one *Zrsr2* allele from the mother to survive. Rescue experiments in which *Zrsr1* mRNA was injected into 1-cell Zrsr1/2^{mu} embryos allowed the development of mutant embryos to blastocyst stage, revealing that minor splicing is essential for ZGA. To investigate the molecular basis of impairing the minor spliceosome machinery during embryo development, 3 pools of 100 *Zrsr1/2*^{mu} 2-cell embryos and 2 pools of 100 wild-type 2-cell embryos were used to purify their RNA and perform RNA-seq analysis. Differential gene expression (DGE) was evaluated with two independent software (DESeq2 and edgeR) (adjusted p-value <0.01) to improve the reliability of our findings. Differential alternative splicing (AS) events was determined using vast-tools software, considering events as modified when the difference in their average inclusion levels was above 10%. DGE analysis showed 3423 upregulated and 1446 downregulated genes in Zrsr1/2^{mu} embryos, which could indicate that the degradation of the maternal mRNAs is impaired. Genes with lower expression in Zrsr1/2^{mu} embryos were enriched in translation, rRNA processing and splicing, and cell cycle GO terms, indicating an essential role of minor splicing during ZGA. Differential AS analysis revealed 2645 upregulated and 1717 downregulated events in *Zrsr1/2*^{mu} embryos, being all categories of alternative splicing affected. Intron retention events were then checked to determine if they were U2- or U12-type intron events, as well as those U2 intron events that occur in genes that contain U12 introns. Seven percent of the intron retention events identified correspond to U12-introns, which represents a significant enrichment when compared with the overall proportion of U12 introns in the mouse genome (7% vs 0.04% expected), showing a crucial role of the U12-type introns during early embryo development. This study identifies paternal ZRSR1 and maternal ZRSR2 as essential factors for efficient U12 intron splicing, highlighting their crucial role on early preimplantation embryo development. Research supported by the Spanish Ministry of Economy and Competitiveness through the project AGL2015-66145-R and BES-2016-077794 grant.

55. Ovulation mediated changes in the transcriptomics of the rabbit isthmus

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Keywords: isthmus, transcriptomics, rabbit

The isthmus is the section of the oviduct where sperm is stored forming the oviductal reservoir, before capacitation takes place and spermatozoa are freed to fecundate the oocytes within the ampulla. This study shows transcriptomics changes in the isthmus shedding light on the molecular processes taking place near the ovulation in rabbit species (*Oryctolagus cuniculus*). Six sexually mature New Zealand rabbit does received 0.02 mg Gonadorelin (im; Fertagyl, Merck & Co., Inc., Kenilworth, USA) at insemination time (t=0 h) to induce ovulation. Tissues were collected after euthanasia at 10 h (pre-ovulatory; n=3) and 20 h (post-ovulatory; n=3). RNA was extracted and samples with a RNA integrity number > 7 were analyzed by microarray platform GeneAtlas System (chip Rabbit Gene 1.1 ST Array Strip, Affymetrix). Data analysis was done with the Partek Genomics Suite software, raw intensities were background corrected and RMA normalized. Differentially expressed genes (DEGs) were defined as those with a fold change > |1.5| and FDR < 0.05 obtained through a variance analysis, and false discovery rate adjustment with RankProd. Biological meaning was assessed with Gene Ontology (GO) enrichment, and pathway analysis with KEGG database. There were 86 genes upregulated at the pre-ovulatory stage. Pathway analysis was enriched in terms such as "protein processing in endoplasmic reticulum", "ECM-receptor interaction" and "MAPK signalling pathway". GO analysis revealed enrichment in extracellular proteins, with 18 gen products labelled as “extracellular exosome” and 28 being secreted proteins. Among the extracellular proteins there were some previously reported as oviductal fluid (OF) proteins in rabbit and other species (cow, pig and mouse). There were upregulated chaperones (HSPA5, DNAJB11, HYOU1 and HSPB7), integrins (ITGA5, IGTA2 and IGTB3), neuropeptide Y (NPY), and protease inhibitor SERPINE1. On the other hand, there was an upregulation of 33 genes at the post-ovulatory stage. GO annotation revealed the presence of eight secreted proteins, among the two proteins associated with fertility, PLAT and SPP1. PLAT was previously reported to be necessary for the success of *in vitro* fertilization in mice, while osteopontin (SPP1) has shown a positive effect on spermatozoa, *in vitro* fertilization and embryos in cattle. In the present study, an overexpression of exosomal proteins was reported for the pre-ovulatory stage. Enrichment in proteins previously detected in murine oviductosomes (OVs), including those integrins mediating spermatozoa-OVs fusion, suggest that this molecular mechanism might be conserved in rabbits, and thus participate in modification of the spermatozoa located in the isthmus. Further experiments are required to characterize these oviductosomes their evolution over the doe reproductive cycle, and investigate their role in the modulation of the spermatozoa fertilization ability. Moreover, there are secreted proteins in the rabbit oviductal fluid previously detected by proteomics that show a differential expression pattern before and after ovulation whose role in reproduction remains unknown, requiring additional functional studies.

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56. Selected physicochemical parameters of serum and fluid from the cavity of corpora lutea and their connection to the mechanisms of its formation in cattle

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It is well known that cows have two morphologically different forms of the corpus luteum - solid and cavitated. In the studies presented in previous years, among others in the AETE meeting (Jaskowski 2016), we have shown that the presence of the cavity is accompanied by an increased level of progesterone in the blood and better results of conception. Perhaps the presence of the cavity is an additional factor that increases the probability of pregnancy in its early stage. The assessment of the nature and some of the fluid components could help to explain the positive effect of cavities on the CL function. The aim of the study was to determine some physicochemical parameters of the fluid from the CL cavities and serum in connection with the presence of the cavitated form of the corpus luteum. The study material from 30 Polish Holstein Friesian cows aged from 43 to 148 months, obtained in one of the cattle slaughterhouses in Poland, was used. After ultrasound examination performed before slaughter and finding the cavity inside CL, blood was collected (tail vein, 7.5 ml tubes, EDTA), and after slaughter liquid from cavities of corpora lutea (eppendorf tubes). The samples were transported to the laboratory under chilled conditions, and the analysates were held at -80 ° C. The specific gravity, total protein content (Danlab refractometer), glucose concentration (Reflovet Plus, Roche) and progesterone (RIA) were determined in the fluid and serum samples. Data were analysed by logistic regression using the STATISTICA 9.0 software PL. The correlation between the size of cavities and the specific gravity, protein and glucose concentration was clearly established. While the increase in cavity diameter corresponded to the increase in glucose concentration (0.5116, p=0.015), specific gravity and protein concentration were negatively correlated with the size of the cavity (-0.5192, p=0.013 and -04813, p=0.023, respectively). There were no significant correlations between the fluid from the cavities and the amount of luteal tissue, as well as serum and fluid from the cavities. There was no correlation between the concentration of progesterone in the cavity and serum fluid, however the mean P4 values obtained from cavity fluid were significantly higher than from serum (4612±3847.6 to 28.6±15.2 ng/ml, p<0.001). It is known that progesterone has an autocrine luteotropic effect on the luteal cells positively affecting its own concentration measured in blood. The P4 values in the cavities, which is many times higher than in serum, may explain the higher concentrations of P4 in the blood of cows with cavitated corpora lutea in relation to those with solid counterparts. Physical parameters of the fluid from the cavities suggest its exudative character. Higher specific gravity and protein concentration in smaller sized cavities compared to the large ones may indicate the angiogenesis related background of cavity formation.

57. Mono (2-ethylhexyl) phthalate induces transcriptomic and proteomic alterations in bovine oocytes

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Keywords: phthalates, oocyte developmental competence, transcriptome

Phthalates are plasticizers, used in a variety of industrial plastics. Di(2-ethylhexyl) phthalate (DEHP), the most commonly used plasticizer, and its main metabolite, mono(2-ethylhexyl) phthalate (MEHP), are known reproductive toxicants. A residual concentration of MEHP (~20 nM) has been found in follicular fluid aspirated from IVF-treated women and DEHP-treated cows. Previously we have reported that exposure of oocyte during maturation to MEHP impairs nuclear maturation, reduces cleavage and blastocyst formation rates. However, the effect of 20 nM MEHP on the transcriptomic profile of oocytes and their derived blastocysts is not entirely clear. Bovine oocytes were in-vitro matured with or without 20 nM MEHP for 22 h. At the end of maturation, they were collected for transcriptomic (by microarray; n = 20 per sample; 4 replicates) and proteomic (n = 200 per group) analyses to examine a possible direct effect of MEHP on the oocyte transcriptomic and proteomic profiles. The remaining oocytes were in-vitro fertilized; embryonic development was recorded 42–44 h and 7 days post fertilization. Blastocysts were also collected for microarray analysis (n = 10 per sample; 4 replicates). Transcriptomic data were analyzed using Partek Genomics Suite software. Control probes were removed; signals were log2 transformed followed by interslide quantile normalization. Genes were considered differentially expressed if the *P*-value by one-way ANOVA was lower than 0.05 and absolute fold change was 1.5 between the control and MEHP-treated group. Proteomic raw data were imported into Expressionist® followed by Mascot software. Data were searched against the bovine sequences from UniProtKB. Proteins were considered differentially expressed at a fold change of ±1.5 with at least 2 unique peptides. Oocyte transcriptome analysis revealed MEHP-induced alterations in the expression of 456 genes. The differentially expressed genes were associated with actin cytoskeleton (n = 47 genes; e.g., *ACTG1*), metabolic pathway (n = 43) including oxidative phosphorylation (n = 12; e.g., *ND5*), oocyte maturation (n = 9; e.g., *PIK3CA*), and embryonic development (n = 14; e.g., *SOX10, NOTCH*); 191 proteins were affected by MEHP in mature oocytes, associated with methylation and acetylation (n = 51), metabolic pathway (n = 33) including mitochondrial oxidative phosphorylation (n = 7; e.g., ATP5E), and cytoskeleton structure (n = 32; e.g., ACTN1, EGFR). In control vs. MEHP-derived blastocysts, 290 genes were differentially expressed, associated with transcription process, cytoskeleton regulation and metabolic pathway; 9 of these genes were impaired in both oocytes exposed to MEHP (i.e., direct effect) and blastocysts developed from those oocytes (i.e., carryover effect). The study explores, for the first time, the risk associated with exposing oocytes to relevant MEHP concentrations (i.e., those found in the follicular fluid) to the maternal transcripts. Although it was the oocytes that were exposed to MEHP, alterations carried over to the blastocyst stage, following embryonic genome activation, implying that these embryos are of low quality.

58. Atrazine-induced DNA fragmentation in bovine spermatozoa is associated with alterations in the transcriptome profile of in-vitro-derived embryos

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Atrazine (ATZ) is an extensively used herbicide, considered a ubiquitous environmental contaminant. ATZ and its major metabolite, diaminochlorotriazine (DACT), cause several cellular and functional alterations in spermatozoa, involving viability, acrosome integrity, and mitochondrial membrane potential. Our aim was to examine the effect of ATZ/DACT exposure on DNA integrity of bovine spermatozoa, fertilization competence, embryonic development and transcriptome profile of in-vitro-produced embryos derived from fertilization with pre-exposed sperm. Three experiments were performed with fresh semen. Statistical analysis was performed using JMP-13 software. Comparison of treatments was performed by one-way ANOVA followed by *t*-test. In the first experiment, spermatozoa (25 x 10^6 cells/mL) were capacitated (4 h) in presence of 0.01% DMSO (solvent; control), 0.1 µM ATZ or 1 µM DACT and were examined for DNA fragmentation (acridine orange dye). Exposure to ATZ and DACT increased the proportion of cells with fragmented DNA compared to control (26 and 25.8 *vs* 84.5% respectively; $P < 0.0001$). Spermatozoa were separated using annexin V micro-bead kit. DNA-fragmentation index was higher among annexin-positive (AV+) vs. negative (AV-) spermatozoa (99.5 ± 0.05 *vs* 20.8 ± 4.29 % respectively; $P < 0.0001$). In the second experiment, fertilization competence of AV+ and AV- spermatozoa was examined with in-vitro-matured (22 h) oocytes (1,051 oocytes, 4 replicates). In the control group, AV+ spermatozoa gave lower cleavage rate (31.3 ± 2.92 %) relative to AV- spermatozoa (78.6 ± 2.18 %; $P < 0.0001$). No blastocysts were recorded in the AV+ group. ATZ/DACT-treated spermatozoa defined as AV- had a significantly lower cleavage rates relative to the control (59.8 ± 4.04 and 65.3 ± 3.33 *vs* 78.6 ± 2.18 %, respectively; $P < 0.005$). The proportion of the developed blastocysts did not differ between groups. In the third experiment, in-vitro fertilization was performed with non-separated spermatozoa. Blastocysts (n=4 per sample; 4 replicates) were collected on day 7 post fertilization and subjected to microarray analysis to identify differentially expressed genes (DAVID). Transcriptome analysis revealed that 139 and 230 genes were differentially expressed (up- or down-regulated) in blastocysts derived from spermatozoa treated with ATZ and DACT, respectively. In particular, alterations were found in genes involved in pregnancy (*IFNT2*, *IFNT3*, *IGFBF5*) and in-utero embryonic development (*YBX3*, *ANKRD11*, *PDGFRA*, *VIM*), pluripotency (*MYF5*), apoptosis (*THEM4*, *BCAD29*, *EIF2AK2*), and methylation and acetylation (*H2B*, *RAB27B*, *H4*, *HIST1H1C*, *LOC616868*). In conclusion, DNA damage induced by ATZ and DACT might explain, in part, the reduced fertilization competence of treated spermatozoa, reflected by lower cleavage rates. Given that blastocyst-formation rate did not differ between groups, other mechanisms cannot be ruled out. Here we report on alterations in the genomic profile of embryos developed from ATZ/DACT-treated spermatozoa, suggesting alterations in some cellular processes, including genetic and epigenetic modifications.

59. Endoplasmic reticulum stress: is it induced in heat-shocked bovine oocytes?

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Keywords: heat stress, salubrinal, in vitro maturation.

The endoplasmic reticulum (ER) is a multifunctional organelle that plays a role in protein synthesis and modification, calcium homeostasis, and lipid synthesis. It also acts as a sensor of environmental stress. Accumulation of unfolded protein in the ER lumen leads to ER stress and activation of the Unfolded Protein Response. The bovine oocyte is very susceptible to environmental stress such as elevated temperature. However, the role of ER stress on heat-shocked oocytes has not been investigated. Therefore, the objective of this study was to determine the role of ER stress on bovine COCs exposed to heat shock (HS) during IVM. The first experiment was conducted to determine whether HS induces ER stress on bovine oocytes. COCs obtained from slaughterhouse ovaries were distributed on the following groups: Control (IVM at 38.5ºC for 22 h) and Heat Shock (IVM at 41ºC for 16 h followed by 6 h at 38.5ºC). After IVM, oocytes were denuded and stored at -80ºC for Western Blotting analysis. This experiment was replicated 5 times using 60 oocytes/treatment/replicate. Exposure of bovine oocytes to HS increased ($P<0.05$) the abundance of spliced X-box-binding protein-1 (sXBP1: ER stress marker) compared to control group, indicating the occurrence of ER stress. The second series of experiments (experiment 2, 3 and 4) were conducted to determine the role of ER stress on fertilization, kinetics and developmental competence of heat-shocked oocytes. COCs were matured in IVM medium containing 0 or 400 nM Salubrinal (ER stress inhibitor) under control and HS temperatures. After IVM, COCs were submitted to IVF and IVC. The fertilization rate was determined at 18 h after insemination (hai) and preimplantation developmental kinetics was determined by evaluating the cell number of each embryo at 26, 29, 32, 35, 38, 41 and 48 hai. These experiments were replicated 5 times using 30 COCs/treatment/replicate. There was not effect of temperature on fertilization rate. However, ER stress inhibition at 38.5ºC increased ($P<0.05$) fertilization rate compared to HS in the absence of salubrinal. Moreover, exposure of oocytes to HS in IVM medium caused a delay on embryonic developmental kinetics reducing ($P<0.05$) the percentage of 2-cell embryos at 29, 35 and 38 hai, as well as the percentage of 4-8 cell embryos at 41 hai and >8-cell embryos at 48 hai. Consequently, HS impaired cleavage rate ($P<0.05$) at 32-48 hai. On days 3 and 8 after insemination, cleavage and blastocyst rates were also reduced ($P<0.05$) in heat-shocked oocytes. Addition of Salubrinal to heat-shocked oocytes had a negative effect on embryonic development reducing ($P<0.05$) the percentage of 3-cell embryos at 38 hai and cleavage rate from 32 to 41 hai compared to 38.5ºC. However, cleavage and blastocyst rates at days 3 and 8 were not different between those groups. In conclusion, the present study demonstrated for the first time that HS induces ER stress on bovine oocytes during IVM. Moreover, HS retarded kinetics of embryonic development, reducing cleavage and blastocyst rates. ER stress inhibition on heat-shocked oocytes with Salubrinal was not as benefic as expected, but further studies are necessary to determine the importance of ER stress on bovine oocytes exposed to HS during IVM.

60. Bta-miR-10b secreted by bovine embryos negatively impacts preimplantation embryo development

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Keywords: Bovine embryos, Secreted miR-10b, HOXA1

In a previous study, we found miR-10b to be more abundant in the conditioned culture medium of degenerate embryos compared to that of blastocysts. In this study, miR-10b mimics, double-stranded and chemically synthesized RNAs ordered from the company, were supplemented to the culture medium (SOF+BSA+ITS) at 21-hours post insemination with a final concentration of 1 μM. The expression of miR-10b in embryos was evaluated by RT-qPCR and was found to be approximately 70 times higher expressed in embryos treated with mimics compared to the control embryos, indicating that miR-10b mimics can be taken up by embryos. Additionally, this uptake results in an increase in embryonic cell apoptosis (2.15 times) using TUNEL staining and aberrant expression of DNA methyltransferases (*DNMTs*) using RT-qPCR. Using several computational methods Homeobox A1 (*HOXA1*) was identified as one of the potential miR-10b target genes and dual-luciferase assay, which measures firefly and Renilla luciferase, confirmed *HOXA1* as a direct target of miR-10b in bovine embryos. Microinjection of si-*HOXA1* into embryos also resulted in an increase in embryonic cell apoptosis (4.44 times) and downregulation of *DNMTs*. Overall, this work demonstrates that miR-10b negatively influences embryonic development and might do this by targeting *HOXA1* and/or influencing DNA methylation.

61. Effects of extracellular vesicles derived from human endometrial mesenchymal stem cells (evEndMSCs) on porcine embryo development in vitro

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Keywords: porcine, IVF, embryo culture

In vitro fertilization in pigs is an assisted reproductive biotechnology that is still developing to optimize its efficiency. Porcine IVF is a very labour-intensive technique that yields inconsistent results between IVF sessions due to low initial oocyte quality, polyspermy and suboptimal composition of embryo culture media among others. Many different approaches are being tested to increase the quality and quantity of embryos produced by IVF in this species. Specific commercial embryo culture media are not available in pigs, being the North Carolina State University 23 (NCSU-23) the most commonly used medium. Combinations of NCSU-23 and different macromolecules, growth factors, hormones or oviductal fluid have been shown to improve blastocysts yield *in vitro*. In this regard, supplementation of extracellular vesicles derived from human endometrial mesenchymal stem cells (evEnd-MSCs) to the zygote culture medium has demonstrated to increase embryo yield and quality in mice (Marinaro F. et al., Biology of Reproduction, 2019, 100(5), 1180–1192). Therefore, in the present work we aimed to improve blastocyst formation in swine using evEnd-MSCs in the embryo culture medium. To test this, EndMSCs were isolated from menstrual blood from four healthy women and characterized according to multipotentiality and surface marker expression. Extracellular vesicles from 4 donors were pooled, purified and characterized by nanoparticle tracking and CD9/CD63 expression by flow cytometry. Porcine oocytes were retrieved at a slaughterhouse, matured *in vitro* and fertilized with 1 x 10^5 spermatozoa/ml for 4 hours in a humidified atmosphere at 38.5°C in a 5%CO_2/95% air incubator. After IVF, the presumptive zygotes were randomly allocated to one of the following groups and cultured for 7 further days: A) Bovine specific medium: BO-IVC medium (IVF Bioscience, Barcelona, Spain); B) BO-IVC + evEnd-MSCs: BO-IVC added with 40 µg/ml of evEnd-MSCs; C) NCSU-23 and D) NCSU-23 + evEnd-MSCs: NCSU-23 added with 40 µg/ml of evEnd-MSCs. The evEnd-MSCs batch used was the same for all the experiments. Four IVF trials were performed and a minimum of 82 oocytes per group were evaluated. Blastocyst rates relative to initial oocyte number were compared among groups by a Chi-Square test, $p < 0.05$ was considered significant; results are presented as embryos obtained/initial oocyte number. Blastocyst rates were: (8/87) for BO-IVC, (5/82) for BO-IVC + evEnd-MSCs, (13/90) for NCSU-23 and (20/87) for NCSU-23 + evEnd-MSCs; no statistically significant differences were observed among groups ($p > 0.05$). In our setting, the use of evEnd-MSCs seemed to yield better results when combined with media specifically developed for porcine, not being able to help to overcome suboptimal culture conditions. More experiments need to be performed to fully corroborate our observations.

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62. Protective effects of Mitoquinone during in vitro maturation of bovine oocytes under lipotoxic conditions

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Oxidative stress and mitochondrial dysfunction in oocytes play a central role in the pathogenesis of several conditions associated with infertility. Upregulated lipolysis during negative energy balance can directly increase oxidative stress and alter mitochondrial functions in oocytes. Furthermore, *in vitro* maturation (IVM) following ovum pick up has been shown to increase gene expression of markers of cellular stress in oocytes. This leads to reduced developmental competence and reduced production efficiency. Mitochondrial targeted treatments containing co-enzyme Q10 are used to increase the anti-oxidative capacity within the mitochondrial matrix and enhance mitochondrial activity, however their efficiency in assisted reproduction to enhance oocyte developmental competence has not been investigated. In the present study, we tested the effect of different concentrations of Mitoquinone (MitoQ; 0, 0.1, 0.5, 1.0 µM) during bovine oocyte IVM, then we tested the effect of MitoQ (0.1 µM) in the presence or absence of palmitic acid (PA)-induced lipotoxicity (150µM) as a model (Marei *et al*. 2019, Sci. Rep. 9:3673). The effect of the carrier molecule of MitoQ, triphenyl-phosphonium (TPP) was also tested. In total, 2823 bovine oocytes from slaughterhouse ovaries were used. All data were derived from at least three replicates and were compared by linear logistic regression (categorical data) or ANOVA (numerical data) with Bonferroni post-hoc corrections. MitoQ supplementation at 1 µM significantly (P<0.05) reduced cleavage (50.8±6.81 vs. 78.7±5.17), and blastocyst rates (6.7±0.98 vs. 27.4±6.07) compared with solvent control (ethanol 0.01%). TPP (1 µM) also induced similar toxic effect (P<0.05). This was associated with, and probably caused by, a reduced mitochondrial inner membrane potential (J-aggregates: monomer intensity ratio of JC-1 staining) (P<0.05). Lower concentrations of MitoQ and TPP had no effects on developmental competence. PA increased the levels of oxidative stress in oocytes (43±2.39 vs. 28.4±2.36, CellRox Deep Red pixel intensity) and reduced cleavage (56.6% vs. 69%) and blastocyst (13.9% vs. 24%) rates compared with the controls (P<0.05). These negative effects were ameliorated in the presence of 0.1 µM MitoQ (CellRox, 30.5±2.30; cleavage, 69.4%; and blastocysts, 24.2%, P<0.05). In contrast, 0.1 µM TPP alone did not enhance cleavage (55.8%) and blastocysts rates (20.2%) compared to the PA group (P>0.1). In conclusion, low concentrations of MitoQ can protect against induced oxidative stress during oocyte IVM, and enhance developmental competence under lipotoxic conditions. These effects are specific to the CoQ10 content of MitoQ since the carrier molecule TPP had no protective effects. In contrast, higher doses of MitoQ and TPP are toxic for oocytes.

63. Prolonged transportation of ovaries negatively affects oocyte quality and in vitro embryo production in sheep

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Keywords: sheep, oocyte quality, ovary transport

The first step during *in vitro* embryo production (IVP) is the collection of developmentally competent oocytes. For wild species, such as Iberian red deer, the recovery of oocytes represents a problem because the slaughtering usually takes place far away from the laboratories and transport times are usually much longer. This may negatively influence developmental competence of immature oocytes and, therefore, their quality. The aim of this work was to examine the effects of different ovary storage times and media composition, using sheep as a model, with a view to achieving better results for wild species in which long transport times are inevitable. Adult sheep ovaries were recovered and randomly assigned to the Control (saline solution) or TCM (medium TCM199) groups and maintained for 13 h at 30 ºC. At 3, 7 and 13 h since ovary collection, sixty cumulus–oocyte complexes (COCs) were denuded and late stages of apoptosis were detected by TUNEL staining. Remaining 889 COCs (467 for Control and 422 for TCM) were matured, fertilized and cultured *in vitro* in order to examine oocyte maturation and sperm penetration rates by staining with Hoechst 3342 fluorescent dye, cleavage and blastocyst rates. Generalized linear model was used to study the influence of medium composition and storage time on oocyte quality and embryo production. When the analysis revealed a significant effect ($P<0.05$), a post hoc test with Bonferroni correction was carried out. Immature oocytes retrieved from ovaries stored during 13 h showed higher apoptosis ($P<0.05$), regardless of the medium composition. After fertilization, the proportion of inseminated oocytes with two pronuclei (2PN) was significantly higher ($P<0.05$) in the Control group compared to TCM (29.43% ± 4.54 vs 13.33% ± 4.54) and after 3 h of storage ($P<0.05$) compared to 7 and 13 h (47.58% ± 5.57 vs 14.46% ± 5.57 and 2.08% ± 5.57, respectively). Although maturation and embryo production did not show differences ($P>0.05$) in terms of medium composition and between 7 and 13 h of ovary storage, the percentage of Metaphase II (MII)-oocytes, cleavage and blastocyst rates were significantly higher ($P<0.05$) when ovaries were stored during a short period of time (3 h) compared to long periods of 7 and 13 h (maturation: 70.83% ± 7.96 vs 32.64% ± 7.96 and 6.25% ± 7.96; cleavage: 73.17% ± 8.43 vs 18.20% ± 8.43 and 1.02% ± 8.43; blastocyst rate: 32.78% ± 2.21 vs 5.82% ± 2.21 and 1.02% ± 2.21, respectively). In summary, although ovary storage medium composition had an influence on oocyte quality, the most prominent effect was found with transport times. Prolonged transportation of ovaries increased oocyte apoptosis and decreased maturation, sperm penetration, cleavage and blastocyst rates. Therefore, for wild species such as Iberian red deer, the optimization of these conditions is necessary to maintain oocyte quality and ensure a successful outcome during IVP.

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64. Evaluation of the cumulus cells viability and its oxidative state by flow cytometry from ovaries subjected to prolonged storage in sheep

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Keywords: cumulus cells, sheep, long storage

Cumulus cells (CCs) have an important role during oocyte maturation and competence acquisition. Assessment of viability and oxidative status of CCs may be potential non-invasive predictors of oocyte quality in *in vitro* maturation (IVM) routines. In addition, the time intervals required by transport of ovaries from the slaughterhouse to the laboratory may adversely affect the oocyte quality. In this context, the current study aimed to evaluated the impact of ovaries storage duration on viability and oxidative status of CCs during *IVM* in sheep oocytes. Adult sheep ovaries were collected in saline solution with antibiotics at 30 ºC and storage for 3, 7 or 13 h. Cumulus-oocytes complexes (COCs, n= 2436, four replicates) were collected after these times and placed in TCM199 supplemented with 10 ng/mL FSH/LH, 100 µM cysteamine and 10% fetal calf serum for 24 h. After IVM, COCs were denuded and CCs were collected. Denuded oocytes were stained with Hoechst3342 to assess *in vitro* maturation rate and CCs were evaluated with flow cytometry using specific fluorophores for viability, intracellular generation of reactive oxygen species (ROS), glutation content (GSH) and mitochondria activity. A general linear model was used to study the influence of storage time on CCs quality and IVM rates. When the analysis revealed a significant effect ($P<0.05$), values were compared by Bonferroni test. The percentage of live CCs was greater ($P<0.05$) when ovaries were stored for 3 h (63.0 ± 8.0) compared to 7 h (19.4 ± 8.0) and 13 h (22.4 ± 8.0) of storage while, at 13 h (56.1 ± 6.5) the dead CCs percentages was significantly higher than for 3h (20.9 ± 6.5) and 7 h (35.6 ± 6.5). After 7h of ovary storage, a significantly higher ($P<0.05$) percentage of apoptosis was observed compared to 3h of storage (45.0 ± 6.6 and 16.2 ± 6.6, respectively). The storage of ovaries for long time period (13 h, 779.3 ± 78.8) produced less ROS levels in CCs than short time periods, 3 h (1 067.8 ± 78.8) and 7 h (1 052.0 ± 78.8). However, no difference in GSH content (670.2 ± 54.3, 687.7 ± 54.3, 543.5 ± 54.3) and mitochondria activity (10.7 ± 2.8, 6. 5± 2.8, 4.7 ± 2.8) was shown for CCs from ovaries stored for 3, 7 and 13 h, respectively. In addition, *in vitro* maturation rate was found significantly higher after 3 h (69.2 ± 6.5) of storage followed by 7 h (34.0 ± 6) and 13 h (4.68 ± 6.5), respectively) ($P<0.001$). In conclusion, ovary storage time negatively influenced CCs viability that may be responsible for alters in oocyte quality and the *in vitro* maturation parameters in sheep.

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65. Effect of vitrification of prepubertal goat oocytes matured with melatonin on embryo development after Parthenogenic Activation.

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Keywords: Melatonin, Vitrification, Goat oocytes

It is known that vitrification negatively affects oocyte quality. Melatonin added to the *in vitro* maturation (IVM) medium helps to improve embryo development of prepubertal goat oocytes (Soto-Heras, Reprod Fertil Dev;30(2):253-261.2018). The aim of this study was to assess the effects of vitrification of IVM-oocytes matured with melatonin on; a) intra-oocyte Reactive Oxygen Species (ROS) levels, b) oocyte apoptosis and c) blastocyst development after Parthenogenic activation (PA). Prepubertal goat oocytes were subjected to IVM in our conventional conditions. Three experimental groups were designed: 1) Control group (CG): oocytes after IVM. 2) Vitrified Group (VG): oocytes vitrified after conventional IVM and 3) Melatonin Vitrified group (MVG): oocytes matured with 10^{-7} M melatonin and vitrified after IVM. After 22 h of IVM, oocytes from VG and MVG groups were vitrified in an open-system using CVM™ Cryologic devices (IVF Bioscience; UK) and vitrification and warming protocol and solutions as described by Kuwayama (Kuwayama, Reprod Biomed Online;11:300-8. 2005). At the end of IVM, intra-oocyte ROS level was measured by staining denuded oocytes during 30 min with 10 µM 2',7' dichlorodihydrofluorescein diacetate (Molecular Probes Inc., OR, USA) (36-37 oocytes per group in 3 replicates). Oocyte apoptosis was analysed using eBioscience™ Annexin V Apoptosis Detection kit (Invitrogen. USA). 98-118 oocytes per group in 5 replicates were classified as viable (unstained), early apoptotic (stained with annexin), dead non-apoptotic (stained with propidium iodide) and necrotic (stained with both: annexin and propidium iodide). PA of mature oocytes was performed by 4 min incubation with 5 µM Ionomicyn followed by 4 h incubation with 2 mM 6-(Dimethylamino) purine (Sigma-Aldrich®Chemical,St. Louis.USA) (83-151 oocytes per group in 4 replicates). Presumptive zygotes were *in vitro* cultured in BO-IVC medium (IVF Bioscience; UK) for 8 days. Data were analyzed by two-way ANOVA followed by Tukey's multiple-comparison test (SAS® software version 9.4). ROS levels were lower in CG than VG oocytes (21327 ± 3309. vs 36959 ± 4336; $P<0.05$). No differences were found between VG and MVG groups. The percentage of viable oocytes after IVM was significantly higher ($P<0.05$) in the CG (67.0%±3.5%) than in both VG (50.8%±2.9) and MVG (39.0%±5.6) vitrified groups. After PA, CG showed higher cleavage rate than VG (80.1 ± 5.3 vs 53.4 ± 9.9, $P<0.05$) and a tendency to higher cleavage rate than MVG (55.6 ± 1.2 %, $P=0.055$), but no differences were found between vitrified groups. However, we did not find any blastocyst development in any of the vitrified groups regardless of the presence of melatonin, whereas in the CG we obtained 19.72%±3.38 blastocysts per oocytes. In conclusion, the vitrification process increased ROS levels and apoptosis in prepubertal goat oocytes and hinder the blastocyst development. Melatonin supplementation during IVM did not prevent these negative effects.

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66. In silico prospection reveals evolutionary divergence in the cattle DNA methylation pathway

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Keywords: bioinformatics, bovine, epigenetics, thermoregulation.

DNA methylation is an epigenetic mechanism that controls gene activity by bookmarking CpG-enriched DNA sequences which recruits methyl-binding trans-acting factors mostly for gene silencing. Multiple reports have demonstrated compelling data that environmental factors – such as heat stress - affect the DNA methylation patterns of germ cells and early embryos. Nevertheless, little is known about the intrinsic epigenetic divergences between taurine - *Bos taurus taurus (B. taurus*) and zebu - *Bos taurus indicus (B. indicus*) cattle, in spite of the greater adaptability of *B. indicus* to harsh environments. Therefore, a preliminary study using *in silico* tools was conducted to prospect evolutionary differences in the components of the DNA methylation pathway (DNA methyl-transferases, transcription factors, and co-factors) by sequence homology approaches between *B. taurus* and *B. indicus*. Initially, DNMTs were scrutinized for fishing possible divergences in DNA methylation activity. The genome anchorage carried out using the "genome data viewer" was similar between subspecies, where DNMT1 is localized on chromosome 7, DNMT2 and DNMT3B on chromosome 13, DNMT3A on chromosome 11, and DNMT3L on chromosome 1. Both *B. taurus* and *B. indicus* hold an identical number of DNMT isoforms (DNMT2: 2, DNMT3A: 2 and DNMT3B: 6), except DNMT1 (*B. taurus*: 10 vs. *B. indicus*: 1) and DNMT3L (B. taurus: 2 vs. *B. indicus*: 1). DNMTs display similar size and sequence between subspecies (DNMT2: 391 Aa, DNMT3A: 909 Aa, DNMT3L: 417 Aa). However, one DNMT3L isoform in *B. indicus* displays four additional amino acids (Aa) in the N-terminus. Six DNMT3B isoforms were found with variable size (*B. taurus*: 773 - 844 Aa vs. *B. indicus*: 733 - 842), with a divergent 10-Aa stretch in the N-terminus and another of 100 Aa in the C-terminus. The larger enzyme is DNMT1 (*B. taurus*: 1,611 Aa vs. *B. indicus*: 1,644). Remarkably, the *B. indicus* DNMT1 N-terminus displays an alanine-rich sequence, which may confer greater structural or thermal stability. No difference was found at the protein domain level of DNMTs between *B. taurus* and *B. indicus* by the CD-search tool. Additional DNA methylation pathway components involved in DNA methylation maintenance were thus addressed. The UHRF1, ZFP57, DPPA3/STELLA, and ZNF445 genes did not show any sequence difference between *B. taurus* and *B. indicus* for the aforementioned protein traits. There is evidence that the expression of DNMT1 is enriched in *B. taurus* spleen and testis within nine tissues investigated (expression atlas - www.ebi.ac.uk). Moreover, DNMT1 mRNA levels have been shown to fluctuate in *B. indicus in vitro* produced embryos and in oocytes and embryos of unknown genotype under heat shock. Even though Dnmt1 protein is found throughout oocyte maturation, it is only present in the cytoplasm of early embryos up to the 8-to-16 cell stage when genome activation is established, probably contributing to imprinting maintenance. In conclusion, the components of the DNA methylation pathway are highly conserved between *B. taurus* and *B. indicus*, although DNMT1 displays evolutionary-driven variation that deserves further experimental investigation.

67. The potential role of isoquercitrin by interfering with cellular reactive oxygen species and growth factors in human ovarian cancer cells

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Keywords: isoquercitrin, OVCAR-3, ROS production

Nowadays, many studies have reported that beneficial properties of flavonoid compounds from medicinal plants can be attributed to isoquercitrin. Isoquercitrin is powerful phytocompound that have been shown to exhibit disease prevention and health promotion properties. The aim of our study was to investigate the impact of isoquercitrin (purity 96.5 %, prepared by selective enzymatic derhamnosylation of rutin) treatment at the concentrations 5, 10, 25, 50, and 100 µg/mL on a human ovarian carcinoma cell culture (OVCAR-3) *in vitro*. Cell viability, cell death, apoptosis, the release of human epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), insulin-like growth factor I (IGF-I), and the production of reactive oxygen species (ROS) by cells after short-term application of 24 h were analyzed. Metabolic activity was determined by AlamarBlue™ assay and apoptosis using flow cytometry; the presence of growth factors was detected by ELISA, and ROS production was quantified by chemiluminescence. One-way ANOVA along with Dunnett's test was used to establish statistical significance at $P<0.05$. All experiments were done in triplicate. Isoquercitrin caused any significant changes neither in metabolic activity of OVCAR-3 cells, nor in the proportion of live, dead and apoptotic cells, nor in the release of EGF, TGF-β1, and IGF-I ($P>0.05$). However, tendency of a slight increase of TGF-β1 level after isoquercitrin application at the highest concentration 100 µg/mL was detected. Interestingly, our results showed, that lower concentrations (5, 10 and 25 µg/mL) significantly ($P<0.01$) inhibited the production ROS. On the other hand, ROS production observed in OVCAR-3 cells after isoquercitrin treatment was significantly ($P<0.001$) increased at the high concentrations (50 and 100 µg/mL). In conclusion, the results of our *in vitro* study show antioxidant and pro-oxidant activities of isoquercitrin in dose-dependent manner. ROS production by OVCAR-3 cells was increased at hight concentrations of isoquercitrin and decreased at lower concentrations. Isoquercitrin at any used concentrations did not interfere with growth factors in human ovarian carcinoma cells, however, further studies are required to better understanding of biological actions of isoquercitrin.

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68. Inhibition of miR-152 during in vitro maturation enhances the developmental potential of porcine oocyte

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Keywords: oocyte, transfection, miRNA

Oocyte and embryo development are regulated by complex molecular mechanisms. Several molecules are involved in these regulation mechanisms including microRNAs (miRNAs). MiR-152 is well known as a tumor suppressor in human cancer by inhibiting cell proliferation and suppressing the PI3K/Akt and MAPK signaling. We have shown previously that miR-152 is upregulated in fully-grown compared to growing oocytes. Several genes required for progression through different stages of meiosis are putatively targeted by this miRNA. However, its specific role in oocyte and embryo development is still unknown. In this study, we evaluated the developmental potential of porcine oocytes after manipulation of miR-152 abundance during in vitro maturation using mimic, inhibitor or random sequence miRNA as negative control. Cumulus-oocytes complexes isolated from 3 – 6 mm follicles of premature gilts have been cultivated in maturation medium TCM 199 (Sigma-Aldrich, Munich, Germany) without hormonal stimulation but with dibutyryladenosine cyclic monophosphate (dbcAMP) and miR-152 mimic (40nM), inhibitor (600nM) or negative control (40nM) in the presence of Lipofectamine 3000 (Thermo Fisher Scientific, Massachusetts, USA) for 4 hours. Then transfection was continued in TCM 199 with hormonal stimulation for 44 hours and metaphase II (MII) rate was calculated. Some of the matured oocytes were parthenogenetically activated and cultivated in porcine zygote medium 3 (PZM 3) for 6 days until blastocyst stage. The abundance of miR-152 was analyzed in MII oocytes using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) on droplet digital PCR (ddPCR) system (Bio-Rad Inc.). Our preliminary results showed that mimic and inhibitor treatments change the abundance of miR-152 in MII oocytes by 2.2 and -2.5 folds, respectively, compared to the negative control group. There were no differences in MII rate among negative control, mimic and inhibitor group (92±1%, 92±2%, and 91±5%, respectively) or in cleavage rate of parthenotes of the same treatment groups (71±12%, 77±3%, and 82±7%, respectively). On the other hand, the blastocyst rate of parthenotes was significantly higher in the inhibitor group (48±3%) compared to negative control (28±5%) or mimic group (22±9%). In conclusion, inhibition of miR-152 during oocyte maturation could enhance the developmental rate of porcine parthenotes. More studies are required to understand the exact function of miR-152 during oocyte and embryo development.

69. Establishment of a single embryo sequential culture system for cell free DNA (cfDNA) based genetic screening in cattle

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Keywords: Culture media, sexing PCR, blastocyst

Recent studies have demonstrated the natural release of genomic DNA by embryo during culture. This cell free DNA (cfDNA) could be employed in whole genome amplification (WGA) strategies to obtain representative quantities of embryonic DNA without the need for invasive techniques, thus minimising damage to the blastocyst intended for embryo transfer, and providing an easily scalable platform for cattle breeders interested in screening large numbers of *in vitro* produced (IVP) embryos for sex or genetic value, a growing trend in the current market. As a result, the development of efficient single embryo culture systems to obtain cfDNA from spent culture media has immense potential for the genetic screening of IVP blastocysts. The present study aims to optimise a culture system, free from foreign DNA contaminants, for the *in vitro* culture of cattle zygotes, to demonstrate presence of amplifiable quantities of cfDNA in this system, and to obtain sexing diagnoses by PCR. Oocytes from abattoir material across two replicates (n=146) were matured for 22 h and fertilised with frozen/thawed bull sperm. Presumptive zygotes derived from the same dam were co-cultured for 3 days in 100 µl of Synthetic Oviduct Fluid (SOF) medium supplemented with 6 mg/ml BSA. The zygotes were then cultured in individual SOF droplets of 25 µl for a further 4 days when blastulation rates were recorded. Following this, individual media samples of different volumes (2 or 5 µl) from each drop and the corresponding blastocysts (n=5) were collected separately on day 7 post-IVF and subjected to WGA by DOPlify® kit (Perkin-Elmer, Waltham, MA), as per manufacturer's instructions. Quantification of the WGA products was obtained by Qubit dsDNA HS fluorometry assay (Thermo Fisher Scientific, Waltham, MA) as per manufacturer's instructions using a 1:200 dilution of the original WGA sample. Following amplification, sexing was performed by PCR using chromosome Y (SRY primers) and bovine autosomal primers (BSPF primers). In this pilot study, culture in single droplets produced a blastocyst rate of 36% per cleaved embryo (n=21 blastocysts and n=58 cleaved embryos). The average cfDNA yield per 25 µl following WGA was 0.92 ± 0.15 µg, while whole blastocysts produced a similar amount of 1.08 ± 0.33 µg (t-test, P= 0.66). There was no statistically significant difference in the total yield of DNA amplified when either 2 or 5µl of spent culture medium were used (paired t-test, P=0.31). The proportion of WGA samples producing a sexing PCR result was 50% and the concordance for sex determination between the blastocyst and its medium was 25% (n=12). While presence of cattle specific DNA was confirmed by PCR, the results presented suggest DNA cross-contamination may affect WGA and PCR efficiency. To address this, future tests will replace BSA with recombinant human serum albumin (HSA) and will include media changes during culture. To improve PCR specificity we will employ shorter amplicons since cfDNA is known to be of very low molecular weight. Following optimisation, we will use these cfDNA samples for single nucleotide polymorphism typing to develop a non-invasive aneuploidy screening strategy for cattle IVP.

70. Timing of pronucleus formation and first DNA replication in porcine IVP zygotes using frozen-thawed spermatozoa

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Keywords: pig, embryo, DNA replication

Improvements in the efficiency and repeatability of porcine IVP is necessary for the application and optimization of new technologies as gene edition of embryos by CRISPR/Cas9. Our lab has optimized IVF procedures like sperm selection by swim-up, the addition of natural fluids (Cánovas, eLife. 6:e23670, 2017) and variations of atmosphere conditions in IVF (García-Martínez, Mol Hum Reprod. 1;24(5):260-270, 2018). For future experimental designs and optimize the IVF system could be useful to know the kinetic of penetration, pronuclear formation and DNA replication. To determinate starting of DNA replication 441 porcine oocytes were *in vitro* matured in NCSU-37 medium (Cánovas, eLife. 6:e23670, 2017) and were inseminated in TALP with frozen-thawed boar spermatozoa selected with NaturARTs-Pig sperm swim-up medium (EmbryoCloud, Murcia, Spain) and cultured at 38ºC, 5% CO_2 and 7% O_2 (García-Martínez, Mol Hum Reprod. 1;24(5):260-270, 2018). Groups of 25 oocytes were fixed every hour from 4 hours to 12 hours and 24 hours after IVF and were staining with Click-iT™ EdU Alexa Fluor™ Imaging Kit (Invitrogen, Spain) to determinate penetration rate (PEN), spermatozoa/oocyte (S/O), male pronucleus formation (MPF) and rate of zygotes with DNA replication. PEN already started at 4h after IVF (15.79%) and increased to 55.00-66.67% at 5-8h, reaching 80.43% at 9h and without significant differences with the following hours. Pronuclei formation started at 6h after IVF (MPF = 12.00%), increasing to 52.78% at 7h and was almost 100% from 8h onwards (93.33%). DNA replication was not detected until 10h after IVF (20.00%) with a slight increase at 11h (37.78%) and 12h (43.48%) to reach 100% at 24h. The kinetic of penetration and MPF depend on various factors like male, sperm preservation (fresh or frozen-thawed), sperm origin (epididymal or ejaculated), sperm selection and capacitation method. Our swim-up method has similar time of penetration rate and pronucleus formation respect other fresh sperm preparation and capacitation method described in the literature but Percoll gradient advance both parameters (Matás, Reproduction. 125(1), 133-41, 2003). Regarding the beginning of DNA replication, our study had a delay of 2h respect the commencement of DNA replication in porcine embryos produced by IVF previously reported (Jeong, Dev Dyn 236(6), 1509-16, 2007). Know the time when first DNA replication takes place in the zygote is important to plan strategies of technology applications like genetic engineering, for which gene edition previous DNA replication is an important objective to reduce mosaicism in the future organism.
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71. In vitro production of the first reindeer (Rangifer tarandus tarandus) blastocysts

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Keywords: ruminant, ex situ conservation

Reindeer herding is economically, societally, culturally and ecologically important livelihood in northern Eurasia. In the future, climate and socio-economical changes in the arctic region may challenge the vitality of the reindeer populations. Optimized reproductive technologies could be used both for the conservation of the reindeer genetic resources and to facilitate successful breeding of reindeer in the future. The aim of this study was to test the suitability of optimized bovine *in vitro* embryo production protocol for reindeer embryo production *in vitro*. Reindeer ovaries were collected during the period of cyclic ovarian activity which extends from September to February in reindeer females. At the slaughterhouse, reindeer ovaries were sliced after collection in EMCARE™ Biofree Flushing solution (ICPbio Reproduction, Spring valley, WI, USA). Recovered and washed oocytes were placed in tubes containing Hepes-buffered TCM199 supplemented with glutaMAX-I (100 × stock solution, Gibco™; Life Technologies Limited, Paisley, UK), 0.25 mM Na-pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 ng/ml FSH (Puregon, Organon, Oss, Netherlands), 1 µg/ml ß-estradiol (E-2257) and 10% heat inactivated FBS (Gibco™, New Zealand) for maturation for 24 h at 38.5°C in air while transported to laboratory. Matured oocytes were fertilized for 20 h with Sperm-TL washed (2×4 ml) frozen-thawed semen (Lindeberg H, Nikitkina E, Nagy Sz, Krutikova A, Kumpula J, Holand Ø 2019, Abstract book of the 10th Circumpolar Agriculture Conference, 13th – 15th March 2019, Rovaniemi, Finland. p. 87) in IVF-TL supplemented with heparin (10 µg/ml) and PHE having 1×10^6 spermatozoa/ml as a final concentration. Denuded zygotes were cultured in G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with FAFBSA (4 mg/ml) and L-carnitine (1.5 mM) at 38.5°C in maximal humidity in 5% O_2, 5% CO_2 and 90% N_2. Cleavage rates were recorded at 42 hpi. Blastocysts were recorded on days 7 and 8 (IVF=day 0). Unless otherwise stated all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The average cleavage rate was 28.4% for the 162 best quality oocytes selected for maturation during the two slaughterhouse visits, one in December and the other in January. Altogether four and two blastocysts (3.7%) were produced by day 7 and 8, respectively. In conclusion, bovine *in vitro* embryo production protocol may also be used to produce reindeer embryos *in vitro*. However, the challenge is the oocyte quality as most of the slaughtered females are either prepubertal or old.

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72. Gene expression profiles of bovine genital ridges during sex determination and early differentiation of the gonads

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Keywords: sex determination, Rna-seq, cattle

Sex determination in mammals depends on a complex interplay of signals that promote the bipotential fetal gonad to develop as either a testis or an ovary. Most knowledge of this process has arisen from experiments in the mouse model. (Sekido and Lovell-Badge 2008; Kim et al. 2007). In mice, the differentiation of supporting cell progenitors into male-specific Sertoli cells or female-specific granulosa cells is controlled by SOX9 presence or absence. However, there is scarce information concerning the process of sex determination in livestock species, especially in cattle. In order to clarify the process of sex determination in cattle, we used an RNA sequencing (RNA-seq) strategy to analyze the transcriptome landscape of male and female bovine fetal gonads collected *in vivo* at important developmental stages before, during, and after *SRY* activation. The estrous cycles of cross-breed heifers were synchronized followed by AI, and heifers were slaughtered at 35 (n=12; bipotential gonad formation), 39 (n=12; *SRY* peak of expression) and 43 (n=9; early gonad differentiation) days later. At each time-point, genital ridges were dissected from mesonephros and RNA was extracted using a Direct-zol™ RNA MiniPrep Kit (Zymo Research, CA, USA) following the manufacturer's protocol. After PCR sexing of the fetuses, RNA-seq libraries were prepared from 3 male and female samples and were sequenced using a HiSeq2500 v4 chemistry system at the Centre of Genomic Regulation (Barcelona, Spain). Differential gene expression analyses were performed independently using DESeq2 v.1.20 (adjusted p-value < 0.05). Firstly, we identified the differentially expressed genes (DEGs) between male and female gonads (sex analysis at D35, D39 and D43). Secondly, we identified DEGs during the period of transition between sex determination and differentiation within each sex (time-couse analysis between D35 and D39, and between D39 and 43). We also used a hierarchical clustering approach to cluster genes with similar expression profiles during the period from D35, D39 and D43. We performed western blot and immunofluorescence analysis of SOX9 and SOX10 to check if expression was also evident at the protein level. Gene analysis identified 143, 96 and 658 DEGs between males and females at D35, D39 and D43, respectively. Regarding the time-course analysis, 767 DEGs were identified in the comparison between D35 vs D39 male gonads, and 545 DEGs in the female gonads. In the comparison between D39 vs D43, 3157 DEGs were identified in males, and 2008 DEGS were identified in females. We found expression of several Y chromosome genes before SRY (that are absent in mice and human), *SOX9* and *SOX10* expression in both somatic and germinal cell lineages in the XY genital ridge during sex determination, the nuclear and higher expression of SOX10 instead of SOX9 in Sertoli cells during male determination and early differentiation, a lack of nuclear internalization of SOX9 in Sertoli cells during early sex differentiation and no early expression of the WNT/β-catenin pathway repressing *SOX9* in gonads. In conclusion, our data indicate that sex determination and early gonad differentiation in cattle exhibit some unique characteristics.

73. Metabolic changes in uterine fluid collected from cyclic heifers at different stages of embryo receptivity

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Keywords: Metabolism, elongation, uterine fluid

Preimplantation embryo development relies on the metabolites, hormones and growth factors present in oviductal and uterine fluids. *In vitro* culture conditions partially recapitulate the natural milieu where embryos develop, but a culture system capable of supporting bovine embryo development beyond the blastocyst has not been established yet, suggesting that medium composition requires further optimization. The objective of this study was to characterize the changes in biochemical compounds in bovine uterine fluid (UF) at different stages of embryo receptivity. To achieve this, 22 crossbred heifers were synchronized and UF was collected post-mortem by uterine flushing using 20 ml of DPBS at specific days after ovulation: 1) Day 0, when UF does not support embryo development; 2) Day 7, when UF supports blastocyst development; 3) Day 10, just prior to when UF triggers conceptus elongation; and 4) Day 14, when UF supports the exponential growth phase during conceptus elongation. UF (n=6, 5, 6 and 5 samples for Days 0, 7, 10 and 14, respectively) were centrifuged at 1500 g for 15 min at 4 ºC to remove cell debris and supernatants were kept at -80 ºC until analyses. Metabolomic analyses were performed by Metabolon Inc., which provides an unbiased metabolite analyses based on Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). Welch´s two-sample t-test was used to identify biochemicals that differed significantly between groups ($p<0.05$). The analysis identified 359 compounds of known identity. The most dramatic changes in the abundance of different compounds occurred between D7 and D14, suggesting that elongation requires significant modifications in UF composition. Biochemicals related to glucose metabolism showed significant changes over time. For instance, glucose, fructose, mannitol/sorbitol and pyruvate increased over time (1.5-, 2.3-, 190- and 5-fold increases between D7 and D14). Significant changes were also noted for glutamate metabolism, with a steady increase of beta-citrylglutamate (24-fold increase between D7 and D14). Compounds involved in Krebs cycle also exhibited significant variation between days, with increases in citrate, aconitate and 2-methylcitrate/homocitrate (8-, 12- and 11-fold increases between D7 and D14). These results highlight that embryo metabolic requirements vary greatly between blastocyst hatching and conceptus elongation and provide relevant insights to develop an *in vitro* system to achieve the cell proliferation, differentiation and migration events involved in this process.

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74. Post-hatching in vitro bovine embryo development inside agarose tunnels or without physical constriction

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Keywords: Elongation, conceptus, hypoblast

The greatest gestational losses in cattle occur during the second week of pregnancy, when critical developmental events take place: hypoblast migrates to cover the entire inner surface of the embryo, and epiblast forms a flat embryonic disk. Previous studies have established an *in vitro* post-hatching development system based on agarose gel tunnels and glucose-enriched medium. This system achieves some expansion of the trophoblast and hypoblast proliferation. However, embryonic disc formation is not achieved and it remains unclear whether the hypoblast covers entirely the inner surface of the embryo. An open question about this system is whether embryo culture inside tunnels is actually required for development or it just shapes the embryo to a tubular shape by mechanical constriction. The objective of this study has been to compare post-hatching development inside agarose tunnels or free-floating in an agarose-coated dish. *In vitro*-produced E11 blastocysts were measured and cultured in Synthetic Oviduct Fluid (SOF) supplemented with 27.7 mM glucose and 10 % FCS inside ~1mm agarose tunnels or over an agarose surface until E15. At the end of the culture period, embryo area was calculated using Fiji software and the development of specific lineages was assessed by immunostaining for SOX2 and NANOG (epiblast), SOX17 (hypoblast), and CDX2 (trophectoderm). No differences were found on embryo survival until E15 and the main factor determining survival was the initial embryo size at E11. In particular, when <0.5 mm E11 were cultured, only 1/16 (6 %) embryos cultured free-floating or 1/18 (6 %) cultured in tunnel survived, while when E11 embryo diameter was ≥ 0.5 mm, 12/17 (71 %) and 16/21 (76 %) survived when cultured free-floating or inside tunnel, respectively. Surviving embryos showed a cylindrical shape when they developed inside the tunnels and spherical when they develop without physical constriction, but area and volume were significantly smaller in embryos cultured inside a tunnel (2.19±0.21 vs. 4.76±1.14 mm^2 and 1.71±0.40 vs. 9.32±2.92 mm^3, for embryos cultured in tunnel or free-floating, respectively, t-test $p \leq 0.05$). A layer of hypoblast cells (SOX17+) was detected inside the trophectoderm, but, irrespective of the culture system, that layer did not cover the entire inner surface of the embryo. Similarly, although a compact cell structure was detected in some developmentally advanced embryos, no SOX2 or NANOG-positive epiblast cells were detected in any of the culture systems. In summary, post-hatching blastocyst culture inside agarose tunnels shapes embryo morphology by physical constriction, but it may restrict embryo growth and does not seem to provide any significant advantage in terms of development of hypoblast and epiblast lineages. The partial hypoblast migration and the absence of embryonic disc highlight that post-hatching culture conditions still requires significant optimization.

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75. Changes in the transcriptome of ovine MII oocytes caused by lipopolysaccharide

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Keywords: Ovine oocyte, Lipopolysaccharide, Differential gene expression

Increasing low fiber high fermentable carbohydrate diets increase the ruminal lipopolysaccharide (LPS) derived from gram negative bacteria cell walls. LPS was detected in plasma and follicular fluid of ruminants with endometritis and mastitis and disturbed the reproduction performance. While several studies have examined the effect of LPS on oocyte maturation and developmental competence, limited knowledge is available on potential effect of LPS on transcript abundance of ovine oocytes. Thus, transcriptome profiles of MII oocytes matured in presence or absence of LPS were compared using 3'tag digital gene expression method. Cumulus oocyte complexes collected from slaughterhouse-derived ovaries were matured either in media supplemented with 0.1 µg/mL, 1 µg/mL, 10 µg/mL of LPS (Sigma Aldrich Inc) or in media without LPS (control). After in vitro maturation, the cleavage and blastocyst formation following parthenogenetic activation were determined for each group. Subsequently, three biological replicates of 36 oocytes cultured in 1 µg/mL LPS and controls were subjected to 3'tag digital gene expression profiling. Differential expression analysis was performed using the R Statistical Programming Language and limma package (using voom method, an animal as a blocking factor and treatment in the model). Functional enrichment analysis of the differentially expressed genes was further performed using Enricher database. Our results showed that maturation rate (determined based on first polar body extrusion), was not significantly different between the groups. The lowest LPS dose that significantly affected developmental competence to blastocyst stage was 1 µg/mL of LPS (unpublished data). After culturing ovine oocytes in vitro for 22 hours in the presence of 0 µg/mL (control) and 1 µg/mL, a total of 7887 gene transcripts were detected and only eight genes were differentially expressed. Of these, seven genes were down-regulated (two-fold or greater) in LPS-treated group (adjusted $p < 0.05$). Down regulated genes were the following: (Tripartite motif containing 25 (TRIM25), Tripartite motif containing 26 (TRIM26), Zona Pellucida glycoprotein 3(ZP3), Family with sequence similarity 50-member A (FAM50), Glyoxalate and hydroxy pyruvate reductase (GRHPR), cornichon family AMPA receptor auxiliary protein 4 (CNIH4) and NADH ubiquinase oxireductase subunit A8 (NDUFA). Functional analysis showed that these genes were significantly enriched in immune response, oxidation-reduction process as well as oocyte development. It is worth to note that *TRIM25* and*TRIM26* were reported as important genes in innate immune response. In addition, *ZP3* is well known as a positive regulator of inflammation, interferon gamma and interleukin 4 production, oocyte and blastocyst development. Moreover, NDUFA8 is involved in the electron transport process which could be related to decreased mitochondrial membrane potential in matured oocytes in the presence of LPS. Accordingly, LPS is associated with impaired developmental competence .In conclusion, our results expand our knowledge of the genes transcribed of non-LPS and LPS treated in MII oocytes, which can shed light on molecular mechanisms of LPS-induced infertility in ruminants.

76. Comparing the ability of epididymal or ejaculated sperm to elicit endometrial transcriptomic changes in cattle

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Keywords: seminal plasma, sperm, endometrium

In mice and pigs there is robust evidence of seminal plasma (SP) modulating the maternal environment, which positively impacts embryo survival and development. However, similar evidence in cattle is sparse. Both mice and boars deposit the ejaculate inside the uterus, while bovine semen deposition takes place in the vagina, and it is questionable whether any SP reaches the uterus. However, at ejaculation, sperm encounter SP, leading to proteins binding tightly to their plasma membrane, which probably allows them to act as a vehicle for SP proteins. Based on the beneficial effect of SP observed in other species, we hypothesised that ejaculated sperm, through SP proteins, elicit a different response in the endometrium than epididymal sperm (which have never been exposed to SP). To test this, a model of endometrial explants, which has been previously used to study embryo-maternal interaction, was used. Six crossbreed heifers were oestrous synchronised and slaughtered 12 h after the onset of oestrus. Three explants from the uterine horn ipsilateral to the preovulatory follicle were obtained from each animal. Epididymal sperm were collected and pooled from the cauda epididymis of three beef bulls slaughtered in a commercial abattoir. In addition, ejaculates were collected by artificial vagina from three Holstein bulls. After pooling, they were washed through a density gradient to isolate ejaculated sperm. Endometrial explants were incubated for 6 h with: 1) medium alone (control); 2) epididymal sperm (10^6 sperm/ml) or 3) ejaculated sperm (10^6 ejaculated sperm/ml). After incubation, they were snap frozen for subsequent RNA sequencing. Strikingly, explants exposed to ejaculated sperm had no differentially expressed genes (DEG) in comparison with control explants. In contrast, explants incubated with epididymal sperm exhibited 48 DEG (32 down and 16 up) in comparison with control explants. For the annotated genes (n=35), the most represented Gene Ontology (GO) terms were “binding” (n=12; 9 down and 3 up) and “catalytic activity” (n=13; 10 down and 3 up) for the molecular function, whereas “cellular process” (n=13; 10 down and 3 up) was the highest represented term in the biological process category. When explants exposed to ejaculated sperm were compared with those exposed to epididymal sperm, 80 DEG were identified (72 up and 8 down). For the 64 annotated genes, “binding” (n=21; 19 up and 2 down), “biological process” (n=29; 26 up and 3 down) were again the most represented GO terms, together with “catalytic activity” (n=17; 16 up and 1 down), and “biological regulation” (n=13; 13 up and 0 down) in the biological process category. In conclusion, these data do not support a role for bovine SP in the regulation of endometrial function. In contrast, the results suggest that SP may mask sperm surface proteins, inhibiting their interaction with the endometrium. Because the media used in this experiment does not support sperm capacitation, it remains to be determined whether this process, which is triggered in the female reproductive tract, can reshape the sperm surface in a way that it enables sperm-endometrium interaction.

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77. Improvement of pig embryonic development after the addition of haptoglobin (Hp) to the culture medium

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Keywords: embryo, haptoglobin, porcine

Haptoglobin (Hp) is an acute phase protein recently detected in female reproductive structures (ovary, endometrium and decidua) and fluids (vaginal and amniotic). We previously described the presence of mRNA and detection by immunohistochemistry of HP in the porcine oviduct along of the oestrous cycle, especially in postovulatory and luteal phases. The upregulation of HP gene in these phases could indicate a function in embryo development. The aim of this study was to evaluate the effect of Hp protein on pig embryonic development. Oocytes were matured in NCSU-37 medium at 38.5ºC, 5% CO_2 and 95% humidity. Later on, *in vitro* matured oocytes were mechanically stripped, transferred to TALP medium and co-incubated at 38.5°C, 5% CO_2 with $1x10^4$ spz/ml porcine sperm selected by a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient (45/90%). At 18h post-insemination putative zygotes were transferred to NCSU23a medium supplemented and incubated for 22-24h. Subsequently, the percentage of cleavage was evaluated and only those zygotes that presented 2 to 4 cells were transferred to NCSU23b medium (in which the sodium pyruvate and lactate of the NCSU23a medium were replaced by D-glucose 5.55mM) for another 120h under the same conditions previously mentioned, to complete a development of 7 days post insemination. In the case of the Hp group, TALP and NCSU23a culture media were supplemented with purified pig Hp protein (HGLB12-N-25. Alfa Diagnostic Internacional, San Antonio, EEUU) at a final concentration of 10 µg/ml. Blastocysts obtained were photographed and their diameter was evaluated by ImageJ® program. Finally, blastocysts were stained with Hoechst 33342 to evaluate the total number of cells per blastocyst. The data were analyzed by Chi-square test ($p<0.05$). Our results showed that cell division was similar in both experimental groups (control: 35.60% vs. Hp: 32.97%). However, the blastocyst development was higher in the Hp group in comparison with the control (control: 37.83 % vs. Hp: 64.50 %). In the case of the embryo quality, both the diameter (control: 357.86 ± 8.98 µm vs. Hp: 373.13 ± 5.40 µm) and the number of cells per blastocyst (control: 52.46 ± 2.73 vs. Hp: 56.11 ± 2.50) were identical in both groups (control: N=14 and Hp: N=20 blastocysts evaluated respectively). In conclusion, adding Hp protein into the culture medium increases the number of embryos that reach the blastocyst stage.

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78. Immunofluorescence analysis of NR3C1 receptor following cortisol exposure during bovine in vitro oocyte maturation

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Keywords: oocyte, cortisol, NR3C1

Glucocorticoid hormones (GCs) play a key role in a various set of important cellular and physiological functions such as stress signaling, lipid and carbohydrate metabolism, apoptosis and mitochondrial activity. However, the mechanisms by which stress and glucocorticoids damage or protect the oocyte are largely unknown. Current knowledge has reported differences in the effect of cortisol exposure depending on the species. As completely opposite examples, pig oocyte in vitro maturation (IVM) was inhibited by cortisol (Yang, Biol Reprod 60:929–936, 1999), whereas equine was not impaired (Scarlet, Dom Anim Endocrin 59:11-22, 2017) and a previous study in bovine reported beneficial effect of cortisol in the IVM medium on blastocyst rate (daCosta, Theriogenology 85(2):323-329, 2016). Here we studied the nuclear maturation rates and the levels of the glucocorticoid receptor (NR3C1) after exposure of bovine oocytes to cortisol during IVM. Briefly, cumulus-oocyte complexes (COCs) were cultured for 24h with cortisol (Control (n=374) (C): 0µg/mL, Control vehicle (n=371) (CV): ethanol, CORT1 (n=372): 0.1µg/mL, CORT2 (n=370): 0.25µg/mL) at 38.5ºC in an atmosphere of 5% CO2 in humidified air. After 24 h of IVM, oocytes were denuded and fixed in 4% paraformaldehyde (30 min at 38.5ºC) and permeated (0.3% Triton-X100, 30 min, room temperature). For nuclear maturation assessment, oocytes were mounted in DAPI medium (Vector labs, Burlingame, USA) for chromosome staining and coverslipped. Metaphase II achievement status was checked. For immunofluorescence determination of NR3C1 glucocorticoid receptor presence, permeated oocytes were incubated overnight at 4ºC with anti-rabbit primary antibody (1:500) (GR/NR3C1 NBP2-42221, Novus Bio, Centennial, USA), washed 5x in PBS and then incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody (1:1000) for 1h at room temperature for subsequent washing and immunofluorescence semi-quantitative assessment. Results showed that nuclear maturation of cortisol treated groups was improved (C: 61.5±1.5; CV: 59.8±3.7; CORT1: 75.3±2.0; CORT2: 76.8±0.5; $p < 0.01$) compared to control ones. NR3C1 expression was 40.1% and 40.9% times more expressed in CORT1 and CORT2, respectively, compared to control groups; while whole intensity of the oocyte was 5.7% and 5.4% increased. Cortisol seems to play a role in the oocyte developmental competence and may be acting directly on oocyte maturation. We hypothesize that this may be due to the preparation of the oocyte for the following stressing phenomenons, acting as a sublethal stress for the acquisition of stress tolerance (Pribenszky, Biol Reprod 563;83:690-7, 2010). Further studies are needed in order to elucidate the specific mechanism by which the glucocorticoid receptor affect the development of oocyte competence on different species.

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79. Production of cloned and fertilized embryos of a cloned bull of buffalo (Bubalus bubalis)

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Keywords: Buffalo, Re-cloning, IVF, and Embryos

Buffalo cloning has gained popularity in India as a valuable tool to make genetically identical copies of superior bulls to mitigate demand of quality semen for breeding schemes. However, it is unknown how the somatic cells of cloned buffalo will behave in re-cloning experiments and fertility of cloned bulls. Also, no data is available about the in vitro fertilization success rate using cloned bull semen. As a step towards answering these apprehensions, we performed re-cloning of the cloned bull that was produced in 2015 by us, and the semen of this cloned bull was used to produce in vitro fertilized embryos. Briefly, the somatic cells of cloned bull and its donor were used for nuclear transfer experiments. Three independent experiment data were used and data were analyzed by Student's t-test using SPSS software (SPSS.com). Following cloning, the blastocyst rate (39.6±1.1 vs 41.2 ±1.2), total cell number (322.0 ±18.2 vs 333.1±28.8) and apoptotic index (3.9±0.5 vs 3.1±0.3) of blastocysts were similar between the donor and cloned bull, respectively. Similarly, there was comparable blastocyst rate (17.5±1.4 vs 16.5±4.8), total cell number (234.8±30.9 vs 202±22.5), and apoptotic index (2.4±0.3 vs 2.5±0.4) of blastocysts produced from IVF procedure using donor and cloned bull semen, respectively. In addition, at the time abstract submission, we established one pregnancy from the transfer of cloned blastocyst of cloned bull and 8 pregnancies following artificial insemination using cloned bull semen. In conclusion, the somatic cells of a cloned bull can be used for re-cloning experiments; whereas, sperms can be used to produce in-vitro fertilized embryos and to impregnate females using artificial insemination.

80. In vivo characterization of pH, CO2 and O2 analytes in the bovine uterus: preliminary study

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Keywords: uterus, pH, oxygen

A precise knowledge of the physiological level of certain environmental parameters such as pH, CO_2 and O_2 are of high relevance for in vitro production of embryos (IVP). The uterus is the site for the transport of sperm, early embryonic development and gestation. However, its physiological environment is poorly defined yet, and in cattle only pH values have been reported (Hugentobler et al., Theriogenology 61, 1419, 2004). By new cutting-edge devices and a non-invasive approach, we aimed to define a new method to record *in vivo* data of pH, CO_2 and O_2 in cows during the ovulation day (Ovul) and luteal (5 days after ovulation, Lut) phases. For this, 3 multiparous Holstein females of 6-8 years under the same conditions of feeding and handling were used. The synchronization of ovulation in all the animals was induced through administration of GnRH at day 0 (0.2 mg i.m., Dalmarelin®, Fatro Ibérica, Barcelona, Spain), together with the application of a progesterone-releasing intravaginal device (1.38 g, CIDR®, Zoetis, Madrid, Spain). On day 7 the intravaginal device was removed and PGF2α (25 mg i.m., Dinolytic®, Zoetis, Madrid, Spain) was administered twice (days 7 and 8, 24 hours interval), plus a final injection of GnRH i.m. (day 9) to induce ovulation. Monitorization of the estrous cycle was verified on a daily basis to detect ovulation time and the existence of corpora lutea through a portable ultrasound scanner (ImaGo®, ECM, Angoulême, France) equipped with a linear transducer from 5 to 7.5 MHz. To facilitate the measurement procedure, all the animals were immobilized in a cattle cage and calmed with 0.20 ml/100 kg of xylazine i.m. 2% (Nerfasin®, Fatro Ibérica, Barcelona, Spain), followed by lidocaine 2% (Anesvet®, Laboratorios Ovejero S.A., León, Spain) epidural (5 ml/animal). Miniaturized (0.3 mm diameter) luminescent probes of pH, CO_2 and O_2 (PreSens®, Regensburg, Germany) were inserted in the caudal-middle part of the ipsilateral uterus horn to ovulation through an insemination steel catheter of 70 cm and 6 mm outer diameter. At Ovul and Lut stages, instant values of these parameters were taken simultaneously every 5 seconds for a total of 15 minutes in one cycle per animal. Data were then processed for basic statistics (mean±SD). No inference tests were used due to the low number of animals. The continuous records of pH, CO_2 and O_2 showed variable oscillatory patterns over time. Average pH was 7.13±0.11 (6.99-7.23 range) and 6.98±0.04 (6.95-7.0 range) at Ovul and Lut phases, respectively; CO_2 was 4.21±0.74 % (3.19-4.81 % range) and 5.75±1.55 % (3.67-6.27 % range); and O_2 was 4.35±0.56 % (2.89-5.21 %) and 10.98±0.78 % (8.64-12.30 %). These preliminary results showed that the methodology used can provide an effective characterization of the uterine environment of cattle with minimal iatrogenesis. Increasing the number of measurements is necessary to better define the oscillatory patterns of each parameter and ascertain potential differences between the stages of the estrous cycle. Finally, this information might be helpful to optimize IVP protocols in cattle.

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81. Embryo quality in relation to endometrial health in cows with problematic reproductive anamnesis intended for MOET

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Keywords: embryo quality, subclinical endometritis, somatic cell count
The quality of embryos is strongly related to the donor cow's overall reproductive health, age and management. Poor health of the endometrium is a detrimental factor for embryo development, quality and harvest. Periparturient reproductive problems may predispose dairy cows to subclinical endometritis (SE). The diagnosis of SE is proven if more than 5% of polymorphonuclear leucocytes (PMNL) are present in a cytology sample of endometrium >8 weeks post parturition (pp) and >14% of PMNL 4 weeks pp. An inflamed uterus is a poor environment for the developing embryo. The aim of this study was to investigate embryo harvest in Latvian native breed donor-cows with problematic reproductive anamnesis in relation to endometrial health and somatic cell count (SCC) in the milk. Ten Latvian native breed donor-cows which had a problematic reproductive anamnesis (repeated artificial insemination, difficult parturition, stillbirth or elevated somatic cell count in the milk (SCC) and with no any signs of illness were included in this study. Milk recording data from Agricultural Data Centre of Latvia were used to establish productivity and milk quality. Cytological samples were obtained using a uterobrush (Mekalasi, SAXO, Finland) and blood samples were taken to establish white blood cells (WBC) count after embryo flushing (7^{th} day after AI). Diff-Quick stain (Sysmex, Japan) was used to visualize cells (PMNL, epithelial cells, lymphocytes, eosinophils (Eo), monocytes). Cytological samples were investigated at 400x magnification using immersion oil. One hundred somatic cells were counted and the percentage of cells was determined in each sample. Results were analysed in relation to obtained transferable and damaged embryos in healthy cows and cows with SE. Average ± standard deviation (SD) was calculated, two-independent samples t-test (Mann-Whitney U test), two-tailed bivariate correlation was performed using *SPSS 17*. Cows were 6.3±2.71 (average±SD) years old (min. 3, max. 12 years), in 3.5±1.90 lactation (min.1 and max. 6 lactation). Productivity was 18.7±6.16 kg/day, milk fat 4.7±0.84%, milk protein 3.6±0.63%, SCC 821.4± 1505.15 thousand/ml (min. 50.0 and max. 5010.0 thousand/ml). Total embryo harvest from all cows was 8.0±6.67 embryos per cow (min. 0, max.18 embryos per cow); 4.7±5.3 embryos per cow were transferable (min. 0, max. 13 embryos) and 2.1±4.10 embryos per cow (min. 0, max. 13 embryos) were degenerated. Subclinical endometritis was diagnosed in 70% of cows on the embryo flushing day. Healthy cows, in comparison to SE cows, had no significant differences regarding WBC in blood (8.0±5.05 vs 7.1±1.40 $x10^3/mm^3$), age (4.8±2.26 vs 6.9±2.80 years), productivity (19.9±6.8 vs 18.2±6.40 kg/day), milk fat (4.9±1.35 vs 4.6±0.65%) and milk protein (3.6±0.25 vs 3.6±0.75%) ($P>0.05$). Cows with SE, in comparison to healthy cows, had statistically significant lower total embryo count (6.1±6.57 vs 12.3±5.50 embryos), transferable embryo count (2.6±4.76 vs 9.7±2.52 embryos) and higher degenerated embryo count (3.6±4.50 vs 1.3±2.30 embryos) ($P<0.05$). SCC in milk strongly correlated with PMNL in the endometrium ($r=0.99$; $P<0.05$) despite an optimal count of WBC in all cows' blood (7.4±2.80 $x10^3/mm^3$). SCC in cows without SE was 253.3±135.24 thousand/ml, but in cows with SE it was 1064.9±1778.09 thousand/ml ($P>0.05$). The count of degenerated embryos correlated with Eo in endometrium ($r=0.97$; $P<0.05$). In conclusion, a cytological investigation of the endometrium should be performed before cows with problematic reproductive anamnesis are considered for use as embryo donors. Studies must be continued to establish at what level increased SCC in milk affects the quality of the embryos in donor cows, because it may be an early marker for successful MOET.
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82. SNP based Preimplantation Genetic Testing for Aneuploidy (PGT-A) to improve pregnancy outcomes in cattle IVP: a blind retrospective study

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Keywords: bovine, pregnancy rates, blastocyst

Currently, the ability to produce Genomic Estimated Breeding Values (GEBVs) from Single Nucleotide Polymorphism (SNP) data acquired from live animals plays a key role in guiding the selection process operated by the cattle breeding industry. Increasingly, this technology is being applied to *in vitro* produced (IVP) embryos as a way to increase genetic gain rates and avoid the birth of unwanted animals. However, a significant proportion of the recipients of a SNP typed embryo will not become pregnant, resulting in a waste of time and resources for breeders. Aneuploidy is the most common cause of early developmental arrest and implantation failure in IVP embryos. Thus, supplementing GEBVs with the use of preimplantation genetic testing for aneuploidy (PGT-A) may ensure the selection of embryos with desirable traits which stand a high chance of returning a pregnancy. Here we employed a new PGT-A algorithm (Handyside *et al*. 2010, J Med Genet, 47:651-8) to obtain ploidy diagnoses from the same SNP data used to establish GEBVs. Heterozygous loci in one parent that are homozygous in the other were used as markers to trace chromosome inheritance across generations. The analysis of haploblock patterns in the chosen embryo was made possible by comparing embryonic and parental SNP information with data acquired from a full sibling (either another embryo or a live-borne). To test the hypothesis that the selection of euploid embryos by our PGT-A algorithm would benefit pregnancy rates, we performed a blind retrospective study analysing the SNP and pregnancy data provided by two commercial cattle breeders: Boviteq (Saint-Hyacinthe, Canada) and Activf-ET (Carlisle, UK). The analysis of 66 embryos revealed that 18.2% of them were aneuploid. When individually transferred, 75.0% of the aneuploid embryos did not result in pregnancy, compared to a rate of just 46.3% for euploid embryos (chi square, P=0.05). One of the pregnancies from aneuploid embryos (n=3) resulted in a miscarriage, effectively increasing aneuploid embryo failure rate to 83.3%. If only euploid embryos had been transferred in this cohort, the average pregnancy rate would have increased from 48.5% to 53.7%. When an embryo transfer resulted in a pregnancy, our PGT-A algorithm identified an euploid embryo in 90.6% of cases; conversely, when there was no pregnancy, aneuploidy was identified in 26.5% of cases. In conclusion, when embryos are euploid, our PGT-A algorithm cannot reliably predict pregnancy outcomes as ploidy is just one aspect of a complex system. Nevertheless, our preliminary study seems to suggest that euploid embryos are more developmentally competent and their elective transfer might offer better value for money to breeders. However, our statistical analysis did not provide a robust answer in this pilot study, and the test of a larger sample is likely necessary to achieve clear statistical significance. In our future work, we will analyse a much larger sample database for a more in-depth analysis. We will then investigate whether certain chromosomal abnormalities are more often associated with reductions in pregnancy rates, and which, if any, can be tolerated by the embryo.

83. Long-term antiapoptotic action of progesterone on bovine oocytes during the second phase of IVM is not mediated through the Bax/Bcl2 pathway

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Keywords: progesterone, oocyte apoptosis, Bax/Bcl2 pathway

Endogenous progesterone (P4) secreted by cumulus cells exerts an antiapoptotic effect on bovine oocytes during IVM through cumulus-expressed proteins (O'Shea et al., Biol Reprod, 89:146, 2013). However, the action of exogenous P4 on the oocytes is not so obvious and may be dependent on the stage of the meiotic maturation. Therefore, the present work was aimed to study: (1) a pattern and duration of effects of P4 and luteotropic hormone prolactin (PRL) on bovine oocyte apoptosis during the second phase of IVM (from M-I to M-II) and (2) a role of the Bax/Bcl2 pathway in these effects. In one-step IVM, bovine cumulus-oocyte complexes (COCs) were matured for 24 h in TCM 199 containing 10% fetal calf serum (FCS), 10 μg/ml FSH, and 10 μg/ml LH (1st IVM medium). In two-step IVM, COCs were cultured in the 1st IVM medium for 12 h and then transferred to TCM 199 containing 10% FCS (2nd IVM medium) and cultured for next 12 h. The 2nd IVM medium was either free of additives (Control) or supplemented with 50 ng/ml P4 or 50 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia). After one-step and two-step IVM, a half of COCs was cultured for additional 24 h in TCM 199 containing 10% FCS to test long-term hormonal effects during aging. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). At the end of culture, oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI. The expression of apoptosis-related genes (*Bax* and *Bcl2*) in oocytes was analyzed by qPCR following RNA isolation by Trizol method and reverse transcription to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA). Levels of the gene expression were normalized by the reference gene *GAPDH*. The data for apoptosis (n=5-6, 87-113 oocytes per treatment) and gene expression (n=3, 30 oocytes per each replicate for each treatment) were analyzed by ANOVA. The rate of M-II oocytes was similar in all the compared groups (83.3-94.3%). Following 24 and 48 h of culture, the apoptosis frequency and the expression of *Bax* and *Bcl2* genes in oocytes did not differ between one-step IVM and the control group of two-step IVM. During 24 h of oocyte aging, the apoptosis frequency increased 2-3 times ($P<0.001$) in all the groups, whereas the relative levels of the transcripts changed only slightly. The addition of P4 (but not PRL) to the 2nd IVM medium resulted in the reduction ($P<0.05$) in the rate of apoptotic oocytes from 11.7±1.2 to 5.9±1.7% after 24 h of maturation. Furthermore, this rate in the P4-treated group was lower than in one-step IVM (17.6±1.6 vs. 24.3±0.4%, $P<0.05$) after 24 h of aging. Meanwhile, P4 did not affect the expression of *Bax* and *Bcl2* genes in matured or aged oocytes. Thus, during the second phase of IVM, exogenous P4 can exert the long-term antiapoptotic effect on bovine oocytes that is not related to modulation of the Bax/Bcl2 pathway. The role of the AVEN-associated pathway is currently under consideration.

84. Bovine oocyte quality when cultured in one-step and different two-step IVM systems

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Keywords: two-step IVM system, MII chromosomes, Bax/Bcl2 pathway of oocyte apoptosis

In vitro matured oocytes are widely used for commercial and research purposes. The quality of oocytes acquired during in vitro maturation is the main limitative factor affecting their capacity for further development. The aim of the present research was to study effects of different conditions of IVM on the state of M-II chromosomes and apoptosis of bovine oocytes. Cumulus-enclosed oocytes (CEOs) were matured in either one-step or two-step IVM systems. In the case of the one-step protocol, CEOs were cultured for 24 h in TCM 199 supplemented with 10% fetal calf serum (FCS), 10 μg/ml porcine FSH, and 10 μg/ml ovine LH (standard medium) at 38.5°C and 5% CO2. In the case of the two-step procedure, CEOs were first cultured for 16 h in the standard medium and then transferred to one of three experimental media and cultured for additional 8 h. The following media for the two-step IVM system were tested: (1) TCM 199 containing 10% FCS (Group 1), (2) TCM 199 containing 3 mg/ml BSA (Group 2), or (3) Fert-TALP medium supplemented with 6 mg/ml BSA (Group 3). Fert-TALP with traditional for IVF concentration of BSA was selected because it can potentially be used throughout maturation and fertilization. At the end of culture, the state of the oocyte nuclear material was evaluated by the Tarkowski's method (N=251). Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI (N=212). The expression of involved in apoptosis genes *Bax* and *Bcl-2* in oocytes was analyzed by real-time RT-PCR (N=332). The data (4 replicates, 69-114 oocytes per treatment) were analysed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rate of M-II oocytes was similar in all groups and reached 84.3-87.7%. No effects of the systems on the frequency of M-II chromosome abnormalities (decondensation, adherence, clumping) were observed, with the frequency after culture was: 29.5±3.0 (one-step IVM), 36.5±3.1 (Group 1), 31.7±2.7 (Group 2) and 33.6±1.9% (Group 3). In the one-step system, the rate of matured oocytes with apoptotic signs was 15.1±2.0%. Transfer of CEOs after 16 h of incubation in the standard medium to TCM 199 containing BSA (Group 2) caused a decrease in the rate of oocyte apoptosis to 6.9±1.3% ($p<0.05$). Moreover, the rate of apoptotic oocytes in Group 2 was lower than in Group 1 (19.4 ± 1.1%, $p<0.01$) and in Group 3 (14.5 ± 2.7%, $p<0.05$). The expression level of pro-apoptotic gene *Bax* after oocyte maturation did not differ between groups or systems. Meanwhile, oocyte culture in Group 2 (but not in Group 1 and 3) led to an increase in the transcript abundance for anti-apoptotic gene *Bcl-2* and a decrease in the ratio of *Bax* and *Bcl-2* transcript levels as compared to the one-step system ($p<0.05$). Our data indicate that bovine oocyte culture in the two-step IVM system including oocyte transfer to TCM 199 containing BSA can increase the apoptosis resistance of the oocytes by enhancing expression of anti-apoptotic gene *Bcl-2* and may be used as an alternative for the standard one-step IVM.

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85. Effect of different culture conditions on bovine embryos derived from metabolically compromised oocytes

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Keywords: FFA-exposed oocytes, in vitro culture conditions, supplementation

Metabolic disorders e.g. obesity lead to elevated saturated (stearic; SA, palmitic; PA) and mono-unsaturated (oleic; OA) free fatty acids (FFAs) in serum and follicular fluid. Exposure of maturing oocytes to these FFAs, particularly to PA, hampers embryo development. Supplementation of embryo culture media with Insulin-Transferrin-Selenium (ITS) or serum is used to enhance embryo production; however the effect of such enrichment on development of metabolically compromised oocytes has not been investigated. Here, bovine oocytes (n=3737) were exposed to either 1) pathophysiological high PA, SA and OA concentrations (150, 75, 200 µM, respectively; **HFA**); or 2) high PA, basal SA and basal OA (150, 28 and 21 µM; **HPA**); compared to 3) basal PA, SA and OA (23, 28, 21 µM; **BASAL**) as a physiological control. Zygotes were cultured in SOF medium containing 1) BSA (2%) only or supplemented with 2) ITS (10 µg/mL insulin; 5,5 µg/mL transferrin and 6,7 ng/mL selenium) or with 3) serum (5%). Cleavage (48h) and blastocyst rates (day 7 (D7) and D8 post insemination) were recorded. D8 blastocysts were analyzed for apoptotic cell indexes (ACI) (caspase-3 immunostaining), embryo metabolism (glucose consumption and lactate production), or mRNA expression of genes involved in ER unfolded protein responses (UPR^{er}) (*Atf4, Atf6*), oxidative stress (*SOD2, GPx, CAT*) mitochondrial UPR (*HSP10, HSP60*) and mitochondrial biogenesis (*TFAM*). Categorical and numerical data were analysed using binary logistic regression and ANOVA, respectively, and were Bonferroni corrected. Cleavage rate was significantly ($P<0.05$) reduced in HPA embryos compared with BASAL when cultured in BSA. However, ITS or Serum in culture alleviated this negative effect. Compared with BASAL, HPA exposed oocytes showed significant lower D7 and D8 blastocyst rates after culture in BSA and Serum, but not in ITS containing SOF medium. Within the PA-treated group, ITS significantly increased D7 and D8 blastocyst rates compared with BSA. HFA did not have significant effects on development under all IVC conditions. For embryo quality, ACI was not different among BASAL, HFA and HPA groups in BSA culture. Surprisingly, supplementation of ITS during IVC significantly increased ACI of HPA and HFA embryos compared to BASAL ($P<0.05$). Serum supplementation also increased ACI of HPA embryos compared with HFA and BASAL ($P<0.05$). Regardless of IVM treatment, embryos cultured in Serum showed increased lactate/2glucose ratio compared with BSA and ITS, confirming the reported preference for Warburg metabolism. In contrast, HPA-derived embryos cultured in ITS or Serum had significantly lower lactate/2glucose ratio compared to BASAL and HFA. At the blastocyst transcriptomic level, HPA increased *HSP60* expression compared to BASAL when cultured in BSA, indicating activation of mitochondrial stress responses. ITS and Serum alleviated this increase in *HSP60*. In conclusion, enrichment of embryo culture media with ITS or serum can improve developmental competence of oocytes after maturation in lipotoxic conditions. However, the surviving blastocysts exhibit higher apoptosis and altered metabolism indicating inferior quality.

86. Relationship between nitric oxide in follicular fluid and ovarian response among oocyte donors

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The identification of a family of enzymes catalyzing the synthesis of nitric oxide (NO) in the oviduct, oocytes and cumulus cells of several species [Rosselli M, Mol Hum Reprod. 2(8):607-12, 1996; Lapointe J, Endocrinology. 147(12):5600-10, 2006; Tao Y, Mol Cell Endocrinol. 222(1-2):93-103, 2004], suggested that NO is a key component of the oocyte microenvironment [Romero-Aguirregomezcorta J, PLoS One. 9(12):e115044, 2014]. Interestingly, it has been reported that NO modulates the granulosa cell function, follicular maturation and ovulation (Yalçınkaya E, J Turk Ger Gynecol Assoc. 14(3):136-41, 2013). The present work aimed to determine if the NO levels in human follicular fluid (FF) correlate with the number of total and MII oocytes retrieved from donors. Seventy-two women participating in the oocyte donation program at IVI-RMA Global Murcia (Spain) took part in this study. FF was obtained at oocyte retrieval in 93 donation cycles to measure the levels of stable-end products of NO oxidation, nitrite (NO_2) and nitrate (NO_3). For each donor, demographic, lifestyle and donation cycle-related data were also recorded. NO_2 and NO_3 were determined by HPLC-UV/VIS. Multivariate mixed Poisson and logistic regression models with random slopes to account for repeated observations within woman were used to compare total and MII oocyte yields for women across tertiles of NO_2, NO_3, total NO and NO_3/NO_2 ratio while adjusting for age, body mass index, hours of sleep, coffee intake, smoking and physical activity. NO_2 levels ranged from 0.7 to 96.1 µM, NO_3 levels ranged from 4.9 to 39.7 µM, total NO levels ranged from 5.6 to 109.5 µM and NO_3/NO_2 ratio ranged from 0.1 to 31.5. NO_2 and NO_3 concentrations were unrelated to each other (r=-0.01). FF NO_2, NO_3, total NO or NO_3/NO_2 ratio were unrelated to total or mature oocyte yield. The multivariable-adjusted MII yield (95% CI) for women in the lowest and highest tertiles of NO_2 was 12.4 (10.2, 15.1) and 13.2 (10.9, 16.0) (p=0.53); 14.1 (11.7, 17.1) and 12.2 (9.9, 15.0) for NO_3 (p=0.21); 13.7 (11.4, 16.5) and 12.2 (10.1, 14.6) for total NO (p=0.26); and 14.1 (11.7, 16.9) and 12.2 (10.1, 14.8) for the NO_3/NO_2 ratio (p=0.15). When MII oocytes were considered as the proportion of total oocytes, however, the proportion of MII oocytes increased with increasing FF NO_2 levels but decreased with increasing NO_3 levels. The adjusted proportion (95%CI) of MII oocytes for women in the lowest and highest FF levels of NO_2 were 68% (58-77%) and 79% (70-85%) (p, linear trend=0.02); whereas the proportion of MII oocytes for women in extreme tertiles of FF NO_3 levels were 79% (70-85%) and 68% (57-77%) (p, linear trend=0.03). In conclusion, NO and its metabolites did not predict the number of mature oocytes retrieved from donors, but NO_2 and NO_3 correlated with the MII proportion. The fertilization rate, embryo quality and pregnancy rates should be analyzed in patients who received these oocytes to determine any correlations with NO levels in FF.

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87. Effect of month of birth on the development of Belgian Blue calves

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Keywords: month of birth, Belgian Blue calves, organs

Intra-uterine growth is important in beef cattle since it determines weight and morphometrics of neonatal calves and hence contributes to ease of calving. Earlier, we have shown in dairy cattle that environmental circumstances like for example season of birth and parity of the mother significantly influence birth weight of calves. The present study aimed to evaluate the effect of month of birth on the intra-uterine development of Belgian Blue calves based on the measurement of 10 body parts shortly after birth: body weight (BW), withers height (WH), oblique length (OL), heart girth (HG), width of the back (WB), shoulders width (SW), circumference of the head (CH), diameter of the head (DH), length of the metatarsus (LM), and length of the underarm at the front leg (LA). Furthermore, we also investigated the effect of month of birth within the sex of calves. The data include 73 records of calves born in the Clinic of Reproduction and Obstetrics at the Faculty of Veterinary Medicine, Ghent University (Belgium), collected between 2016 and 2017. The results show that the average BW was 52.46 kg, WH 70.08 cm, OL 64.78 cm, HG 80.08 cm, WB 25.95 cm, SW 24.45 cm, CH 50.49 cm, DH 13.39 cm, LM 30.57 cm, and LA 25.28 cm. There was a significant association between the month of birth and the length of the metatarsus as well as the length of the underarm at the front leg. In comparison to calves born in winter, calves that were born in autumn had both a longer metatarsus (31.13 versus 29.82 cm; $P<0.05$) and a longer underarm at the front leg (26.13 versus 24.14 cm; $P<0.05$). Furthermore, the data showed an effect of the gender of the calves, as the male calves had a significantly ($P= 0.026$) bigger HG (81.69 cm) than their female counterparts (78.67 cm). Based on these results, it appears that the season of birth and the gender of the calves both have a significant effect on some of the neonatal morphometrics in Belgian Blue calves, which is important to know in terms of calving ease.

88. Dynamic transcriptome changes during embryonic diapause and reactivation in the embryo and endometrial epithelium of the European roe deer

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The European roe deer pre-implantation embryo development is characterized by a four-month period of embryonic diapause, after which the embryo rapidly elongates and implants. Pre-elongation developmental pace is 10 times slower than in cattle. In roe deer, endometrial secretions at implantation are 1.5-fold higher than during diapause, and morphological changes of the embryo coincide with changes of the uterine fluid composition. To identify the reactivation initiating mechanism, we investigated the embryonic and endometrial transcriptome changes during diapause and following reactivation. Samples were collected at regular huntings between September and January 2015-2017. A total of 360 animals was sampled and 537 pre-implantation embryos were collected (77% recovery rate). A group of six day 14 *ex vivo* flushed embryos from captive roe deer represent the early blastocysts. Embryonic DNA was extracted for DNA content determination and cell number estimation. Endometrial luminal epithelial was collected by laser-capture micro-dissection. Total embryonic RNA from 87 embryos and total endometrial RNA from 56 different females was subjected to RNA-sequencing. Raw sequence reads were analyzed using a customized Galaxy pipeline. A pseudotime analysis (CellTree) was performed to gain insight into the transcriptome dynamics of diapausing embryos, in which the number of embryonic cells was used as proxy for developmental progression. Differentially expressed transcripts (DET) were identified in a time-course dependent manner with the ImpulseDE2 algorithm with an FDR <1%. To elucidate dynamic gene expression changes, a self-organizing tree algorithm (SOTA) was used. Gene set enrichment analysis and gene ontology were used to identify enriched hallmarks between diapause and elongation. As determined by a rise in DNA content, embryonic cells divide every two weeks during diapause. With developmental progression, an overall increase in the number of embryonically expressed transcripts was observed. The pseudotime analysis of both embryos and luminal epithelium showed grouping of the early blastocysts on one end of the trajectory, the elongated embryos on the other end with diapausing embryos dispersed heterogeneously in between. Embryonic time-course analysis revealed 13,193 DET out of 29,575 transcripts. The DET grouped into 7 SOTA clusters. Gene set enrichment analysis and gene ontology revealed an enrichment of MYC targets, MTORC1 signaling, PI3K, AKT and MTOR signaling, unfolded protein response, peroxisome and the glycolytic pathway in elongated embryos. In the luminal epithelial cells, changes were less dynamic; 2,754 DET grouped into 2 clusters and lacked any enriched biological pathway. Taken together, roe deer embryos divide at a slow pace and are transcriptionally active during diapause. Enriched pathways indicate cell proliferation following reactivation. Targeted transcript analyses will emphasize on the identification of diapause-related regulatory pathways and aim at identifying conserved mechanisms of cell cycle control.

89. Effect of the zona pellucida removal on the developmental competence of domestic cat embryos generated by in vitro fertilization

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Keywords: felids, in vitro embryo production, zona pellucida

The domestic cat is a valuable model for the development of assisted reproductive techniques that might be used in the conservation of endangered felids. However, the efficiency of the *in vitro* embryo production in the domestic cat remains low compared to other species. In the bovine and equine, the zona pellucida removal enhance the developmental competence of the embryos generated *in vitro*, allowing the birth of live offspring (Gambini *et al.*, 2012; Rodriguez *et al.*, 2008). The objective of this research was to evaluate the effects of zona pellucida removal in domestic cat embryos generated by in vitro fertilization (IVF). To achieve this purpose, two experimental groups were made, 1) domestic cat embryos generated by IVF and *in vitro* cultured (Zona-included), 2) domestic cat embryos generated by IVF and cultured without zona pellucida (Zona-Free). To evaluate the effect of the zona removal, the developmental capacity and morphological quality of the embryos generated in the Zona-free group were compared against the Zona-included group. For this, the ovaries of domestic cats were collected by ovariohysterectomy and the cumulus-oocyte complexes (COCs) were recovered by slicing. The COCs were *in vitro* matured in supplemented TCM-199 Earle's salts medium for 26-28 hours, in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5ºC. The IVF was realized using epididymal refrigerated sperm. 1.5 – 2.5 x 10^6 spermatozoa /mL were incubated with 20-30 COCs in supplemented TALP medium for 18 hours, in a 5% CO_2 atmosphere, at 38.5ºC. After cumulus cell removal, the zona pellucida of the presumed zygotes was removed by 2-4 minutes incubation in 2 mg/mL of pronase (Sigma-Aldrich, P8811, USA). The presumed zygotes were cultured using the well of the well system (Vajta *et al.*, 2000) in supplemented SOF medium, in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5ºC, for 8 days. The cleavage, morulae and blastocysts rates were estimated at day 5 and 8. The Zona-included and Zona-free groups were performed at different times, with nine and six replicates respectively. The diameter and total cell number of the blastocysts were evaluated. The Wilcoxon non-parametric test was used to analyze the developmental competence and the t-test was used to analyze the diameter and total cell number. Regarding to the results, no statistical differences were observed between the Zona-included and Zona-free groups in the cleavage rate: 155/239 (64.9%) and 116/177 (65.5%), morulae rate: 115/155 (74.2%) and 68/116 (58.6%), and blastocysts rate: 51/155 (32.9%) and 36/116 (31.0), respectively ($P > 0.05$). No differences were observed in the total cell number (mean ± SD) of the blastocysts generated in the Zona-included (279.9 ± 148.1) and Zona-free group (313.1 ± 164.9) ($P > 0.05$). Finally, the diameter (mean ± SD) of the blastocysts from the Zona-free group (253.4 ± 83.3 µm) was significantly higher than the diameter of the blastocysts from the Zona-included group (210.5 ± 78.5 µm). In conclusion, the zona pellucida removal did not affect negatively the morphological quality and developmental competence of domestic cat embryos in our cultured conditions. However, more studies are needed to evaluate the *in vivo* competence of these embryos.

90. Effects of serum and serum substitutes on in vitro maturation (IVM) and embryo development of porcine oocytes

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Keywords: Embryo development, blastocyst, pig

Porcine *in vitro* embryo production (IVP) protocols have traditionally relied on the use of follicular fluid and serum, which results in undefined media with undetermined levels of growth (and other) factors. Media composition can alter the efficiency of embryo development, and more importantly gives rise to a potential biohazard. Moreover, the use of serum in IVP has been linked to alterations in embryo transcriptional activity (Oliveira *et al*. 2006, Reprod Domest Anim. 41:129-36). Hence, our aim was to establish whether a serum substitute (formed by a combination of cytokines currently under NDA) could be used efficiently in pig IVP. Here, we compared the use of this serum substitute during *in vitro* maturation (IVM) vs. sow follicular fluid (sFF) as stand-alone treatments or in combination, and followed the development of the resulting zygotes.

Oocytes collected from abattoir-derived ovaries were matured for 44 h in supplemented or non-supplemented Porcine Oocyte Medium (POM). There were four treatment groups: 1) Non-supplemented (control), 2), 10% sFF, 3) serum substitute, or 4) combination of 10% sFF + serum substitute (n=100 oocytes/group). Fertilisation of matured oocytes was carried out using extended boar semen (JSR, Driffield, UK). *In vitro* culture (IVC) of zygotes across treatment groups remained consistent, and occurred in defined Porcine Zygote Medium 5 (PZM5) supplemented with our serum substitute and with partial media changes at 48 h and 96 h post-IVF. Cleavage and blastocyst rates were assessed at 48 h and 144 h post-IVF, respectively. Differences between the groups were analysed using a comparative General Linear Model followed by Tukey's *post-hoc* test. Cleavage rates, as compared to the control group (36.7%), were significantly higher in both serum substitute (57.4%, p=0.02) and combination groups (70.4%, $p=6x10^{-6}$). There was no significant effect of using 10% sFF compared to controls (50.4%, p=0.25). Interestingly, there was no significant difference in the proportion of blastocysts per cleaved embryo between the control (30.3%), 10% sFF (26.8%), serum substitute (26.8%) and combination groups (30.9%)(F_3=1.85, p=0.14).

The use of serum in combination with cytokines might have resulted in a higher proportion of growth factors, providing a possible explanation to the combinatory effect here described for cleavage rates. Moreover, the fact that cleavage but not blastocyst rates differed between these groups advocates that the effects of the supplementation in IVM did not extend to IVC. Certain IVM treatment groups produced a higher proportion of developmentally competent oocytes, as highlighted by the difference in cleavage rates. At the same time, the use of a standardised IVC protocol following IVM might have had a role in ensuring consistent development to the blastocyst stage following successful fertilisation. To further characterise this observation, future experiments will assess the effects of supplementation with sFF and serum substitutes on IVC, while IVM will be kept consistent. While our serum substitute appeared suitable for IVM and IVC, the combinatory effect observed suggests that the development of a more complex and efficient serum substitute should be possible.

91. Using a time-lapse system to study the morphokinetics of blastocysts derived from heat-shocked oocytes

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Keywords: heat shock, embryo morphokinetics, time-lapse system

Embryonic development involves multiple dynamic events that are remarkably sensitive to environmental changes. We used a non-invasive time-lapse system (TLS) to continuously monitor the kinetics of embryonic development. The objectives were to: (1) compare embryonic development in a conventional incubator and in an incubator equipped with a TLS; (2) characterize the precise cleavage time and duration (i.e., morphokinetics) of individual in-vitro-derived embryos; (3) examine whether exposing oocytes during maturation to heat shock affects the morphokinetics of the developing embryo. Ovaries were collected from a local abattoir. Cumulus-oocyte complexes (COCs) were aspirated, matured (22 h) and fertilized (18 h) in a conventional incubator (humidified air, 5% CO_2 at 38.5˚C). Statistical analysis was performed using JMP-13 software. In the first experiment, putative zygotes were cultured in a conventional incubator (n = 192, control) or an incubator equipped with a TLS (n = 126, TLS) for 8 days. The proportion of oocytes that cleaved to the 4-cell stage (74.3±7 vs 89.2±2.3%, respectively) and that of blastocysts (20.6±6.9 vs 25.9±5.8%, respectively) did not significantly differ between control and TLS groups. In the second experiment, the morphokinetics of embryos (n = 427) cultured in the TLS-equipped incubator were individually recorded. Findings revealed that the median of cleavage into 2-cells stage was 27.5 h post fertilization (pf): from 2- to 4-cell stage was 37.5 h pf, from 4- to 8-cell stage was 50.5 h pf and to the blastocyst stage was 127.5 h pf. In the third experiment, COCs (n = 421) were matured for 22 h in an incubator under normothermic conditions (5% CO_2, 38.5˚C, control) or exposed to heat shock (6% CO_2, 41.5˚C, HS) in a conventional incubator. Following fertilization (18 h), embryos were cultured for 7 days under normothermic conditions in the TLS-equipped incubator and embryo morphokinetics was recorded. In the control group, embryo cleavage was characterized by two waves of divisions; the first between 22 and 28 h pf and the second between 28 and 36 h pf. In the HS group, cleavage was characterized by one wave of divisions that occurred between 28 to 36 h pf. The median of the cleavage into 2-cell stage was 31.5h pf and 27.5h pf for HS- and control groups, indicating a delay in cleavage. Blastocysts from both control and HS groups were collected for real-time PCR assay to evaluate the expression of selected transcripts (*OCT4*, *NANOG*, *SOX2*, *DNMT1*, *PTGS2*, *GDF9*, *STAT3*). In summary, use of a TLS adds to our understanding of the mechanisms by which heat stress can impair oocyte developmental competence. We documented the precise morphokinetics of bovine embryo development from oocytes matured under normothermic or heat-shock conditions during maturation. These were associated with reduced developmental competence in the HS group. PCR findings will enable a comparison of gene expression in blastocysts developed from control and heat-shocked oocytes.

Cloning, Transgenesis and Stem Cells

92. RS-1 increases CRISPR-mediated Knock-in rate in bovine embryos

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Keywords: CRISPR, bovine, homologous recombination

The insertion of genomic sequences at specific loci (targeted Knock-in, KI) has been challenging due to the low efficiency of homologous recombination (HR). This efficiency has been boosted by the use of endonucleases, such as CRISPR, that generate a double-strand break (DSB) at the target locus. However, CRISPR-generated DSB can be repaired by one of two mechanisms: 1) HR, which can lead to the intended targeted KI if a donor DNA is provided or 2) non-homologous end Joining (NHEJ), which generates random mutations. The objective this study has been to test the effect of an enhancer of HR pathway, RS-1, on the KI rates following CRISPR injection in bovine zygotes. A preliminary study (3 replicates) was conducted to evaluate the highest concentration of RS-1 compatible with normal percentages of developmental. Bovine zygotes were incubated in SOF supplemented with 0, 7.5 or 15 µM RS-1 for 24 h and subsequently cultured in the absence of RS-1 for 8 days. RS-1 at 15 µM significantly reduced embryonic cleavage and blastocyst development, whereas 7.5 µM resulted in similar percentages of development to the control group (cleavage: 71.8±3.0; 83.6±1.8; 84.0±3.4 %; blastocysts 16.9±3; 30.3±3; 37.8±6.5 %, for 15, 7.5 and 0 µM groups, respectively; mean±s.e.m. logistic regression and ANOVA $p<0.05$). In a second experiment, zygotes where injected with CRISPR components (300 ng/µl Cas9 mRNA and 100 ng/µl sgRNA) and a single-stranded donor DNA (100 ng/µl) to mediate the insertion of an XbaI restriction site on a target non-coding region. Following microinjection, zygotes were transiently incubated for 24 h in SOF containing no RS-1 or RS-1 at 3.75 µM or 7.5 µM. As expected, the three groups displayed similar percentages of embryonic cleavage (77.8±3.3; 78.8±2.5; 73.7±4.0 %, for 0, 3.75 and 7.5 µM groups, respectively; mean±s.e.m., 5 replicates) and development to blastocyst (25.8±1.7; 27.0±2.0; 23.2±1.8 %, for 0, 3.75 and 7.5 µM groups, respectively; mean±s.e.m., 5 replicates). Resulting blastocysts were genotyped to detect genome edition (Sanger sequencing of PCR product) and targeted KI (XbaI digestion of PCR products). A significantly higher incidence of targeted insertion was achieved in embryos exposed to 7.5 µM RS-1 following microinjection compared with other groups (53.1% -17/32- for 7.5 µM vs. 26.5% -9/34- and -9/39- 23.1 % for 0 and 3.75 µM, respectively; Fisher's exact test $p<0.05$). In conclusion, transient exposure of bovine embryos to 7.5 µM RS-1 following CRISPR microinjection enhances targeted insertion of genomic sequences, highlighting its potential for animal research.

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93. First production of Calpain3 KO pig embryo by CRISPR/Cas9 technology for human disease modelling: efficiency comparison between electroporation and intracytoplasmic microinjection

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Limb-Girdle Muscular Dystrophy Type2A is an autosomal recessive myopathy caused by mutations in the Calpain3 gene. Currently the disease has not treatment and lacks good animal models. Thus, the study of this disease in pig would offer a great valuable tool to understand the disease and its possible treatments. In porcine species, somatic cell nuclear transfer and CRISPR microinjection (MI) are the main techniques to produce genetically modified embryos (GME). Previous studies have demonstrated that electroporation (EP) allows the production of GME embryos and further offspring (Tanihara, Sci. Adv. 2, e1600803, 2016). In this work we compare the use of MI and EP to produce GME for Calpaine3 evaluating embryo quality and mutation rate. In vitro matured porcine oocytes were treated before insemination with 100ng/µl of CRISPR/Cas9 ribonucleoprotein (RNP) by using two Calpain3 RNAguides. Five groups were used to compare mutation efficiency and embryo development achieved by MI and EP: oocytes microinjected with RNP (MI group), oocytes microinjected without RNP (sham group); oocytes electroporated with 4 (EP4 group) or 6 (EP6 group) pulses (30mV, 1 ms); and non-treated oocytes (Control group). All treatments were performed before IVF. Oocytes were inseminated in TALP medium with frozen-thawed boar spermatozoa selected with NaturARTs-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). Eighteen hours after insemination, putative zygotes were cultured (NCSU-23 medium) for additional 156 h to evaluate the blastocyst yield, regarding the total number of oocytes, and the gene deletion by PCR previous digestion of zona pellucida to remove bound spermatozoa. Experiment was repeated 4 times with 50-55 embryos per group and variables were analyzed by one-way ANOVA. Results showed similar cleavage rate among groups (63.2-72.9%) except for EP6 group which was the lowest (42.9; $p<0.01$). Blastocyst yield decreased in all treatment compared to control group (32.9±3.2%), being similar between MI (22.5±2.9%), sham (21.1±2.8%) and EP4 groups but again the EP6 was the lowest (11.1±2.2%; $p<0.01$). Regarding mutation rate, 41.5% (17/41) of MI derived blastocysts had a large gene deletion whereas EP4 showed 20.7% (6/29) and EP6 was 17.65% (3/17). As for the biallelic KO, it was similar in all blastocysts independently of the treatment applied: 14.6% (6/41) for MI; 17.2% (5/29) for EP4 and 11.8% (2/17) for EP6. These results confirm that EP is a valuable technique to produce KO embryos using CRISPR/Cas9 technology. Despite the low efficiency, the easiness to produce a greater number of embryos in a shorter time as well as the requirement of less high-qualified personnel and high-value equipment than with MI increase the possibility of its use to generate KO embryos. This is the first report about production of Calpain3 KO pig embryos, opening the doors to generation of KO big animals and promising further advances in the relevant field of human disease study. Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.

94. Generation of a polled phenotype in cattle using CRISPR/Cas

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Keywords: genome editing, somatic cell nuclear transfer, cattle

In modern livestock farming horned cattle pose an increased risk of injury for each other as well as for the farmers. Dehorning without anesthesia is associated with stress and pain for the calves and raises concerns regarding animal welfare. Naturally occurring mutations causing polledness are known for most beef cattle but are rarely distributed within dairy populations such as Holstein-Friesians and Brown Swiss. The propagation of polled Holsteins and Brown Swiss is limited due to the rather low genetic merit of the offered polled bulls which originate from a few founder bulls. In beef cattle, a mutation consisting of a 210 bp insertion and an 8 bp deletion (*Celtic mutation*) causes the polled phenotype while in Holsteins an 80 kbp duplication accompanied by several single point mutations is causative (Medugorac, Seichter et al. 2012). In this project, we used the CRISPR/Cpf1 system (Cas12a) to introgress the *Celtic mutation* into the *horned locus* of Holstein-Friesian and Brown Swiss fibroblasts derived from horned individuals with the aim of producing polled clones from originally horned bulls. The *Celtic mutation* was isolated from an Angus cow via PCR and cloned into a transfection vector as a knock-in template. Editing efficiencies in this locus were low, so multiple CRISPR/Cpf1 target sites were evaluated in order to improve knock-in efficiencies. Furthermore, we used the CRISPR/Cas9 system to create a novel knock-out mutation in the *horned locus* to examine whether also a deletion in this genomic area causes a polled phenotype. For this purpose, two target sites flanking a 300 bp sequence were used to create a large knock-out mutation. Cell clones carrying the desired mutation were propagated further to serve as donor cells for the somatic cell nuclear transfer (SCNT). In the fetus, horn buds are histologically detectable from day 90 of the gestation (Allais-Bonnet et al. 2013), hence the first pregnancy of each experiment will be aborted prematurely to examine the development of horn buds. All other pregnancies will be carried to term. Sequencing data and PCR results showed the desired integration for both the knock-in and knock-out experiment. First cloning experiments showed that development rates of edited embryos (blastocyst rate: 23,68 %; (9/38)) were comparable with those of wild type embryos (blastocyst rate: 20,29 %; (14/60)). The edited embryos were recently transferred into surrogate mothers. In conclusion, we successfully edited the genome of bovine fibroblasts by using different variants of the CRISPR system to introgress a complex mutation (*Celtic mutation*) and also create a novel knock-out mutation. Furthermore, we were able to reliably produce embryos from edited cell lines using SCNT. Once the embryo transfers result in pregnancies the fetuses and offspring, respectively, will be examined for polledness.

95. Establishment of several cloned pregnancies of buffalo breeding bulls

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Keywords: Buffalo, Cloning, Breeding bull, Embryo

Buffalo cloning is a valuable tool to improve the genetic potential of buffalo. Despite reported births of cloned buffaloes worldwide, the birth of several clones (more than 5 clones) of an individual buffalo have not yet been reported. Thus, in the present study, we attempted to produce multiple clones of an individual buffalo. The skin-derived fibroblast cells of two buffalo breeding bulls, namely M-29 and NR-480, were used as nuclear donors. The cloned embryos were produced using optimized handmade cloning (HMC) of our laboratory. The blastocyst production rate ranges from 35-40% in each experiment for both bulls. We transferred one or two cloned blastocysts on day 7 and 8 post oestrus into recipient buffaloes. We used transrectal ultrasonography to confirm the pregnancies at Day 30 post embryo transfer, and reconfirmed it again at day 60. We established 13 pregnancies (13 pregnant/56 recipient buffaloes, represents 23% conception rate) of M-29 bull, of which three were aborted at the first trimester. For NR-480 bull, we established 3 pregnancies (3 pregnant/7 recipient buffaloes, represents 42% conception rate). These established pregnancies are continuing at six to two months of gestation. To establish more cloned pregnancies, the embryo production and embryo transfer experiments are on-going. In conclusion, we established multiple cloned pregnancies of an individual buffalo using HMC embryos. HMC can be used to multiply elite buffalos in short period.

Support Biotechnologies: Cryogenesis and Cryobiology, Diagnosis through Imaging, Molecular Biology and "Omics"

96. Comparisons of lipid content and genes of lipid metabolism in follicular cells and fluid in follicles of different size in bovine.

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Keywords: Lipid metabolism, FA metabolism, follicle-size, mass spectrometry

Ovaries of mammals have thousands of follicles intended for atresia and only a few become dominant and designated to ovulate. The energy cost for follicular growth is high and requires different substrates, including fatty acids (FA). Somatic follicular cells and oocyte have molecular machinery to metabolize FA into energy. Within bovine biotechnologies, oocytes of the large follicles are more competent for the in vitro embryo development compared to the small ones. The objective of our study was to elucidate the specificity of the lipid composition and the metabolism of FA in antral bovine follicles of different sizes. MALDI-TOF mass spectrometry (MS) imaging allowed the mapping of 281 lipid characteristics in ovarian compartments. Lipid analysis using Red Nile demonstrated differential size dependent distribution of neutral lipids in granulosa (GC) and theca (TH) cell layers. MALDI-TOF MS lipid fingerprints of isolated follicular cells and follicular fluid (FF) of small (SF, medium size 5 mm) and large follicles (LF, mean size 13 mm) acquired by MALDI-TOF MS revealed drastic changes in follicular fluid lipidome (more than 55% of the detected characteristics varied more than twice between LF and SF, Student's t-test, $p<0.05$). The size of the follicle significantly influenced the lipid composition of TH, GC and cumulus cells (ranged from 5%, 15%, and 10%, respectively) in contrast to oocytes that had less than 2% of lipid profile modulation between SF and LF. Identified differential lipids (in total 17%) revealed potential changes of membrane lipids in both somatic follicular cells and fluid along with follicular growth. Among them, the phospholipids containing long and very long chain FAs were preferentially found more abundant in the cells of the LF. In the oocytes from SF., two identified phosphatidylcholines (PC29:1, PC31:1) and 1 sphingomyelin (SM 32:1) were more abundant than in LF. Analysis of gene expression in TH and GC in LF compared to SF suggests a significant increase of FA beta-oxidation and oxidative stress, respectively (observed by expression of *ACADVL*, *HADHA*, and *GPX4*). Gene *ACOT9*, coding for a thioesterase catalyzing the hydrolysis of long-chain Acyl-CoAs, showed overexpression in TH of the SF. In summary, FA metabolism in follicular cells changes through follicular growth and significantly modulates lipid composition of FF. Differential distribution and abundance of lipids, including signaling molecules may, therefore, influence either follicular atresia or dominance; also, an increase in long-chain FA can provide substrates for post-ovulation body-luteal progesterone production.

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97. A new contribution to the improvement of human embryo culture media: a comparative study of low-abundance proteins of reproductive fluids and plasma of fertile women

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Keywords: low-abundance proteins, human reproductive fluids, salpingectomy
The improvement of the embryo culture media is gaining relevance as demonstrated by the growing number of publications describing its influence on successful implantation rates, pregnancy, neonatal outcomes and potential effects in the adult life. The ideal conditions for embryo development are those naturally occurring in the female reproductive tract, i.e., the oviductal and uterine fluids. These fluids provide all the nutrients, hormonal and non-hormonal factors, electrolytes, macromolecules as well as precisely regulated volume, pH and osmolality required for the gametes, zygotes, and later, embryo development. In order to shed light on the differences between chemical and natural media, a detailed study of the composition of the female reproductive fluids is imperative. Here, we performed the first comparative study of the low abundance proteins in plasma, uterine and oviductal fluid collected from January 2016 until June 2018, simultaneously, from healthy and fertile women that underwent a salpingectomy. In order to select the most homogenous samples for this study, 3 women (out of 62 initially recruited) were selected based in the following criteria: similar age (31, 33 and 39 years old), evidence of healthy progeny and phase of their menstrual cycle (secretory phase). Samples were collected with a modified Mucat® device. The amount and quality of the collected samples allowed us to perform an efficient antibody-based depletion of the most-abundant serum proteins to facilitate the detection of the lower-abundance proteins of each fluid. The rationale for this design derives from the fact that high-abundant proteins in these fluids usually come from blood serum and usually mask the detection of low abundant proteins, which presumably could have a significant role in specific process related with the reproductive function. Differential regulation was measured using label-free quantitative shotgun proteomics, and statistical significance was measured using q-values (FDR). All analyses were conducted using software from Proteobotics (Madrid, Spain). The proteomic analysis by 1D-nano LC ESI-MSMS has shown a higher number of differentially expressed proteins in the oviductal fluid (131) than in the uterine fluid (22) when compared to plasma. From these 131 proteins, 92 were upregulated and 39 downregulated. Regarding the up-regulated proteins identified, they were predominantly involved in cellular catabolic processes, biosynthesis of aminoacids and organic substances, organic and aromatic compounds and catabolic acid signalling. The differentially expressed proteins of uterine fluid were mainly proteins implicated in immune response and granulocyte activation. In conclusion, this study presents a high-throughput analysis of female reproductive tract fluids, which constitutes a novel contribution to the knowledge of oviductal and uterine secretome.
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98. Transcriptome of porcine blastocysts stored in liquid state for up to 48 h

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Keywords: embryo, pig, liquid storage, transcriptome

Recently, we have developed a method for the liquid storage of *in vivo*-derived porcine blastocysts. Storage is done at 25 ºC in NCSU-BSA medium without controlled CO_2 gassing for up to 48 h, thus facilitating the commercial application of embryo transfer (ET) in pigs. In a preliminary study with few ETs, 30% liquid stored blastocysts for 48 h were able to develop *in vivo* until day 38 of pregnancy. However, subsequent ET-studies with liquid-stored blastocysts, showed impaired farrowing rates compared to that of fresh or 24 h-stored blastocysts. Therefore, to elucidate the causes of these pregnancy losses, we evaluate hereby the transcriptional patterns of *in vivo*-derived blastocysts stored in liquid state for 24 and 48 h. Blastocysts were collected by laparotomy at Day 6 of the cycle (D0=onset of estrus) from weaned cross-bread sows (N=7). Some fresh blastocysts (control group) were frozen immediately after collection and stored at -80ºC until transcriptome analysis. The rest of the blastocysts were stored in 1 mL of NCSU-BSA in Eppendorf tubes at 25ºC for 24 or 48 h upon which they were morphologically evaluated under a stereomicroscope. A total of 30 viable blastocysts (three pools of 10 blastocysts) per group were transcriptomically analyzed. Transcripts (24,123) were evaluated in a microarray (GeneChip Porcine Genome Array, Thermo Fisher Scientific). A False Discovery Rate adjusted analysis p-adjusted <0.05 and a fold change cut-off of ±1 were set to identify differentially expressed genes. Data were analyzed using Partek Genomic Suite 7.0 software, which also identified altered KEGG pathways. None of the stored blastocysts had hatched by the end of storage. The blastocyst survival rates at 24 (97 %) and 48 h (94 %) of storage were similar to those achieved in control blastocysts (100%). However, the number of differentially expressed genes of stored blastocysts compared to controls dramatically increased during storage, from 127 genes by 24 h to 4,175 genes by 48 h). Blastocysts stored for 24 h displayed 70 down-regulated and 57 up-regulated genes. Only seven pathways (Axon guidance, PPAR signaling, Long-term potentiation) had an enrichment score >4, with less than 5% of their genes modified with respect to the control blastocysts. In contrast, in blastocysts stored for 48 h, 2,120 genes were down-expressed and 2,055 over-expressed. Thirty-six pathways had an enrichment score >4. In addition, 12 pathways showed more than 30% of their genes altered, related to pathways fundamental for embryonic development and pregnancy as: Protein processing in endoplasmic reticulum, Metabolic pathways, Cell cycle, Oxidative phosphorylation, Notch signaling pathways, Mismatch repair, Nucleotide repair and DNA replication. These results would not only certainly explain the very low pregnancy rates obtained with 48 h-stored blastocysts in our previous studies but also help designing novel target strategies to improve liquid storage systems for porcine embryos.

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99. Influence of different extenders on post-thaw quality of cryopreserved yak (Poephagus grunniens) semen

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Keywords: extender, post-thaw, semen, yak

Yak is a unique livestock of high altitude surviving under hypoxic and extreme cold conditions above 3000 metre from mean sea level. They are considered as multipurpose animal as they provide milk, meat, fibre/wool, hide, fuel and the much needed transportation to the highlanders. Yak breeding face a lot of challenges under field conditions due to geographical isolation of the herds and repeated use of same breeding bull for generations. Cryopreservation of semen and use of Artificial Insemination (AI) can be one of the effective tools for overcoming the breeding problem in yaks. Yak semen has been successfully cryopreserved using Tris-citrate-fructose-egg yolk-glycerol (TFYG) extender (S. Deori, SAARC. J. Agric., 15, 215-218, 2017). Therefore, present study was designed with an objective to compare commercially available soybean based and liposome based extenders with TEYG extender on post-thaw quality of yak semen following cryopreservation. Semen was collected from 4 mature yak bulls (aged between 4 to 5 years) using artificial vagina. A total of twenty ejaculates (5/bull) having initial motility >70 percent were used for the study. Each ejaculate was split and diluted with BioXCell®, OPTIXCell® (IMV Technologies, France) and TFYG extenders to achieve a concentration of 30 x 10^6 per 0.5 mL straw. TFYG extender consists of 20 percent egg yolk and 6.4 percent glycerol. The straws were cooled to 5°C, equilibrated for 4 hours before freezing and cryopreserved in liquid nitrogen (BKD Borah et al., Int. J. Chem. Stud., 6, 509-511, 2018). The straws were thawed at 37°C for 10 seconds and evaluated for post-thaw sperm motility under phase contrast microscope (400x) and recorded from 0 to 100 based on the percentage of progressive motile sperm, sperm viability by Esoin-Nigrosin stain and acrosomal integrity by Giemsa stain. For sperm viability and acrosomal integrity a total of 200 sperm were counted and recorded in percentage. The mean values of the post-thaw sperm motility (%) in TFYG, BioXCell® and OPTIXCell® extenders were 53.50 ± 0.53, 54.50 ± 0.34 and 56.75 ± 0.55 respectively. The corresponding values for percent sperm viability and acrosomal integrity were 63.95 ± 0.53, 64.55 ± 0.51 and 66.35 ± 0.43, and 87.95 ± 0.67, 88.50 ± 0.66 and 91.10 ± 0.35 respectively. Analysis of variance indicated that post-thaw sperm motility, sperm viability and acrosomal integrity were significantly ($P<0.05$) higher in OPTIXCell® extender in comparison to BioXCell® and TFYG extenders. The values did not differ significantly between BioXcell and TEYG extenders. In conclusion, OPTIXCell® – a liposome based extender may serve as an alternative for successful cryopreservation of yak semen.

100. Combined embryo and recipient metabolomics improves pregnancy prediction in cattle

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Keywords: Embryo, recipient, metabolomics

Pregnancy prediction within IVP embryos or recipients often fails, as competence of the embryo is not defined in recipient studies and vice-versa. We designed a multi-variate study with controlled factors (i.e. embryo breed, recipient breed, Day-6 embryonic stages) to identify pregnancy biomarkers in recipient plasma and embryo culture medium (CM; SOFaaci). Abattoir oocytes matured and fertilized with Asturiana de los Valles (AV) or Holstein sperm were first cultured in groups, and singly from Day-6 to Day-7 (12 µL CM). Expanded blastocysts were vitrified/warmed (V/W) and the CM was stored at -150°C until metabolomic analysis by GC-qTOF/MS. V/W embryos (N=24 [AV] and N=12 [Holstein]; N=6 bulls) were transferred to synchronized recipients (N=13 AV, N=17 Holstein and N=6 crossbred), and blood plasma was collected on Day-0 (PD0; N=35) and Day-7 (PD7; N=36). Independent Holstein embryos and recipients (N=13 ETs) were used for validation. Pregnancy was diagnosed on Day-62 and birth. Metabolites identified were N=36 (CM) and N=71 (plasma). Metabolite values in CM were subtracted from incubated blank controls. Data were transformed by Pareto scaling and weighed by embryo breed, bull and embryonic stage on Day-6 (embryos), and recipient breed. Pregnancy-regulated metabolite concentrations were identified by GLM ($P<0.05$ and FDR ($P<0.05$)). Biomarkers were obtained in two ways: 1) singly, by ROC-AUC>0.650 ($P<0.05$; FDR<0.05); and 2) by F1 score, as a Boolean product of 1 metabolite from CM and 1 metabolite from plasma. Thus, concentrations of metabolites in pregnant plasma and CM were paired (True (T)*T = T), and open samples outside the pregnant range considered T (one, another, or both, plasma and CM, outside the pregnant range) or false (F; both CM and plasma in the pregnant range). Biomarkers in CM at birth were capric acid (C, P=0.021) and monostearin (M; P=0.016); CM at Day-62: no metabolite. PD0 at birth: creatinine (CR, AUC: 0.690, P=0.024) and azelaic acid (AZ, AUC: 0.694, P=0.047). PD7 at Day-62: Leucine (L, AUC: 0.744, P=0.029). Fold changes in CM were >3.0, while in plasma were <2.0. Combining non-significant PD0 metabolites as glycine (G, 22 –correct- /35 –total- samples), hydrocinnamic acid (HY, 23/35) and hippuric acid (HI, 21/35) with the CM biomarker C improved sample classification (GxC: 28/35; HYxC: 30/35; HIxC: 28/35), and their respective F1 score (0.780, 0.872, 0.829; $P<0.003$) over C AUC (0.755; 23/36). Similar increases were observed with the CM biomarker M (not shown). In contrast, PD0 significant biomarkers as CR and AZ did not improve F1 accuracy as combined with C or M. Target metabolomics using pure analytical standards coinjection confirmed the identity of C, M, HY and HI in the analyzed samples. Validation of M in Holsteins led to AUC=0.769 (10/13), while C showed AUC=0.615 (8/13). Reliable birth predictions are feasible once: 1) biomarkers are identified in CM and plasma; and 2) embryo and recipient biomarkers are confronted. Acknowledgements: MINECO (AGL2016-78597-R and AGL2016-81890-REDT). GRUPIN 2018-2020 (IDI/2018/000178). FEDER. COST Action 16119 (Cellfit). ASEAVA. ASCOL.

101. Pregnancy after short exposure of cryopreserved porcine embryo to cryoprotective agents

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Keywords: porcine embryo, vitrification, short exposure

The pig industry has nowadays an increasing demand for a reliable and cost-effective porcine embryo cryopreservation allowing long-term conservation, transport and widespread dispersion of high-quality genetics resources. Progress in embryo vitrification process made it possible to use the method in pigs, but lower and variable pregnancy rates are achieved with frozen embryos compared to fresh one. High concentrations of cryoprotective agents (CPAs) used for vitrification are believed to negatively affect developmental competence (Woelders et al, Cryobiology, 2018). The aim of the present study was to test the viability of cryopreserved porcine embryo after short exposure to CPAs during vitrification process. Embryos were surgically recovered 6 days after ovulation from Large White sows. Only embryos at the blastocyst stage were selected and vitrified in superfine open pulled straw (SOPS). Embryos were firstly placed in equilibration solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% DMSO, and then in vitrification solution (VS), containing 16% EG, 16% DMSO and 0.4M sucrose. Embryos were incubated 2 min in ES and 30 sec in VS (short exposure to CPAs) or 3 min in ES and 1 min in VS (control, Cuello et al, RFD, 2010). Embryos were then loaded into straws and plunged into liquid nitrogen. After thawing, they were transferred to Talp-Hepes PVA with decreasing sucrose concentrations (0.13 and 0M) for 5 min each. In vitro and in vivo survival were tested. For in vitro survival, embryos were cultured for 3 days in 50µL of NCSU-23 + 10% FCS at 38.8°C in a humidified atmosphere of 5% CO_2 in air. For in vivo survival, embryos were surgically transferred in uterine horn of synchronised Meishan recipient (30 blastocysts per recipient). Three hundred and four embryos were used to test in vitro embryo survival after short and control exposure to CPAs. In the first experiment, the survival rate was better with shorter exposure to CPAs (66.2% vs 45.6%, p=0.008, n=145), but in the second experiment, it was identical (47.4% vs 60.6% ; p=0.101, n=159). New embryos were produced and collected (n=157) to test in vivo survival. Transfers were performed with embryos vitrified according to short exposure to CPAs. Among 4 recipients, one is pregnant. Farrowing is expected in next weeks. Our results show that a short exposure to CPAs is as efficient as the longer exposure usually employed for porcine embryo vitrification. As short exposure decreases the embryos contact with high level of toxic CPAs, reducing potential harmful and epigenetic modification of embryonic genome, this short exposure to CPAs should be chosen for porcine embryo vitrification.

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102. Cumulus-oocyte complexes-like 3D models to analyze sperm binding

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Keywords: zona pellucida, gamete interaction, magnetic beads

The oocyte encapsulated by a glycoprotein matrix named zona pellucida (ZP) is surrounded by cumulus cells forming the cumulus oocyte complexes (COCs) that are ovulated in a plug in polytocous mammalian species. In recent years, a new 3D model to study gamete interaction in depth under *in vitro* conditions has been developed (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). The model, consists of porcine ZP recombinant proteins conjugated to magnetic Sepharose® beads (B_{ZP}), supports sperm binding and resembles oocyte's size and shape being a valuable tool to simulate gamete interaction studies. In this work, we have taken a step forward to improve the model by better imitating the shape and possible function of the native COCs by incubating the B_{ZP} with cells cumulus thus generating cumulus-oocyte complexes-like 3D models ($CB_{ZP}C$) to evaluate whether they support further sperm binding. In order to obtain the $CB_{ZP}C$ models, B_{ZP} were generated as previously described with recombinant porcine ZP2, ZP3 and ZP4 proteins (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). B_{ZP} were incubated for 24 h with cumulus cells isolated from *in vitro* matured porcine COCs (2,500 cells/ B_{ZP}). Then, groups of 50-55 $CB_{ZP}C$ were incubated for 2 h with fresh ejaculated porcine sperm separated by double centrifugation method (200,000 sperm/mL) at 38.5 ºC, 20% O_2, 5% CO_2, and saturated humidity. After co-incubation period, $CB_{ZP}C$ were washed twice in PBS, fixed and stained with Hoechst 33342. The mean number of sperm bound per $CB_{ZP}C$ ($S/CB_{ZP}C$) was scored by epifluorescence microscopy. Data was analyzed using Systat v13.1 (Systat Software, Inc San Jose, CA, USA) by one-way ANOVA and the values compared by Tukey's test when P value <0.05. The preliminary results (3 replicates) show that $S/CB_{ZP}C$ was significantly higher for the $CB_{ZP}C$ conjugated to ZP2 (11.88 ± 0.72, N=176) than beads conjugated to ZP3 (5.82 ± 0.41, N=195) and ZP4 (8.83 ± 0.61, N=176). These results are consistent with those previously reported for the 3D models without cumulus cells (B_{ZP}) (Hamze, Animal Reprod 13: 647, 2016) as well as the ones described in human and mice (Avella, J Cell Biol. 205(6):801-9, 2014; Avella, Sci Transl Med. 27;8(336):336ra60, 2016) suggesting that ZP2 could act as a sperm receptor in porcine species as well. In conclusion, this study offers a more physiological 3D model offering data consistent with previous observations thus reinforcing the viability of these models as a valuable tool to study gamete interaction. Moreover, as recombinant technology it is easily transferable to other species.

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103. Effect of highly dispersed silica nanoparticles on the functional activity of actin cytoskeleton in native and devitrified bovine oocytes during IVM

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Keywords: oocyte, cytoskeleton, vitrification

Actin takes over various essential function during oocyte meiosis (UrajiJ, et al., J Cell Sci,131 22: 1-6, 2018). Nanoparticles are widely used in various fields including reproduction. The mechanisms of the influence of *highly dispersed silica nanoparticles* (HDSns) on the functioning of intracellular organelles are still not clear. The aim of the present study was to identify the effects of HDSns (Chuiko Institute of Surface Chemistry, Ukraine) on the functional activity of the actin cytoskeleton [the intensity of fluorescence of rhodamine-phalloidin (IFRF) conjugated with actin filaments] in dynamics of meiosis of native **(unfrozen)** and devitrified (DV) oocytes. IFRF was evaluated in: native oocytes; native oocytes were cultured with 0.001% of HDSns; DV oocytes; DV oocytes pre-treated with 0.001% of HDSns before vitrification (20 min) and were cultured with 0.001% of HDSns. Vitrification was performed by equilibration of cumulus oocyte complexes (COCs) **before IVM** in: CPA1:0.7 M dimethylsulphoxide (Me2SO) +0.9 M ethylene glycol (EG), 30 sec; CPA2:1.4 M Me2SO + 1.8 M EG, 30 sec; CPA3:2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose, 20 sec and loading into straws. After thawing COCs washed in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199. COCs were cultured 24 h in TCM 199 + 10% (v/v) FCS + 50 ng/ml PRL with 10*6granulosa cells /ml. For assessment of chromatin and IFRF fixed oocytes were incubated sequentially in rhodamine-phalloidin(RF, R415 Invitrogen, Moscow, Russia), 1 IU/ml, for 30 min to label actin. Then oocytes were incubated in 4',6-diamidino-2-phenylindole, 10 |µg/ml, for 10 min to label chromatin. Oocytes were examined using confocal laser scanning system Leica TCS SP5 with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543nm laser lines were used for fluorochrome excitation. IFRF were expressed in arbitrary units. All chemicals, except for RF, were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by ANOVA. Chromatin status and IFRF of 391 native and DV oocytes (in 3 replicates, 30-34 oocytes/group) were evaluated during IVM. There were no differences between the IFRF in native oocytes and native oocytes treated with HDSns before and in dynamic of culture (23±1.1 vs 21.1±1.08; 14 h of IVM - 55.8±5.6 vs 49.2±6,7; 24 h of IVM - 29.8±5,8 vs 21.3±7.3, respectively). The lowest level of IFRF were tested in DV oocytes before, after 14 h and 24 h of IVM (14.7±4.4, 16.1±3.8, 10.5±6.1, respectively). Treatment of DV oocyte with HDSns increased the IFRF after 14 h and 24 h of IVM (16.1±3.8 vs 37.8±5.9 and 10.5±6.1 vs 23.5±4.9, respectively, $P < 0.01$, $P < 0.05$). The data of study showed that the treatment of COCs with 0.001% of HDSn influences on actin cytoskeleton integrity of bovine oocytes during vitrification. The mechanisms of the realization of this effect are under the further investigation.

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104. Quality of bovine in vitro produced embryos derived from frozen-thawed oocytes

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Keywords: bovine, cryopreservation, in vitro fertilization

Bovine oocyte cryopreservation has recently been a promising method of preserving genetic resources. A prerequisite for the proper development of preimplantation embryos after IVF is good quality of thawed oocytes. The aim of the study was to compare quality of IVP embryos derived from vitrified-warmed (V) oocytes with those obtained from fresh (C; control) oocytes. Embryo quality was evaluated basing on blastocyst cell number. Bovine cumulus-oocyte complexes were matured *in vitro* and then frozen by an ultra-rapid cooling vitrification technique in a minimum volume of vitrification solution (E-199 medium, 25 mM HEPES, 30 % ethylene glycol, 10% FBS) using 300 mesh electron microscopy nickel grids as a carrier. After warming the oocytes were fertilized in vitro and cultured in a Menezo B2 medium on the cumulus cell monolayer until the blastocyst stage. Embryo cleavage rate was counted on the Day 2 and blastocyst rate - on the Day 7 of embryo culture. For cell number counting, DAPI staining on nuclei observed under a Leica fluorescent microscope was used. For evaluation of differences between the experimental and control groups in embryo cleavage and blastocyst rate a Chi-square test, and in blastocyst cell number the t-test were used. Cleavage rate (Day 2) in V oocytes was lower (55.81%) compared to C (72.5%) group. Similarly, the blastocyst rate (D7) in V oocytes was different (11.82%) compared to C group (23%). In the V group, the higher incidence of asynchronous division at 3-cell stage, irregularly divided blastomeres at advanced embryo stages, and occurrence of blastocyst-like embryos (appearance of a blastocoel, but only a few nuclei) were observed in contrast to fresh oocytes. However, the cell number in truly D7 blastocysts in the V group (85 ± 10.16) was not statistically different from the control (97.3 ± 6.43) blastocysts. In conclusion, although the quality (the cell number) of V-derived blastocysts is comparable with those of fresh control, their development is affected by an asynchronous/irregular division resulting in a lower blastocyst rate. Further optimization of an oocyte cryopreservation regimen is required.

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105. Effect of different egg yolk extenders on in vitro fertility of thawed ram semen

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Keywords: thawed semen, egg yolk extenders, in vitro penetration.

An attempt to optimize sperm freezing extenders in ram was made by the replacement of fresh egg yolk (FEY) by powdered egg yolk (PEY). Therefore, ejaculates from 8 rams (2 years old) were collected by artificial vagina and mixed immediately. Pooled semen was centrifuged twice and the pellet was split into two aliquots and resuspended in a Tris-citric acid-glucose solution with 5% glycerol and 15% of PEY or FEY for 4 h at 5ºC before freezing in liquid nitrogen vapours. Secondly, cumulus-oocyte complexes were obtained by slicing from prepubertal sheep ovaries, selected and matured in 100 µL drops of BO-IVM® medium (IVF Bioscience, UK) plus estrous sheep serum (ESS, 10%) for 24 h at 38.5ºC in 5% CO_2 atmosphere. Then, FEY and PEY semen samples were thawed and spermatozoa were selected by 2 mL density gradient (40%/80%), using BoviPure® and BoviDilute® (Nidacon, Mölndal, Sweden) for its preparation. Once the column was centrifuged at 1200g for 10 min, the supernatant was discarded, the pellet resuspended with 2 mL of BoviWash® and then centrifuged again at 1200g for 5 min. After discarding the supernatant, total and progressive motility was assessed from the selected sperm populations by a CASA system (ISAS®, PROISER SL, Valencia, Spain). Then, the selected sperm were co-cultured with the in vitro matured oocytes in 100 µL drops of BO-IVF® medium (IVF Bioscience, UK), supplemented with 2% ESS, at a final concentration of $2x10^6$ sperm/mL. After 17 h of co-culture, oocytes inseminated (n=126 for FEY and n=127 for PEY) were mechanical decumulated, washed and fixed in 4% paraformaldehyde (v/v) at room temperature for 1 h approximately. Afterwards, oocytes were stained with Hoechst 33342 for 15 min in the dark at 4ºC. Then, oocytes were mounted on slides and kept at 4ºC in darkness until analysis under an epifluorescence microscopy (ZEISS Axioskop 40, Oberkochen, Germany). The parameters assessed were the penetration rate (number of oocytes penetrated by at least one spermatozoon of the total number of the potential mature oocytes) and the monospermy rate (number of oocytes penetrated by a single spermatozoon of the total penetrated oocytes). Statistical analysis was performed using a General Lineal Model procedure (SPSS® 20, IBM® Corporation, Armonk, NY, USA). The results (mean ± SD) did not show significant differences between extenders on penetration rate (82.6 ± 6.7 and 88.2 ± 3.9, for FEY and PEY, respectively) neither on monospermy rate (58.5±13.6 and 60.4±13.4, respectively). As well, no significant differences were found between extenders on total sperm motility (41.6±13.1 and 37.1±12.1 for FEY and PEY, respectively) and progressive motility (14.9±5.3 and 9.2±5.6 for FEY and PEY, respectively). In conclusion, powdered egg yolk can substitute successfully the conventional fresh egg yolk in ram sperm freezing extenders, providing higher biosecurity, due to its pasteurization process, and greater homogeneity in the composition of the diluents.

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106. Incubation with seminal plasma after thawing reduces immature sperm in Blanca de Rasquera goats

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Keywords: cryopreserved sperm, seminal plasma, male age

Addition of seminal plasma (SP) to cryopreserved sperm has been suggested as a potential alternative for improving sperm quality after thawing. DNA status, measured by the Sperm Chromatin Structure Assay (SCSA) provides an accurate measurement of sperm with a high DNA stainability (HDS), which has been related to sperm immaturity. Based on this, a preliminary study was conducted to evaluate the addition of SP to post thawed goat sperm on HDS levels in cryopreserved sperm from 1 and 2 years old bucks, thus studying a potential male age effect. Briefly, ejaculates from 8 bucks were collected via artificial vagina twice a week in 2 consecutive breeding periods. Fresh ejaculates were immediately pooled, centrifuged twice and diluted in a Tris-based media containing 15% powdered egg yolk (NIVE, Nunspeet Holland Eiproducten, Ochten, The Netherlands). Then, diluted samples were refrigerated for 4 h at 5°C before freezing in liquid nitrogen vapours. After thawing at 37°C for 30 seconds, sperm samples were selected by a single layer centrifugation using BoviPure® (Nidacon, Mölndal, Sweden), according to the manufacture instructions. Then, selected sperm sample was split in 2 aliquots and incubated in different media consisting on: a) *in vitro* fertilization commercial media (BO-IVF, IVF Bioscience, UK) and b) BO-IVF media plus 20% SP, for 3 h at 38,5 ºC in a 5% CO_2 atmosphere at a final concentration of $40x10^6$ sperm/mL. Then, HDS sperm proportion was determined using flow cytometry after acid-detergent treatment for 30 s and acridine orange staining according to SCSA methodology described by Evenson et al (Journal of Andrology, 2002; 23, 1, 25-43). Viability was also assessed by flow cytometry using SYBR14 and PI probes. Statistical analysis was performed using a General Lineal Model procedure (SPSS 19.0). Results showed no significant differences on HDS sperm proportion (mean % ± SE, n=6) between samples collected from 1 (4.4±0.8) and 2 years old males (3.7±0.6) after incubation for 3h in BO-IVF media. Likewise, no differences were found between sperm samples from 1 (3.0±0.6) and 2 years old males (1.6±0.2) after 3h incubation in BO-IVF media plus seminal plasma. However, this HDS population was significantly lower in sperm from 2 years old males incubated in the presence of seminal plasma compared to the sperm incubated only in BO-IVF media, while in 1 years old males no significant differences were observed. However, neither the male age nor the incubation media had a significant effect on thawed sperm viability, showing values (mean % ± SE) of 12.0±2.6 and 16.6±4.8 in 1 year old male samples and 15.3±3.6 and 14.8±1.8 in 2 years old male sperm incubated in BO-IVF or BO-IVF+SP for 3h, respectively. In conclusion, this study showed that the age of the donors had no identifiable beneficial effect on the HDS-parameters in goat sperm. Further studies are needed to elucidate the potential beneficial effect of seminal plasma on HDS levels in cryopreserved, especially in older male sperm. Supported by INIA (RZP2014-00001-00-00) and PIVEV (AGL2016-81890-REDT).

107. Intracytoplasmic sperm injection using sex-sorted sperm in the bovine

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Keywords: ICSI, paternal contribution, cattle

Intracytoplasmic sperm injection (ICSI) involves the fertilization of oocytes at the metaphase II stage (MII) by direct injection of a sperm cell. Despite the similar success rates between ICSI and conventional IVF in humans, the success rates in cattle are poor with low developmental rates compared to those obtained via IVF. One reason might be the need of an artificial activation of the oocyte after sperm injection. This activation could also lead to parthenogenetic embryonic development up to the blastocyst stage and beyond without paternal contribution. Checking the morphology of the obtained blastocysts is not sufficient to distinguish between parthenogenetic and fertilized embryos after ICSI. Therefore, we used Y-sorted sperm for ICSI. Sexing the subsequent blastocysts employing bovine and Y-chromosome specific primers will provide reliable results regarding the sex of the embryos. Bovine cumulus-oocyte-complexes collected from follicles out of slaughterhouse ovaries were in vitro matured employing a standard protocol (Stinshoff et al. Reprod Fertil Dev. 2014;26(4):502-10). After denudation, only MII oocytes were used for ICSI. Bovine Y-sorted sperm from four (bull 1-4) different bulls (1.5-2 years of age) with proven fertility (ICSI Y1, Y2, Y3, Y4) was prepared via a SpermFilter™ centrifugation. After immobilization of the sperm cell, it was sucked into the ICSI pipette using Eppendorf manipulators mounted on an Olympus microscope and injected into the oocyte. Chemical activation was performed with 5 µM ionomycin for 5 minutes followed by a 3 h culture period and an additional incubation for 3 h in 1.9 mM 6-DMAP. After activation, oocytes were cultured in SOFaa for 8 days. Oocytes which have been fertilized conventionally by co-culturing oocytes and sperm (IVF), which have only been activated (CA) and those which have been injected with non-sorted (ICSI non) and X-sorted (ICSI X) sperm served as controls. Experiments were repeated at least four times with an average of 25 oocytes per run. Cleavage (day 3) and developmental rates at day 8 (mean±SD) ranged from 15.8±5.4 to 40.9±13.1 % and 0.0 to 11.0±3.6 % in the ICSI Y1-Y4 groups. Within the control groups of embryos the following data could be obtained: 66.7±9.7, 33.0±7.4 % (IVF); 44.0±11.9, 9.7±3.5 % (CA); 49.2±18.0, 11.6±10.6 % (ICSI non); 51.4± 7.7, 7.1±2.8 % (ICSI X). After sexing, only 4 of the tested 21 embryos stemming from the ICSI Y1-Y4 group were male. Only 1 male embryo could be obtained using non- sorted semen for ICSI (1/24 embryos tested). Embryos out of the CA (16/16) and ICSI X groups (3/3) were all female as expected. After IVF, 38 out of the total number of 59 tested embryos were female. Taken together, these data indicate that there is a bull-specific suitability of sperm to be used for ICSI. Furthermore, even when an advanced developmental stage has been reached, the paternal contribution needs to be verified.

We acknowledge Stephane Alkabes (Masterrind GmbH, Germany) providing the sorted semen.

108. Histological cut of a paraffin-embedded blastocyst: optimized protocol for murine blastocysts

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Paraffin-embedded tissues have been used for research and therapeutic applications for decades, as they represent a valuable tool in histology and for molecular analysis, as well as being a way to preserve tissue samples for long periods at a low cost. The information currently available to embed blastocysts into paraffin blocks include: protocols using gelatin and paraffin, protocols to embed a piece of uterus containing the blastocyst already implanted, and a few protocols for *Xenopus* or bovine embryos, using specific equipment that might not be available in every laboratory. Nonetheless, little information is available on easy protocols to embed mouse blastocysts into a paraffin block to then make histological sections. The purpose of this work was to create an optimized protocol to embed mouse blastocysts into paraffin blocks, without using gelatin, and to perform histological cuts of the sample with the morphology well preserved, which can then be used for subsequent analysis. For this protocol we used 3 in vivo mouse blastocysts and performed 20 cuts in total with the microtome. Each one of them was fixed with 4% PFA for 30 min, permeabilized with 0.2% of Triton X-100 for 30 min, stained with 2 % eosin for 15 seconds to facilitate its visualization, dehydrated using different concentrations of ethanol (96% and absolute) for 10 min each, immersed in xylene for 5 min and embedded into the paraffin block. The next day, the paraffin block was cooled down and cut into 6 µm sections with a microtome, and the sample was processed to remove the paraffin, stain the cellular structures and be visualized under a microscope. Out of the 20 histological cuts performed, 65% of the sections contained just some cells of the blastocyst or the blastocyst folded onto itself, but the protocol was optimized based on the results and problems encountered until good histological cuts of the blastocyst with the morphology well preserved were obtained. Sections were considered of good quality when the samples had a circular shape and the nucleus and cytoplasm of the cells could be identified. This optimized protocol can be used to obtain good quality histological sections of a blastocyst, which can be used for studies involving *in situ* hybridization, immunohistochemistry, enzyme histochemistry, DNA, RNA or protein extractions, analysis of biomarkers, characterization of surface markers of stem cells integrated into the embryo, to analyze the effect of potential compounds that could be used to improve the embryo culture media, to prepare histological material for educational purposes, etc. Some of these studies could represent a valuable source of new information for the field of reproductive biology.

Workshop I: The impact of heat stress on dairy cow fertility – The management point of view.

Efficient cooling management and remedial hormonal treatment to alleviate the effect of heat stress on ovarian function in dairy cows

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Keywords: Heat stress, cooling management, hormonal treatments, reproduction

The impact of elevated temperature on the reproductive performance of dairy cows is becoming a worldwide problem, as a result of climate change and intensive genetic selection for high milk production. Reduced fertility is no longer confined to subtropical and tropical climates; adverse effects of elevated temperature have been reported in dairy farms located in cooler regions, such as Europe, and in temperate and Mediterranean climate zones. Furthermore, the effects of heat stress are not limited to the hot months: they carry over to the following cooler months, resulting in long-term effects throughout the year.

Various heat-abatement strategies have been developed in the last four decades; these include providing shade to block direct solar radiation, ventilation, and indirect and/or direct cooling with water. The direct cooling approach is based on short-term spraying of water followed by its evaporation from the skin) constituting one cycle). Efficient cooling requires several cooling windows per day, each consisting of several cycles and lasting about 30–50 min. The efficiency of cooling on commercial farms can be conveniently compared by calculating the ratios between summer and winter milk production and conception rate. Calculations demonstrate that by using efficient cooling management, it is possible to maintain 98% of the winter milk production in summer; however, summer conception rate reaches only 68% of that in winter. Findings indicate that the reproductive system is highly susceptible to thermal stress and additional means are required to improve conception in the summer.

Understanding the mechanism by which thermal stress impairs ovarian function led to the development of supporting hormonal treatment. The long-term effects of seasonal heat stress on the hypothalamus–pituitary–ovarian axis involve various impairments. These include alteration of the ovarian pool of follicles and their enclosed oocytes and impaired function of the corpus luteum, expressed by reduced plasma progesterone concentration. Induction of three consecutive 9-day follicular waves during the summer and fall improved conception rate (37 vs. 53% for control and treated cows, respectively) in primiparous cows. Treatment was more effective for multiparous cows with a high body condition score (BCS) and low somatic cell count. Administration of a controlled intravaginal progesterone-releasing device on day 5 ± 1 post-AI for 13 days was found beneficial for a subgroup of cows with low BCS at peak lactation compared to their control counterparts (53 vs. 27%, respectively) and for cows exhibiting both low BCS and postpartum reproductive disorders, compared to their control counterparts (58 vs. 14%, respectively). In summary, the reproductive tract, and in particular the ovarian components (i.e., follicles, oocytes, corpus luteum), are highly sensitive to elevated temperatures. Using an efficient cooling system to maintain normothermia in cows is a prerequisite for any additional remedial approach. Given that the effect of heat stress on fertility is multifactorial in nature, a combination of several treatment approaches might be most effective.

Embryo transfer- a promising tool for improving fertility during heat stress

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Keywords: embryo transfer, heat stress, cattle
The major cause of infertility caused by heat stress is damage to the oocyte and early embryo by direct effects of elevated temperature and the physiological changes in the cow caused by heat stress. The developing embryo develops increased resistance to maternal hyperthermia by Day 3 of pregnancy. It is this characteristic of embryonic development that makes embryo transfer, typically performed at Days 6 to 8 after estrus, an effective tool to increase fertility during heat stress. Pregnancy rates following embryo transfer in the summer can be twice as high as pregnancy rates after artificial insemination. Moreover, differences in pregnancy rates between summer and winter are much less for embryo transfer than for artificial insemination. Coupling embryo transfer with an ovulation synchronization scheme like Ovsynch can make it possible to bypass effects of heat stress on estrus detection. The major limitation to the economic use of embryo transfer in commercial dairy and beef system is the cost of the procedure, which must be kept low to make implementation of an embryo transfer program economical.

Using thermoprotective factors to alleviate the effects of heat stress on the ovarian pool of oocytes: lessons from the lab bench to the field

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Keywords: antioxidant, anti-apoptotic, survival factors

Growth and development of ovarian follicles and oocytes require a series of coordinated events leading to successful ovulation, oocyte maturation, fertilization and preimplantation embryonic development. In cattle, such events can be disrupted by high environmental temperature leading to heat stress. Adverse environmental temperatures observed during the hot months of the year in subtropical and tropical climates reduce fertility in lactating dairy cows. Heat stress compromises follicular development, hormonal secretion, endometrial and oviductal function, oocyte and preimplantation embryonic development. Oocyte susceptibility to heat stress has been demonstrated during the germinal vesicle (GV) and maturation periods. In vivo and in vitro studies indicated that exposure of bovine oocytes to elevated temperature affects the cellular and molecular machinery required for proper oocyte function. For example, heat stress increased mitochondrial production of reactive oxygen species (ROS) altering the oocyte balance between ROS accumulation and removal by intracellular antioxidants. Heat stress also compromised mitochondrial function, nuclear and cytoplasmic maturation, induced cytoskeleton disorganization and apoptosis. Many efforts have been employed to alleviate the low fertility associated with heat stress. Therefore, the objective of this work is to highlight basic and applied strategies to protect oocytes from heat stress. Recently, molecules such as insulin-like growth factor I, astaxanthin, melatonin, epigallocatechin gallate, caspase inhibitors and sphingosine-1-phosphate were identified as thermoprotective factors for bovine oocytes. These factors rescued several cellular functions damaged by heat stress enhancing the ability of the oocyte to be fertilized and reach the blastocyst stage. Moreover, in vivo administration of antioxidants improved reproductive performance in heat stressed animals. Therefore, manipulation of themoprotective molecules has the potential to mitigate the deleterious effects of heat stress on the bovine oocyte improving fertility during summer.

Adaptation to heat stress in sows: an unfinished business

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Keywords: animal welfare, heat stress, pigs

Animal welfare and production efficiency are impaired by stress. Exposure to heat (measured by the Temperature Humidity Index) can lead to physiological and behavioral changes indicative of stress. According to recent predictions on climate change, severe heat events are forecasted to increase in intensity and frequency throughout the next decades, likely affecting animal production. This is of special interest in pig production as according to FAO, more than 50% of pig production occurs in warm climates, with predicted faster temperature growth than temperate areas.

Heat stress response is a multisystemic adaptive mechanism triggered to cope with high environmental temperature. When heat exceeds the adaptation capacity of pigs, the body temperature increases, unbalancing homeostasis, which has negative consequences for production and welfare. Intensive breeding have led to a reduction of the adaptation capacity that confer a reduction in resilience towards environmental challenges. Pigs are particularly sensitive to heat because they lack functional sweat glands and the presence of a thick layer of subcutaneous adipose tissue that prevents heat dissipation. Besides the previous limitations, pigs kept in intensive conditions are prevented to perform most of natural behavior regulating body temperature, such as wallowing, which exacerbates the diminished adaptation capacity.

The effects of heat stress in pigs are diverse, perturbing several body systems. Respiration rate increases to optimize the transpiration capacity and reduce body temperature. Activity decreases in order to reduce the metabolic source of heat due to muscle contraction. An increase in satiety during heat events alter feeding behavior and reduce the feed intake. Reproduction performance is linked to this reduction in feed intake, as lower access to nutrients leads to a loss of body condition and a negative energy balance, provoking reproductive problems associated with inadequate ovarian function. These effects are readily apparent on-farm, including anestrus, reduced farrowing rates, increased abortion rates, and reduced litter size. Heat is the environmental stressor that produces the highest (negative) impact on pig production, and it is likely that reproduction plays a major role.

Strategies of heat stress mitigation can either focus on reducing the environmental challenge to which pigs are submitted or alleviating the consequences of the stress response on the organism. In the first approach, the reduction of ambient temperature (5–7°C) inside swine barns using evaporative cooling systems is the most extended strategy in intensive pig production. Alleviating the consequences of heat stress, stimulating feed intake using dietary strategies (i.e. additives) and feeding management are, to date, the most efficient strategies. Out of housing and management strategies, improving pig resilience to heat stress through breeding and epigenetic strategies are promising approaches addressing the fundamental root, which is adaptation.

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Workshop II: Social acceptance of reproductive technologies in livestock

Social acceptance of Reproductive Technology in livestock

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Keywords:

The manipulation of animal fertility has been essential for the development of current agricultural practise. The practice of utilising reproductive performance of animals can be traced back thousands of years, and on the whole, there is widespread acceptance of the strategic use of animal fertility in modern agriculture, even if this acceptance is borne of a certain 'rational ignorance'.

Reproductive technology is a rare example of scientific research that has made immediate translational impact into society. However, its position in society is more broad than 'science' since it touches on an emotive subject – about which almost everyone has an opinion. Indeed, few other areas of biological research have captivated front page headlines in quite the same way as reproductive breakthroughs; from the birth of the first IVF baby in 1978, through to successful cloning in the mid 90s and to the current era of genomic editing. There are a number of studies that explore the societal and ethical aspects of reproductive technology in human clinical medicine, however its use in agriculture can present even more challenging topics for society. The concept of combining of reproductive technology with agricultural practices can stir evocative and powerful views in wider society. However, these views may arise from limited information, or sensationalist representation in the media and online.

It therefore becomes an imperative to take a proactive approach to defining and describing the use of reproductive technologies in a responsible and considered manner in livestock. Terms that are familiar to practitioners and researchers may be unclear and, even alarming to individuals with less understanding. The AETE seeks to embark on a journey to critically reflect on the real and perceived impacts of modern assisted reproductive practices on animal welfare and to consider the potential perception by the wider public. Moreover, this workshop will seek to consider the representation and appearance of reproductive technologies as they are used in livestock management and will run a workshop at the upcoming annual meeting. The aspiration from this workshop is to improve the readiness of the AETE to become involved in playing a wider role in informing public opinion.

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