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Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

39ème COLLOQUE SCIENTIFIQUE

39th SCIENTIFIC MEETING

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Dr. Sabine Meinecke-Tillmann and

Dr. Burkhard Meinecke

Special Celebration

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Heraklion, Greece, 7th and 8th September 2023

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Dear colleagues and supporters,

As the long and torturing summer is gradually becoming milder, we will have the chance to be together for the 39th AETE meeting in Heraklion – Crete, under the refreshing atmosphere offered by the Aegean Sea winds. Heraklion is a vibrant city; throughout the year, more than 3.5mil tourists arrive, while it is the host city for 20000 University students. The nice five-star Galaxy hotel is going to be our home for the three days of the meeting. Here, around the pool, we will have our Welcome Reception, we will enjoy the scientific program, which apart of the presentations of the five distinguished invited speakers, it includes short oral presentations and four sessions of electronic -for the first time in this meeting-, poster presentations, student competition and of course the always interesting practitioner's forum.
As the social events are on the top of interests for any scientific meeting, the LOC decided that no gala dinner will be offered this year. Instead, we'll try to introduce delegates to the past Cretan lifestyle, with our visit to Arolithos, which is the recreation of a traditional Cretan village. Here, will taste a variety of Cretan cuisine plates, local wines, and spirits, under the sounds of Cretan music. Don't worry, the Friday night farewell party will be westernized…In the Garden -a typical city club- we will enjoy drinks, music and dancing even until early morning.
This year the LOC cooperated with ARTION a Greek PCO. The people of ARTION namely, Despina, Valentini and Efi, through their professionalism provided valuable services to the organization of this meeting.
We would like to thank all sponsors of this event, especially the contribution of the Greek companies is highly appreciated, because their business interests are by no way related to the topics and the aims of our association.
Dear friends, colleagues, and sponsors, we hope you will meet your expectations of this meeting and you will leave Heraklion with unforgettable experiences.

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Dr. Sabine Meinecke-Tillmann and Dr. Burkhard Meinecke

A.E.T.E. Medalists 2023

Commendation of Dr. Sabine Meinecke-Tillmann and Dr. Burkhard Meinecke for the AETE Pioneer Award 2023

The Association of Embryo Technology in Europe (AETE) has decided to give the AETE Pioneer Award 2023 to

Sabine Meinecke-Tillman and Burkhard Meinecke Joint AETE Medalists

Burkhard Meinecke was born on 11th January 1948. His father was a veterinarian running a mixed practice typical for that time and it was probably this surrounding in which Burkhard grew up that inspired him to study veterinary medicine. He graduated as a veterinarian at the Justus Liebig University Giessen (JLU, Germany) where he also received his doctoral degree in 1976 and his graduation as Dr. habil. in 1983. He had been appointed Professor of Physiology and Pathology of Reproduction (extracurricular) at the JLU in 1989 and received and accepted an appointment to be the chair of the Institute for Reproductive Medicine (later retitled as Institute for Reproductive Biology) at the University of Veterinary Medicine Hannover in 1992. He declined an appointment from the JLU in 1998 and stayed in Hannover until his retirement in 2016 where he devoted his energy to teaching and training reproductive biology and medicine including the vice-presidency of research for two years (2005-2007). From 2008 to 2011 Burkhard provisionally managed the Institute of Wild Animal Research. He was accepted as Founding Diplomate of the European College of Animal Reproduction in 2002. He always emphasized the importance of clinical veterinary medicine and surgery and his own experience encompasses many different species. Besides his clinical work, his general research activities focused on oocyte maturation, embryo transfer and associated technologies in pigs, sheep, goats, horses and cattle; and his main research interest went to the porcine oocyte or more precisely to the porcine cumulus-oocyte-complex. He was able to add some pieces to the puzzle of oocyte maturation showing that the cross-talk between the maternal environment and the oocyte has a balanced relationship. Due to his enormous experience and knowledge in female reproduction he is well-known for asking questions at the meetings "I do not completely understand what you said, could you please describe?" or as we say in German "I hab' da mal 'ne Frage!".

Burkhard was a member of the AETE nearly from the beginning onwards and visited the annual meetings on a regular basis. He became AETE president in 1995 which clearly shows his esteem for the society. One of his accomplishments was to organize the annual meeting in Hannover in 1995. This was the second time that the meeting was not held in Lyon and starts the tradition to host the conference all over Europe, which makes the AETE a truly “European Society”.

Sabine Meinecke-Tillman was born on 14th March 1950. Both of her parents were veterinarians, her father being the head of the Clinic for Reproduction and Obstetrics at the University of Berlin and later at the Justus-Liebig-University Giessen (JLU), Germany. Consequently she came into close contact with the clinical and scientific aspects of reproduction at a very early stage in life. She graduated as veterinarian at the Justus-Liebig-University Giessen (JLU), Germany, finished her doctoral thesis (Dr. med. vet.) with a focus on embryo transfer in sheep in 1977, and became a veterinary specialist for reproductive medicine two years later. She worked as research fellow (German Research Foundation, DFG) from 1977 to 1984 and thereafter, until 1993, as C1-professor and research fellow at the Institute for Animal Breeding and Genetics of the Faculty of Agricultural Sciences, Nutritional Sciences and Environmental Management of the JLU. In 1994, Sabine completed her habilitation thesis focusing on the developmental capacity of early embryos in sheep and goats, and received the Venia legendi in reproductive biology. This was followed by the appointment as private lecturer in 1994. From 2002 to 2015 she was professor (extracurricular) at the agricultural faculty of the JLU, and from 1993 to 2015 associate professor and research fellow at the Institute for Reproductive Medicine (later retitled as Institute for Reproductive Biology) of the University of Veterinary Medicine Hannover. Teaching focused on topics of reproductive medicine and reproductive biology, whereas her scientific projects concentrated on sheep and goats but also other species, interactions between mother and conceptus or ultrasonography. From 2006 to 2007 Sabine Meinecke-Tillmann was the 1st Speaker of the AET-d (Association of Embryo Transfer of German speaking Countries) and from 2007 to 2014 she was a member of the Scientific Advisory Board of the Leibniz Institute for Zoo and Wildlife Research in Berlin.
Sabine was a member of the AETE nearly from the beginning onwards. She attended the annual meetings for many years and contributed lectures and posters.

Sabine and Burkhard met each other during their studies at university and married in 1974. They not only enjoyed the scientific programs but also the social events and the networking at the annual conferences. Beside their research, they never lost the contact to the practical and clinical side of their work aiming to translate science into practice. Their vast knowledge in reproductive biology and medicine across species has made them to excellent teachers in the field and well-recognized experts for practitioners to contact.
They are true and internationally well-respected scientists and mentors. Both stayed at the famous Roslin Institute for a research fellowship which had a great influence on their further work. Some of the work on ART in small ruminants is outstanding and still state of the art. Most probably the outstanding scientific achievement of the two was the production of chimeras of sheep and goats by means of micromanipulation, the first worldwide. Based on their scientific merits and in particular their efforts towards the processes during oocyte maturation and the generation of chimaeras and twins, Sabine Meinecke-Tillmann and Burkhard Meinecke are truly worthy recipients of the AETE Pioneer Award 2023!

Animal Reproduction

Thematic Section: 39th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Lab partners: oocytes, embryos and company. A personal view on aspects of oocyte maturation and the development of monozygotic twins

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How to cite: Meinecke B, Meinecke-Tillmann S. Lab partners: oocytes, embryos and company. A personal view on aspects of oocyte maturation and the development of monozygotic twins. Anim Reprod. 2023;20(2):e20230049. https://doi.org/10.1590/1984-3143-AR2023-0049

Abstract

The present review addresses the oocyte and the preimplantation embryo, and is intended to highlight the underlying principle of the "nature versus/and nurture" question. Given the diversity in mammalian oocyte maturation, this review will not be comprehensive but instead will focus on the porcine oocyte. Historically, oogenesis was seen as the development of a passive cell nursed and determined by its somatic compartment. Currently, the advanced analysis of the cross-talk between the maternal environment and the oocyte shows a more balanced relationship: Granulosa cells nurse the oocyte, whereas the latter secretes diffusible factors that regulate proliferation and differentiation of the granulosa cells. Signal molecules of the granulosa cells either prevent the precocious initiation of meiotic maturation or enable oocyte maturation following hormonal stimulation. A similar question emerges in research on monozygotic twins or multiples: In Greek and medieval times, twins were not seen as the result of the common course of nature but were classified as faults. This seems still valid today for the rare and until now mainly unknown genesis of facultative monozygotic twins in mammals. Monozygotic twins are unique subjects for studies of the conceptus-maternal dialogue, the intra-pair similarity and dissimilarity, and the elucidation of the interplay between nature and nurture. In the course of in vivo collections of preimplantation sheep embryos and experiments on embryo splitting and other microsurgical interventions we recorded observations on double blastocysts within a single zona pellucida, double inner cell masses in zona-enclosed blastocysts and double germinal discs in elongating embryos. On the basis of these observations we add some pieces to the puzzle of the post-zygotic genesis of monozygotic twins and on maternal influences on the developing conceptus.

Keywords: oocyte maturation, soma-germ interactions, monozygotic twins, demi-embryos, maternal effects.

Introduction

"Knowledge of history allows the conquest of the future. This is especially true for embryo transfer with all its ramifications, which has become one of the most challenging frontiers in theriogenology" (Jöchle, 1983, p. 293). About 50 years ago, physiological aspects of oocytes and embryos came into research focus, mainly driven by questions on bovine embryo transfer (ET), which enhanced the interest in reproductive biology in farm animals. Depending on the species, each year thousands of embryos are produced in vitro or collected in vivo and transferred to final recipients. Basis of these events is the physiological and undisturbed maturation of fertilizable oocytes. Resulting preimplantation embryos,

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Received: March 31, 2023. **Accepted:** June 12, 2023.
Financial support: None.
Conflicts of interest: The authors have no conflict of interest to declare.
#These authors contributed equally to this work.

regardless of their in vivo or in vitro origin, show tremendous regulatory capacities, and in some cases more than half of the blastomeres can be lost before further development completely fails. The ability to compensate for a reduced blastomere count is the basis of facultative or induced polyembryonic development. Artificial splitting of high quality embryos and single transfer of the resulting halves allows, in dependence on the manual skills of the operator, a 30% to 40% greater embryo transfer success in cattle compared to the transfer of intact embryos.

Despite the successful implementation of the newly developed reproductive technologies into animal production and human reproductive medicine, essential questions remained only incompletely answered. Two of the most puzzling processes, the preovulatory maturation of the oocyte and the origin of monozygotic twins, accompanied our research and are covered by the following sections of the review.

Oocyte maturation: the perspective of the pig

Animal models

Experiments of Pincus and Enzmann (1935) and of Edwards (1965) initiated a countless number of studies about in vitro maturation (IVM) of mammalian oocytes. Many of these studies were conducted to improve the quality of IVM-oocytes for use in animal biotechnology but also to decipher basic mechanisms of mammalian oocyte maturation. Female meiosis is an outstanding model for studying the cell cycle since oocytes become arrested in prophase1 (equivalent to the G2-phase of mitotic cells) during fetal development until just before ovulation when they proceed to metaphase 2 (M2). Newly developed methods have led to a significant broadening and deepening of knowledge about the regulation of oogenesis. It became evident that key molecules of this complex process appeared rather early in vertebrate evolution (Dalbies-Tran et al., 2020). At the same time, these new findings also shed light on special features of the animal species and the necessity to verify observations in each of them. The ability to manipulate the mouse genome makes mice the premier model organism for genetic approaches to study the molecular mechanisms underlying the maturation of oocytes. However, different adaptations of physiological regulatory circuits make it necessary to develop new model systems according to the respective questions. For example, in contrast to mice, pig oocytes depend on mRNA- and protein synthesis for germinal vesicle breakdown (GVBD) and show a chromosome dependent spindel formation (Fulka et al., 1986; Meinecke and Meinecke-Tillmann, 1993; Miyano et al., 2007). The following review about some aspects of oocyte maturation focuses on the pig. Additional information derived from other species will be used in order to complete the description of the relevant processes.

Physiological considerations

Porcine follicular development from the primordial to the preovulatory stage lasts about 120 days (Hunter, 2000). In the course of follicular maturation it is inevitable that the countless factors influencing this evolvement cause a considerable heterogeneity of follicles and their oocytes. (Moor and Dai, 2001). However, in vivo a cohort of heterogeneous oocytes is being transformed to a selected few of widely homogenous oocytes before ovulation occurs. Maturation of pig oocytes is initiated by the preovulatory endogenous LH surge at the end of a follicular phase of 4 to 6 days. Recruitment from the pool of about 100 developing follicles occurs after the pulsatile GnRH-LH secretion changed from a lesser frequency/greater amplitude to a greater frequency/lesser amplitude pattern. In pigs FSH is important for increasing the number of follicles that reach the medium/larger sized category, whereas LH is necessary for the further growth of these follicles to preovulatory size. Luteinizing hormone (LH) stimulates follicular estrogen synthesis in theca interna and granulosa cells which have expressed sufficient LH receptors. Two to 3 days before ovulation,

LH pulsatility and FSH secretion decrease to hardly detectable levels, while estrogen concentrations reach their maximum. On the first day of standing estrus (Day 0 of the estrus cycle) and on Day 1 estrogens dominate follicular steroid hormone synthesis and output. About 12 h before ovulation occurs on Day 2, estrogen synthesis has ceased and follicular levels have dropped to a tenth of their original maximum levels (Eiler and Nalbandov, 1977; Meinecke et al., 1987; Soede et al., 2011; Knox, 2019). Ovulations take place 44 h ± 3 h after the onset of the LH surge and last about 3 h (Soede et al., 1994). Compared to adult sows, induction of ovulation in prepubertal gilts with an equine chorionic gonadotropin/human chorionic gonadotropin (eCG/hCG) regimen is accompanied by an altered pattern of follicular growth and steroid hormone synthesis, as well as a greater variability in oocyte maturation, and a prolonged ovulation process (Ainsworth et al., 1980; Meinecke et al., 1984; Foxcroft and Hunter, 1985; Wiesak et al., 1990; Soede et al., 1998). The considerable aberration of morphological and biochemical parameters is also reflected in reduced developmental competence of oocytes from prepubertal gilts (Pinkert et al., 1989; Bagg et al., 2004, 2007).

Nuclear changes

Nuclear changes in follicular oocytes at various times after the onset of estrus were reported by Spalding et al. (1955), or after hCG application by Hunter and Polge (1966). A first report on IVM of pig follicular oocytes revealed a coinciding time line between in vitro and in vivo nuclear maturation (Edwards, 1965). However, it soon became evident that the presence of a M2 figure is in no sense an adequate indication of normal maturation of the oocyte and that complete maturation comprises two separate but interacting entities (Leman and Dziuk, 1971; Motlík and Fulka, 1976; Mattioli et al., 1989). It turned out that in pigs, the transfer of IVM oocytes as well as of oocytes recovered from atretic follicles into previously mated recipients revealed a reduced and retarded cleavage rate of IVM oocytes. In contrast, the sperm penetration rate of oocytes from atretic follicles was equivalent to IVM oocytes, indicating that under in vivo conditions spermatozoa do not discriminate between in vitro matured, atretic, and ovulated oocytes (Meinecke and Meinecke-Tillmann, 1978a, b).

Aquisition of meiotic competence

Although oocytes of follicles ≤ 1 mm in diameter are able to resume meiosis in vitro, only those of > 2 mm in diameter can complete the first meiotic division indicating a close relationship between follicular growth and oocyte maturation. During pig folliculogenesis, the ability to undergo GVBD and to proceed to metaphase 1 (M1) is acquired earlier than the ability to reach M2 (Tsafriri and Channing, 1975; Motlik et al., 1984). The competence to transit through post-GVBD stages undisturbed is only achieved following transcriptional silencing (McGaughey et al., 1979; Crozet et al., 1981; Motlik et al., 1984; Motlík and Fulka, 1986; Meinecke and Meinecke-Tillmann, 1998; Bjerregaard et al., 2004; Pan et al., 2018). Subsequent to removal of the nucleolus, fully grown porcine oocytes resume meiosis and proceed undisturbed to M2. Removal of the nucleolus induces GVBD in growing oocytes which normally are unable to resume meiosis, and the cell cycle proceeds to M2 (Fulka et al., 2003). These observations challenge the concept of the nucleolus as a mere ribosome factory and indicate an active role in preventing GVBD in growing oocytes. The changes in transcriptional activity are reflected by chromatin appearance and can be used to differentiate transcriptionally active oocytes from transcriptionally silent ones (Motlík and Fulka, 1986). The chromatin in porcine oocyte nuclei is initially decondensed in a non-surrounded-nucleolus (NSN) configuration, but subsequently condensed, forming a surrounded nucleolus (NS) configuration with a heterochromatin rim around the nucleolus (Sun et al., 2004). In the meantime, the morphological criteria have been further refined (Guthrie and Garrett, 2000; Sun et al., 2016; Pan et al., 2018; Lee et al., 2019). Although growing (≤ 90 µm in diameter) and fully grown pig oocytes (≥ 115 µm) contain comparable amounts of the two subunits of the maturation promoting factor (MPF), they are not able to activate the MPF to a sufficient extent. One of the causes of this incompetence is the

continuous activation of a MPF-inhibiting kinase (WEE1B) which became phosphorylated by a persistent activity of cAMP-dependent protein kinase (PKA) (Christmann et al., 1994; Nishimura et al., 2009, 2012). Furthermore, growing oocytes are unable to establish an intact mitogen-activated protein kinase-pathway (MAPK3/1) required for full meiotic competence (Kanayama et al., 2002). Extracellular signal-regulated kinases (ERK1/2) are involved in early porcine folliculogenesis as evidenced by the marked intensity of activated MAPK3/1 immunolabeling in the cytoplasm of oocytes from primordial/primary, secondary and tertiary follicles (Moreira et al., 2013). Furthermore, Sun et al. (2016) highlighted the signaling pathways of MAPK3/1 required for the transition of chromatin from a decondensed to a condensed condition in growing pig oocytes.

Prophase1 arrest

Since prophase1 arrest of mammalian oocytes could be maintained in vitro by a membrane-permeate form of cAMP or a cAMP phosphodiesterase inhibitor, early ideas of prophase1 arrest were based on the concept that premature resumption of meiosis is prevented either by cAMP which is produced by follicle cells and diffuses into the oocyte or by cAMP synthesized by the oocyte itself (Jaffe and Egbert, 2017). This concept held until the demonstration of a constitutive active G-protein-coupled-receptor 3 (GPR3), stimulating adenylyl cyclase in mice oocytes. The resulting elevated cAMP levels in the oocyte prevent resumption of meiosis (Kalinowski et al., 2004; Mehlmann et al., 2004). Creation of mouse oocytes defective in synthesis and degradation of cAMP has shown that both, GPR3 and phosphodiesterase 3A, are the most important regulators of intra-oocyte cAMP concentrations necessary to block resumption of meiosis (Vaccari et al., 2008; Norris et al., 2009). Phosphodiesterase 3A is competitively inhibited by cyclic guanosine monophosphate (cGMP) which is provided by granulosa cells and diffuses through gap junctions into the oocyte. Granulosa cell production of cGMP is controlled by natriuretic peptide stimulation of natriuretic peptide receptor 2 (NPR2) which is coupled to a guanylyl cyclase (Zhang et al., 2010). Convincing evidence of cAMP production by the oocyte itself, and the meiosis-inhibiting effect of cAMP have also been demonstrated in the pig (Rice and McGaughey, 1981; Mattioli et al., 1994). Likewise, proof has been given of the presence of the GPR3-adenylyl cyclase-cAMP system and cGMP inhibition of phosphodiesterase 3 A in porcine oocytes (Laforest et al., 2005; Sasseville et al., 2006; Morikawa et al., 2007; Yang et al., 2012; Zhang et al., 2012). Pig granulosa cells produce and secrete both B-type brain natriuretic peptide (BNP) as well as C-type natriuretic peptide (CNP), and are endowed with NPR1 and NPR2. Moreover, meiotic resumption of porcine oocytes has been inhibited by CNP signaling (Kim et al., 1992; Ivanova et al., 2003; Hiradate et al., 2014; Santiquet et al., 2014; Zhang et al., 2015).

The combined data suggest that subsequent to binding of CNP and BNP to NPR2, production of cGMP in porcine granulosa cells is activated which then passes through gap junctions into the oocyte. Prophase1 is maintained by high cGMP concentrations which competitively inhibit phosphodiesterase 3, thus retaining high intra-oocyte cAMP levels. The cAMP activates PKA which holds MPF in an inactive state by phosphorylating WEE1 (Shimaoka et al., 2009; Jaffe and Egbert, 2017). The cooperations between somatic and germinative compartments remain stable until increasing and persistent estrogen blood concentrations support the LH surge.

Resumption of meiosis by LH signaling

Gonadotropin receptor expression of porcine ovarian follicles during the estrous cycle depends on both follicular development and stage of the estrous cycle. In small and medium ovarian follicles of prepubertal and adult pigs, follicle stimulating hormone receptors (FSHR) are highly expressed in granulosa cells and decline in mature follicles at estrus. In contrast to the luteinizing hormone receptor (LHR), the number of FSHR per granulosa cell and their binding affinity do not increase in the course of follicular development (Nakano et al., 1977).

Luteinizing hormone receptor (LHR) expression in granulosa cells weakly starts in medium sized follicles and is increased in large follicles on Day 0 (Nakano et al., 1977; Daguet, 1979; Liu et al., 1998). Porcine cumulus cells express low numbers of LHRs and show no cAMP response upon LH stimulation (Mattioli et al., 1994). In vitro LHR-expression of porcine cumulus cells increases only after additional FSH stimulation (Shimada et al., 2003; Ozawa et al., 2008). Simultaneously with the follicular development, the adenylyl cyclase-system of porcine granulosa cells reacts increasingly responsive to hCG. Following LH stimulation of follicles (6-10 mm in diameter) about 7.000 molecules of cAMP are formed/sec/granulosa cell (Lee, 1976). Since follicles of that size class contain about 2 to 3 million granulosa cells (Foxcroft and Hunter, 1985) one can imagine that follicles become submerged by cAMP molecules. Moreover, LH signaling is extended by internalization of the receptors after LH binding, such that signaling may continue from endosome compartments even in the absence of extracellular LH (Johnson and Jonas, 2020). Prophase1 of oocytes remains arrested when pig ovarian follicles are isolated and subsequently cultivated without gonadotropins, whereas addition of LH/FSH or eCG/hCG to the culure medium induces resumption of meiosis (Gérard et al., 1979; Meinecke and Meinecke-Tillmann, 1981). The LHR is a G protein-coupled receptor and, upon binding of its ligand, the activation of cAMP-dependent targets is stimulated (Mattioli and Barboni, 2000; Choi and Smitz, 2014). Furthermore, signal transduction also takes place via additional G protein-independent pathways (Johnson and Jonas, 2020). However, the primary targets of the LH signal, initiating the ovulation process and the resumption of meiosis are a.) the steroidogenesis of the preovulatory follicle, b.) the gap junctional communication between follicle cells and the oocyte, c.) the natriuretic peptide system, and d.) the epidermal growth factor network.

a) Steroidogenesis of the preovulatory follicle

In early studies, porcine follicles were cultivated in vitro after ovariectomy of eCG/hCG treated prepubertal gilts. It was shown that stimulation of progesterone secretion already started in follicles removed 15 min after intravenous hCG application, whereas resumption of meiosis was induced in follicles removed after at least 4 h following hCG (Meinecke and Meinecke-Tillmann, 1979). The time window for in vivo LH signaling targeting oocyte maturation corresponds with the results obtained by IVM of porcine cumulus oocyte complexes (Ebeling et al., 2007; Sasseville et al., 2009). In further investigations the relative amount of progestagens, androgens, and estrogens in follicular fluid and in follicular wall samples, revealed the change from estrogen to progesterone synthesis of the preovulatory follicle (Eiler and Nalbandov, 1977; Ainsworth et al., 1980; Meinecke, 1981; Foxcroft and Hunter, 1985; Meinecke et al., 1987). The role of steroid hormones in oocyte maturation has been debated for decades (Tsafriri and Motola, 2007) but recently became a subject of interest again. Estrogen promotes the natriuretic peptide driven production of cGMP in mouse granulosa cells and in cumulus cells, thus assisting meiotic arrest (Liu et al., 2017), whereas progesterone signaling via its receptor is essential for the resumption of meiosis and cumulus expansion in pigs (Yamashita et al., 2010). Our own investigations have demonstrated that an inhibition of MAPK3/1 by U0126 in porcine cumulus cells during gonadotropin induced IVM resulted in a cessation of progesterone synthesis by suppression of 3ß-hsd gene expression and an increase of estradiol synthesis by stimulating Cyp 19 a1 gene expression (Ebeling et al., 2011). The same effect had been noticed in cultured granulosa cells and cumulus oocyte complexes (COCs) of mice (Su et al., 2006). Thus, it can be assumed that the LH signal induces a differential expression of genes essential for estrogen and progesterone synthesis in cumulus cells as well as in granulosa cells and that this process is mediated by a MAPK3/1-dependent signaling pathway.

b) Gap junctional communication between follicular cells and the oocyte

Pigs strongly express connexin 43 in cumulus cells, connexin 60 in oocytes, and connexin 45 in both oocytes and cumulus cells (Santiquet et al., 2013). Following hCG application to eCG pretreated pigs, coupling of cumulus cells and oocytes as determined by [3H]uridin uptake remains unchanged until about 32 h. At this point of time pig oocytes reach M1 and exhibit complete cumulus cell expansion (Motlik et al., 1986; Mattioli et al., 1988). Gonadotropin

treatment (FSH/LH; eCG/hCG) causes a rise in the amount of connexin 43 protein in pig COCs corresponding to an increase in gap junctional communication during the initial phase of IVM, whereas GVBD is accompanied by closure of gap junctions (Shimada et al., 2001; Sasseville et al., 2009). During the first few hours of IVM the presence or absence of gonadotropins has hardly any effect on the gap junction network between porcine cumulus cells. Currently, gap junctional communication during porcine IVM is explained in terms of increasing and decreasing the total number of connexin 43 molecules in cumulus cells, thus varying the number of gap junctions and hence the gap junctional communication flow rate (Santiquet et al., 2013). However, it should be stressed that the capacity of cumulus cells to promote resumption and completion of oocyte meiosis is gonadotropin-dependent and that a rapid and profound decrease of gap junctional communication accompanies GVBD in porcine oocytes (Sasseville et al., 2009; Santiquet et al., 2012).

c) The natriuretic peptide system

In rodent follicles, LH inhibits gualylyl xyclase activity of NPR2 via dephosphorylation by a rapid still unknown way, thus lowering the cGMP levels in the somatic compartment (Robinson et al., 2012; Egbert et al., 2014; Shuhaibar et al., 2015). Additionally, the LH-cAMP-PKA system of granulosa cells activates phophodiesterase 5 which also contributes to a reduction of somatic cGMP concentrations via hydrolytic cleavage (Egbert et al., 2016).

In pigs, the application of hCG to eCG pretreated animals decreased CNP as well as BNP concentrations (~ 80%) in follicular fluid at 18 h and 36 h after treatment, respectively. These observations indicate that under physiological conditions BNP and CNP jointly contribute to meiotic arrest, and LH attenuates this inhibitory effect by decreasing the expression levels of BNP and CNP in vivo (Hiradate et al., 2014; Zhang et al., 2015). The timely upregulation of the principal phosphodiesterase 3 activity in porcine cumulus cells requires FSH signaling (Sasseville et al., 2009), and so both, the CNP-NPR2 down-regulation and the upregulation of phosphodiesterase 3 activity lowers the cGMP concentrations in the somatic compartment. As a consequence, cGMP diffuses out of the oocyte down its concentration gradient. Consequently, the competitive inhibition of phosphodiesterase 3 by high cGMP levels in the oocyte is released and hydrolysis of cAMP begins (Jaffe and Egbert, 2017).

d) The epidermal growth factor network

Activation of the EGF receptor after the LH surge is mediated by amphiregulin and epiregulin (Park et al., 2004). Synthesis of both peptides increases after LH stimulation in porcine granulosa cells, and after EGF receptor activation cGMP levels decrease in granulosa cells and in cumulus cells (Zhang et al., 2014). It is suggested that amphiregulin and epiregulin are released from granulosa cells into the extracellular space and diffuse to cumulus cells, where they lower cGMP concentrations. In addition, LH related EGF receptor signaling induces MAPK3/1 activation, mucification of the cumulus matrix, gap junction closure, and oocyte meiotic resumption in several mammals including the pig (Liang et al., 2005; Prochazka and Blaha, 2015). During porcine folliculogenesis, responsiveness to EGF signaling develops concomitantly with follicular growth (Marchal et al., 2001; Procházka et al., 2000). Porcine cumulus cells from small (<4 mm in diameter) and large (>4mm in diameter) follicles contain similar quantities of EGF receptor protein but following EGF stimulation only expanding cumulus cells from large follicles contain EGF receptors capable to activate intrinsic tyrosine phosphorylation (Prochazka et al., 2003). In addition, porcine COCs from small follicles expressed equivalent amounts of EGF receptor mRNA compared to COCs from large follicles. However, the former had less total EGF receptor protein, resulting in failed activation of phospho-EGF receptor and phospho-ERK1/2, despite of equivalent total ERK1/2 protein levels (Ritter et al., 2015). This underlines the importance of an intact EGF receptor signaling pathway, since MAPK3/1 in particular is of importance for cumulus expansion, resumption of meiosis, and ovulation.

Germinal vesicle breakdown (GVBD)

The MPF complex consists of two components, a catalytic subunit, namely the cyclin dependent kinase 1 (CDK1), and a regulatory subunit, cyclin B (Dunphy and Newport, 1988;

Nurse, 1990). Phosphorylation on threonine 161 by a Cdk activating kinase (CAK1) and dephosphorylation on Thr 14 and thyrosine 15 by the cell division phosphatase 25 (CDC25) activates CDK1 (Krek and Nigg, 1992; Solomon et al., 1992). The necessity of threonine 161 phosphorylation of CDK1 for MPF activation at meiotic resumption of porcine oocytes has been confirmed (Fujii et al., 2011). In immature porcine oocytes, high cAMP levels activate WEE1B which subsequently inactivates CDK1 (Nishimura et al., 2009). After in vivo/in vitro induction of meiotic resumption, declining cAMP levels in the oocyte inactivate WEE1B, followed by activation of CDC25 and the conversion of pre-MPF to MPF. It is suggested that the MPF activity during this early period is not sufficient to induce meiotic resumption because of still low cyclin B concentrations. The moderate MPF activity starts the cyclin synthesis which results in a further MPF activation and provokes GVBD (Shimaoka et al., 2009). Activation of MPF during IVM of pig oocytes occurs in close correspondence to GVBD but is influenced among other factors by media composition and quality of the oocytes (Naito and Toyoda, 1991; Wehrend and Meinecke, 2001; Setiadi et al., 2009). Since inhibition of protein synthesis as well as suppression of CDK1 activation prevents porcine GVBD, it was assumed that cyclin B synthesis is required for GVBD (Naito et al., 1995; Kubelka et al., 2002). However, the trigger of GVBD in pig oocytes might not be the cyclin synthesis but the dephosphorylation of pre-MPF. When porcine oocytes were injected with antisense cyclin RNAs (B1 and B2), they gradually underwent GVBD in the absence of cyclin B synthesis. Despite the resulting low MPF activity in these oocytes, they were able to activate a small amount of pre-MPF to induce GVBD, although the time line was retarded. This suggests that pig oocytes do not require cyclin synthesis for GVBD induction per se but they need either cyclin B1 or B2 synthesis for GVBD in a correct time course (Kuroda et al., 2004).

During IVM, the activities of MPF and MAPK3/1 increase around the time of GVBD. Maturation promoting factor exhibits two maxima at M1 and M2 with a temporary drop during M1 to M2-transition, whereas MAPK3/1 activities remain stable at peak levels until M2 (Mattioli et al., 1991; Inoue et al., 1995; Wehrend and Meinecke, 2001; Ye et al., 2003). In COCs connected to a piece of the mural granulosa cell layer, spontaneous maturation is prevented unless a combination of LH/FSH is present in the medium (Motlik et al., 1991; Ebeling et al., 2007). This in vitro model allows for a distinction between spontaneous and gonadotropin induced resumption of meiosis. In cumulus cells, FSH/LH induces an early and rapid U0126-insensitive MAPK3/1 phosphorylation, while U0126-susceptible MAPK3/1 phosphorylation occurs in the oocyte itself at GVBD (Ebeling et al., 2007). Since chromosome condensation can occur in the absence of MPF-activity, and GVBD can take place without MAPK3/1 activation, the specific roles of MPF and of MAPK3/1 in the oocyte have yet not fully been elucidated (Kubelka et al., 2002; Ye et al., 2003; Prochazka and Blaha, 2015; Kalous et al., 2018).

Cytoplasmic maturation

Cytoplasmic maturation is an ill-defined process providing the oocyte with the ability to navigate through fertilization and early embryonic cleavage until completion of zygotic genome activation. Previous experiments have demonstrated that follicle size affects the competence of the oocyte to develop to the blastocyst stage (Marchal et al., 2002; Bagg et al., 2007). Induction of cytoplasmic maturation requires signal exchange between somatic cells and the oocyte as shown by the first successful IVF of porcine in vitro matured oocytes (Mattioli et al., 1989).

At present the efficiency of porcine in vitro production (IVP) of embryos is very low. Despite the many improvements of maturation and fertilization of the oocyte as well as cultivation of the early embryo under in vitro conditions, it has yet not been possible to achieve a significant enhancement in the overall process (Grupen, 2014; Chen et al., 2021). Involvement of the MAPK3/1 in cytoplasmic maturation had been demonstrated by our group through the role of the kinase during the oocyte aging process. By prolonging the culture period of porcine COCs from 46 h up to 72 h to induce oocyte aging, a significant decrease of the MAPK3/1 activity occurred during the first 12 h of aging and stabilized during a further prolonged culture time (Ebeling et al., 2010). Prematurely decreasing MAPK3/1 activities in

aged MII porcine oocytes seem to hamper subsequent early embryonic development. Furthermore, a proportion of oocytes with abnormal anaphase II significantly increased after parthenogenetic activation of aged oocytes (Ma et al., 2005). However, our attempts to use MPF/MAPK3/1 monitoring as an indicator of cytoplasmic maturation during porcine IVM showed that this method is not suitable (Setiadi et al., 2009).

Conclusion

Maturation into a developmentally competent oocyte under in vitro conditions seems to be particularly difficult in swine as compared to cattle. Despite the tremendous efforts during the past decades only limited success has been achieved. Main problems like heterogeneity of oocytes, polyspermic penetration, and aberrant early embryonic development, to name the most obvious examples, have yet not been fully understood. In almost all studies, oocytes from slaughtered prepubertal animals are used, which may explain part of the limited competencies of the oocytes seen following IVM. On the other hand, these oocytes represent an indispensable source for deciphering fundamental phenomena. Advances in the systematic analysis of the signals generated in the somatic and germinative compartments of the follicles will help to solve the problems. The prerequisite, however, is that the results are checked against the in-vivo conditions.

Monozygotic twins and multiples

Facultative and obligatory polyembryony in mammals

In mammals, sexual reproduction is the rule. In contrast to monotocous/uniparous species with singleton pregnancies, polytocous/multiparous animals normally produce more than one progeny per gestation which might be achieved by multiple ovulations with fertilization of the resulting oocytes or by polyembryony. The latter can be classified as a reproductive strategy in which sexual reproduction is combined with asexual splitting of the fertilization product: During this natural cloning process one fertilized oocyte gives rise to more than one individual. In most mammalian species, regardless of whether uni- or multiparous, this natural process occurs only occasionally (facultative polyembryony), resulting in monozygotic (MZ) twins or multiples, such as in humans (Bulmer, 1970), horses (Meadows et al., 1995; Govaere et al., 2009), cattle (Hancock, 1954; Johansson et al., 1974; Silva del Río et al., 2006; Rogberg Muñoz et al., 2020), pigs (Ashworth et al., 1998; Bjerre et al., 2009), and dogs (Joonè et al., 2016). In contrast, polyembryony seems to be obligatory at least in two of the living armadillo species (Kölliker, 1876; Fernandez, 1909, 1915; Prodöhl et al., 1996; Loughry et al., 1998; Enders, 2002).

Presumably due to the high dizygotic (DZ) twinning frequency in sheep and goats, scientific studies on natural MZ twins are missing in these species and only anecdotal reports are available. However, from observations during preimplantation development in vivo (Assheton, 1898; Rowson and Moor, 1964; Meinecke-Tillmann, 1993) and from the occurrence of conjoined twins (Dennis, 1975; Ahmad et al., 2020) it can be concluded that facultative polyembryony also occurs in small ruminants.

Data on naturally occurring MZ twins indicate a low frequency of about 0.2% to 0.33% per calving in cattle (Johansson et al., 1974; Silva del Río et al., 2006) which corresponds well with the reported frequency of 0.4% per birth in humans (Steinman, 2001).

Historical aspects

In the 19th century the concept was developed that MZ twins are derived from a single fertilized egg or oocyte. Despite many advances in the field of embryology, there is still uncertainty about the precise mechanisms in which MZ twins or multiples arise. The common

hypotheses had been developed on the basis of retrospective analyses of the fetal membranes. Chorionicity and amnionicity allowed the classification into dichorionic-diamniotic (DC/DA), monochorionic-diamniotic (MC/DA) and monochorionic-monoamniotic twins (MC/MA) and gave some indications on the initiation of MZ twin formation via separation of blastomeres or subdivision/splitting of embryos during early development (Corner, 1955), as it was the case with X-chromosome inactivation studies in human embryos (Chitnis et al., 1999).

In contrast, López-Moratalla and Cerezo (2011) and Herranz (2015, 2014) suggested that all types of MZ twins originate from the constitution of "two zygotes" through one longer fertilization process and the subsequent fusion of membranes rather than from the separation of two compartments of an original embryo. However, at least in human embryos (Asami et al., 2022; Perry et al., 2022), first embryonic genes are already activated after male and female nuclear syngamy in the one-cell stage, hence indicating the beginning of embryonic development.

In vivo, separate development of blastomeres or groups of blastomeres in early cleavage stages was postulated to occur in about one-third of human MZ twins and to result in DC/DA individuals, whereas the most common form of MZ twinning, the MC/DA twins, should originate from two separate ICMs (ICM: inner cell mass, embryoblast) in the blastocyst stage (Corner, 1955; Boklage, 1981; Sadler, 2012).

MC/MA MZ twins are not topic of the present review. They are rare and were assumed to emerge after amnion formation, resulting in the growth of two primitive streaks (Corner, 1955) in about 2% to 4% of the cases (Bulmer, 1970; Derom et al., 1995).

These possible modes of MZ twin formation were adopted for other species including farm and companion animals, too.

DC/DA monozygotic twins

DC/DA monozygotic twins associated with atypical or with assisted hatching

The MZ twinning frequency in humans seems to be higher in assisted reproduction programs than during natural MZ twinning and reaches about 1.2% to 4.9% (Blickstein et al., 1999, 2003; Nakasuji et al., 2014; Scaravelli et al., 2022). Data on domestic animals are scarce: A frequency of 1.6% monozygotic multiples had been reported after transfers of single in vitro produced (IVP) equine blastocysts (Dijkstra et al., 2020).

Traditionally DC/DA MZ twin pregnancies were believed to originate early in development, i.e. before the first differentiation into trophoblast and embryoblast (Corner, 1955). This first possibility of twin formation was suggested because of obstetrical evidence, but it has been questioned by experienced embryologists and reproductive physicians: In association with artificial reproductive technologies (ART) the development of DC/DA MZ twins was not uncommon after blastocyst transfer in humans (Peramo et al., 1999; Costa et al., 2001; Kyono, 2013; Sundaram et al., 2018; Li et al., 2020; Dallagiovanna et al., 2021; Brouillet et al., 2022; Chu et al., 2023). This observation was mainly attributed to a disturbed hatching process in the blastocyst stage, with herniation of trophoblast and some ICM cells (Malter and Cohen, 1989). Accordingly, spontaneous MZ twinning or development of MZ multiples in animals occurred after ET of in vivo collected, cultured, frozen, or in IVP-embryos, such as in horses (McCue et al., 1998; Mancill et al., 2011; Roberts et al., 2015; Dijkstra et al., 2020; Peere et al., 2022), cattle (Moyaert et al., 1982; Kraay et al., 1983; Smith et al., 1991), or mice (Chida, 1990; Yan et al., 2015).

Interestingly, in own experiments on assisted hatching (AH) in commercial ET in cattle (Rüther et al., 2002; Rüther, 2005) 3 sets of MZ twins were born after single transfers of in vivo collected embryos in superovulatory cycles but only in the control group (334 transfers of zona-intact embryos, 150 animals calving) and not in the experimental group (324 transfers of zona-manipulated embryos, 177 animals calving).

In contrast to other studies with small zona openings [human zona: <10-30 µm as summarized by Alteri et al. (2018), Sills et al. (2000) and Liu et al. (2022); bovine zona: 7-15x40 µm or at least 40x40 µm: (Schmoll et al., 2003)], a wide slit of the zona pellucida (~ 120 µm slit) was produced by zona dissection in own experiments in order to avoid a disturbed hatching process and, thus, the development of MZ twins in the experimental group (Rüther et al., 2002; Rüther, 2005).

Whereas significant differences in zona pellucida thickness were recognized between human patients (Schiewe et al., 1995), zona pellucida thickness measured at eight different points of the zona, respectively, was homogenous in cattle but differed highly significantly ($P < 0.0001$) between the developmental stages (Rüther, 2005). Zona thickness was not associated with pregnancy rates (Rüther, 2005), but transfer success was significantly higher after assisted hatching, particularly when fresh first quality rank or frozen/thawed embryos were transferred [pregnancy rates: Rüther et al. (2002); calves born: Rüther (2005)]. Thus, zona hardening during IVC and too small openings in the zona pellucida seem to be associated with disturbed hatching processes in bovine embryos (Schmoll et al., 2003).

In a new study, blastocyst transfers after IVF and AH are confirmed as risk factors for MZ twinning in humans, whereas intracytoplasmic sperm injection, preimplantation genetic testing, and frozen embryo transfer do not appear to be associated with MZ twinning (Chu et al., 2023).

Another own observation after in vivo collection of small ruminant embryos shed some additional light on a possible origin of DC/DA MZ twins or multiples: In a sheep blastocyst which had been collected for embryonic stem cell isolation on D10 of pregnancy, an atypical hatching process in vivo was observed. The zona-entrapped embryo had initiated incomplete hatching and outgrowth of three strangulated vesicles at different areas via tiny openings in a thinned zona pellucida which had not been lost in time (Meinecke-Tillmann, 1993). Provided that each or at least two of these vesicles contained numerically enough ICM cells to support further embryonic development, DC/DA MZ twins or even multiples are conceivable. In this special case the spreading of ICM cells was observed from two of these vesicles during IVC, i.e. from the zona-entrapped blastocyst and from the largest outgrowth. Thus, independent from AH or other in vitro techniques, an altered hatching process in in-vivo developed embryos might result in DC/DA MZ twins or multiples, although the first differentiation into trophoblast and ICM had already occurred.

This assumption is supported by observations in humans (Van Langendonckt et al., 2000; Konno et al., 2020; Brouillet et al., 2022), horses (Dijkstra et al., 2020) and cattle (Massip et al., 1983), and by attempts to induce MZ twins in cattle via a zona-perforation technique (Skrzyszowska et al., 1997, 1999). Linear apoptosis in the ICM might support the twinning process (Ménézo and Sakkas, 2002).

DC/DA monozygotic twins associated with double blastocysts

Based on microsurgical experiments with early cleavage stages in small ruminants (Meinecke-Tillmann and Meinecke, 1984b) and on observations on in vivo collected D7 to D12 embryos in sheep (Meinecke-Tillmann, 1993; D0 = day of estrus), it was possible to add some further evidence for the developmental mechanisms of MZ twinning.

Of special interest was the first hypothesis related to the induction of MZ twins during early cleavage but before reaching the compaction stage. This possibility has been questioned because of the easiness of common embryonic development after chimeric embryo aggregation and on the basis of DC/DA MZ twinning seen after single blastocyst transfers when MC/DA MZ twinning had been expected.

Nonetheless, a double blastocyst within a common zona pellucida had been observed which led to the authors' question: "Monovular twin bovine blastocysts before hatching? Do identical twins sometimes separate this early ...?" [ADRI photo unpublished, in Betteridge (1977, p. 78). Unfortunately, the zona pellucida of this specimen was broken as well as partially inverted at the contact area of the two "blastocysts", and for the smaller structure

the presence of an ICM cannot be verified on the basis of the photo. Therefore, it cannot be excluded that a disturbed hatching process had been the origin of a blastocyst connected to a constricted trophoblastic vesicle, pseudo-blastocyst or small blastocyst. This might have occurred via a minor herniation and the subsequent collapse of the original blastocyst with retraction of the strangulated prolapse during the embryo collection procedure.

However, in own investigations on small ruminants, the presence of two separate blastocysts within an intact single zona pellucida was realized in one in vivo developed specimen after its collection on D6 of pregnancy (Meinecke-Tillmann, 1993). After zona removal and separation of these blastocysts, their IVC with the intention to isolate embryonic stem cells resulted in outgrowths of both ICMs. Although the blastocysts had not been transferred into recipient ewes and therefore no twins were born, the observation of two blastocysts within one zona pellucida refutes the assumption of Herranz (2015, p. 5) that "the splitting and growth of twins within the pellucida has been never observed or live-recorded."

Moreover, based on earlier microsurgical experiments in farm animals for the induction of intra- and interspecies chimeras in sheep and goats (precisely intergeneric chimeras), some further observations with regard to the possible mechanism of twinning were made (Meinecke-Tillmann and Meinecke, 1984b): When sheep blastomeres of developmentally asynchronous early cleavage stages were combined within a common zona pellucida – in this case a single blastomere of the 4-cell stage (1/4 embryo) with two blastomeres of the 8-cell stage (2/8 embryo) representing together 1/2 embryo – the developing cells formed a single composite blastocyst in 21% of the aggregates after transfer into an intermediate recipient. However, in several cases (39%) the regulation failed, and the parts of the original embryos remained separated and formed two small blastocysts or only one blastocyst without further cleavage of the other component(s) within their common host zona pellucida. The original embryos had been collected on D2 (4-cell stage; D0 = day of estrus) and D3 of pregnancy (8-cell stage), and therefore, the chronological difference between the aggregated blastomeres was about 24 h. A double-zona technique was used to prevent wastage of cells through the slit in the host zona (a small zona pellucida from porcine slaughterhouse material was used as blastomere host, whereas a larger pig zona served as a clamp in order to firmly close and cover the slit in the first one and to prevent blastomere loss; Meinecke-Tillmann and Meinecke, 1984a, b). After blastocyst transfer to the final recipients, some of the half- as well as quarter-embryo derived blastocysts were able to develop into lambs (11/28; 39.3%). On the basis of these results it was suggested that an asynchronous cleavage of the first blastomeres in non-manipulated embryos might lead to the formation of two separate blastocysts within a single zona pellucida, and finally to the development of DC/DA twins (Meinecke-Tillmann and Meinecke, 1984b). Nonetheless one limitation of the observation has to be mentioned retrospectively: Blastomeres of the original 4- and 8-cell embryos had been separated mechanically after a short treatment with Ca- and Mg-free medium. This might have influenced the aggregation readiness, although the blastomeres had been carefully washed after the separation procedure.

As a cause of a naturally occurring blastomere asynchrony, intra- or extrafollicular aging of oocytes had been discussed which might interfere with the quality of the developing embryo (Meinecke-Tillmann and Meinecke, 1984b), whereas superovulation, particularly in combination with ovulation induction, might induce precocious ovulation and extrafollicular aging of oocytes when timed artificial insemination takes place.

Other studies on the aggregation of asynchronous blastomeres also demonstrated difficulties with the regulation of a common embryonic development [rhesus monkeys: Schramm and Paprocki (2004)]. Already Mintz (1965) advised against asynchronous blastomere combination in order to avoid such "parabiotic embryos" which had sporadically been observed by Stern and Wilson (1972) in mice. In this context it should be kept in mind that differences between species exist, particularly with regard to regulative and regenerative competency and capacities of the early embryo [e.g., Kohri et al. (2019)]. Furthermore, differences in adhesiveness of blastomeres might play a role in the possible formation of aggregates (Kimber et al., 1982).

Asynchronous blastomere cleavage after IVF was observed in humans and mice and was associated with lower ICM quality and higher abortion rates (Mashiko et al., 2022). A safe cryopreservation of “synchronous” as well as “asynchronous” embryos (cryopreserved 2 or 3 days after oocyte aspiration) was possible in women, but, unfortunately, detailed information is missing, although twins had been born (Wiener-Megnazi et al., 2014).

Furthermore, Bomsel-Helmreich (1974) and Bomsel-Helmreich and Papiernik-Berkhauer (1976) reported MZ twin blastocysts within the same zona pellucida after delayed ovulation in rabbits. Delayed ovulation results in intrafollicular aging of the oocytes. Their minor quality was associated with high embryonic mortality and chromosomal anomalies as well as the occurrence of monozygotic twins. On the basis of cytogenetic investigations the authors hypothesized that twins of the same sex must have their origin in the 2-blastomere-stage, i.e. at the same time when mixoploids arise.

MC/DA monozygotic twins

In MC/DA MZ twins other mechanisms must be active than in those with DC/DA membranes. It was suggested that two inner cell masses might occur within a single zona-enclosed blastocyst via ICM-splitting or -duplication. Accordingly, three in vivo developed normal sized sheep blastocysts, each with two separate inner cell masses at opposite poles of the zona-enclosed preimplantation embryos were collected from different superovulated ewes on D7 of pregnancy (Meinecke-Tillmann, 1993).

The development of two separate ICMs might be induced by disturbances of blastocoel formation or purely mechanically. The latter had been suggested on the basis of blastocyst collapse and re-expansion in human embryos (Payne et al., 2007; Mio and Maeda, 2008). Factors controlling cavity formation and the positioning of the eccentric fluid-accumulation are not completely understood. In mice, a hydraulic flux fractures cell-cell contact in a network of microlumina which empty themselves into larger ones until a single cavity results. Thus, blastocoel formation in mice and presumably in other species such as cattle or humans depends on functional ion transport through a polarized epithelium as well as hydraulic and osmotic phenomena as indicated by Dumortier et al. (2019) and Le Verge-Serandour and Turlier (2022). Usually, trophectoderm cells flatten under increased pressure which is believed to ensure that there are no asymmetric divisions or additional ICM cells formed after the blastocyst cavity reaches a certain size (Chan et al., 2019). This stresses the importance of mechanical influences during early development.

The formation of multiple blastocoelic cavities was considered to be abnormal (Alikani et al., 2000). It is unknown if such a situation can result in an altered positioning of prospective ICM cells. The development of strings and bridges between ICM and trophoblast during human blastocyst growth [summarized by Hardarson et al. (2012)] might influence blastocyst quality and might further be involved in the shaping of the ICM and therefore in the process of twinning. Mechanical influences on blastocyst shaping are gaining increasing interest (Özgüç and Maître, 2020; Firmin and Maître, 2021), and their actions should also be considered during the peri-implantation period.

Monozygotic double ICMs have been reported in mouse blastocysts, too. They were recognized after in vivo or in vitro fertilization and subsequent in vitro culture from the 2-cell up to the blastocyst stage (Chida, 1990), or after in vivo fertilization and subsequent in vitro culture of blastocysts up to the egg cylinder stage (Hsu and Gonda, 1980). In the latter example the double ICMs were induced purely mechanically. In this context it should be kept in mind that in vitro situations are prone for artifacts, although it cannot be excluded that a disturbed intrauterine embryo-orientation might also be involved in monozygotic twinning processes in vivo.

Furthermore, Otsuki et al. (2016) recommended the exclusion of in vitro produced blastocysts that contain decompacting ICMs from transfer in order to avoid monozygotic MC/DA twinning via a doubling of the ICM. On the basis of time-lapse photography, it was possible to ascribe one case of human MC/DA twins to the transfer of a blastocyst with a

decompacted ICM of at least eight cells (Otsuki et al., 2016). Inner cell mass morphology is associated with embryo quality and ET success (Subira et al., 2016; Ai et al., 2021; Yaacobi-Artzi et al., 2022), and the appearance of blastocysts with "loosely arranged" inner cell mass cells and tightly packed trophectoderm (Shi et al., 2021), or with tightly packed trophectoderm (Ge et al., 2022) is related to the development of human monozygotic twins. Even though an assessment of the latter studies is difficult since relevant data is missing, both investigations indicate that the fate of the ICM in in vitro produced preimplantation blastocysts remains labile, thus making a reorganization and development into singletons or twins possible. Interestingly, in humans the extremely rare event of familial MZ twinning was reported which seems to be associated with cell junction-signaling pathways (Liu et al., 2018).

In a further human blastocyst, the presence of two ICMs was recognized, both differing in the stage of development (Noli et al., 2015a). This indicates a certain autonomy of a group of pluripotent inner cells with the growth of a second ICM rather than the splitting of the first one. In this context it is known that the trophectoderm of human (Paepe et al., 2013) and cattle embryos (Berg et al., 2011) can under special conditions still contribute to the ICM. Despite these observations, Herranz (2014) postulated that all human MZ twins start as dichorionic twins and may become monochorionic via trophectoderm fusion.

Interestingly, even a monochorionic triamniotic pregnancy resulted after transfer of an 8-shaped hatching blastocyst with two ICM structures (Sutherland et al., 2019). In contrast to the above mentioned publications, Gu et al. (2018) stated that an ICM incarceration in 8-shaped blastocysts does not increase the incidence of MZ twins in humans. This might indicate that the duration of an ICM incarceration is a relevant factor.

It should be emphasized that MZ human twins carry a robust DNA methylation signature in adult somatic tissues at genes involved in processes including cell adhesion, WNT signaling and cell fate (Van Dongen et al., 2021). As indicated above, cell adhesion might also play a role in the development of either DC/DA MZ twins derived from early cleavage stages or of MC/DA MZ twins derived from blastocysts with double ICMs. The Wnt/ß-catenin pathway plays a role during early development and maintenance of pluripotency (Denicol et al., 2013; Sidrat et al., 2020; Kinoshita et al., 2021; Liu et al., 2021; Xiao et al., 2021).

A human blastocyst with two ICMs, atypically resulting in DC/DA MZ twin embryos, was described by Meintjes et al. (2001). Although the authors stated that "[...] this case of monozygotic twinning can not be explained by in vitro zona alteration" Meintjes et al. (2001, p. S173), a disturbed hatching process with sequestration of a trophoblastic vesicle containing ICM cells is the most obvious cause for this atypical dichorionicity.

In addtion to the above mentioned pre-hatching sheep blastocysts with two ICMs, one in vivo developed elongating ovine blastocyst with two embryonic discs was collected from a superovulated donor ewe on D11 of pregnancy (Meinecke-Tillmann, 1993). Such specimens which theoretically might result in MC/DA monozygotic twins were already described by Assheton (1898) in an elongating sheep embryo which can be estimated to be about 10 to 11 days old [Bindon, 1971; Meinecke-Tillmann, 1993; although an age of D7 had been published by Assheton (1898)], and by Rowson and Moor (1964) in four sheep embryos collected between D6/7 and D14 of gestation. In our D11 embryo it cannot be completely ascertained whether the twin embryonic discs originated from ICM duplication or fission, or from a fusion of two zona-free blastocysts. Regarding the position of the embryonic discs and the absence of any trophoblastic strictures in the D11 conceptus, a previous ICM duplication within a single blastocyst is most likely, and MZ twins would result.

Blastocyst fusion and twinning

In the case of blastocyst fusion MC/DA DZ twins instead of MZ twins would be expected, as long as the ICMs remain separated. This event results in a temporary primary chimerism and must be extremely rare in in-vivo grown developmental stages since pre-hatching embryos are preferred for commercial ET in ruminants. Even after the transfer of two embryos, fusion cannot be expected because the blastocysts are usually transferred into

different uterine horns, avoiding a close contact to each other. Likewise we never observed fusion of hatching or early post-hatching blastocyst stages during IVC of ruminant embryos.

The readiness for aggregation and development of firm interconnections might be dependent on the species, the physiological time of embryo-attachment to the endometrium, and the type of implantation. Mouse and human blastocysts show very little expansion before early implantation soon after zona shedding. In contrast, implantation in ungulate species is superficial and delayed. Therefore, an early fixation of the conceptus would hamper further development. Accordingly, we recognized conjoined blastocysts which were flushed from the uteri of three superovulated ewes not before D10/D11 of pregnancy. In two pairs of these still spherical D11-blastocysts obtained from two of these ewes, respectively, a large superficial attachment zone between the trophoblasts was present, including about one sixth of their surface and resulting in a local flattening of the connected spheres. Both blastocyst pairs could be pulled apart without any tissue loss with the help of two microtools. In contrast, a separation without severe tissue damage was not possible in a group of five spherical D10-blastocysts recovered from the third ewe. They were conjoined via cell projections at punctuated trophectodermal contact areas. The central one was interconnected with every other blastocyst, whereas the lateral ones showed firm connections with the central one as well as with their direct neighbor. Confluence of blastocoels was absent in every entity, and each of the involved blastocysts contained a normal sized ICM. It is realistic to assume that both of the more loosely interconnected and otherwise normal blastocyst pairs might have resulted in physiological pregnancies, although the growth into a filamentous conceptus and the orientation of the embryo in relation to the uterine luminal epithelial layer might have been hampered if the conjunction between the two blastocysts would have stabilized.

The group of five firmly interconnected zona-free blastocysts resembled the above mentioned embryo with atypical hatching in different small areas of the thinned zona pellucida (see 3.3.1). Possibly such a zona entrapment is not permanent as long as the embryo is viable and proceeds in development. However, each of these five equal sized spheres possessed a normally developed ICM and trophoblast which suggests secondary fusions of blastocysts during the post-hatching period. It is difficult to speculate on a possible further development of these firmly interconnected zona-free structures. Trophoblast fusion might have a negative influence on embryo attachment, implantation and placentation. Furthermore, permanent interconnection might disturb embryo spacing and result in a crowding-effect in one uterine horn with negative consequences for embryonic survival.

Only few reports on early blastocyst fusion are available in the international literature. In mice, fusions during in vitro culture were induced with fusogenic viruses (Tarkowski and Wojewodzka, 1982: inactivated Sendai virus) or electrofusion (Ozdzeński et al., 1997; Tarkowski et al., 2005). In this context it should be noted that the electrofusion of zona-free mouse blastocysts allowed the development of common trophoblastic vesicles containing either one aggregated or two separate ICMs which was dependent on the prior orientation of the inner cell masses (Tarkowski et al., 2005). Thus, fusion of blastocysts might be a mechanism of twinning but - as mentioned above – of MC/DA DZ twins. This rare event had also been recognized in group-cultured human blastocysts after laser dissection of the zona pellucida (Schiewe et al., 2015), and spontaneous trophectoderm amalgamation was observed twice between two hatching blastocysts, respectively. Spontaneous fusions of group-cultured human blastocysts were further described by Swain (2021). Thus, it would be advantageous to avoid group-culture systems which allow a close contact between the individual embryos.

Moreover, fusion of blastomeres occurred after freezing and thawing of early human cleavage stages through membrane destabilization induced by cryoprotectants (Balakier et al., 2000).

Interestingly, the development of human MC/DA MZ twin pregnancies had also been reported after zona-free blastocyst transfer (Frankfurter et al., 2004) but, unfortunately, the data presented are incomplete and do not allow a final interpretation. Although Frankfurter et al. (2004) stated that monozygotic twins resulted from zona-free blastocysts, they disregarded the possibility of blastocyst fusion which may result in temporary or

permanent chimerism and DZ twinning. Data are missing which are related to the number of blastocysts that had been transferred to the individual women getting pregnant with twins (presumably two blastocysts since "normal" DZ multiples were also reported). Furthermore, the possible monozygosity had only been determined on the basis of ultrasonic scans during early pregnancy, whereas monozygosity at birth or pathology was only confirmed "when possible", and the report of appropriate criteria is also missing.

From conjoined oocytes which have occasionally been seen after oocyte retrieval in humans and which can result in successful pregnancies (Magdi, 2020; Wang et al., 2022), only singletons, DZ twins or chimeras would be expected after fertilization, unless other mechanisms which have been reported above contribute to twin formation

Artificially induced monozygotic twins or multiples

Splitting of early preimplantation embryos

As could be demonstrated, the early mammalian embryo exhibits a remarkable plasticity, and its cells are able to respond rapidly to damaging conditions. Interest in the tremendous regulative capacities of early embryos led to investigations including experimental blastomere isolation, blastomere isolation and aggregation, embryo halving, quartering, or separation into eights, and trials related to the artificial induction of monozygotic twins or multiples. This has been accomplished in a variety of species, such as humans (experiments not further than up to preimplantation stages: Hall et al., 1993; Van de Velde et al., 2008; Illmensee et al., 2010; Noli et al., 2015b; Omidi et al., 2020), monkeys (Mitalipov et al., 2002), horses (Allen and Pashen, 1984; Skidmore et al., 1989), cattle (Willadsen et al., 1981; Willadsen and Polge, 1981; Ozil et al., 1982; Voelkel et al., 1985; Warfield et al., 1987; Johnson et al., 1995; Rho et al., 1998; Skrzyszowska et al., 1999; Hashiyada, 2017), sheep (Trounson and Moore, 1974; Meinecke-Tillmann et al., 1979; Willadsen, 1979, 1980, 1981; Meinecke-Tillmann, 1980, 1993; Meinecke-Tillmann and Meinecke, 1981, 1983b, 1987), goats (Meinecke-Tillmann and Meinecke, 1983a, 1987; Tsunoda et al., 1985; Udy, 1987; Nowshari and Holtz, 1993), pigs (Nagashima et al., 1989; Reichelt and Niemann, 1994; Dang-Nguyen et al., 2011), rabbits (Yang and Foote, 1987), rats (Matsumoto et al., 1989), and mice (Mullen, 1971; Moustafa and Hahn, 1978; Gärtner and Baunack, 1981; Tsunoda and McLaren, 1983; Nagashima et al., 1984; Tsunoda et al., 1987; Carstea et al., 2007; Katayama et al., 2010; Tarkowski et al., 2010; Zhang et al., 2018; Krawczyk et al., 2021; Maemura et al., 2021). Success rates differed according to species, manipulated developmental stages and manual skills of the operator.

In farm animals the first artificially induced monozygotic twins were reported in sheep after separation of blastomeres of very early cleavage stages (Willadsen, 1979), or after bisection (splitting) of morulae and blastocysts (Meinecke-Tillmann, 1980; Meinecke-Tillmann and Meinecke, 1981).

Although, at least in farm animals, demi-embryos are nearly as suitable for the establishment of pregnancies as intact embryos, the developmental potential of single blastomeres of the 4- or 8-cell stage is more limited than that of the 2-cell stage (Willadsen, 1981; Krawczyk et al., 2021).

Whereas simple division of preimplantation embryos has occasionally been successful for producing up to monozygotic quadruplets, serial splitting was introduced in the hope to create higher order multiples, and in vitro trials on serial splitting of mouse or bovine cleavage stages were reported (Illmensee et al., 2006; Silvestri et al., 2022). In this context it might have been overlooked that, despite of the impressive regulative capacities, a developmental clock regulating polarization and blastocyst formation is present in preimplantation embryos. This was already indicated, for example, by Tarkowski (1959) and Tarkowski and Wroblewska (1967), and demonstrated by other authors (Pratt et al., 1981; Johnson et al., 1984; Dean and Rossant, 1984; Prather and First, 1986; Modliński et al., 2002; Lorthongpanich et al., 2012; Noli et al., 2015b; Zhu et al., 2020; Maemura et al., 2021). Until now, this developmental clock prevents the multiplication of animals via serial splitting of cleavage stages.

Monozygotic multiples via chimeric cloning

The only theoretical possibility to produce monozygotic multiples of higher order is "chimeric cloning". This can be performed either via blastomere complementation (sheep quintuplets: Fehilly and Willadsen, 1986; sheep triplets: Meinecke-Tillmann, 1993), or via stem cell complementation. In mice, embryonic (ESC), parthenogenetic or induced pluripotent stem cells (iPSC) were successfully combined with blastomeres, tetraploid blastomeres, tetraploid blastocysts, or trophoblastic vesicles (Nagy et al., 1990, 1993; Modliński et al., 2004; Huang et al., 2008; Boland et al., 2009, 2012; Chen et al., 2009; Zhao et al., 2009, 2010; Sumiyama et al., 2018), whereas in cattle only low-grade chimeric progeny resulted after aggregation of ESC-like cells with presumably tetraploid embryos (Iwasaki et al., 2000). Unfortunately, in contrast to mice, a high grade of mosaicism between diploid and tetraploid cells occurred after electrofusion of blastomeres in cattle (Curnow et al., 2000).

Our experimental approaches with regard to embryonic stem cells, for example in sheep and goats, dated back to 1991 (Meinecke-Tillmann and Meinecke, 1991, 1996; Meinecke-Tillmann, 1993), but trials to establish ESC-lines from farm animals posed technical problems. Nowadays, some breakthroughs have also been achieved in farm animals [see reviews of Navarro et al. (2019); Kim and Roh (2021); Kumar et al. (2021); Aguila et al. (2022)]. This might enhance the technique of chimeric cloning.

Meanwhile, the suitability of trophoblastic vesicles with ICM-exchange (i.e. the induction of a temporary chimerism) was demonstrated as a promising approach for endangered species conservation, although until now, in contrast to earlier experiments with asynchronous blastomere aggregation (Meinecke-Tillmann and Meinecke, 1984a) only intraspecies progeny has been born (Loi et al., 2018). Before, successful ICM-replacement was performed between differing mouse stains (Bi et al., 2003; Zheng et al., 2005) and with ICMs derived from bovine nuclear transfer embryos and trophoblastic vesicles of bovine IVP-embryos (Murakami et al., 2006). However, it might become possible to generate gametes from iPSC of endangered animals that can be used to create IVP-blastocysts and to transfer their isolated ICMs into trophoblastic vesicles derived from a suitable species which carries the interspecies chimeric blastocysts to term (Saragusty et al., 2020). A similar approach is conceivable with ICMs from IVP-blastocysts after interspecies cloning with somatic cells or iPSC of an endangered species and oocytes from another closely related and compatible but unthreatened species. Unfortunately, in these cases mtDNA of a foreign species is undesirably transmitted.

Since ectogenesis is far from reality it is necessary to find suitable and common species that can serve as foster mothers for the interspecies approach in animals that are on the brink of extinction. In the context of endangered animals it should not be forgotten that non-mammalian species deserve attention, too (Lipke et al., 2009b, a; Strand et al., 2020; Bolton et al., 2022).

The zona pellucida in micromanipulated embryos

The zona pellucida (Moros-Nicolás et al., 2021) protects pre-compaction embryos from lysis, immobilization, aggregation, disaggregation or loss of blastomeres, and also from contact with immune cells or infectious material (Modliński, 1970; Nichols and Gardner, 1989; Ueno et al., 2007). Additionally, the zona is necessary for the establishment of a special microenvironment. Furthermore, it eases handling during ET procedures. Thus, for the protection of zona-injured cleavage stages before reaching the compacted morula stage, different methods were tested or established such as encapsulation in agar (Willadsen, 1979; Willadsen et al., 1981; Tsunoda and McLaren, 1983), agarose (Blakewood et al., 1989), or agar/agarose cylinders (Meinecke-Tillmann, 1993), sodium alginate (Adaniya et al., 1987; Cosby and Dukelow, 1990; Hall et al., 1993; Watanabe et al., 1995; Yániz et al., 2002), or poly-l-lysine (Krentz et al., 1993), protection with a double zona pellucida (Meinecke-Tillmann and Meinecke, 1984a, b; Meinecke-Tillmann, 1993), gelatin embedding (Warfield et al., 1987), or zona substitution with special agarose capsules (Nagatomo et al., 2017) or sodium

hyaluronate gel (Song et al., 2022). In the case of encapsulation with non-degradable material the embryos had to be freed in the late morula or early blastocyst stage.

Before embedding in foreign material, manipulated embryos / blastomeres normally were surrounded with a host zona pellucida. These host zonae were usually taken from unfertilized / degenerate oocytes or embryos that were recovered during embryo collection in vivo or during in vitro culture, or from oocytes collected from abattoir material. In our own studies fresh zonae pellucidae derived from oocytes of prepubertal pigs were preferred (Meinecke-Tillmann and Meinecke, 1983b, 1984a, b, 1987; Meinecke-Tillmann, 1993) because of their smaller size which made them more suitable for splitted or otherwise treated embryos, and because of the absence of pre- and post-fertilization hardening which might unfavorably interfere with blastocyst hatching [zona hardening: see Coy et al. (2008)]. The double zona technique allowed, if desired, the direct transfer into final recipients. Nevertheless, it cannot be excluded that the mechanics during hatching might have been of negative influence on pregnancy and embryo survival rates, since these were not as high as after agar/agarose embedding/removal and ET to intermediate recipients (Meinecke-Tillmann, 1993). On the other hand it has to be taken into account that embedding in agar/agarose cylinders allowed a preselection of embryos and therefore higher success rates after ET.

Hygienic precautions had been taken into account since the pig zonae were derived from slaughterhouse material. Therefore, they were carefully denuded, evacuated and washed in order to remove possible contaminants. In contrast to former observations (Moore et al., 1969), a foreign zona pellucida does not negatively influence the pre-hatching development.

The necessity of zona-envelopment had been questioned by Feltrin et al. (2014) since low embryonic survival rates were observed after oviductal transfers of both zona-free and zona-enclosed cloned goat D1- to D2-embryos on D30 of pregnancy (presence of an embryo proper: 5.6% vs. 5.8%; with heartbeat: 0.7% vs. 0.6%). In this context it should be kept in mind that cloned embryos are a suboptimal approach to investigate the problem of a missing or injured zona pellucida. Even when advanced IVC- methods are used (Park et al., 2015), the enclosing in a host zona pellucida might still be of interest, depending on the species, the preimplantation stage, and the type of manipulation. In general, however, zona-free embryos develop in vitro at a similar rate to blastocysts as zona-enclosed embryos (Lagutina et al., 2007), and zona-hardening in vitro and its consequences are undesirable (Madani et al., 2022). Thus, the necessity of the presence of the zona pellucida in modern embryo culture systems (Hashiyada, 2017; Fan et al., 2022) has to be questioned. Nonetheless, further investigations are required, since it had been shown that zona removal affects, for instance, blastomere conformation (Katayama et al., 2010) and gene expression as well as pre- and postimplantation development in mice (Fan et al., 2022), although influences might again be species specific (cattle: Velásquez et al., 2013).

Stickiness of zona-free blastocysts can be overcome by proper handling of the embryos, and, thus, ET of micromanipulated zona-free post-compaction stages did not lower pregnancy rates in comparison to the control group (Warfield et al., 1987).

Maternal effects

Embryo technologies per se (Betteridge, 1977, 1981; Biggers, 2012; Hansen, 2020a, b) and artificial production of monozygotic twins can be involved in breeding programs or in comparative experiments (e.g. Wassmuth and Meinecke-Tillmann, 1980; Biggers, 1986; Kippax et al., 1991; Weppert, 2006; Hashiyada, 2017; Casser et al., 2019a; Mueller and Van Eenennaam, 2022).

One aspect that shall be discussed here in some more detail is the influence of the maternal genotype or phenotype on the phenotype of the offspring (Wolf and Wade, 2009) since birth weight is a relevant factor for progeny survival and health.

Studies on maternal effects initially involved ET between large and small breeds or reciprocal exchange of embryos between strains or breeds in different species, including

sheep (Hunter, 1956; Karihaloo and Combs, 1971; Meinecke-Tillmann and Wassmuth, 1977; Hinkelman et al., 1979; Anderson et al., 1981; Naqvi et al., 2006; Emsen et al., 2012; Sharma et al., 2012; Oliver et al., 2015), pigs (Smidt et al., 1966; Steinbach et al., 1967), and mice (McLaren and Michie, 1958; Brumby, 1960; Cowley et al., 1989) which were later followed by experiments on horses (Allen and Pashen, 1984; Tischner, 1987; Tischner and Klimczak, 1989; Allen et al., 2002; Peugnet et al., 2017) and cattle (Guilbault et al., 1990; Gregory and Maurer, 1991). They were mainly performed to investigate the impact of a more "comfortable" uterine environment in comparison to a restricted one on the pre- and postnatal development of body weight and size of the fetus/newborn, and on the duration of pregnancy. In contrast, studies related to birth weight in cattle primarily concentrated on the effects of artificial reproductive techniques (King et al., 1985; Lopes et al., 2022).

Although Emsen et al. (2012) observed no recipient breed effect in sheep, significant influences were recognized in most of the investigations and reflected the regulatory effects of the uterine environment on either birth weight or weaning weight of lambs.

Depending on the breed, larger/heavier genotype lambs were smaller/lighter when born to smaller/lighter genotype dams (e.g., Hunter, 1956; Karihaloo and Combs, 1971; Meinecke-Tillmann and Wassmuth, 1977; Sharma et al., 2012). However, contradicting results had been obtained with regard to birth weight or body dimensions of small genotype lambs that were born to larger embryo recipients. Whereas significant differences were seen in comparison to the controls when smaller/lighter genotype lambs were delivered by larger/heavier genotype foster mothers (e.g., Karihaloo and Combs, 1971; Meinecke-Tillmann and Wassmuth, 1977), this was not the case in another study (Sharma et al., 2012). Similar to sheep, birth weight of calves was higher in Ayrshire dams bearing Limousin fetuses than in those bearing Ayrshire fetuses (Guilbault et al., 1990) which was compensated within 5 months of postnatal development. Dependent on the breed, differences between control lambs and lambs that were delivered by foster mothers were compensated between 14 d and 8 weeks of age (Meinecke-Tillmann and Wassmuth, 1977) or after 8 months (Hunter, 1956), whereas in horse fillies (Pony and Thoroughbred), differences in birth weight as well as in other parameters resulting from transfer to host mothers were still obvious at 3 years of age (Allen et al., 2004). Similar to sheep, compensation of the influence of the intrauterine environment on prenatal development of piglets already took place during the first 4 to 5 weeks of life (Smidt et al., 1966).

Since postnatal growth of progeny is influenced by milk production of the individual dams, and since milk production itself depends on the breed, birth type and sex of the newborn (Guilbault et al., 1990; Hinde et al., 2014; Abecia and Palacios, 2018) as well as the breed of the dam, it was sought to objectify the results obtained during the time period from birth until weaning in a few studies. This was achieved by artificial rearing of the lambs (Meinecke-Tillmann and Wassmuth, 1977), or by uniform foster mothers and a defined number of pups per female in mice (Cowley et al., 1989).

Pregnancy duration was longer in dams bearing progeny with higher birth weight (e.g. sheep: Meinecke-Tillmann and Wassmuth, 1977; cattle: Guilbault et al., 1990). In this context, it should be kept in mind that in contrast to several other mammals the strategy of timing of parturition in sheep depends solely on the fetal hypothalamic-pituitary-adrenal axis (Liggins, 1974; Rokas et al., 2020). Thus, in other species differing results might be expected.

In order to specify effects of the maternal environment, matching MZ demi-embryos had been transferred into a single or into two different foster mothers (sheep: Meinecke-Tillmann, 1980, 1984; horse: Allen and Pashen, 1984; Allen, 2005) since a mere transfer of embryos between large and small breeds left questions open. Although only small groups of monozygotic twins were available in these studies, results indicated that lambs derived from demi-embryo pairs which had been transferred to two ewes of similar size were more similar to each other than after transfer to different uterine horns of a single mother. In the latter case, restricted growth of the smaller MZ twin was compensated within three month after delivery. Pregnancy duration was identical in MZ twin pairs born from two different but similar sized ewes (same day, difference of only few hours; Meinecke-Tillmann, 1984). In contrast, MZ fillies were born 23 d apart to two mares of different body size and showed a marked dissimilarity in birth weight which was not completely compensated in later life (Allen

and Pashen, 1984). In another group of MZ horse twins the growth rate was either enhanced or curbed depending on the genetic background of the foals in the first 6 months post partum, and a minor effect of the uterine environment persisted in the mature animals (Allen et al., 2004).

Since in sheep the size of each twin at birth might largely be determined during early gestation (Hancock et al., 2012), further investigations on monozygotic twins carried by a single or by different foster mothers are recommendable. Ultrasonography is suitable to follow the intrauterine development noninvasively (Meinecke-Tillmann and Meinecke, 2007; Elmetwally et al., 2016a, b; Meinecke-Tillmann, 2017). In this context it would be of particular interest that matching demi-embryos can successively be carried by the same or by different dams, such as in different seasons or under other differing environmental circumstances. With regard to animal behavior, reciprocal embryo transfers between anxious or non-anxious individuals or between breeds of different temperament would offer new perspectives, particularly when MZ twins would be investigated.

Monozygotic sheep and cattle twins of different age were already born after one demi-embryo had been transferred directly after collection and micromanipulation, whereas the other demi-embryo had been stored at -196°C for several weeks (Willadsen, 1980; Seike et al., 1991). This approach also allowed successful pregnancies in goats carrying their MZ twin conceptus (Oppenheim et al., 2000). Similar to goats, a syngeneic pregnancy was also successful in horses, although in cloned individuals (Galli et al., 2003). The MZ twin model has further been used for investigating the embryo-maternal dialogue in cattle (Klein et al., 2006).

Artificial monozygotic twinning would be helpful for the differentiation between true maternal effects [see Wolf and Wade (2009)] and other factors such as maternal inheritance or genomic imprinting. That maternal effects can override any genetic control was impressively demonstrated with regard to the development of endometrial cups in a mare and a jenny donkey, each pregnant from one of two matching MZ mule embryo halves (Allen et al., 1993).

Blastocysts/conceptus derived from demi-embryos might differ from each other, for instance since dissimilarities between cleavage products [Casser et al. (2019b), discussed by Denker (2020)] or universal epigenetic inter-individual dissimilarities (Planterose Jiménez et al., 2021) may exist, the maternal environment (Ollikainen et al., 2010) or assisted reproductive technologies may influence DNA methylation/gene expression (Urrego et al., 2014; Velásquez et al., 2016, 2017; Barberet et al., 2022; Håberg et al., 2022), or postzygotic mutations may occur in somatic cells as well as germ cells (Jonsson et al., 2021).

In this context, the transfer of MZ demi-embryos into MZ twin mothers might be of special interest for further investigations.

Conclusion

The mammalian preimplantation embryo is equipped with amazing regulative capacities which seem to be both species and stage specific. This reproductive strategy allows the compensation of a loss of defective blastomeres even at two-cell stages or the regulation of ploidy without endangering the whole pregnancy, especially in uniparous species with their long generation intervals. Therefore, the cellular and embryological basis for twinning might vary between species. The high regulative capacity also allows the artificial splitting of preimplantation embryos in order to increase the number of transferable embryos per donor animal.

Regarding the process of spontaneous monozygotic twinning in association with natural pregnancies as well as artificial reproductive technologies, hypotheses on the development of dichorionic/diamniotic and monochorionic/diamniotic twins are discussed on the basis of selected international publications and of own observations of in vivo developed or in vitro manipulated embryos in small ruminants. Contrary to the general view that twin blastocysts cannot occur within a common zona pellucida, we describe such cases in native and in manipulated embryos. Furthermore, possible mono- or dizygotic twinning related to

blastocysts with double ICMs or to fused blastocysts are reported. MZ twins offer the possibility to perform comparative studies in which one twin serves as the control. This is of special interest with regard to maternal influences on the developing conceptus.

Acknowledgements

We would like to thank our teachers, mentors, co-workers, and students who accompanied us during our scientific life. These are, just to name a few of them, Rudolf Wassmuth, to whom this review is dedicated on the occasion of his 95th birthday, Harry Tillmann, Emmanuel C. Amoroso, Wolfgang Jöchle, Diedrich Smidt, Edita Podhajsky and Heidrun Lewalski.

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Author contributions

BM: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing; SMT: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing.

President´s letter

Dear friends and colleagues,

It is with great pleasure that I invite you to the 39th Annual Scientific Meeting of AETE. The meeting will take place on the magnificent island of Crete, in its capital city of Heraklion from 7th to 8th September. The venue, Hotel Galaxy, is located close to the city center. With comfortable and spacious rooms, a nice restaurant and coffee area, and a beautiful pool, it is an excellent venue for our meeting. While writing this, the preparations of the next AETE meeting are proceeding with pace.

The Local Organizing Committee, chaired by Georgios Amoiridis, together with Artion, have worked hard to provide us ideal settings for our meeting as well as an outstanding social program. The theme of our 2023 AETE meeting is ***The Interaction of ARTs and the Environment***. We address this interaction from two perspectives: how the environmental changes might affect the reproduction of livestock and how our activities in ART might affect the environment. We have put together an exciting scientific program and invited excellent speakers to discuss these challenging topics.

Our meeting will start with a Welcome Reception that takes place in the evening of Wednesday, September 6th, at the hotel's pool area. After this reunion, the scientific program will be launched on Thursday by Urban Besenfelder with a title "*The interaction between the environment and embryo development in assisted reproduction".* The talk of our second invited speaker, Hilde Aardema will address a current topic titled *"The potential impact of microplastics on reproduction"*. Pietro Baruselli, representing our Brazilian sister society SBTE, will cover *"The contribution of assisted reproductive technologies and reproduction management to reducing the CO_2 equivalent emissions of dairy and beef industry"*. Conservation biology will be addressed in William Holt's talk entitled *"Environmental effects on developmental plasticity in conservation biology"*.

The AETE board is honored to announce Sabine Meinecke-Tillmann and Burkhard Meinecke will be presented with the Pioneer Awards of 2023 for their pioneering, wide and versatile work in the field of assisted reproduction, especially in sheep and goat. Burkhard Meinecke served as a president of AETE in mid 1990's.

We thank everyone for sharing high quality abstracts for this year's meeting, to compete for the best oral presentation, best poster and student competition, where for the first time, the winner from the finalists will be selected by a jury composed of you among others, and not by the board as usually.

The practitioners' forum, *"SOPs for Donors and Recipients"* will be sponsored and coordinated by Calier and will present the outcome of the work of an international expert committee, followed by a round table discussion. On Friday, the scientific program will culminate in a Workshop on *"Animal Welfare in ARTs"*. This interactive workshop aims to: discuss the perception of animal welfare; present current situation of regulations and; highlight some

initiatives, including some research studies, to evaluate impacts of some ARTs on animal welfare.

The social program will take us to Arolithos Traditional Cretan Village and our meeting will end in a Farewell Party in a club downtown on Friday. This meeting would not be possible without the valuable support of our sponsors! On behalf of the whole board, I want to express our gratitude to all of them, old and new ones.

I want to thank the board of governors for their dedicated work for the society as well as our members for support. I hope that you all enjoy the AETE meeting in Heraklion.

Marja Mikkola
President of the AETE

Commercial Embryo Transfer Activity in Europe 2022

Collated by Helene Quinton

National data collectors

Country	Collector	Comment
Belgium	Isabelle DONNAY	
Czech Republic	Jiri SICHTAR	
Denmark	Henrik CALLESEN	
Finland	Anna OKSA-PULLIAINEN	
France	Serge LACAZE	
Germany	Hubert CRAMER	
Greece	Foteini SAMARTZI	
Hungary	Ferenc FLINK	
Italy	Giovanna LAZZARI	
Latvia	Ilga SEMATOVICA	
Lithuania	Rasa NAINIENE	
Netherlands	Anna BEKER VAN WOUNDENBERG	
Netherlands	Erik MULLAART	
Netherlands		
Norway	Tjerand LUNDE	
Poland	Jedrzej JASKOWSKI	
Portugal	Joao Nestor CHAGAS E SILVA	
Romania	Stefan CIORNEI	
Russian Federation	Victor MADISON	
Serbia	Aleksandar MILOVANOVIC	
Slovakia	Dalibor POLAK	No activity
Slovenia	Aleksandar PLAVSIC	
Spain	Pablo BERMEJO	
Sweden	Renee BÅGE	
Switzerland	Sarah Wyck	
Turkey	Hakan SAGIRKAYA	
United Kingdom	Peter MAY	
United Kingdom	Jake OLIVIER	

Declared bovine *In vivo* embryo production

	Dairy				Beef				All		
Country	Collec-tions	% Coll. with sexed semen	Embryos & ova	Transferable embryos	Collec-tions	% Coll. with sexed semen	Embryos & ova	Transferable embryos	Collec-tions total	Transferable embryos total	Embryos/ collection
Austria	526	8%	5 048	3 595	13	8%	174	40	539	3 635	6,7
Belgium	13	0%	78	22	652	0%	3 677	2 407	665	2 429	3,7
Czech Republic	410	0%	2 939	2 043	0		0	0	410	2 043	5,0
Denmark	773	0%	6 170	4 336	50	0%	535	402	823	4 738	5,8
Finland	365	7%	3 492	2 553	2	0%	16	10	367	2 563	7,0
France	4 836	16%	40 105	25 092	1 486	5%	15 160	8 450	6 322	33 542	5,3
Germany	3 970	0%	37 465	25 826	340	0%	4 048	2 364	4 310	28 190	6,5
Greece	4	100%	23	14	0		0	0	4	14	3,5
Hungary	389	98%	1 780	1 573	38	34%	225	181	427	1 754	4,1
Italy	2 145	56%	21 085	19 410	112	0%	1 098	796	2 257	20 206	9,0
Lithuania	2	0%	5	5	0		0	0	2	5	2,5
Netherlands	2 466	1%	19 762	12 879	0		0	0	2 466	12 879	5,2
Norway	370	1%	2 436	2 230	10	0%	93	65	380	2 295	6,0
Poland	235	52%	2 034	1 322	3	0%	29	16	238	1 338	5,6
Portugal	85	87%	781	364	30	0%	302	160	115	524	4,6
Romania	11	0%	68	31	4	0%	3	3	15	34	2,3
Russian Federation	358	55%	1 840	867	753	1%	7 259	4 545	1 111	5 412	4,9
Slovenia	15	20%	60	21	2	0%	7	7	17	28	1,6
Spain	179	86%	1 721	782	112	7%	1 241	757	291	1 539	5,3
Sweden	123	7%	971	655	0		0	0	123	655	5,3
Switzerland	408	54%	4 664	2 876	3	67%	69	34	411	2 910	7,1
Turkey	315	56%	2 824	1 791	0		0	0	315	1 791	5,7
United Kingdom	748	0%	7 431	4 138	3 296	0%	9 516	4 374	4 044	8 512	2,1
Total général	**18 746**	**18%**	**162 782**	**112 425**	**6 906**	**1%**	**43 452**	**24 611**	**25 652**	**137 036**	**5,3**

Declared bovine *In vitro* embryo production (OPU-IVP)

	Dairy				Beef				All		
Country	OPU	% OPU with sexed semen	Oocytes	Embryos	OPU	% OPU with sexed semen	Oocytes	Embryos	OPU	Embryos	Embryos /OPU
Austria	25	96%	237	38					25	38	1,5
Finland	521	0%	4 749	1 075	0		0	0	521	1 075	2,1
France	1 544	2%	19 448	4 872	38	3%	924	392	1 582	5 264	3,3
Germany	2 650	0%	32 038	8 323	0		0	0	2 650	8 323	3,1
Hungary	1	0%	6	1	1	0%	4	1	2	2	1,0
Italy	177	71%	1 798	488	28	0%	114	28	205	516	2,5
Netherlands	5 871	1%	62 410	11 773	0		0	0	5 871	11 773	2,0
Norway	39	0%	447	70	0		0	0	39	70	1,8
Poland	150	85%	2 242	553	0		0	0	150	553	3,7
Romania	40	0%	209	84	0		0	0	40	84	2,1
Russian Federation	97	100%	985	147	0		0	0	97	147	1,5
Serbia	32	91%	412	141	0		0	0	32	141	4,4
Spain	748	77%	7 888	2 316	196	10%	2 339	765	944	3 081	3,3
Switzerland	228	34%	2 414	918	0		0	0	228	918	4,0
Turkey	144	0%	1 843	239	0		0	0	144	239	1,7
United Kingdom	787	0%	12 296	3 867	0		0	0	787	3 867	4,9
Total général	**13 054**	**9%**	**149 422**	**34 905**	**263**	**8%**	**3 381**	**1 186**	**13 317**	**36 091**	**2,7**

Declared bovine *In vitro* embryo production – abattoir

	Dairy				Beef			
Country	Donors	% with sexed semen	Oocytes	Embryos	Donors	% with sexed semen	Oocytes	Embryos
Greece	6	14%	192	16	12	0%	151	29
Netherlands	2	0%	75	27	0			
Slovenia	164	0%	26 890	2 565	0			
Spain					947	0%	13 173	3 562
United Kingdom	229	0%	2 478	837	64	0%	555	195
Total	**401**	**0%**	**29 635**	**3 445**	**1 023**	**0%**	**13 879**	**3 786**

Declared bovine embryo technologies – embryo genotyping

	Sexed embryos		Genotyped embryos	
Country	In Vivo	In Vitro	In Vivo	In Vitro
France	266	0	678	88
Germany	163	9	0	0
Netherlands	0	0	0	4 156
Total	**429**	**9**	**678**	**4 244**

Declared bovine embryo transfers and exports– *In vivo*

Country	Dairy				Beef				Total embryos transferred
	Fresh embryos transferred	Frozen embryos			Fresh embryos transferred	Frozen embryos			
		Domestic transferred	Foreign transferred	Exported		Domestic transferred	Foreign transferred	Exported	
Austria	1 515	1 786	48	24	16	94	0	0	3 459
Belgium	2	20	0	0	493	1 914	0	0	2 429
Czech Republic	1 253	914	0	0	0	0	0	0	2 167
Denmark	3 147	1 519	0	18	103	106	0	9	4 875
Finland	722	1 875	83	573	3	12	50	0	2 745
France	12 031	10 525	877	124	2 863	3 375	156	256	29 827
Germany	10 861	15 403	0	853	836	1 340	0	0	28 440
Greece	14	0	7	0	0	0	0	0	21
Hungary	644	621	0	0	62	83	13	0	1 423
Italy	6 037	1 755	0	0	132	0	0	0	7 924
Netherlands	2 290	9 596	0	0	0	0	0	0	11 886
Norway	43	2 063	150	0	30	35	80	0	2 401
Poland	783	286	46	0	4	0	0	0	1 119
Portugal	124	170	0	0	74	53	88	0	509
Romania	61	0	0	0	4	0	46	0	111
Russian Federation	312	587	1 045	0	10	4 586	121	0	6 661
Slovenia	30	5	0	0	3	4	0	0	42
Spain	353	478	61	31	132	322	100	0	1 446
Sweden	420	1 099	0	0	0	0	0	0	1 519
Switzerland	671	1 952	270	152	0	0	0	0	2 893
Turkey	1 128	113	0	0	0	0	0	0	1 241
United Kingdom	1 738	2 097	814	4	795	2 240	438	135	8 122
Grand Total	**44 179**	**52 864**	**3 401**	**1 779**	**5 560**	**14 164**	**1 092**	**400**	**121 260**

Declared bovine embryo transfers and exports – *In vitro*

Country	OPU				Abattoir		Total embryos transferred
	Fresh embryos transferred	Domestic frozen embryos transferred	Foreign frozen embryos transferred	Embryos exported	Fresh embryos transferred	Domestic frozen embryos transferred	
Austria	0	19	0	0	0	0	19
Finland	0	747	0	281	0	0	747
France	2 373	2 138	208	0	0	0	4 719
Latvia	9	10	80	0	0	0	99
Netherlands	3 907	2 228	0	0	0	0	6 135
Poland	89	57	0	0	0	0	146
Portugal	0	1	78	0	0	0	79
Romania	33	51	0	0	0	0	84
Russian Federation	6	80	0	0	0	0	86
Serbia	0	3	0	0	0	0	3
Spain	845	1 068	30	29	1 394	980	4 317
Turkey	0	239	0	0	10	61	310
United Kingdom	302	843	1	130	0	0	1 146
Grand total	**7 564**	**7 484**	**397**	**440**	**1 404**	**1 041**	**17 871**

Declared embryo production, transfer and export in other species – *In vivo*

		Embryo collection		Embryo transfer			
Species	Country	Collections	Viable embryos	Fresh embryos	Frozen domestic	Frozen foreign	Exported embryos
Sheep	Hungary	7	7	7	0	0	0
	Portugal	12	16	7	0	0	0
	Turkey	5	13	7	6	96	0
	United Kingdom	290	1296	1095	175	0	0
	Total	**314**	**1332**	**1 116**	**181**	**96**	**0**
Goat	Turkey	2	24	24	0	0	0
	United Kingdom	1	22	22	0	16	0
	Total	**3**	**46**	**46**	**0**	**16**	**0**
Horse	Czech Republic	23	22	7	0	0	0
	Hungary	3	3	3	0	0	0
	Poland	7	7	7	7	0	0
	Portugal	87	49	49	0	0	0
	Romania	3	0	2	0	0	0
	Russian Federation	42	28	25	2	0	0
	Sweden	33	0	23	0	0	0
	Total	**198**	**109**	**116**	**9**	**0**	**0**
Rabbit	Romania	31	209	0	0	0	0

Declared embryo production, transfer and export in other species – *In vitro*

		Oocyte collection				IVP embryo transfer			Exported embryos
Species	Country	OPU conv	OPU sexed	Oocytes	Embryos	Fresh embryos	Frozen domestic	Frozen foreign	
Sheep	Serbia	2	0	22	6	0	0	0	0
	Sweden	0	0	0	0	0	0	295	0
	United Kingdom	12	0	48	0	0	0	0	0
	Total	**14**	**0**	**70**	**6**	**0**	**0**	**295**	**0**
Horse	Italy	1595	0	47321	7036	9	796	0	3609
	Poland	21	0	210	21	0	0	0	21
	Portugal	39	0	419	74	0	0	2	0
	Sweden	0	0	0	0	23	0	0	0
	Switzerland	40	0	114	22	0	14	8	8
	Total	**1 695**	**0**	**48 064**	**7 153**	**32**	**810**	**10**	**3 638**

INVITED LECTURES

The interaction between the environment and embryo development in assisted reproduction

Urban Besenfelder, Vitezslav Havlicek

Animal Reproduction 2023, vol 20.

Animal Reproduction

Thematic Section: 39th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

The interaction between the environment and embryo development in assisted reproduction

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How to cite: Besenfelder U, Havlicek V. The interaction between the environment and embryo development in assisted reproduction. Anim Reprod. 2023;20(2):e20230034. https://doi.org/10.1590/1984-3143-AR2023-0034

Abstract

It can be assumed that the natural processes of selection and developmental condition in the animal provide the best prerequisites for embryogenesis resulting in pregnancy and subsequent birth of a healthy neonate. In contrast, circumventing the natural selection mechanisms and all developmental conditions in a healthy animal harbors the risk of counteracting, preventing or reducing the formation of embryos or substantially restricting their genesis. Considering these facts, it seems to be obvious that assisted reproductive techniques focusing on early embryonic stages serve an expanded and unselected germ cell pool of oocytes and sperm cells, and include the culture of embryos outside their natural habitat during and after fertilization for manipulation and diagnostic purposes, and for storage. A significant influence on the early embryonic development is seen in the extracorporeal culture of bovine embryos (in vitro) or stress on the animal organism (in vivo). The in vitro production per se and metabolic as well as endocrine changes in the natural environment of embryos represent adequate models and serve for a better understanding. The purpose of this review is to give a brief presentation of recent techniques aimed at focusing more on the complex processes in the Fallopian tube to contrast in vivo and in vitro prerequisites and abnormalities in early embryonic development and serve to identify potential new ways to make the use of ARTs more feasible.

Keywords: assisted reproduction, embryo development, environment, oviduct, endoscopy.

Introduction

The establishment and use of reproductive techniques represent a potential way beyond natural selection to obtain a higher number of offspring from genetically selected parents for breeding, including for pre-implantation diagnostics. These techniques make an enormous contribution to accelerating breeding progress, allow a better consideration of several complex breeding traits and have thus become a central component of sustainable animal breeding programs (Berglund, 2008; Ferré et al., 2020; Georges et al., 2019). One success that has dominated breeding so far has been the steady increase in milk yield. Meanwhile, however, the high level of milk production with all its metabolic consequences is also held responsible for drastic losses in reproduction (Lucy, 2001; Diskin and Morris, 2008). This development shows that achieved breeding goals inevitably have a negative impact on some traits and thus reveal an antagonism (competition between fertility and production traits) (Roxström et al., 2001; Berglund, 2008).

If assisted reproduction techniques are used for breeding of genetically valuable animals and early embryogenesis is shifted to the laboratory, trait antagonisms affecting fertility and possible environmental influences are opposed to breeding success. It affects precisely those developmental stages that are particularly sensitive to environmental changes, which becomes manifest both, in the short term during embryo development as well as in the long term

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Received: March 8, 2023. Accepted: June 28, 2023.
Financial support: UB received funding for a part of this research from the Federal Ministry of Agriculture, Forestry, Environment and Water Management and the Federal Ministry of Education, Science and Culture (grant number 1227, GZ 24.002/80-IIA 1a/00).
Conflicts of interest: The authors have no conflict of interest to declare.

postpartum through modifications in the phenotype (Duranthon and Chavatte-Palmer, 2018). There is much evidence that these generated embryos are of inferior quality and therefore both ex vivo and in vitro impairments similarly exert influence on these vulnerable stages. In general, more or less all reproductive techniques have been shown to result in the birth of a calf. The gold standard of comprehensive understanding is based on physiological embryogenesis, i.e. the complete development of the gametes in the animal, followed by fertilization and embryo development to succeed in a competent fetal growth and finally the birth of a healthy calf. Development of early embryos appears to be a sensitive indicator for disorders, which becomes evident in a reduction of embryo survival during the first days and weeks after fertilization (Wiltbank et al., 2016; Diskin and Morris, 2008), in long-term consequences (Farin et al., 2006; Duranthon and Chavatte-Palmer, 2018) and finally in a limited suitability of embryo technologies for the application of ARTs (Fèrre et al., 2020).

Among most ARTs, in vitro production aims at culturing oocytes and embryos under artificial conditions in the laboratory for a long time and poses one of the greatest challenges to early embryo development. Interestingly, IVP has resulted in the strongest economic benefit among ARTs in recent years. Where in 2009 the proportion of embryos produced in vitro accounted for half of the embryos obtained via superovulation (Stroud, 2010), in 2018 there were already twice as many and in 2020 three times as many embryos obtained via IVP compared to superovulation (Viana, 2019, 2021). Accordingly, over the past 40 years, much research has been carried out on the IVP, resulting in an extensive literature (Lonergan, 2007). In the meantime, the technology has reached a level that allows, within a very short time, detection and visualisation of a large number of traits and details, scanning micro-structures and below, displaying complex molecular-genetic and metabolic correlations and spectra of effects and to cluster functional areas, which can be assigned to embryo activity. Nevertheless, practical aspects directly related to the application of IVP should also be prioritized as many analytical details in cattle and basic prerequisites still have to be explored. There are still open questions between the scientific knowledge obtained in this field and its implementation for application regarding important key points of environmental factors, plasticity of the embryos during their early development phase and methodological challenges, as the following examples might illustrate:

1) Quality of in vitro-produced embryos is still significantly lower compared to ex vivo (Merton et al., 2003; Ferré et al., 2020; Ealy et al., 2019).
2) Recipient animals do not necessarily have to have contact with the embryos for the first 7 (up to 16) days (Betteridge et al., 1980).
3) On day 3 after insemination, there was no detectable response of the epithelium in the Fallopian tube to the presence of the embryo (Rodríguez-Alonso et al., 2019).
4) Results from heterologous in vivo culture in sheep oviduct resulted in the development of high-quality bovine embryos (Lazzari et al., 2010).
5) The addition of substantial amounts of oviduct fluid to in vitro culture media negatively affects embryo development (Lopera-Vasquez et al., 2017).

In this context, processes in the Fallopian tube have been repeatedly emphasized as the decisive basis and orientation for the needs of early embryonic development (Leese et al., 2001; Ferré et al., 2020; González-Brusi et al., 2020; Saint-Dizier et al., 2020; Dissanayake et al., 2021).

More than 25 years ago, our working group established an endoscopic approach to access the bovine oviduct in order to perform comparative studies in early embryo development in vitro and in vivo (Besenfelder and Brem, 1998). The idea behind this was to identify factors that optimize the feasibility of ART´s in bovine breeding. Therefore, in the following, the anatomical features and the basic tasks of the oviduct as a physiological template for embryonic development are briefly outlined, before moving on to possibilities that show the use of the Fallopian tube in connection with various environmental conditions and embryo growth. Most of the experiments shown below were done by our team or were performed in collaboration.

Physiological requirements

Reproduction techniques have always been measured against physiological processes in animals. Early embryo development takes place mainly in the oviduct before the embryo reaches the uterus and sends signals to prevent luteolysis. Oocytes and sperm cells enter the Fallopian tube from different directions, meet and fuse via the fertilization process [see Hunter (2008)]. In the Fallopian tube, the embryos undergo further cleavage (Besenfelder et al., 2008), during which some peculiarities take place such as epigenetic tuning (Reik et al., 2001) and timing of major genome activation in the 8 to 16 cell stage (Graf et al., 2014). Mitochondria, with which the oocyte has already been equipped, are adequately distributed to the blastomeres during embryo cleavage and migration phase in the oviduct and first undergo de novo synthesis again in the blastocyst stage (May-Panloup et al., 2005). Transition to the uterus is expected to occur from around day 4 (Croxatto, 2002).

The Fallopian tube is divided into three sections (infundibulum, ampulla, isthmus) which are responsible for embryo nutrition and migration [see Hunter (2012), Yániz et al. (2000)]. The embryo is surrounded by tubal fluid [see Leese (1988); Hunter (2012)], which creates the physical conditions for the processes in the Fallopian tube providing the proper viscosity, pH value and osmolarity (Menezo and Guerin, 1997; Hugentobler et al., 2004). Overall, the oviduct is equipped with secretory and ciliated cells whose ratio and activity are subjected to cyclic changes (Uhrín, 1983). Cells carrying cilia, the circular and longitudinal muscles of the tube ensure cycle-dependent and stage-specific transportation of the embryo along the tubal sections up to the tip of the uterine horn (Ruckebusch and Bayard, 1975; Kölle et al., 2010). The epithelial cells, which have secretory properties, are responsible for the nutritional supply such as carbohydrates, fatty acids, proteins, enzymes and amino acids, and ions (Killian et al., 1989; Menezo and Guerin, 1997; Hugentobler et al., 2007a, b, 2008, 2010; Jordaens et al., 2017). Cytokines and growth factors are thought to have a decisive modulating effect on successful embryogenesis (Neira et al., 2010; Tribulo et al., 2018). Furthermore, microvesicles acting as a carrier for diverse biomolecules (i.e. mRNAs, miRNAs, proteins) are ascribed an important guiding function (Salilew-Wondim et al., 2020).

Impact of the Zona pellucida

A special feature that characterizes the passage of embryos through the Fallopian tube is their covering, the zona pellucida (ZP), which surrounds and protects them (Van Soom et al., 2010). It seems important to note here that the embryos do not have direct contact with the tubal epithelium during the migration phase through the oviduct due to the ZP that surrounds them. The ZP is an important prerequisite for embryo development, passage and later implantation, from which the embryo in the blastocyst stage hatches after uterotubal passage (Negrón-Pérez and Hansen, 2017).

Impact of the oviduct fluid

For all processes taking place in the Fallopian tube, the fluid plays a central physical role. All active substances are either in dissolved form in this fluid or are packed in vesicles and are exchanged between the epithelium and the embryo. The fluid facilitates migration of the embryo through the oviduct. Reports on the amount of oviduct fluid vary between species and are significantly influenced by the status of the ovarian cycle. Overall, it can be assumed in cattle that about 1.0 ml of fluid is produced in the oviduct in 24 h during the estrus phase and 0.1 – 0.2 (0.4) ml/24 h of fluid during diestrus (Roberts et al., 1975; Kavanaugh and Killian, 1988; Killian et al., 1989; Kavanaugh et al., 1992; Dickens and Leese, 1994). Based on the surface area (Yániz et al., 2000) and the density of the secretory cells in the individual tubal sections, the ampulla appears to have the greatest secretory capacity. The Fallopian tube is described as a small tube. However, the inner surface area reaches a considerable extent through multi-layered folds and crypts but there is only a capillary gap for the embryos to migrate and to exchange molecules (Kölle et al., 2010; Yániz et al., 2000). The epithelium of the Fallopian tube is covered with a film of fluid to ensure maximum humidity and to fill up the small and narrow capillary spaces.

Non-physiological environmental conditions

The structure of the Fallopian tube shows that complex processes take place correctly and according to a strict time pattern in this microenvironment. In this context, the diverse approaches in the in vitro production of bovine embryos are to be understood, which make it impossible to follow the entirety of all processes in the oviduct, but which have set priorities for culture imitation in specific biological as well as technical fields:

The aim of using only chemically defined media is to control and ensure proper functioning and to make unbiased statements about changes in the composition of the culture media based on Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP). In addition, these media are offered commercially and comply with international standards and guidelines that are also required by international societies (Van der Valk et al., 2010). Static monocultures are easier to manage, while medium renewal or use of different media during embryo culture (sequential media) aim to remove metabolites from the embryo environment or to adapt to the changing culture requirements of the cleaving embryos [see Ferré et al. (2020)].

Due to the large number of signaling molecules and metabolic peculiarities for in vitro cultures of embryos, media with more complex additives are applied that can be used via co-cultures (Van der Weijden et al., 2017; Carvalho et al., 2017) or obtained directly from the animal (such as follicular fluid, oviduct fluid, serum) (Van der Valk et al., 2018;). Other, more sophisticated, systems mimic the microtubular anatomy of the Fallopian tube (Beebe et al., 2002). Ferraz et al. (2018) aimed to improve the quality and genetic integrity of IVF embryos by developing an instrumental approach to providing a Fallopian tube on a chip. Finally, Rizos and co-worker transferred the whole oviduct into the petri dish for the culture of bovine embryos (Rizos et al., 2010b).

All in all, the in vitro approaches developed over decades focus on a multitude of elementary peculiarities of early embryo development in vitro. However, combining all these achievements and findings appears to be very complex, extremely costly and hitherto impossible and confirms the uniqueness of the Fallopian tube in early embryo development (Leese et al., 2001). Reproductive techniques, especially the in vitro production of bovine embryos, are still far from optimal at this sensitive stage of development (Sirard, 2017, 2021) and should be further refined to be more efficient and sustainable for use in animal breeding (Ealy et al., 2019). In the following, some in vivo approaches are presented in order to meet demands that directly show the influence of environmental changes on the embryonic stages that are used in assisted reproductive technology (González-Brusi et al., 2020).

Physiological environment - reflections of environmental disorders in embryo development

Surgical methods have been applied in various ways (Rowson et al., 1969) and have also been successfully used mainly in sheep (Lazzari et al., 2010), which, however, have not been established or maintained in practice to date. Currently, there is a transvaginal endoscopic access to the bovine oviduct, which has been further developed over several years, constantly improved and adapted to various forms of application (Besenfelder et al., 2010). This technique is now easy to use from a technical and anatomical point of view, is minimally invasive and can be performed within a short time. Moreover, the use of transvaginal endoscopy in cattle, which was first presented more than 25 years ago (Besenfelder and Brem, 1998; Besenfelder et al., 2001), is now being applied more and more to determine environmental influences on early embryo development. This technique is thus available for routine applications in practice as well as for processing of scientific questions (Lonergan and Fair, 2008).

Zona pellucida properties

The nature and properties of the zona pellucida are not only designed for the mechanical stability of the embryo during transport through the Fallopian tube, but also for protection against microorganisms and viruses (Van Soom et al., 2010) and modulate the transzonal

exchange of nutrient substrates and messenger substances in the direction of the embryo and back (Clark, 2010; Kölle et al., 2010).

In order to illustrate these transzonal activities, the zonae pellucida of different stages of bovine embryos were obtained endoscopically from the Fallopian tube, morphometrically recorded and compared to in vitro produced embryos. The analysis was performed using scanning electron microscopy. When measuring the pores on the in vivo zonae, it was shown that up to the morula/blastocyst stage, the number and size of the pores became smaller. Compared to the zonae of in vitro cultured embryos, the surfaces of the zonae obtained ex vivo were almost completely covered with secreted granules and the pores were no longer visible. In contrast, 30 to 50% of in vitro embryos showed partial degeneration of the outer zona layer (Mertens et al., 2007). In order to display the zona in its layers, a 10 - 20 µm hole was drilled with a laser. It was found that in in vitro embryos, the outer layer of the zona accounted for 7.5% of the total zona thickness in the zygote and approximately 10% in the later stages. In contrast, the zonae of ex vivo flushed embryos showed a proportion of 18% in zygotes, which increased up to 30% in progressive stages, which was also associated with the disappearance of the reticular structure. This study shows that in vitro and in vivo zonae show significant differences, which are seen as a crucial influence of transzonal exchanges of nutritional and signaling factors in the oviduct (Mertens et al., 2006, 2007).

Gene expression outlines

Merton et al. (2003) impressively illustrated how the gradual transfer of embryo development from in vivo to the laboratory affects culture results. It was shown how the origin of the oocyte and embryos affects the culture result when embryos are produced either by natural cycle, or by superovulation with/without partial in vitro culture, or by ovum pick-up of slaughterhouse ovaries following in vitro maturation, fertilization and culture. It becomes clear from this presentation that with each successive step performed in vitro, the blastocyst rate decreases significantly (Merton et al., 2003).

In a large-scale study, alternative in vivo and in vitro culture conditions have been examined at the time of fertilisation, major embryonic genome activation and blastocyst formation. Embryos were flushed out of the Fallopian tube at different time points and cultured in vitro up to the blastocyst stage. Vice versa, embryos from in vitro culture were transferred into bovine oviducts at different time points and re-collected from the uterine horn on day 7. Embryos whose development took place exclusively in the animal for 7 days served as a control. From this large-scale study, it was found that changing culture conditions from in vitro to in vivo and vice versa had no effect on embryo development rates (Gad et al., 2012). However, the origin of the oocyte per se had a marked impact on the culture outcome in favor of embryos originating from the oviduct, as also confirmed by other studies (Rizos et al., 2002). The ontological analysis showed mainly contrasting expression patterns in the area of lipid metabolism and oxidative stress between in vivo and in vitro obtained blastocysts. Embryos in the 8-cell stage, around the time of major EGA, were particularly sensitive to cultural environmental change. The study revealed molecular mechanisms and signaling pathways that are especially influenced by in vitro culture (Gad et al., 2012).

It is also known that hormonal stimulation of the ovaries to induce multiple follicles and ovulations also significantly affects the environment in which the embryos develop for the first 7 days. To learn more about the impact of blastocyst development under abnormal endocrine conditions, a study was performed in which heifers were superovulated. On day 2, in half of the animals the oviducts were flushed and these embryos were transferred to heifers having a single ovulation. From both groups, the embryos were flushed on day 7. Here it could be shown that the development up to the morula or blastocyst clearly depends on the endocrine environment. The ratio of recovered blastocysts to morulae was approximately 0.5 in the superovulated heifers, whereas this ratio was 1.8 in the heifers having only one ovulation. These results provide evidence that hormonal use during superovulation negatively impacts embryo cleavage and slows down blastocyst development. Additionally, the embryos were subjected to global gene expression analysis (Bovine Genome GeneChip 100 Format Array).

The superovulation treatment triggered higher cellular and metabolic activities in the embryos. Oxidative phosphorylation genes, which are involved in various metabolic, translational and transcriptional processes, were highly expressed in superstimulated heifers compared to the embryos from unstimulated animals (Gad et al., 2011).

Epigenetic effects

In addition to the different expression signatures in response to environmental effects, these changes in embryos can also be identified using DNA methylation patterns [see Sirard (2021)]. In order to also show epigenetic effects on in vitro/in vivo culture, the DNA methylation pattern of bovine embryos obtained ex vivo was determined in an experimental design before, during and after major embryonic genome activation (EGA). For this purpose, 2-, 8- and 16-cell stage embryos were flushed out of the Fallopian tube and subjected to in vitro culture up to the blastocyst stage and a genome-wide DNA methylation analysis was performed. As expected, not as many blastocysts developed from the flushed 2- and 8-cell embryos compared to the obtained 16-cell stages. These differences were also reflected in the increased number of differentially methylated genomic regions (DMRs) found in blastocysts cultured longer in vitro (from 2- and 8-cell stages) compared to control embryos which developed in vivo. A total of 1623 genomic loci, including imprinted genes, were hypermethylated in blastocysts from all groups (2-, 8- and 16-cell flush), indicating genomic regions sensitive to in vitro culture at each stage of embryo development (Salilew-Wondim et al., 2018).

Chromosome instability (CIN) in cleavage-stage embryos

There are numerous studies showing that chromosomal aberrations occur more frequently in in vitro produced embryos (Viuff et al., 2002). However, little is known about the comparison of chromosomal stability of in vivo and in vitro embryos that are in the cleavage stage. Therefore, the rate and nature of chromosome instability (CIN) between embryos obtained in vivo and cultured in vitro was examined and compared in a study design. Five Holstein-Friesian heifers were used to isolate single blastomeres from embryos obtained from the same animals (i) ex vivo, (ii) produced in vitro (IVM-IVF), and (iii) from ovarian stimulation with subsequent in vitro production (OPU-IVF).

The individual blastomeres isolated from the embryos were processed for genome amplification and hybridized together with the total DNA of the donor cows (mothers) and the bull (father) on Illumina BovineHD BeadChip arrays. In addition, DNA was analyzed from the parents of the cows (paternal and maternal grandparents respectively) and from the parents of the bull. A genome-wide haplotyping and copy number profiling was then carried out to record the genomic structure of 171 individual bovine blastomeres from the three study groups. The blastomeres from the embryos of both in vitro groups (CIN: 69.2% of the OPU-IVF embryos; 84.6% of the IVM-IVF embryos) showed a strong impairment of the genomic stability. In embryos produced in vitro, the frequency of whole chromosomal or segmental aberrations was significantly higher than in those obtained ex vivo (18.8%). Although the occurrence of CIN was also seen in in vivo embryos, this study illustrates that in vitro production exacerbate chromosomal abnormalities during early embryonic development, thereby significantly impairing the developmental competence and viability of the embryos (Tšuiko et al., 2017).

Embryo development in heifers and dairy cows

Similarly, dairy cows are subjected to an enormous metabolic stress and, accordingly, loss of weight. It is well accepted that embryo development is very much affected by these environmental conditions and these restrictions are comparable to various IVF procedures (Sirard, 2017). Most importantly, they have a significant impact on postpuerperal fertility, including early embryonic loss (Diskin and Morris, 2008; Wiltbank et al., 2016). A key function is assigned to progesterone, which plays an important role in both folliculogenesis and the

establishment and maintenance of pregnancy. As a result of high metabolic activity post partum, high milking dairy cows are not able to provide enough progesterone to support embryo implantation [see Lonergan and Sánchez (2020)]. In order to investigate this limitation and thus the importance of progesterone, embryos generated in vitro were endoscopically transferred into the Fallopian tubes of (i) single-ovulating heifers (controls and progesterone tuned), (ii) nulliparous Holstein heifers and postpartum lactating Holstein cows and (iii) in postpartum Holstein cows (dried-off vs. milking cows) and recovered on day 7 post-oestrus.

The experiments in heifers demonstrated that development to the blastocyst stage is not affected by progesterone administration, but at the molecular level, progesterone-induced changes in the embryonic transcriptome may become manifest later in the post-hatch period (Carter et al., 2010). In the second experiment embryos were transferred into nulliparous Holstein heifers and postpartum Holstein dairy cows which differed in their blood serum progesterone concentrations. In the heifers, 79% of the embryos could be recovered, while cows showed a recovery rate of 57%. Based on the number of transferred embryos, approximately 3 times as many blastocysts could be obtained from the heifers compared to the cows. Of the embryos recovered, 33.9 ± 3.6% had developed to the blastocyst stage in heifers compared to 18.3 ± 7.9% in the post partum cows. There was no evidence of a difference in blastocyst quality as illustrated by the total cell count in the blastocysts (71.2 ± 5.7 vs. 67.0 ± 5.3) (Rizos et al., 2010a). Cows in lactation and cows that were not milked after birth showed significant differences in body weight and metabolic profile. In the first 90 days pp, the cows that were not milked had higher body weights and thus a higher BCS, higher insulin, glucose and IGF1 concentrations in the blood, but lower ß-HBA and NEFA values. The transfer of embryos into the oviducts of these animals resulted in a similar recovery rate, however, the development of the embryos to blastocysts was higher in the non-milked cows at day 7 (39.6 vs. 26.3%) and 8 (49.3 vs. 32.6%) (Maillo et al., 2012).

In summary, the reproductive tract of the postpartum lactating dairy cow is less able to support early embryo development compared to non-lactating heifer and this may contribute to low pregnancy rates observed in such animals. All of these experiments indicate that dairy cows display severely impaired environmental conditions for early embryo development and point to the reason for early embryonic mortality (Rizos et al., 2010a; Maillo et al., 2012).

Finally, it should be pointed out that even asynchrony between the embryo and the recipient limits embryo development. To demonstrate this as well, matured and fertilized oocytes were transferred ipsilaterally into each Fallopian tube of day 1 (n=20) or day 3 (n=20) synchronized recipients. The animals were slaughtered on days 3, 6 or 14 after transfer and the developmental status of the embryos was determined. On days 3 and 6 of slaughter, a greater number of degenerated and retarded embryos was found from asynchronous transfer than from synchronous transfer. On day 14 of slaughter, a clear elongation of the embryos could already be determined. However, only 50% of the asynchronous transfers delivered elongated embryos, whereas all recipients with synchronized ET resulted in conceptuses (Rodríguez-Alonso et al., 2020).

Cryo-resistance

Post-freezing results also show the impact of the environmental effects and thus the cryoresistance of embryos originating from different culture systems. Thus, Lazzari et al. showed that bovine IVM/IVF embryos cultured in the ovine oviduct prior to cryopreservation were similar to ex vivo embryos and hardly differed from them in terms of the pregnancy rate after transfer (Lazzari et al., 2010). Lonergan et al. (2003) divided the embryo culture into an in vivo and an in vitro culture period. Bovine embryos, which were first cultured for 4 days in the sheep oviduct and then maintained in vitro, did not differ from their in vitro counter partners after thawing. However, embryos that stayed in the oviduct for the last 4 days were significantly better (Lonergan et al., 2003).

Experiments performed with bovine oocytes obtained in vitro and transferred in the bovine oviduct clearly showed that the duration of in vivo culture of embryos is crucial for survival

after cryopreservation. To demonstrate this fact, matured oocytes were co-incubated with sperm cells in a glass capillary for 3-4 hours before transfer into oviducts of cattle that had just ovulated. A second set of in vitro embryos was transferred to Fallopian tubes at the 4-8 cell stage. Embryos of both groups were retrieved on day 7 and frozen directly (day 7 embryos) or one day later (day 8 embryos). After thawing, it was clearly shown that the duration of the stay in the Fallopian tube affects cryosurvival. The longer the embryos were in the Fallopian tube, the higher their rate of re-expansion and hatching (Havlicek et al., 2010).

Conclusion

There is no doubt that embryos can be obtained in vitro and successfully transferred again. The numerous holistic studies carried out to date are seen as a significant contribution to the understanding of fertility and are just as important in the context of assessing fertility problems. From a qualitative point of view, the question still remains open what factors are needed by an embryo and what conditions have to be provided to a conceptus to enable it to develop into a healthy organism in the long term. It is worth mentioning that not only from a scientific point of view the in vivo and vitro development of bovine embryos are still far apart, but also from a practical point of view there is a large gap between these two culture forms. Attempts to close this gap with fluid from the Fallopian tube failed, since the direct use of oviduct fluid in relevant concentrations for IVC does not provide any advantages for embryo culture (Lopera-Vasquez et al., 2017). Tubal fluid constantly collected during the oviduct culture period to be used as a sequential media replacement is not yet available.

To bridge the gap between in vivo and in vitro produced embryos, new ways and more viable approaches should be sought that identify those components from the complex regulated processes in the Fallopian tube [see Ghersevich et al. (2015)] that represent key molecules (metabolites, messenger substances, stimulants) to be efficiently used for ARTs in breeding and research.

The endoscopic approach described here has now been further expanded. It has been used to determine oestrous cycle-dependent alterations of pro-inflammatory factors in the bovine oviductal epithelium post partum in dairy cows (Neubrand et al., 2021; Pothmann et al., 2022), for the intratubal insemination of sperm from various treatments (Radefeld et al., 2018), as well as the collection of fluid from the oviduct for the determination of stage-specific tubal components (Pothmann et al., 2017; Papp et al., 2019; Havlicek et al., 2022; Neubrand et al., 2022).

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Author contributions

UB: Conceptualization, Writing – original draft, Writing – review & editing; VH: Conceptualization, Writing – original draft, Writing – review & editing.

The potential impact of microplastics on reproduction

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Animal Reproduction 2023, vol 20.

Animal Reproduction

Thematic Section: 39th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

The impact of microplastics on female reproduction and early life

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How to cite: Yang J, Kamstra JH, Legler J, Aardema H. The impact of microplastics on female reproduction and early life. Anim Reprod. 2023;20(2):e20230037. https://doi.org/10.1590/1984-3143-AR2023-0037

Abstract

Plastic pollution in our environment is one of the most important global health concerns right now. Micro- and nanoplastics (MNPs) are taken up by both humans and animals, mainly via food and water, and can pass important epithelial barriers. Indications of plastics in the blood circulation have recently been shown in both humans and farm animals, but standardized methods to quantify the exact levels of MNPs to which we are exposed are currently lacking. Potential hazards of MNPs are being investigated very recently, including the impact that MNPs may have on reproduction. However, studies on mammalian reproduction are scarce, but a wealth of data from aquatic species indicates reproductive effects of MNPs. The first studies in rodent models demonstrate that MNPs reach the gonads after oral exposure and may impact offspring after maternal exposure during the gestational period. These effects may arise from the particles themselves or the presence of plastic contaminants that leach from plastics. Plastic contamination has been detected in human placentas, fetal fluid and the meconium of newborns, indicating the presence of plastics from the very first start of life. Currently there is a lack of studies that investigate the impact of MNP exposure during the periconception and embryonic period, whereas this is an extremely sensitive period that needs considerable attention with the growing amount of plastics in our environment.

Keywords: microplastics, reproduction, genital tract, oocyte, embryo.

Introduction

Plastic pollution is an increasing global health concern, particularly the ever-increasing amount of tiny plastic particles commonly referred to as micro- and nanoplastics (MNPs). Human activities with a major impact on our 'global plastic foot print' are the behavioral and common use of e.g., littering of single-use plastics, packaging and food waste, the release of car tire particles, textile fibers, paint particles, but also inadequate waste management (e.g., open landfills), and environmental conditions (e.g., flooding), including the dispersion of agricultural soils and farmland treated with sewage sludge, and the utilization of agriplastic mulching and fruit protection foams (Huang et al., 2020; Fakour et al., 2021; Yang et al., 2021; Tian et al., 2022). In the environment, plastics remain present for a very long time and disintegrate into smaller pieces, eventually becoming microplastics (MP; < 5 mm) or nanoplastics (NP; < 1 µm), resulting in the current ubiquity of MNPs in global ecosystems. Plastics pollute all environmental compartments, including surface water, sediment, groundwater, soil and the atmosphere (Alimi et al., 2018; Hurley et al., 2018; Ng et al., 2018; Fan et al., 2022; Wang et al., 2021). Recent estimates indicate that we will reach levels of plastic

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Received: March 16, 2023. **Accepted:** June 14, 2023.
Financial support: JY is financed by Chinese Scholarship Council (No. 202207720070).
Conflicts of interest: All authors have no conflicts of interest to declare relevant to this study.

waste of 11,000 Mtons by 2025 (Dong et al., 2023). Unfortunately, once present in the environment, plastics remain in the environment for a long time. Current estimates report that recycling is limited to around 9% of plastic waste, indicating that the remaining 91% of plastic waste enters and remains in the environment (Dong et al., 2023). The widespread presence of MNPs has been demonstrated in many different species and as the environmental levels of MNPs rise, the exposure levels for human, animal, and plant will inevitably increase (Barboza et al., 2018; De-la-Torre, 2020; Azeem et al., 2021). Not surprisingly, plastic pollution has been listed as one of the top 10 environmental problems by the United Nations Environment Programme (UNEP). The UNEP recently issued a statement warning that plastic leaking into farmer's fields may endanger food security (UNEP, 2022). There are many different types of plastics present in the environment. The most frequently observed polymer types in, for example, freshwater are in decreasing order polyethylene (PE) and polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC) and polyethylene terephthalate (PET) (Koelmans et al., 2019). The presence of MNPs has widely been detected and also appears to be present on human skin and hair, in saliva and sputum, and in stool (Schwabl et al., 2019; Abbasi and Turner, 2021; Huang et al., 2022). Recent data indicates that MPs are even present in blood of humans, and in human urine, breastmilk and placenta, indicating that MPs circulate through the body (Leslie et al., 2022; Pironti et al., 2023; Ragusa et al., 2021, 2022a, 2022b). However, the current lack of standardized methods to analyze the levels of MNPs in different matrices makes it complicated to determine exposure levels of MNP in humans and animals, and to compare different studies (Koelmans et al., 2019; van Mourik et al., 2021). Since, plastics have also been detected in the meconium of new-borns (Zhang et al., 2021), it seems no longer a question whether early life is exposed. To this end, it is important to investigate the potential effects of MNPs on human and animal reproduction, in particular, as some rodent studies show effects of maternal MNP exposure on reproduction (Figure 1). This review aims to give an overview on the current knowledge of the potential effects that MNPs have on female reproduction and early life.

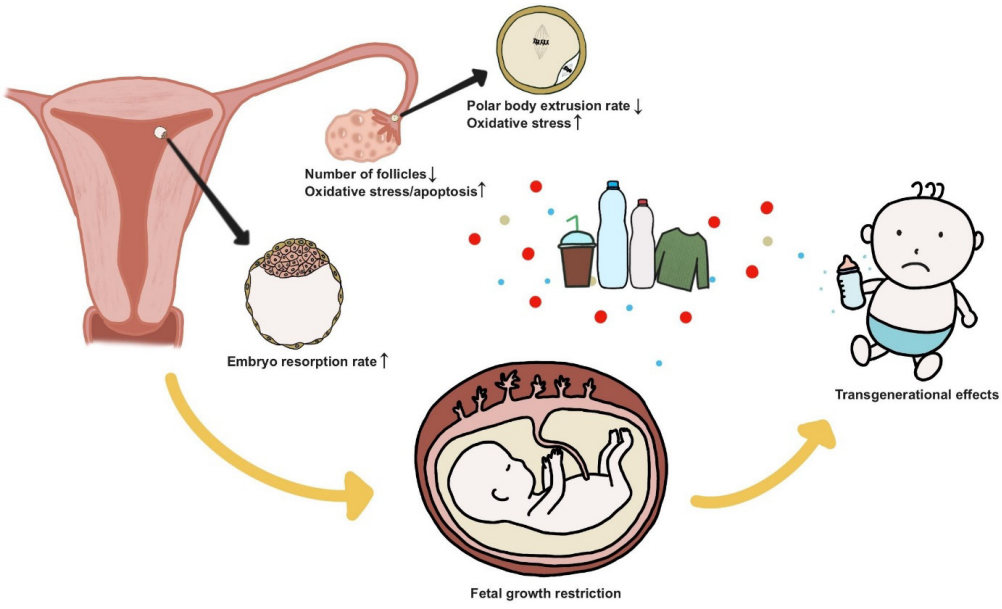

Figure 1. Potential impact of micro and nanoplastics (MNPs) on female reproduction and early life.

Thus far the rodent model is the most often used model to study the potential impact of maternal MNP exposure in mammals. Studies in rodents show that MNPs reach the genital tract and appear to decrease the number of follicles, while ovarian oxidative stress and apoptosis in granulosa cells are increased. Furthermore, maternal MNP exposure increased the oxidative stress in oocytes and reduced polar body extrusion. In pregnant mice oral MNP exposure resulted in a higher embryo resorption rate and placental and fetal growth

restriction, indicating a potential carry-over effect of maternal MNP exposure on offspring. Chemical toxins leaking from MNPs have been demonstrated in human fetal fluid, which stresses the importance to investigate the impact of MNPs on future generations.

Plastic particles are detected in many species including livestock

Plastic particles can enter the body through ingestion, inhalation, and dermal contact. Ingestion appears to be the main exposure route (Galloway, 2015). MNPs appear to be widely distributed throughout the food chain and have been found in many consumption products like salt, sugar, honey, soft drinks, beer, milk, fruit and drinking water (Pironti et al., 2021). Currently, MNPs and plastic contaminants have been detected in a wide range of different species, with a primary focus on aquatic species, including zooplankton, amphipods, worms, molluscs, fish, sea birds, and whales (Sá et al., 2018). Already back in 2008 it has been demonstrated that the Mussel, Mytilus Edulis, ingested MPs of 3 and 9.6 µm from seawater in an experimental setup (Browne et al., 2008). Mussel ingested MPs ended up in the gut, entered the circulation after 3 days and were present in the mussel until 48 days later (Browne et al., 2008). Lately, the presence of MNPs has also been demonstrated in terrestrial animals, including chickens, sheep, pigs and cattle and appears to originate from their direct (food) environment via plastic waste, via agricultural fields with plastic mulch, food fed to chickens and livestock (Huerta Lwanga et al., 2017; Beriot et al., 2021; Wu et al., 2021), or MNP uptake from water resources (Koelmans et al., 2019). The UN Environment Program (UNEP, 2022) recently issued a statement warning that plastic leaking into farmer's fields may endanger food security. Plastics have also been detected in the blood of livestock in a pilot study, demonstrating that MNPs can enter the body after uptake and are able to pass important epithelial barriers and may potentially reach different organs including the genital tract (van der Veen et al., 2022).

Potential risks of microplastics for cells

Pristine, commercially available spherical, PS in different sizes is currently the most often used plastic type for *in vivo* and *in vitro* studies that investigate the impact of MNPs on cells. Reported cell responses after exposure to MNPs, are an inflammatory and oxidative stress response, which may potentially result in cell damage and apoptosis, depend on dose, exposure time and the type of plastic to which the cells are exposed.

MNPs that enter the body can harm cells via three potential hazardous routes;

(1) Physiological effects attributed to the MNP particle itself;

In vivo experimental studies on MNP toxicity in mammalian and other animal species demonstrate systemic exposure and adverse health effects that are related to physical effects of particles, including immunomodulation and apoptosis, the production of reactive oxygen species and peroxidative damage, impaired neurotransmission, metabolic effects as well as continuous inflammatory activation (Vethaak and Legler, 2021). Responses that are in general also observed after in vitro exposures to MNPs (Silva Brito et al., 2022).

(2) Hazardous reactions from the leakage of plastic substances from the MNPs;

Additives such as plasticizers, antioxidants, flame retardants, pigments etc. incorporated during the manufacture of plastic may be leached into body tissues, resulting in induced changes or bioaccumulation (Issac and Kandasubramanian, 2021). Moreover, many of these additives have been reported to be endocrine disrupting chemicals associated with effects on reproduction via estrogenic or (anti)androgenic properties (Wiesinger et al., 2021).

(3) Noxious reactions to pollutants and/or pathogens absorbed by microplastics;

Metals or chemicals that stick to the surface of the plastic, and depending on the physical and chemical properties of a polymer such as diffusivity, surface area, crystalline, and

hydrophobic nature the quantity and type are affected (Rochman et al., 2019; Issac and Kandasubramanian, 2021). Besides this, small plastics may be rapidly colonized by microorganisms including harmful pathogens. Biofilms on MNPs have been reported to be different from the ambient environment, meaning that if ingested, plastics may promote a shift in the structure of microorganisms present in for example the digestive tract (Fackelmann and Sommer, 2019; Santos et al., 2022).

MNPs that enter the blood circulation can be transported to tissues in the body. The active uptake of nanoparticles of cells is by endocytosis, via two main mechanisms either phagocytosis, for particles of > 500 nm, and pinocytosis for smaller particles via a clathrin or caveolin mediated route (Zhao and Stenzel, 2018). Both active and passive transfer are potential options for the uptake of MNPs. Smaller NPs can enter cells via passive transfer when they are small enough to pass the pores of membranes (Lai et al., 2022). MNPs taken up by cells accumulate in the cytoplasm, and phagosomes containing particles may fuse with endosomes and end up in lysosomes (Galloway, 2015). Even particles with a size of around 50 µm appear to be able to enter tissues, which was reported after a 30-day oral MP exposure in mice and subsequent MP uptake in the gut (Deng et al., 2020). MPs with a size between 4-20 µm show the highest level of accumulation in the body, according to a recent review of Dong et al. (2023), possibly due to more potential uptake routes for cells, as both phagocytosis and (macro) pinocytosis are options for uptake. However, another reason for the seemingly lower abundance of smaller NPs in tissues could be the limitation of proper detection methods for the smaller MNPs. An *in vivo* study in a zebrafish cell line demonstrated a dynamin dependent endocytosis route for 50 nm NPs and phagocytosis for 1 µm MPs, both the 50 nm and 1 µm MNPs were distributed towards lysosomes after cellular uptake (Sendra et al., 2021).

Apart from the three previously described potential hazards of MNPs, another potential danger is the enhancing impact that MNPs may have during co-exposures with other stressors. In zebrafish, combined exposure to MNPs and an infection with a pathogen significantly decreased the survival rate. Exposure of zebrafish to the pathogen A. hydrophila, alone resulted in a survival rate of 76%, while combined with 50 nm or 1 µm MNPs the survival rate of zebrafish dropped to respectively 29% and 34%, whereas exposure to MNPs alone had no effect on the survival rate of zebrafish larvae (Sendra et al., 2021). Likewise, the survival rate of immunodeficient zebrafish larvae significantly decreased from 92% towards 6% and 43% in the presence of respectively 50 nm and 1 µm MNPs (Sendra et al., 2021). Furthermore, in a study of He et al. in zebrafish, it was shown that the presence of NPs, and not MPs in this study, enhanced the impact of the toxic compound triphenyl phosphate on the male and female gonad and increased the negative effect on spermatogenesis and oogenesis (He et al., 2021). The enhancing impact of MNPs in the presence of other stressors in the examples above, highlights the importance to investigate the potential effect of MNPs also during co-exposures.

Microplastics enter the testis and ovary

The information that we have today on the potential impact of MNPs on reproduction is predominantly based on aquatic species and soil fauna and, more recently, also studies in rodents (Yuan et al., 2022). Oral ingestion of MNPs appears to be the major route for uptake of MNPs and once ingested in the gut, MNPs can travel through the body (Galloway, 2015). In a study with oviparous zebrafish, 30 days of oral exposure to 70 nm pristine PS resulted in the accumulation of the NPs in intestine, brain and liver, and an abundant accumulation in male gonads (Sarasamma et al., 2020). Furthermore, in Poecilia Reticulata, used as a representative for viviparous fish and better known as Guppy, 30 days of oral exposure of pregnant fish to 23 nm PS-NPs at 50 µg/L resulted in the accumulation of NPs up until the level of the embryo (Malafaia et al., 2022), indicating that the NPs passed important barriers including the genital tract. In zebrafish, 21-day waterborne exposure to 2 mg/L of 46 nm PS-NPs and 5.8 µm PS-MPs had no effect on the ovarian index, which is a major indicator of reproductive activity in fish (He et al., 2021). However, exposure of zebrafish to 46 nm NPs reduced the number of spawned eggs, while 5.8 µm MPs had no effect on spawning number (He et al., 2021).

Studies in rodents reveal that MNPs accumulate in several tissues in the body after oral uptake, including the lung, spleen, liver, kidney, intestine, brain, and uterus (da Silva Brito et al., 2022). The cellular uptake and distribution of nanoparticles significantly depends on the size, shape, stiffness and surface area of the particle (Zhao and Stenzel, 2018), which is presumably also the case for the uptake and distribution of MNPs in cells. In studies with rodents that were orally exposed, from 20-44 days, to varying PS-MNPs sizes of 50 nm, 80 nm, 500 nm, 4 µm, 5 µm and 10 µm at different concentrations (from 0.015 until 30 mg/kg/d), PS-MNP accumulation in testis and ovarian tissue was demonstrated (Liu et al., 2022; Wei et al., 2022; Jin et al., 2021; Hou et al., 2021a, 2021b). This indicates that MNPs are able to pass the blood-testis and blood-follicle barrier. In male mice a 30 and 45-day exposure period to 5 µm PS-MPs (0.1 mg/d) resulted in a reduction in sperm count and increased number of abnormal sperm cells (Wei et al., 2022). These data are in line with a study by Hou et al. where a 35-day exposure period to 5 µm PS-MPs via water, with a daily consumption of 6-7 mL per mouse (at concentrations of 0.1, 1 or 10 mg/L) resulted in a drop of sperm counts, but in this study only the highest concentrations resulted in an abnormal sperm morphology (Hou et al., 2021a. In a study with male mice that were orally exposed to different sizes of PS-MNPs (10 mg/ml) of 500 nm, 4 and 10 µm during 28 days, all groups showed accumulation of PS-MNPs in the testis, a reduced level of testosterone in serum, and a reduction in viable sperm count and an increased number of abnormal sperm cells (Jin et al., 2021). In all three studies exposure to PS-MNPs was linked with an increased inflammatory response in testis tissue, which may be linked to the observed negative impact on sperm cells (Wei et al., 2022; Hou et al., 2021a; Jin et al., 2021). In the study of Wei et al. both male and female mice were orally exposed to 5 µm PS-MPs (0.1 mg/d) for two days, which resulted in a higher accumulation of PS-MPs in ovaries in comparison to testes (Wei et al., 2022).

In rats it has been demonstrated that after oral exposure to 500 nm PS-NPs (concentrations of 0.015, 0.15 or 1.5 mg/kg/d) for 90 days the PS-NPs entered the ovary and reached the cytoplasm of granulosa cells (Hou et al., 2021b). Rats exposed to PS-MNPs showed a reduced number of follicles and AMH levels, and a dose-dependent higher level of malonyldialdehyde and reduced levels of the anti-oxidants glutathione peroxidase, catalase and superoxide dismutase in ovarian tissue, which is indicative for an oxidative stress response (Hou et al., 2021b; An et al., 2021). Furthermore, oral exposure to PS-MNPs at a concentration of 1.5 mg/kg/d increased the levels of the cytokines 1L-18 and IL-1β, suggesting an inflammatory response, and the by MNP induced oxidative stress appeared to activate the Wnt/β-Catenin signalling pathway and apoptosis in granulosa cells (Hou et al., 2021b; An et al., 2021). When granulosa cells were *in vitro* exposed to 500 nm PS-NPs (0.025 mg/ml) for 24h this resulted in an elevated expression of NLRP3 and cleaved caspase-1 and increased level of apoptosis (Hou et al., 2021b). The aforementioned studies suggest potential negative impacts of PS-MNPs on both testis and ovary after oral exposures, and indicate inflammatory and oxidative stress responses in cells after exposure to PS-MNPs. However, we should take into account that the levels of PS-MNPs used in these studies may be far beyond the levels that are present in blood, and that there may be significant cross species differences. Nevertheless, the demonstrated accumulation of MNPs in the ovary and passage of MNPs over the blood-follicle barrier suggests that they are able to reach the oocyte in the ovarian follicle and request for further investigations into the potential effects of MNPs on the oocyte.

Do microplastics pose a risk for oocytes?

Whether MNPs affect or enter the oocyte presumably depends on the potency of the granulosa and cumulus cells to temper and prevent the exposure of the oocyte to MNPs, and the extracellular matrix that surrounds the oocyte. The potential mechanisms through which MNPs can enter the oocyte are via 1) passage over the zona pellucida or via 2) gap junctional exchange from cumulus cells towards the oocyte. Oocytes of all animal species are surrounded by a thick extracellular matrix, called the vitelline envelop in most animals, which helps to prevent polyspermy and protects the oocyte and early embryo against influences from outside, like the undesired invasion of viruses (Vanroose et al., 2000). In mammalian species this matrix is called the zona pellucida and it consists of glycoproteins that form a

network of overlapping meshes (Clarke, 2022). However, apart from the necessary protective shield of the oocyte and embryo against potential harm from the environment, it also prevents the passage of metabolites needed for growth and development. To this end, there is a connection established from the early stages of follicular growth onwards, most presumably from the primary stage when the zona pellucida is formed, which bridges the contact between cumulus cells and the oocyte via transzonal projections (TZPs) formed by cellular extensions that penetrate the zona pellucida (Clarke, 2022). These TZPs appear to be essential for the transfer of small metabolites (< 1 kDa), like pyruvate from cumulus cells as oocytes lack the machinery to break down glucose, RNA transfer and factors like GDF 9 and BMP 15 that support the bilateral communication between the oocyte and adjacent cumulus cells (Gilchrist et al., 2008; Albertini and Barrett, 2003; Macaulay et al., 2014). The average diameter of pores in the zona pellucida of bovine oocytes appears to be 182 nm, suggesting that passage of particles with a size just below 200 nm may be possible (Vanroose et al., 2000). In pig and rat oocytes, these pores appear to be around 50-100 nm (Stringfellow, 2009). The zona pellucida surrounding the oocyte and the transzonal projections most presumably dictate the maximum size of the MNPs that are able to enter the oocyte. Interestingly, the TZPs and thus the exchange and communication between cumulus cells and the oocyte appears to be tightly regulated by hormonal influences, of follicle-stimulating hormone (FSH) and anti-mullerian hormone, which was described in a recent review paper (Buratini et al., 2022). However, apart from the necessity of TZPs between cumulus cells and the oocyte, together forming the cumulus-oocyte-complex (COC), the TZPs also pose a risk for passage of undesired particles like potentially MNPs, which may not be able to pass the zona pellucida without the TZPs. Until the end of final oocyte maturation when cumulus cells are retracted, the TZPs connect cumulus cells and the oocyte.

After mice were orally exposed to large PS-MPs of 5.0-5.9 µm there was a reduction observed in ovarian size in the study of Wei et al. (Wei et al., 2022), but this was not the case in the studies of Liu et al. (2022) where PS-NPs of 790 nm were used, which may relate to a size-dependent responsiveness of organs to MNPs. When mice were exposed to PS-NPs of 790 nm by oral exposure for 35 days (30 mg/kg), this resulted in the accumulation of PS-NPs in uterus and ovary and resulted in a reduction of the number of antral follicles, whereas the ovarian index (ovary/body weight; an indicator of developmental status of gonad) was not affected (Liu et al., 2022). This data is in line with the studies of Wei et al. and Hou et al. where oral gavage during respectively 44 days to 5.0-5.9 µm PS-MPs (0.1 mg per mouse/d) and 90 days exposure to 0.50 µm PS-NPs 0.15 mg/kg/d and 1.5 mg/kg/d resulted in a reduced number of follicles (Wei et al., 2022; Hou et al., 2021b). Furthermore, in the study of Wei et al. a reduced hormone level of estradiol was reported and an increased level of FSH and luteinizing hormone (LH), but whether this is an indirect effect due to the lower number of follicles and lack of negative feedback on the hypothalamic-pituitary system or a direct effect needs further investigation (Wei et al., 2022). In contrast, the study of Liu et al. (Liu et al., 2022) reported a reduction in antral follicles, but no effect on FSH and LH levels after exposure to 790 nm PS-NPs. Additionally, after exposure to PE-MPs of 10-40 µm for 7 times via intratracheal administration (6, 60 µg/administration), or 90 days of oral gavage of 40 µm PE-MPs (3.75, 16, 60 mg/kg/d) there was no specific ovarian histopathological lesion found (Park et al., 2020; Han et al., 2021). This could indicate that not only the size, but also the type of plastic, PS versus PE, may have a distinct impact on the outcome for ovarian exposure. Furthermore, in the above reported studies pristine MNPs were used, whereas current data demonstrate that more environmental relevant weathered MNPs appear to have a more negative impact on cells (van den Berg et al., 2022). The type and size of MNPs, including pristine versus weathered MNPs, and their potential impact on reproduction certainly needs more attention.

MNPs also appear to affect oocyte competence, as oral exposure of mice to 790 nm PS-NPs resulted in a decrease in first polar body extrusion (PBE) rate, which is the parameter for successful oocyte nuclear maturation at the end of maturation (Liu et al., 2022). In line with this, exposure to 50 nm PS-NPs at 100 µg/mL during *in vitro* oocyte maturation in mice also resulted in a decreased PBE rate whereas no effects were observed at lower exposure concentrations (10, 50 µg/mL) (Park et al., 2022). In addition, after exposure to MNPs the

oxidative stress and inflammatory response were increased in the ovary and granulosa cells, and the level of apoptosis was increased in granulosa cells (Liu et al., 2022; Wei et al., 2022; Hou et al., 2021b). Furthermore, there was an increase of oxidative stress in the oocyte after MNPs exposure (Liu et al., 2022; Park et al., 2022). However, in the study of Liu et al., there were no differences observed in embryo cleavage and blastocyst rates after maternal exposure to MNPs, which suggests that oocyte developmental competence was not affected (Liu et al., 2022). Recent pilot data in our group demonstrate that bovine COCs take up 50 and 200 nm PS-NPs, which stresses the importance to investigate the effect of MNPs on oocyte developmental competence (Yang et al., 2022).

Co-exposure may increase the potential impact of MNPs. In a study with zebrafish, co-exposure of diethylstilbestrol (DES), a synthetic estrogen, and NPs to zebrafish during 21 days exacerbated the impact of the solitary exposures on the gonads, and affected the hormone levels of estradiol and testosterone, decreased the cumulative egg number and reduced the fertilization and hatchability and significantly increased the number of abnormalities in offspring (Lin et al., 2023). Furthermore, a recent paper in mice demonstrated that metabolic stress significantly increased the impact of MNPs (Okamura et al., 2023). This indicates that apart from studying the direct impact of MNPs on early life, it is of crucial importance to investigate the effect of MNPs in co-exposures and in a more physiological context with other potential (metabolic) stressors.

Are embryos hampered by MNPs?

In copepods it has been demonstrated that 50 nm, 500 nm and 6 µm pristine PS-MNPs were ingested and affected fertility resulting in dose-dependent mortality in the F0 and F1 generations, and impaired fecundity in response to 500 nm and 6 µm PS-MNPs (Lee et al., 2013).

In female and male zebrafish fed a diet that contained 42 nm PS-NPs, a dose of 1 mg per gram fish, there was no effect observed on reproduction, despite the induced reduction of glutathione reductase in brain, muscle and testis. However, a worrying finding was that the 42 nm PS-NPs were maternally transferred to embryos, during the 7-day parental exposure period that started the day after mating, and were found in the yolk sac, gastrointestinal tract, liver and pancreas of the offspring (Pitt et al., 2018). Another study in zebrafish demonstrated that large PS-MPs of around 160 µm were not able to pass the chorion barrier of zebrafish embryos, and only a few of the smaller 100 nm PS-NPs passed the chorion and were visible in the periovular fluid, with the majority of the lipophilic MNPs adhering to the chorion (Duan et al., 2020). It was only after hatching that 100 nm NPs were taken up by the zebrafish larvae and accumulated in brain, gills, blood, liver and digestive tract. Zebrafish larvae that were exposed to 50nm NPs and 1 µm MPs for 24h at 120 hours post fertilization (hpf), demonstrated accumulation of the MNPs in mainly the gut, but also in the skin and caudal fin, and the smaller 50 nm NPs were also transferred to the eyes of the zebrafish (Sendra et al., 2021). Furthermore, in vivo exposure of zebrafish larvae at 120 hpf resulted in an increased oxidative stress response in mainly the stomach and gut (Sendra et al., 2021). In the viviparous fish Poecilia reticulata the transfer of PS-NPs, after a 30-day exposure period of females to 23 nm PS-NPs at 50 µg/L, towards embryos was demonstrated and resulted in a reduced pregnancy success rate and offspring number (Malafaia et al., 2022). Although the outcomes of these studies with aquatic species at the level of the embryo are interesting, they may considerably differ from the impact that MNPs have on mammalian embryos. Unfortunately, until now there are only a few mammalian studies reported, which are based on rodent models, that investigated the impact of MNPs during gestation.

Female and male mice receiving a daily gavage of 40-48 µm PE-MPs, at a dosage of 3.75, 15, or 60 mg/kg body weight during 90 days, and mated from day 80 till 89, were not hampered in their reproduction, but there was a slightly lower birth weight of the pups after exposure of the parents to the highest dosage of PE-MPs (Park et al., 2020). Another study where pregnant mice (n=9) were intraperitoneally injected with 10 µm PS-MPs during day 5.5 and 7.5 of gestation, with a dosage of in total 250 µg in a 200 µL saline solution, resulted in a significantly higher rate of implanted embryos and a higher percentage of embryo resorption at day 11.5

after MP exposure in comparison to the control (Hu et al., 2021). Resorption of embryos was also observed in another study after intravenous injection of 300 µg 900 nm PS-NPs at days 9.5 and 15 of gestation, but was not observed after intravenous injection of 60 nm PS-NPs in this study (Nie et al., 2021). However, due to the relatively low numbers of mice that were used in both studies it is difficult to draw strong conclusions out of it. There is one early study that investigated the impact of a 24h exposure period to mixed-sized PS-NPs of 40 till 120 nm in 2-cell stage mouse embryos, where no effects were observed on the competence of embryos to develop into a blastocyst after NP exposure (Bosman et al., 2005). At this point there are, as far as we know, no other studies available that investigated the impact of MNPs during the periconception and early embryonic period. Currently, there is a major lack in information on the potential effects of MNPs during early embryonic development in mammals, while this is an extremely sensitive period in early life. Also indicated by a study in chick embryos where exposure to 60 or 900 nm PS-NPs resulted in malformations and congenital abnormalities after early embryonic exposures (Nie et al., 2021).

Microplastics enter the placenta and reach the fetus

Plastic particles may also reach and pass the blood-placenta barrier, suggested by the presence of plastics associated chemicals, recognized as endocrine disruptors, in human amniotic fluid and the human placenta (Dusza et al., 2022; Dusza et al., 2019; Ragusa et al., 2021, 2022a). MPs of 5-10 µm in size have been detected in different parts of the human placenta, on the fetal side, maternal side, and in the chorioamniotic membranes (Ragusa et al., 2021). In a recent study of Ragusa et al., MPs were located via transmission electron microscope in pericytes and endothelial cells in the chorionic villi, indicating that MPs are able to reach the fetal blood circulation (Ragusa et al., 2022a). Despite the still limited number of studies on the effect of MNPs on the placenta, the findings are concerning. In *in vivo* mice studies, oral exposure to different sizes of PS-MNPs (50 nm, 100 nm, 5 µm) did not affect the placenta weight (Aghaei et al., 2022, 2023; Chen et al., 2023). Whereas in another study with mice intravenous injection of 60 nm or 900nm PS-NPs, of 300 µg at day 9.5 and 15 of gestation, resulted in a decreased placenta weight and reduced weight of the pups at birth, with an earlier exposure to PS-NPs resulting in increased negative effects on placental and birth weight (Nie et al., 2021). Furthermore, the study of Nie et al., demonstrated more cellular damage in both the placental tissue and fetus after exposure to the smaller NPs of 60 nm in comparison to the 900 nm PS-NPs (Nie et al., 2021). The disruption of immune balance in the placenta, demonstrated by the alteration of macrophage polarization, the population of T cells and cytokine secretion on the fetal-maternal interface-decidua was also observed after exposure to 10 µm PS-MPs (Hu et al., 2021). The placenta plays a critical role for the fetus during gestation in the transport of nutrients and oxygen delivery, which is limited by the maternal blood flow, from the mother to the fetus (Ridder et al., 2019). Exposure to 10 µm PS-MPs during the pre-implantation period decreases the diameter of uterine arterioles from the first trimester onwards; and during gestation exposure to 100 nm PS-NPs results in disruption of the coagulation cascade (Hu et al., 2021). The demonstrated changes in uterine arterioles and the effect of MNPs on the coagulation cascade, may both affect the uterine blood supply and may impact fetal growth and survival. Furthermore, exposure to 50 nm PS-NPs or 5 µm PS-MPs during gestation resulted in a shorter umbilical cord length, which has been related to fetal growth restriction (Aghaei et al., 2022). Fetal growth restriction, has indeed been observed after maternal exposures to MNPs (Aghaei et al., 2022; Chen et al., 2023; Ridder et al., 2019). Moreover, it has been suggested that MNPs may alter placental metabolism, which is related to fetal nutrient supply (Chen et al., 2023; Aghaei et al., 2023). The Barker hypothesis, already introduced in 1995, advocates that an abnormal maternal environment during the susceptible periconception and gestational period can predispose offspring for metabolic aberrances and disease in later life future diseases (Barker, 1995). To this end, further investigation on the potential impact of MNPs on early life are vital.

Transgenerational effects of MNPs on offspring via maternal exposure

Transgenerational effects have been observed in the past with many different stressors, such as stress, radiation and chemical pollution (Babenko et al., 2015; Fitz-James and Cavalli, 2022; Rebuzzini et al., 2022). Even more, these effects are thought to be inherited via epigenetic mechanisms (Fitz-James and Cavalli, 2022), as many of such stressors do not have the capacity to induce genetic damage. Few studies up to now have been conducted to assess genuine transgenerational effects, i.e. an effect not caused by any carry-over effects of MNP exposures, and mostly in aquatic species (Junaid et al., 2023). On the other hand, multigenerational effects, which might involve carry-over effect of maternal PS-MNPs exposure during gestation and lactation to the next generation, have been reported with effects that include aberrant physiologic behaviour in progeny, reproduction and a higher risk for metabolic disorders in studies with rodents (Liu et al., 2022; Jeong et al., 2022; Wei et al., 2022; Luo et al., 2019a, 2019b). Similar to the toxic mode of action of these particles, these multigenerational effects could be due to altered physiology due to particle toxicity, pathogens of chemicals. Although more extensive research to study genuine transgenerational effects of MNPs are warranted, these results indicate that progeny may not be protected against the harm of MNPs.

Discussion

Despite the world wide health concern of plastic pollution in the environment, there is currently a lack of information on the impact of MNPs on reproduction. Exposure studies in aquatic and rodent models demonstrate that MNPs travel through the body and can reach the gonads. MNPs are able to pass important epithelial barriers and can reach the circulation, also in humans and farm animals. Until now, most studies are performed with pristine PS particles and studies in mice demonstrate that exposure to MNPs can result in an inflammatory and oxidative stress response of the ovary, including the granulosa cells. Depending on the size of the MNP some mice studies show a dose-dependent effect on oocyte nuclear maturation, and smaller NPs appear to be taken up by the oocyte (Figure 1). However, more environmental relevant MNPs, weathered and plastics of distinct sizes and shapes might have a different impact than what has been found in pristine PS particles (Vethaak and Legler, 2021). Today's information on the impact of MNPs during the embryonic phase is very scarce. However, in a chicken study it was demonstrated that early exposures to PS-NPs dose-dependently resulted in congenital malformations. Furthermore, zebrafish models demonstrate transfer to embryos after maternal MNP exposure, and based on mice models there are indications for a multigenerational effect on offspring after dams were exposed to MNPs. Plastics are indeed taken up by placental cells and are able to pass the blood-placental barrier and reach the fetus, which was demonstrated by the presence of plastic contaminants in human fetal fluid (Dusza et al., 2022; Ragusa et al., 2021, 2022a; Dusza et al., 2019). However a major lack is the current absence of peer reviewed articles with other than rodent models that report on the impact that MNP exposures may have on mammalian reproduction. Our own pilot data demonstrate that 50 and 200 nm PS-NPs are also taken up by the bovine COC, and may indeed impact early life (Yang et al., 2022). Based on the similarities between bovine and human reproduction during oocyte and embryo development, this may also apply to human COCs.

Conclusion

It is not a question whether we need to be concerned about early life exposures to plastics and the potential effects of plastics exposure on the next generation, as plastics already invade early life processes (Figure 1). However, currently a major burden is the lack of studies that investigate the impact of MNP exposure during the periconception and embryonic period, whereas this is an extremely sensitive period that needs considerable attention. Therefore, with the daily expanding plastic pollution there is an urgent need to better understand the impact of MNPs on reproduction, early life and next generations.

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Author contributions

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The contribution of assisted reproductive technologies and reproduction management to reducing the CO_2 equivalent emissions of dairy and beef industry

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Animal Reproduction 2023, vol 20.

Animal Reproduction

Thematic Section: 39th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Applying assisted reproductive technology and reproductive management to reduce CO_2-equivalent emission in dairy and beef cattle: a review

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How to cite: Baruselli PS, Abreu LÂ, Paula VR, Carvalho B, Gricio EA, Mori FK, Rebeis LM, Albertini S, Souza AH, D'Occhio M. Applying assisted reproductive technology and reproductive management to reduce CO_2-equivalent emission in dairy and beef cattle: a review. Anim Reprod. 2023;20(2):e20230060. https://doi.org/10.1590/1984-3143-AR2023-0060

Abstract

Methane emission from beef and dairy cattle combined contributes around 4.5-5.0% of total anthropogenic global methane. In addition to enteric methane (CH_4) produced by the rumen, cattle production also contributes carbon dioxide (CO_2) (feed), nitrous oxide (N_2O) (feed production, manure) and other CH_4 (manure) to the total greenhouse gas (GHG) budget of beef and dairy production systems. The relative contribution in standard dairy systems is typically enteric CH_4 58%, feed 29% and manure 10%. Herds with low production efficiency can have an enteric CH_4 contribution up to 90%. Digestibility of feed can impact CH_4 emission intensity. Low fertility herds also have a greater enteric CH_4 contribution. Animals with good feed conversion efficiency have a lower emission intensity of CH_4/kg of meat or milk. Feed efficient heifers tend to be lean and have delayed puberty. Fertility is a major driver of profit in both beef and dairy cattle, and it is highly important to apply multi-trait selection when shifting herds towards improved efficiency and reduced CH_4. Single nucleotide polymorphisms (SNPs) have been identified for feed efficiency in cattle and are used in genomic selection. SNPs can be utilized in artificial insemination and embryo transfer to increase the proportion of cattle that have the attributes of efficiency, fertility and reduced enteric CH_4. Prepubertal heifers genomically selected for favourable traits can have oocytes recovered to produce IVF embryos. Reproductive technology is predicted to be increasingly adopted to reduce generation interval and accelerate the rate of genetic gain for efficiency, fertility and low CH_4 in cattle. The relatively high contribution of cattle to anthropogenic global methane has focussed attention on strategies to reduce enteric CH_4 without compromising efficiency and fertility. Assisted reproductive technology has an important role in achieving the goal of multiplying and distributing cattle that have good efficiency, fertility and low CH_4.

Keywords: cattle, enteric methane, efficiency, fertility, assisted reproductive technology.

Introduction

The global population of beef and dairy cattle combined is approximately 1.5 billion. Amongst domestic herbivores globally, cattle contribute about 20% of meat and 85% of milk. The global

*Corresponding author: barusell@usp.br
Received: May 16, 2023. Accepted: July 31, 2023.
Financial support: FAPESP (15/19563-0 and 19/14679-1), CNPq (306759/2016-0 and 315978/2021-0) and CAPES (Grant number 001).colarfnaqui
Conflict of interest: The authors have no conflict of interest to declare.

demand for meat and milk is projected to increase by 57% and 48%, respectively, between 2005 and 2050 (Alexandratos and Bruinsma, 2012). Cattle, therefore, will continue to have a very important role in future global food security (Davis and White, 2020). Beef and dairy production occur across diverse environments and in both extensive and intensive systems (Faverdin et al., 2022). Extensive cattle grazing is found in rangelands and savannas that are suited to low-input and low-cost animal production. Intensive beef and dairy systems utilize, and add value to, feed sources that are either unsuitable or surplus to human consumption. Grazing lands cover about 25% of the global landmass (Mottet et al., 2018) and intensive beef accounts for <15% of global beef production (Mottet et al., 2017). The environmental footprint of cattle production has received increased attention globally (Knapp et al., 2014; Faverdin et al., 2022). Methane emission from cattle has been recognised for around 30 years (Johnson and Johnson, 1995) and has become a particular focus as cattle contribute around 4.5-5.0% of total anthropogenic global methane (Wallace et al., 2015; Hayes et al., 2016; de Haas et al., 2021; Faverdin et al., 2022; Hossein-Zadeh, 2022; Galyean and Hales, 2023). Most methane produced by cattle is from enteric fermentation of complex carbohydrates into simple sugars by methanogenic protozoa (Bowen et al., 2020). The biology and function of the rumen has been well reviewed (Ross et al., 2013; Knapp et al., 2014). The ability to digest cellulolytic material into usable energy and protein is arguably the greatest advantage but also the greatest disadvantage of cattle. The relative abundance of ruminal methanogenic and non-methanogenic microbes influence the amount of methane produced by an individual animal (Bowen et al., 2020). The population of ruminal microbes can now be determined by microbial gene abundance (Roehe et al., 2016).

Assisted reproductive technologies can have a major impact on improving productivity in beef and dairy cattle. Artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) increase the rate of dissemination of animals with traits that have high genetic merit and high productive capacity. However, the mature technologies of AI and MOET do not increase the rate of genetic gain from one generation to the next. The latter is controlled by generation interval which is relatively long in cattle (Schefers and Weigel, 2012; Kasinathan et al. 2015). Generation interval can be shortened in cattle by utilising oocytes from heifers early in life to produce IVF embryos (Baruselli et al., 2016; Baldassarre and Bordignon, 2018). This review seeks to demonstrate how assisted reproductive technology (ART) and reproductive management can be used to generate cattle that have improved efficiency and produce less methane.

Reproductive efficiency in cattle and application of artificial insemination to improve efficiency and reduce methane emission

In beef cattle, the cow-calf unit utilizes approximately 70% of resources. Selection for reproductive efficiency therefore has a major bearing on both efficiency and profitability. With high reproductive efficiency, fewer cows are required to produce the next generation of calves, and this reduces resource requirement, herd methane production, and costs (Hegarty and McEwan, 2010). Also, reproductively inefficient cows are removed from herds. In a United States beef production system, an improvement in reproductive efficiency (0.5 to 1 calves/year) resulted in a 34% reduction in water use, 44% reduction in land use, and 39% reduction in the CO_2-equivalent (CO_2-eq) footprint (Davis and White, 2020). ART can be incorporated into beef breeding programs to further improve efficiency and reduce CO_2-eq emission intensity. In Brazil, the use of timed artificial insemination (TAI) in a breeding herd reduced age at first calving from 48 to 24 months and increased weaning rate from 60% to 80% compared with natural mating (Abreu et al., 2022). There was a 37.7% reduction in pasture required and 85.4% reduction in CO_2-eq to produce 400 calves (Abreu et al., 2022). The CO_2-eq was calculated according to livestock units (1 LU=450 kg of live weight) and a stocking rate of 1 LU per hectare of pasture was estimated to produce calves (Figueiredo et al., 2017). The low reproductive efficiency system (natural mating) emitted 3,714.5 tons of CO_2-eq per year while the high reproductive efficiency system (TAI) emitted 2,311.3 tons of CO_2-eq annually. The TAI system generated US$84,196 in credit for reducing CO_2-eq emissions (quoted at US$60 per 1-ton CO_2-eq). TAI has been applied in beef heifers to reduce age at first pregnancy and calving (Baruselli et al., 2017) which impacts lifetime reproductive efficiency and CO_2-eq emissions. TAI

can also be utilized to manage inter-calving intervals so that cows produce a calf annually (Sá et al., 2013; Baruselli et al., 2018a).

The same basic principles addressed above apply in dairy cattle (Hutchinson et al., 2013). For example, lowering the age at first calving and culling frequency reduced the number of replacement heifers needed and enteric methane emission per unit of kg energy-corrected milk (CH_4/ECM; Knapp et al., 2014). Improving the fertility of dairy herds can potentially reduce methane emission by up to 25% (Garnworthy, 2004). We recently studied the influence of calving interval (CI, i.e. reproductive efficiency) on the CO_2-eq footprint of lactating dairy cows using life cycle assessment methodology (Abreu et al., 2023). A comparison was made between production and CO_2-eq/milk (corrected for fat and protein content) of cows with a CI of 13 or 15 months. The lactation period was estimated at 11 and 13 months for cows with a CI of 13 or 15 months, respectively (Cole and Null, 2009; Biassus et al., 2010). Total greenhouse gas emissions for 1 kg of milk (CO_2-eq/milk) was 0.657 when the CI index was 13 months and 0.703 (7% increase) when the CI index was 15 months.

Embryo technology to mitigate methane emission

Dairy cattle can suffer heat stress (HS) during summer which decreases dry matter intake (DMI), daily gain, milk yield, and fertility (Kadzere et al., 2002; Hansen, 2007). During HS, milk production decreases more than dry matter intake which increases the CO_2-eq emission/kg energy-corrected milk (Rhoads et al., 2009). HS contributes to culling and death of cows (St-Pierre et al., 2003). The reduction in fertility is associated with altered ovarian folliculogenesis and oviductal function and increased embryonic mortality. The latter can be managed during periods of HS by replacing natural mating and artificial insemination (AI) with the transfer of either *in vivo* or *in vitro* derived embryos to cows on day 7 of the estrous cycle (Hansen, 2007; Baruselli et al., 2020).

We developed a simulation model which compared the use of AI or embryo transfer (ET) in HS dairy cows (Figure 1). The model assumed that pregnancy per AI (P/AI) and P/ET during HS were around 17 and 40%, respectively, and the service rate was 60% for AI and 50% for ET (ET was performed only on animals with a corpus luteum) (Baruselli et al., 2018b). The pregnancy rate following 105 days of breeding was 34.6% for AI and 53.1% for ET (53.6% increase). Cows subjected to AI had a greater number of days open (59.3 days) than cows exposed to ET (52.5 days) after the beginning of the breeding program. This shows that it is possible to increase the 21-day pregnancy rate by eight percentage points using ET in place of AI in HS dairy cows. As noted earlier, shorter inter-calving intervals are associated with a reduced CO_2-eq budget in cattle.

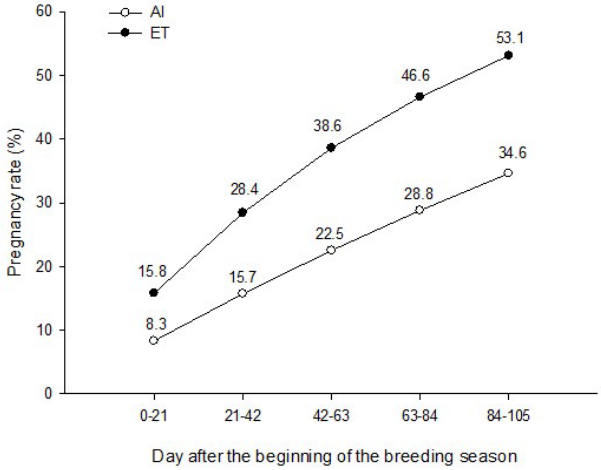

Figure 1. Survival curve assuming 60% service rate, 17% conception rate (P/AI) and 10% pregnancy rate every 21 days in repeat breeders and heat stressed dairy cows during 105-day AI program (pregnancy loss of 19% between 30 and 60 days gestation). For ET program, it was assumed 50% service rate (ET only in recipients with a corpus luteum), 40% conception rate (P/ET) and 15.3% pregnancy rate every 21 days in heat stressed dairy cows during a 105-day ET program (pregnancy loss of 21% between 30 and 60 days gestation). Adapted from Baruselli et al. (2018b).

As noted earlier in this review, the mature technologies of AI and MOET do not increase the rate of genetic gain. The latter is controlled by generation interval which is relatively long in cattle (Schefers and Weigel, 2012; Kasinathan et al., 2015). Generation interval can be shortened in cattle by utilizing oocytes from heifers early in life. Waves of follicular growth occur before birth and in the first weeks after birth in heifers (Evans et al., 1994a, 1994b; Monteiro et al., 2009). Oocytes can be recovered before birth (velogenesis; Betteridge et al., 1989; Georges and Massey, 1991; Kauffold et al., 2005) and well before puberty (Onuma et al., 1970; Baruselli et al., 2016), and used to generate viable embryos in the laboratory using *in vitro* embryo production (IVEP) (Baruselli et al., 2021). Prepubertal heifers show a good ovarian follicular response to FSH superstimulation and a relatively large number of oocytes can be retrieved for IVEP (Baruselli et al., 2021). IVEP is less efficient for oocytes from young heifers compared with mature heifers and cows and further research is needed to optimize IVEP in prepubertal heifers (Baruselli et al., 2021). Notwithstanding, IVEP with oocytes from young heifers has emerged as a fundamental enabling technology for the exploitation of genomic selection to produce cattle defined by efficiency, fertility and low CH_4 emission.

Balancing feed efficiency in meat and milk production with fertility and low CO_2-eq emission

Cattle consume a relatively large amount of biomass and have a low feed conversion ratio compared with other livestock (FAO, 2018; Mottet et al., 2018). The provision of feed typically accounts for 70-80% of production costs in both extensive and intensive systems (Mottet et al., 2018). There is considerable interest, therefore, in identifying and multiplying cattle that have improved feed efficiency (Løvendahl et al., 2018; Davis and White, 2020). This applies to both extensive and intensive systems (Hietala and Juga, 2017; Kava et al., 2023). Associations between feed efficiency, methane production, and sustainability, have been known for more than 20 years (Arthur and Herd, 2005; Nkrumah et al., 2006; Freetly and Brown-Brandl, 2013). The relatively high heritability of growth and feed efficiency in cattle was recognised some 70 years ago and subsequently confirmed (Knapp and Nordskog, 1946; Berry and Crowley, 2013; Gonzalez-Recio et al., 2014; Sypniewski et al., 2021).

More recently, single nucleotide polymorphisms (SNPs) have been identified for feed efficiency in cattle and have been used in genomic selection (Arthur, 2015; Seabury et al., 2017; Sypniewski et al., 2021; Madilindi et al., 2022; Buss et al., 2023). As noted earlier, the relative abundance of ruminal acetogenic and methanogenic microbes influences methane emission by individual animals. There is a significant host effect on the ruminal microbe population, and it has been proposed that microbial gene abundance can be used to select cattle for feed efficiency and growth (Roehe et al., 2016). The genome of cattle can influence the population of ruminal microbes and hence the ruminal microbe genome profile which determines methane production (Difford et al., 2018; O'Hara et al., 2020; Gonzalez-Recio et al., 2023). Characterization of the ruminal microbe gene profile has been proposed as an alternative to expensive, time consuming methods for measuring feed efficiency in individual cattle (Arthur and Herd, 2005; Basarab et al., 2013; Kenny et al., 2018; Terry et al., 2021).

Growth and feed efficiency genes show single nucleotide polymorphism (Abo-Ismail et al., 2013; Seabury et al., 2017; Madilindi et al., 2022; Buss et al., 2023). Methane emission also shows single nucleotide polymorphism in cattle (Sarghale et al., 2020). The advent of molecular gene markers has created the opportunity to accurately identify cattle with desirable genes and to then use ART to rapidly multiply and disseminate cattle with improved feed efficiency and growth performance. Efficient cattle were reported in one study to have reduced CH_4 (g/day) and CO_2-equivalent (g/day) emissions (Callegaro et al., 2022). The breeding technology used to generate efficient cattle will be governed by the production system and resources available. For example, AI and ET are already utilized in intensive dairy systems. Artificial insemination can be adopted in extensive beef systems as demonstrated in Latin America (Baruselli et al., 2004; Ferraz et al., 2012; Sartori et al., 2016; Mapletoft et al., 2018; Bó et al., 2018). Low-input, low-cost beef systems (North and South America, northern Australia, South Asia, Sub-Saharan Africa) will continue to rely on natural mating. For these regions, central breeding facilities will utilize genomic selection and ART to produce male embryos and/or bulls for dissemination for natural mating.

Whilst feed efficiency is undoubtedly a commercially important trait in beef and dairy cattle, selection for feed efficiency should not be at the expense of other important traits (Mu et al., 2016). As this review has argued, fertility has a major impact on enterprise productivity and profit in both beef and dairy systems. Studies in young growing British and European (*Bos taurus*) bulls consistently showed negative associations between feed efficiency and fertility measures including testicular growth and morphology and the characteristics of seminal plasma and spermatozoa (Awda et al., 2013; Fontoura et al., 2016; Montanholi et al., 2016; Bourgon et al., 2018). In contrast, a study in growing composite bulls (*Bos taurus* x *Bos indicus*) found that fertility measures did not differ for bulls of different feed efficiency (Kowalski et al., 2017).

Heifers with improved feed efficiency were reported to be leaner and reached puberty later than heifers with lesser feed efficiency (Randel and Welsh, 2013). In another study, heifers with good feed efficiency attained puberty earlier than heifers with poorer feed efficiency (Canal et al., 2020). Other studies in female cattle have also shown either a negative effect of feed efficiency on fertility (Mu et al., 2016; Ferreira et al., 2018) or no effect (Crowley et al., 2011; Davis et al., 2016). A study in dairy cows under commercial conditions reported that cows with high feed efficiency had a greater inter-calving interval (Vallimont et al., 2013). Dairy cattle selected for milk yield and feed efficiency had a reduced methane budget resulting from increased milk yield (Knapp et al. 2014). The impact of this selection strategy in an intensive dairy system was estimated to be a reduction of 9-19% in CO_2-eq emission/kg energy-corrected milk (Knapp et al. 2014). In another study in dairy cows, selection based on genetic potential for milk production was associated with a decline in fertility, an increase in non-productive cows, and overall increase in CO_2-eq emission for the production system (O'Brien et al., 2010). Another study in dairy cows reported low genetic correlations between methane production and fertility traits (Zetouni et al., 2018). Given the contrasting reports there is a need for further studies on feed efficiency, methane production, and lifetime fertility in cattle. The above studies have also demonstrated the importance of multi-trait selection in cattle breeding programs and the need to balance feed efficiency with other commercially important traits, in particular fertility (Bonamy et al., 2019).

Enteric methane in production system life cycle assessment

Enteric methane forms part of the broader greenhouse gas (GHG) budget of beef and dairy production systems (Ibidhi and Calsamiglia, 2020). The broader GHG budget includes methane, nitrous oxide (N_2O) and CO_2 emission from manure, feed production, vehicles and transport, and other plant and equipment. The total GHG budget of a production system is determined by life cycle assessment (LCA) methodology standardized by ISO 14040 (ISO, 2006a) and ISO 14044 (ISO, 2006b) (de Vries et al., 2015; Kyttä et al., 2022). The relative contribution of different components of production systems to the GHG budget can vary greatly for different beef and dairy systems. One estimate for milk production was enteric methane 58.5% (CH_4), feed production 29.4% (CO_2, N_2O) and manure 9.5% (CH_4, N_2O; FAO, 2018). The relative contribution of enteric methane can reach 91% in low efficiency systems (Chhabra et al., 2013). The digestibility of feed can also have a major impact on enteric methane contribution to the overall GHG budget (Pinares-Patiño et al., 2007; FAO, 2019; Eugéne et al., 2021; Congio et al., 2022). Herds with high fertility and high production efficiency have a reduced GHG budget (Strandén et al., 2022). In low fertility herds, replacement heifers can contribute up to 27% to the GHG budget (Garnworthy, 2004). The contribution of replacement heifers decreases to 10-12% in high fertility herds. High fertility herds with fewer replacement heifers require less feed production and have reduced manure, which lowers methane and nitrous oxide emission.

Conclusions and future direction

The global attention on enteric CH_4 production in cattle requires a response that involves collaboration between researchers and industry. Future generations of cattle will be characterized by better efficiency and fertility, which may reduce CH_4 emission intensity. This will result from balanced multi-trait selection. There has been progress in the discovery of SNPs for efficiency and methane emission in cattle. These SNPs will be incorporated into assisted

reproductive technology such as AI and ET for targeted multiplication and dispersal of cattle with defined production and environmental credentials. The urgency in moving to the next generation of cattle will see an increase in the production of embryos from genomically defined prepubertal heifers. This will reduce generation interval and accelerate the rate of genetic improvement to cattle defined by better efficiency and fertility and lower CH_4 emission. The opportunity for cattle to be a part of ecosystem management was recently highlighted (Thompson et al., 2023). The challenge remains to communicate the importance of cattle for food security and the environment (Manzano et al., 2023).

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Environmental effects on developmental plasticity in conservation biology

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Animal Reproduction

Thematic Section: 39th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Biobanks, offspring fitness and the influence of developmental plasticity in conservation biology

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How to cite: Holt WV. Biobanks, offspring fitness and the influence of developmental plasticity in conservation biology. Anim Reprod. 2023;20(2):e20230026. https://doi.org/10.1590/1984-3143-AR2023-0026

Abstract

Mitigation of the widely known threats to the world's biodiversity is difficult, despite the strategies and actions proposed by international agreements such as the United Nations Framework Convention on Climate Change (UNFCCC) and the Convention on Biological Diversity (CBD). Nevertheless, many scientists devote their time and effort to finding and implementing various solutions to the problem. One potential way forward that is gaining popularity involves the establishment of biobank programs aimed at preserving and storing germplasm from threatened species, and then using it to support the future viability and health of threatened populations. This involves developing and using assisted reproductive technologies to achieve their goals. Despite considerable advances in the effectiveness of reproductive technologies, differences between the reproductive behavior and physiology of widely differing taxonomic groups mean that this approach cannot be applied with equal success to many species. Moreover, evidence that epigenetic influences and developmental plasticity, whereby it is now understood that embryonic development, and subsequent health in later life, can be affected by peri-conceptional environmental conditions, is raising the possibility that cryopreservation methods themselves may have to be reviewed and revised when planning the biobanks. Here, I describe the benefits and problems associated with germplasm biobanking across various species, but also offer some realistic assessments of current progress and applications.

Keywords: biodiversity, extinction, cryopreservation, amphibians, corals, genetic resources.

Introduction

The past century has seen the rise of important and severe environmental changes, most of which are attributable to human activities. Well known examples include changes in land and water use, the industrialization of agriculture, fishing and tourism, not to mention global warming and climate change. These influences have had profound, and usually negative, impacts on every aspect of the world's biodiversity. For up to date and detailed information see the reports of the International Panel on Climate Change (IPCC): (https://www.ipcc.ch/report/sixth-assessment-report-working-group-ii/). Global warming and climate change have far-reaching effects on human and animal populations alike, often facilitating the transmission of viral, bacterial and fungal diseases that affect a multitude of species. Apart from the upsurge in pandemics, such as the ongoing and devastating Covid 19 outbreak across the world, many amphibian species have been decimated by the global spread of the lethal and highly infectious fungal disease, Chytridiomycosis (Rollins-Smith, 2020). A 2004 assessment of global amphibian populations (Stuart et al., 2004) estimated that 34 species had disappeared completely and nearly 2000 were under threat of extinction. Unfortunately, these figures have increased over the last two decades, and the number of amphibian species threatened with extinction is now about 2,500 (out of a total of 7,296, assessed by the International Union for the Conservation of Nature's "Redlist" (IUCN, 2022). As discussed later in this paper, considerable scientific

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Received: February 19, 2023. Accepted: July 5, 2023.
Financial support: None.
Conflicts of interest: The author has no conflict of interest to declare.

and technical effort has been invested in rescuing some of these amphibian populations via assisted breeding techniques, and important advances have been made over the past 3 decades.

A recent assessment of global fish diversity (Miranda et al., 2022) estimated that the number of recognised fish species is now over 36,000, of which about 20,000 are regarded as being under some level of threat. Significantly this assessment showed that freshwater fishes are more vulnerable than marine species to the impacts of pollution, much of which is attributable (among other reasons) to contamination from agriculture, mining effluents containing heavy metals, pesticides, chemicals involved in the manufacture of both plastics and cosmetics, as well as the release of human sewage with its content of endocrine disrupting chemicals (Kanda, 2019, Marlatt et al., 2022) and pharmaceuticals (Sanderson et al., 2004). Many of these chemicals are known for their undesirable impacts on embryonic development in wild species and impaired reproductive functions, not only in fishes (Tyler and Jobling, 2008), but also in birds, reptiles, amphibians and many marine crustaceans (Beyer et al., 2022).

As reproductive biologists we may not be able to play a major role in mitigating or reversing the negative impacts of climate change, global warming and industrial practices, but we can try to understand why and how these processes exert their impacts. Moreover, reproductive biotechnologists may be able to contribute their skills to the mitigation efforts, especially through technologies such as cryobiology, the biobanking of reproductive cells and tissues for future use in supporting, or even regenerating, threatened populations (Holt and Comizzoli, 2021). Some of the available technologies have their origins in agriculture and fisheries, where it is now commonplace to use artificial insemination (AI) for breeding domestic mammals and some birds, and to use cryopreserved spermatozoa for breeding over 200 commercially important fish species (Boe et al., 2021; Mayer, 2019). However, it is very clear that for wild species, sperm cryopreservation techniques have to be refined for every target species (Comizzoli and Holt, 2022; Holt and Comizzoli, 2021). The diversity of species, their reproductive behaviors, social systems and respective anatomical peculiarities, is responsible for the technological difficulties that may be encountered when attempting to develop a novel cryopreservation protocol for a conservation-sensitive species. In addition to this caveat, it is now becoming apparent that some reproductive technologies, such as gamete cryopreservation, may inadvertently lead to inadequate post-fertilization offspring growth and survival, while disease resistance may also be suboptimal.

Some of these considerations would have been almost unthinkable as little as 40 years ago, when the possibility that assisted reproductive technologies (ARTs) might cause alterations in the way that genomic DNA sequences are transcribed and translated (i.e., the science of epigenetics) was still unrecognized. The purpose of this review is twofold: on the one hand to outline the undoubted conservation benefits that have, and will be, provided by the use of ARTs, but on the other, to examine the growing evidence that reproductive fitness in some species may be negatively impacted by the laboratory procedures involved.

Principles of biobanking for conservation

Wildlife conservation actions are undertaken at a wide range of scales. These include projects that aim to improve the biodiversity in a local area such as a lake, woodland or even a small garden, up to the establishment of government-backed national parks or marine protected areas. In some cases, large and small zoos, wildlife parks and aquariums focus their attention on a particular species or group of species, and establish a captive breeding program that aims to minimize the deleterious risks associated with the inbreeding that eventually occurs in small populations (Howell et al., 2021). The captive breeding programs are usually managed carefully, often with different organizations collaborating across international boundaries, in order to ensure the prevention of inbreeding. However, such “live” breeding programs have to operate in a world where animals are not immortal, and it may eventually be impossible to form genetically ideal breeding pairs if one of the breeding animals dies. This situation can be overcome to some extent by establishing a so-called genetic resource bank (GRB) (Holt et al., 1996; Watson and Holt, 2001), or biobank (Comizzoli, 2017; Herrick et al., 2016), which contain stored and preserved germplasm (e.g., spermatozoa, oocytes and

embryos), and might still be used for breeding, thus contributing to the genetic aims, long after the animal's death (See Figure 1).

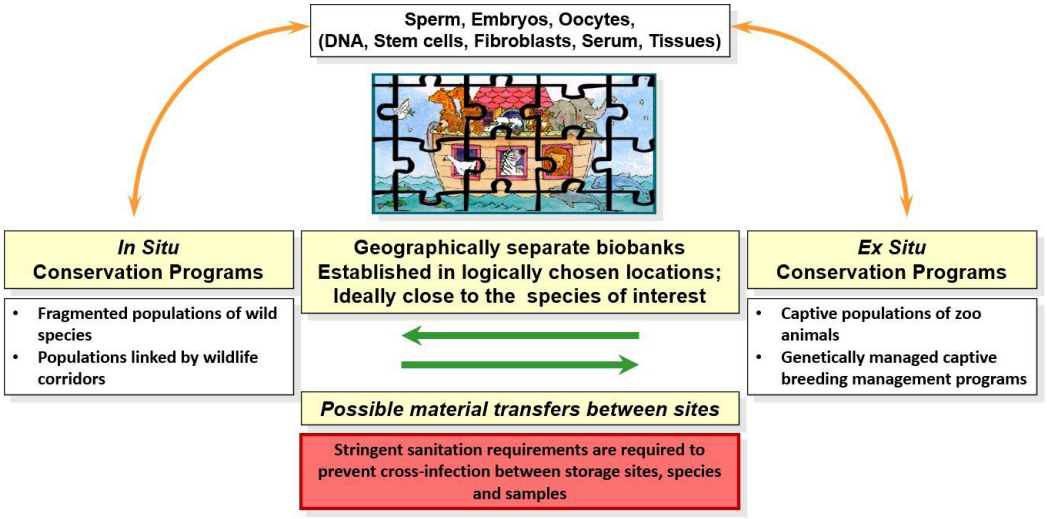

Figure 1. Schematic illustration of the general principles of biobanking for species conservation. Long-term storage of gametes, embryos and other genetic material is accomplished via cryopreservation or other methods such as desiccation. Cell storage takes place at multiple sites in a system of containers for sanitary, geographical and regulatory limitations. Biobanks located in different countries/continents, must comply with stringent international import/export regulations, transportation and political climates that preclude translocations and exchanges. It is also worth noting that storage capability of genetic materials near to the relevant site(s) of eventual use, would represent considerable logistical advantages, by avoiding the expense and difficulty of transporting them by land or air. An additional, advantage of keeping the stored materials close to the relevant wild populations would mean that in the future, when ART techniques for wild species are more reliable and efficient, the biobank may also be considered as a feasible analogue of a natural wildlife corridor, thus permitting isolated and fragmented populations to exchange and share their genetics via the use of advanced technology.

There are other reasons for attempting to preserve germplasm from threatened species, including capturing genetic snapshots of the dwindling populations before they succumb to extinction. While a database of DNA sequences is undoubtedly useful for evolutionary research, it would miss the diverse associated RNA species that are now known to be functionally important for many aspects of development and survival.

Maintaining a carefully curated collection of viable cells and tissues contributes towards one of the most important aims of conservation biology, namely, to support and augment the ever-disappearing genetic diversity that occurs within endangered populations (Comizzoli, 2017; Holt and Comizzoli, 2021). Viable germplasm in effective long-term storage facilities is expected to remain intact and undamaged for hundreds, if not thousands, of years if maintained appropriately (Whittingham et al., 1977), which means that the genetic properties of gametes and embryos (and stem cells when available) could still be used to counter the deleterious impacts of inbreeding long after the donor animal has died. The use of such conservation-focused biobanks is therefore realistic, provided the appropriate preservation technologies (for example, conventional freezing, vitrification and drying (Anzalone et al., 2018)), and appropriate assisted fertilization methods (including techniques to synchronize the availability of sperm and eggs, and in the case of species with internal fertilization and embryonic development (for example, mammals, birds,, some fishes and reptiles), the ability to synchronize embryonic development with the physiological status of the recipient female reproductive tract. Ensuring that these technologies are all available when required is not trivial, but neither is it impossible. The highly endangered black-footed ferret (*Mustela nigripes*) was rescued from near extinction (Santymire, 2016; Santymire et al., 2014) by intensive and successful research, including the combined use of sperm cryopreservation, biobanking and artificial insemination to restore genetic diversity (Howard et al., 2016).

The financial burden of establishing species and conservation-directed biobanks, in support of associated captive breeding programs, has typically proven to be a strong disincentive for commercial zoos, given that it is not possible to plan for a future time when such support will no longer be needed. In addition to supporting the live, captive populations, of necessity the biobanking costs will include a maintenance requirement for buildings, equipment, electrical power, laboratory reagents and staff. Howell and his colleagues recently published a unique economic cost-benefit analysis in which the financial costs were considered in the context of conserving a particular amphibian species, the Oregon spotted frog (*Rana pretiosa*), for which robust financial data exists (Howell et al., 2021).

The authors of that study made a very strong case for the inclusion of biobanking as a conservation strategy in support of captive breeding programs. It is worth quoting the first paragraph of the discussion section within their paper, which concisely sums up their findings very well.

> We present powerful theoretical support for the proposition that biobanking technology should be considered as a complementary tool in captive breeding programs. The use of such technology would address the two most common criticisms of captive breeding: cost and loss of wild-type genetic diversity and fitness. We showed this with models integrating biobanking scenarios into estimates of costs and inbreeding in the captive management of a colony of R. pretiosa. These models demonstrated huge reductions in costs and required sizes of the live colony while achieving reductions in the rate of inbreeding sufficient to achieve and exceed the suggested standard for captive genetic diversity (< 10% loss in heterozygosity after 100 years). (Howell et al., 2021, p. 1).

Although this study was focused on amphibian conservation, the theoretical modelling is more widely applicable and will be of considerable use to biologists justifying similar programs for other taxonomic groups.

The widespread storage, exchange and use of frozen semen and embryos from commercially important mammalian, avian and fish species relies on the observance of strict health and hygiene practices for its success. National and international regulations have been established in order to prevent the inadvertent transmission of diseases (for example, see the detailed regulations that govern terrestrial species from OIE Terrestrial Animal Health Code, 2018). These regulations are based on a detailed understanding of the risks associated with natural and assisted animal breeding practices. However, applying these principles to the biobanking of germplasm from threatened species is typically much more difficult. The risks of disease transmission between species are heightened if the frozen germplasm samples from different species, carrying different and possibly unknown micro-organisms, are maintained within the same liquid nitrogen container (Bielanski, 2014). This complicates, and probably precludes, the popular concept of a cryopreservation container filled with frozen samples from a range of different species. The establishment of biobanks in support of particular species or populations is nevertheless entirely feasible if samples are prepared and stored as near as possible to the population requiring genetic support. In essence this means that, where possible, it is best to avoid transporting samples across international borders and fulfilling the sometimes onerous and complex import and export requirements imposed by animal health authorities.

An emerging issue that is likely to gain more importance in the future concerns the use of animal-derived products such as egg yolk, skimmed milk and bovine serum albumin, in cryopreservation media. The regulatory frameworks governing the international movement of germplasm for agriculture and fisheries require assurances that these products are not themselves infected with transmissible disease vectors. As biobanking becomes more widely applied across a range of wild species, practitioners will need to remain mindful of these risks and avoid them where possible by finding alternative, possibly synthetic, additives. To date this search is still ongoing as the reliability of substitute preparations is usually questionable (for further information see references (Akhter et al., 2021; Kucuk et al., 2021; Li et al., 2005; Sicchieri et al., 2021).

What is the current status of mammalian biobanking for conservation?

Currently, two well-organized wildlife biobanks containing frozen semen have contributed to the genetic support of threatened wild animal populations; as discussed above; one is focused on the black-footed ferret recovery program, and the other supports the Giant panda breeding program in China.

The Giant panda breeding program in China has amassed sufficient data to examine success rates of AI with frozen semen and to compare them with natural breeding outcomes (Li et al., 2017). An analysis published in 2012 found that the success rate of AI with frozen semen up to the year 2011 was about 25%; (5/20 AI events with cryopreserved semen resulted in live offspring). This is slightly higher than a later estimate (18.5%) published in 2017 (Li et al., 2017), which was based on 65 AI events, carried out using both fresh and cryopreserved semen, although lower than the birth rate of 60.7% obtained after natural mating. Successful use of frozen semen for the giant panda AI has also been achieved outside China (Comizzoli, 2020).

Interestingly, the factors determining AI success in the giant panda (Huang et al., 2012; Li et al., 2017, Martin-Wintle et al., 2019; Spindler et al., 2006) have revealed that the quality of frozen semen is less important than the timing of the insemination itself.

As with many other mammals, matching the timing of insemination and the occurrence of ovulation requires precise information about ovarian status. The lifespan of frozen-thawed semen within the female reproductive tract may be very short (possibly less than 4 hours in some species, and therefore if ovulation occurs 5 hours or more after insemination, fertilization would not take place. While information about ovulation status can be obtained using ultrasound scanning in readily handled domestic species, this is not practical in most wild species that, of necessity, require sedation or anesthesia for such examinations (See, for example, a recent study of brown bear ovarian dynamics (Torii et al., 2020). Successful AI in the giant panda is known to depend on very precise estimations of ovarian status, either by the regular examination of cellular morphology in vaginal smears (Durrant et al., 1998) or the regular (8-hourly intervals) measurement of urinary oestrogen, progesterone and luteinizing hormone (Martin-Wintle et al., 2019). Greatest success using the hormonal measurement method was obtained when AI was performed within 40 hours of the decline in urinary oestrogen concentration.

A number of European zoos have supported an EAZA (European Association of Zoos and Aquariums) initiative to ensure that the genetic diversity of wild felid species is protected and preserved (Fernandez-Gonzalez et al., 2019) in a dedicated biobank. Male and female gonads from 74 females and 67 males, from 36 different zoological institutions, have been donated to the project, which is organized by the Leibniz Institute for Zoo & Wildlife Research in Berlin, Germany, whose reproductive biology department has a specific focus on biobanking for conservation (https://www.izw-berlin.de/en/biobanking-for-assisted-reproduction-techniques.html.). The 2019 publication (Fernandez-Gonzalez et al., 2019) explains that epididymal spermatozoa have been collected from 42 males (63%) and that samples from 36 males (54%) were suitable for freezing. This project has used the spermatozoa and oocytes to generate 47 felid embryos by oocyte maturation and *in vitro* fertilization, including one Asiatic golden cat embryo and 8 from two Northern Chinese leopards. As this is an ongoing project, and can be regarded as an active biobank, these numbers are expected to increase as more zoos donate samples.

Research progress leading towards the establishment of biobanks for other mammalian species is still at an early stage, possibly because of the technical difficulties associated with effective sperm cryopreservation despite extensive research efforts (see, for example, the difficult problems around cryopreserving marsupial spermatozoa (Johnston, 2019; Johnston and Holt, 2019). Some researchers have argued that because sperm cryopreservation is so unlikely to succeed by the use of traditional cooling and freezing methods, it is now time to develop alternative approaches to genetic biobanking based on the use of cloning and stem cell technologies (Rodger, 2019). It is at least theoretically possible to derive gametes from induced pluripotent stem cells (iPSCs), which could then be used for the production of embryos from a range of somatic cells. (Dicks et al., 2021; Stanton et al., 2019; Sukparangsi et al., 2022). Some biobanks, see for example, a project in Thailand aimed at the development of a biobank

specifically to support fishing cat (*Prionailurus viverrinus*) conservation (Sukparangsi et al., 2022), are employing this approach as their primary option and the same principles are being explored in relation to the conservation of some aquatic species (Rivers et al., 2020; Temkin and Spyropoulos, 2014). It will be instructive to see whether mammalian offspring that are generated by the exploitation of stem cell technologies show comparable viability and survival as those produced more conventionally. There is currently too little evidence to make informed predictions around this topic, but a number of authors have pointed out the risks associated with epigenetic reprogramming and development (Borges and Pereira, 2019; Wang et al., 2020). Cloning itself, or somatic cell nuclear transfer (SCNT), was popularly expected to revolutionize and facilitate the breeding of endangered species, using the frozen somatic cells contained in biobanks as nuclear donors. However, this approach has become less popular because of problems with the requisite nuclear reprogramming, as well as matching the species of the available nuclear donor with oocytes of the same species. This has led to high levels of embryonic death, placental incompatibility and perinatal mortality (Borges and Pereira, 2019; Dicks et al., 2021; Mastromonaco et al., 2014; Niemann, 2016).

Biobanks, conservation and the significance of developmental plasticity

The evolutionary literature is replete with examples of species where both males and females have evolved and adapted their physiological, behavioral, and morphological characteristics, optimizing their survival, reproductive success and fitness over centuries and millennia. However, while many of these long-term adaptations have been effected via gene mutation and various kinds of evolutionary selection, many such adaptations are induced by changes in the diverse ways in which gene expression is controlled. Such changes, caused via developmental plasticity, need not be solely determined by the precise nature of the DNA sequences involved. In fact, changing the way that the DNA is expressed permits species to respond quickly to environmental changes. The relevant control of gene expression during development is effected via processes such as DNA methylation, histone and microRNA modifications. These developmental processes have become especially sophisticated in social insects, where they control the behavior, morphology and social roles of different insect castes, all of which develop from eggs produced by a single female (known colloquially as “the Queen”) for review, see (Oldroyd and Yagound, 2021).

Given the enormous influence that epigenetic changes can induce during development, without the necessity of gene mutations, it is worth asking whether the technical processes needed for gamete and embryo cryopreservation, even including the nature of incubation media, might have the capacity to induce functionally significant epigenetic changes that might affect the future survival of offspring. This is not impossible, even though the cryopreservation of spermatozoa has been used successfully in agriculture and human medicine for more than five decades.

The application of ARTs in various human infertility treatments is estimated to have resulted in the birth of over million children in Europe (Schroeder et al., 2022; Wyns et al., 2021). Evidence suggests that a small proportion of the children born following *in vitro* fertilization and embryo transfer suffer from genomic imprinting diseases (Beckwith-Wiedemann, Angelman, Prader-Willi and Silver-Russell syndromes), slightly elevated risk of infant mortality in the first year of life, exhibit signs of large size for gestational age, high birthweight and other problems (for reviews, see Mani et al., 2020; Reyes Palomares and Rodriguez-Wallberg, 2022; Wyns et al., 2021). While some of these problems may be related to the original causes of the infertility, the various technologies used in clinical ART have also been implicated. The situation with agriculturally important animals undoubtedly differs from that with humans, conservation-significant wild animals and farm animals. While agriculturally important animals are subjected to continuous and commercially-driven selection pressures, these do not apply to humans or threatened species. In fact, conservation biologists strive to conserve as much genetic variability and thereby avoid selection processes as far as possible. This means that breeders and farmers would not usually maintain animals that show poor performance characteristics (e.g., milk yield, semen quality, feed conversion efficiency, etc.), which therefore

may not be recognized and studied. Nevertheless, one of the first negative impacts of ART to be reported, was the recognition of Large Offspring Syndrome in cattle and sheep (Young et al., 1998); many other aspects of genetic inheritance in agricultural species have been reviewed by Thompson et al. (2020).

Such considerations also raise valid questions about the eventual re-integration of preserved and stored cells into conservation-focused species support and recovery programmes. If spermatozoa, oocytes or embryos have been preserved by cryopreservation, desiccation or other means, and stored carefully for half a century, is there any likelihood that ART applications would fail for technical reasons? This question was investigated about 40 years ago (Whittingham et al., 1977), when frozen mouse embryos maintained at −196 ◦C were exposed experimentally to background radiation that simulated the equivalent of several thousand years. Fortunately, no deleterious impacts were detected. Investigating the potential occurrence of technically-induced genetic mismatches between genetic materials in long-term storage and their use in contemporary breeding programmes undoubtedly requires other approaches. One interesting possibility involves comparing the epigenetic signatures obtained from contemporary species of conservation interest with historical genomes derived from museum specimens; such studies are currently being carried out with black-footed ferret genomes (Personal Communication: Dr Klaus-peter Koepfli, Smithsonian Conservation Biology Institute, Washington, DC.).

Biobanking and the genetic rescue of threatened amphibians

The world's amphibian populations have been in decline over the last few decades, largely owing to the transmission of several infectious diseases (Daszak et al., 1999; DiRenzo and Campbell Grant, 2019), including, among others, both a fungal (Chytridiomycosis) and a viral (Ranavirus) infection (Rae and Murray, 2019; Sutton et al., 2014). These infectious diseases have been responsible for the diminution, or even complete disappearance, of amphibian populations from diverse habitats across the world. However, in turn, this problem has stimulated considerable research effort aimed at either eradicating the disease vector (*Batrachochytrium dendrobatidis* (Bd) (Sigafus et al., 2014), or finding ways that support the wild amphibian populations, and possibly permit them to co-exist with the disease (DiRenzo et al., 2018; Scheele et al., 2019). Current evidence suggests that co-existence is a possibility, given the remarkable developmental plasticity of amphibians and their ability to adapt and survive under diverse, and adverse, conditions (Jonsson et al., 2022; Sun et al., 2020).

The amphibian extinction crisis has also stimulated reproductive biologists to investigate the feasibility of cryopreserving amphibian spermatozoa, storing them in cryobanks and producing offspring that could be used in support of dwindling populations (Browne et al., 2011; Clulow et al., 2014; Kouba et al., 2013; Silla and Byrne, 2019; Uteshev et al., 2019). Besides developing appropriate sperm cryopreservation methods, researchers have developed a range of hormonal treatments for the controlled and reliable collection of male and female gametes for use in *in vitro* fertilization (summarized by Clulow et al., 2019). It is worth mentioning here that, because the amphibian male reproductive tract releases spermatozoa into a cloaca, the spermatozoa are released together with urine. Moreover, the collected spermic urine can be cryopreserved, stored and subsequently used to fertilize eggs (Kouba et al., 2012; Uteshev et al., 2012).

Unlike the situation with wild, but endangered, mammals and birds, a body of preliminary evidence about the development and survival of tadpoles and adults derived from frozen-thawed spermatozoa, is accumulating. In some cases, there is no suspicion that the use of cryopreserved amphibian spermatozoa might result in offspring with compromised survival or fertility (Lampert et al., 2022). Indeed, the successful use of cryopreserved spermatozoa in breeding programs for threatened amphibian species, and for establishing biobanks and insurance populations as a hedge against extinction, is now widely regarded as an important aspect of amphibian conservation (Kouba et al., 2013; Silla and Byrne, 2019; Upton et al., 2021). However, a detailed developmental study of Fowler's toad reproductive success (Poo et al., 2022) showed that cryo-derived tadpoles and post-metamorphic toadlets were consistently

smaller than their naturally-derived counterparts at the same stage. Interestingly, the authors did not detect any negative impacts on post-metamorphosis survival. These results mirror a study which found that the post-hatching growth of wild brown trout (*Salmo trutta)* (Nusbaumer et al., 2019) derived from cryopreserved spermatozoa was significantly lower than control embryos derived from fresh spermatozoa. At present there is no explanation for this effect, although one explanation put forward by Nusbaumer et al. (2019) suggested that these effects might be easily overlooked. It is also worth considering that the effects of amphibian sperm cryopreservation could vary between species, and might even show evidence of heritability. Although there are now a number of other studies in which phenotypically normal embryos were produced by artificial fertilization with cryopreserved frog and toad spermatozoa, and where metamorphosis resulted in normal adults (i.e. (*Xenopus laevis* (Pearl et al., 2017) and Golden Bell frog (*Litoria aurea*) (Upton et al., 2021)), it seems that more research into the heritability question is needed.

Biobanking for the restoration and support of coral reefs

Considerable attention has recently been focused on the establishment of successful cryopreservation technologies for coral species (Daly et al., 2018; Grosso-Becerra et al., 2021; Hagedorn et al., 2012, 2019) and a number of focused gene banks have already been established around the world (Caribbean, Hawaii, French Polynesia and the Great Barrier Reef; (Hagedorn et al., 2019). Spermatozoa from 31 different coral species have been cryopreserved successfully using a standard technique involving dimethylsulfoxide as the cryoprotectant, and a novel technique for the successful cryopreservation of coral larvae has been developed (Daly et al., 2018). Compared with other organisms there is an added complication to the cryopreservation of coral cells in that many co-exist with dinoflagellate symbionts (see Jiang et al., 2021) for review), whose biochemical functions are also being compromised by climate change, toxic chemicals and other environmental problems. This presents a multidimensional problem, which is nevertheless being addressed successfully.

Integration of biobanks and biodiversity conservation

Successful sperm cryopreservation has now been achieved in many wild species (Prieto et al., 2014) but success has, in many cases, been recognised mainly by the recovery of cellular structure and function (i.e., motility, plasma membrane and acrosomal integrity and, more recently, the retention of functional mitochondria and undamaged DNA (Paoli et al., 2019; Pollock et al., 2018). While these parameters are informative, they do not necessarily guarantee that spermatozoa will have the capacity to both fertilize oocytes and support appropriate embryonic development. It is therefore pointless to invest in major biobanking projects that rely entirely on the use of cryopreserved, but untested, sperm samples. However, it is worth mentioning that the IUCN Species Survival Commission recently established an Animal Biobanking Specialist Group in 2022, thus endorsing the value that is currently being placed on biobanking as a further resource for species recovery programs (https://www.iucn.org/our-union/commissions/group/iucn-ssc-animal-biobanking-conservation-specialist-group)

Agricultural and fisheries biobanks that focus on commercially significant species and breeds (Blackburn, 2018; Boe et al., 2021), as well as those focused on basic sciences and that store particular genetic lines of mice (Kaneko and Serikawa, 2012; Landel, 2005) and small fishes (Tiersch et al., 2011; Yang and Tiersch, 2009) ensure that their stocks of spermatozoa are reliably fertile. This level of assurance is not currently possible with most wild species, and it is for that reason that I regard only the Black-footed ferret and Giant panda projects as possessing well-established sperm biobanks. It is also true that, despite the current enthusiasm for banking somatic cells in the expectation that they will be used for the production of iPSC cells, artificial gametes and hence embryos (Pessôa et al., 2019), these expectations have yet to be convincingly demonstrated. Biobanks that aim to support the genetic diversity of threatened populations should ideally contain as much of the existing gene pool as practical. This means storing sufficiently large collections of somatic cells to capture

the maximum possible genetic diversity (Herrick, 2019; Herrick et al., 2016) and using them to produce genetically diverse offspring that represent the founder populations. Very small numbers of individual animals have so far been produced by this method (Stanton et al., 2019) and, as such, are not yet able to contribute much genetic diversity to existing populations.

Studies of the relationships between climate change and species survival have detected that many species, including birds, amphibians, reptiles, mammals and fishes (Hermes et al., 2018; MacNeil et al., 2010; Morris and Dupuch, 2012; Tiberti et al., 2021) are responding by either going locally extinct (Albano et al., 2021), or by shifting their preferred habitats towards cooler, polar and higher altitude regions. This undoubtedly imposes different selection pressures and disease risks upon populations experiencing those shifts. One relevant, and possibly surprising prediction anticipates that by 2070, 35% of mammals and 29% of birds will have over half of their 2070 climatic niche in countries where they are not currently present (Titley et al., 2021). The organization of biobanks may have to take these predictions into consideration, especially if the relevant germplasm samples intended for the provision of genetic support unexpectedly turn out to have become more suitable for supporting populations in a foreign country. Interestingly, some authors have expressed the view that if an endangered species is already unable to cope with the negative impacts of climate change, translocating the population elsewhere may help it to survive (Schwartz and Martin, 2013; Thomas, 2011) if a suitably hospitable area could be provided.

Conclusions

The concept of biobanking for conservation is not new, and indeed detailed proposals for a variety of biobanks targeted at different species, were published in the late 1970s (Veprintsev and Rott, 1979). In the 50 years that have elapsed since those early proposals, millions of biological samples, including cells of human, animal or bacterial origin, viruses, serum/plasma or DNA/RNA, are maintained in well-organized international collections. Unfortunately, the technology for exploiting these biobanks for use with wild animal conservation and breeding has been less effective than it might have been, given better financial support. To some extent, this shortcoming can be attributed to conservation biologists themselves, who have frequently dismissed, or ignored, the possible supportive role of cryopreserved biomaterials. This is in sharp contrast to the attitudes of the botanical community, who have been able to develop well-funded, long-term projects for the establishment of seed banks. (See, for example, The UK Millennium Seed bank at Kew in London in the United Kingdom (Chapman et al., 2018; Liu et al., 2018), which now contains seeds from nearly all native UK plants and many from outside the UK).

Over the last 20 years there have been many developments in reproductive technology, driven to a great extent by the requirements of agriculture, aquaculture and human reproductive medicine. The possibility that mammals, such as camels, horses and even sheep, could be cloned (Mastromonaco et al., 2014; Wani et al., 2017), or that fishes of one species can be engineered to produce the gametes of another (Morita et al., 2012), were matters for science fiction. As I have pointed out, there are some question marks over the health and fitness of the offspring that result from these techniques. Nevertheless, it is highly likely that those obstacles will eventually be overcome in time to save at least some of the world's most endangered species.

Acknowledgements

I am grateful to Dr Nana Satake for her valuable help with preparation of both the manuscript and Figure 1.

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Author contributions

This article was conceived and written solely by the author, Professor William Holt.

SHORT COMMUNICATIONS

SHORT ORAL COMMUNICATION COMPETITION

Uptake and effects of nanoplastics in maturing oocytes

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Keywords: nanoplastics, oocyte maturation, bovine

Micro- and nanoplastics (MNPs) have raised increasing concerns due to their widespread presence in the environment. The detection of plastic particles in human blood implies that MNPs are circulating in our body and are able to reach organs, including the reproductive tract (Leslie et al., Environ Int, 163:107199, 2022). However, the impact of nanoplastics (NPs) on oocyte development in mammals remains largely unknown, as the focus has mostly been on aquatic animals. In this study, the uptake and impact of NPs in oocytes was investigated with a bovine cumulus-oocyte complex (COC) model. This model is superior to study human reproduction compared to other models, due to the large similarities between bovine and human reproduction during oocyte and embryo development (Sirard, In: Animal Models and Human Reproduction, 127-144, 2017). To determine the uptake of NPs by the cumulus cells and oocyte, bovine COCs collected from slaughterhouse ovaries were in vivo matured (IVM) in medium (NaHCO3-buffered M199 supplemented, with 100 IU/ml Penicillin-streptomycin, 0.05 IU/mL FSH, 0.1 µM c-ysteamine, and 10 ng/mL EGF) containing 10 µg/mL of 50 nm or 200 nm fluorescently labeled pristine polystyrene (PS) NPs (CD Bioparticles, New York, USA; Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany) for 23 h at 39°C and 5% CO2 in air. The dose-response effect on oocyte maturation was studied by exposing COCs during IVM to 50 nm or 200 nm non-fluorescently labeled pristine PS NPs (Polysciences Europe GmbH) at the concentrations of 0, 0.3, 1, 3, 10, and 30 µg/mL. After IVM the uptake and oocyte nuclear maturation stage, defined by the metaphase-II stage of meiosis, was examined via Olympus IXplore SpinSR. One-way ANOVA followed by Holm-Sidak multiple comparisons test was used for statistical analysis. A p-value < 0.05 was considered statistically significant. Confocal microscopy showed that 200 nm NPs were only taken up by cumulus cells, while the 50 nm NPs entered both cumulus cells and oocytes (≥ 80 COCs were imaged per group). In total, 1426 and 1468 COCs in ≥ 5 replicates were analyzed for maturation rate in response to 50 nm and 200 nm NPs respectively. After exposure to 50 nm NPs, there was a significant decrease in oocyte nuclear maturation rate at 3 µg/mL (66.1%) compared to the control group (81.0%, P=0.01). Exposure to 200 nm NPs during COC maturation was not affecting the oocyte nuclear maturation rate at any of the tested concentrations. In conclusion, exposure to 200 nm PS NPs during COC maturation resulted in uptake by cumulus cells, but did not affect oocyte nuclear maturation. Exposure to 50 nm PS NPs resulted in uptake by cumulus cells and oocytes and hampered oocyte maturation at a concentration of 3 µg/mL. The mechanism of 50 nm NPs affecting oocyte maturation needs to be further investigated. Future research will also focus on the endpoints of the oocyte including the competence to develop into an embryo, to further study the impact of NPs exposures on oocyte developmental competence.

Mitochondrial dysfunction during oocyte maturation and its impact on embryo epigenetic programming: mechanistic insights

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Keywords: oocyte, mitochondria, ATP

During final oocyte maturation, mitochondrial activity increases and epigenetic changes are highly dynamic, making this a sensitive window to metabolic and environmental stressors. This may impact fertility and offspring health. Somatic cell studies show that mitochondrial ATP is important for the biosynthesis of substrates and activation of regulatory (co)enzymes required for epigenetic programming. The extent by which early epigenetic programming is dependent on oocyte mitochondrial ATP is not known. Therefore, we aimed to reduce oocyte mitochondrial ATP production using a specific ATP synthase inhibitor (oligomycin A, OM) during bovine *in vitro* maturation (IVM) and assess the impact on global DNA methylation and histone modifications in the exposed oocytes and resultant embryos. For this, a bovine in vitro production model was used, where cumulus-oocyte complexes were exposed to control media (CONT; TCM-199 with 0.4mM L-glutamine, 0.2mM sodium pyruvate, 50µg/ml gentamicin, 0.1µM cysteamine and 20ng/ml epidermal growth factor) or CONT + 5nM OM during IVM (24h). *In vitro* fertilization (IVF) was performed in Fert-TALP + 0.72U/ml heparin (20h) and presumptive zygotes were cultured in synthetic oviductal fluid + 2% bovine serum albumin (7d) (14 replicates, 2110 COCs/treatment). Oocytes, zygotes, 4-cell embryos and morulae were collected at 24h IVM, 20h post insemination (p.i.), 48h p.i. and 4.7d p.i., respectively. Oocytes (34/treatment, 3 replicates), zygotes (26/treatment, 3 replicates) and morulae (30/treatment, 4 replicates) were fixed for 5mC and H3K9ac/H3K9me2 immunostaining and confocal microscopy to assess global DNA methylation and histone acetylation/methylation, respectively. Mitochondrial ATP production rate was measured in oocytes and 4-cell embryos using a Seahorse XF HS Mini Analyzer (Agilent, ATP rate assay kit) (20/pool, 3 replicates). Data were analyzed with t-test or Mann-Whitney U test depending on homogeneity of variance. ATP production rate was significantly reduced (by 33.8%, P=0.018) in OM-exposed oocytes (at 24h IVM), but returned to normal levels in the produced 4-cell embryos (P=0.547), compared to the CONT group. OM-exposure significantly decreased 5mC staining intensity in oocytes (9.8% reduction, P=0.019) but increased 5mC in zygotes (22.3% increase, P<0.001) and morulae (10.3% increase, P=0.041). H3K9ac staining was not detectable in oocytes. OM significantly increased H3K9ac in zygotes (10.3% increase, P=0.023) but not in morulae (P=0.414). Finally, H3K9me2 staining intensity was increased in OM-exposed oocytes (10.9% increase, P=0.024), but was not changed in zygotes (P=0.819) and morulae (P=0.509). We conclude that oocyte and embryo epigenetic patterns are dependent on oocyte mitochondrial ATP production. Maturation under metabolic stress conditions that are known to impact oocyte mitochondrial functions may thus result in persistent oocyte and embryo epigenetic alterations even if the stress is alleviated and mitochondrial functions are restored at subsequent stages.

Embryo-induced alterations in the protein profile of bovine oviductal extracellular vesicles

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Keywords: oviductal extracellular vesicles, maternal-embryonic communication, protein signature

The oviduct provides the optimum environment for early embryonic development. Maternal-embryonic communication, which is essential for embryo quality, is mediated partly via extracellular vesicles (EVs). This study aimed to investigate the protein cargo of EVs obtained from the oviductal fluid (OF) of pregnant and cyclic heifers and their implications for maternal-embryonic communication in vivo. Oestrous cycles of crossbred beef heifers were synchronized, following which they were artificially inseminated (pregnant; n=13) or not (cyclic; n= 8) and slaughtered 3.5 days after insemination. The oviduct ipsilateral to the corpus luteum was flushed and the OF was examined to confirm the presence of a 6-8 cell embryo in pregnant animals. OF-EVs were isolated using size exclusion chromatography, concentrated by ultrafiltration, while EVs presence were characterized by flow cytometry using antibodies for specific EV markers (CD63, CD81, and CD44). Proteomic analysis was carried out using nanoLC-MS/MS with spectral counting to identify and quantify the proteins present in the EVs. Five animals from each group were used and statistical analysis was performed using ANOVA for flow cytometry data or T-test for proteomic data, both with a significance level of 5%. Bioinformatic analysis was performed with the DAVID and STRING tools. Flow cytometry analysis confirmed EV presence and no significant differences in EV markers between groups. A total of 1,101 proteins were identified: 5 unique to OF-EVs from cyclic heifers, 611 unique to pregnant heifers, and 485 in common. Among the common proteins, 93 were upregulated and 42 were downregulated in OF-EVs from the pregnant group. Functional enrichment analysis demonstrated that proteins exclusive to pregnant OF-EVs are involved in the Ras and Hippo pathways. Of note, Ras signaling is critical for mouse embryo development at the time of embryonic genome activation, which in cattle occurs in the oviduct during 8- to 16-cells transition. Furthermore, LLGL1, PATJ, and PARD6GB, members of the Hippo pathway exclusively found in pregnant OF-EVs, can regulate cell polarity and establishment of pluripotency. Additional pathways related to unique and upregulated proteins in pregnant OF-EVs include tight junction, cell adhesion molecules, and focal adhesion, which are essential for proper oviductal cell functioning and embryo development. Gene ontology analysis also revealed that upregulated proteins in pregnant OF-EVs, in comparison to EVs from cyclic animals, are associated with the immune response. In conclusion, although our model does not exclude a potential effect of sperm on the OF-EVs in the inseminated group, the study characterized a specific protein signature in OF-EVs from pregnant animals, which is likely due to the interactions established between the mother and the embryo.

ZP2 is essential for embryo development in golden hamster (Mesocricetus auratus)

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Keywords: CRISPR-Cas9, zona pellucida, fertility

The zona pellucida (ZP) is an extracellular glycoproteic matrix made-up by three to four different glycoproteins (ZP1, ZP2, ZP3 and ZP4) in eutherian mammals that surrounds the oocyte and early embryo, and it is crucial in several mammalian reproductive processes. Genetic ablation of ZP2 in mice, a species whose ZP lacks ZP4, produced oocytes without a ZP, showing an impairment in fertility (Rankin T.L. et al., Develop., 128, 1119–1126, 2001). However, some ZP2 mutations found in infertile women resulted in a thinner ZP surrounding oocytes that could be fertilized by ICSI but not by IVF (Weimin Jia, J Assist Reprod Genet, 39, 1205–1215, 2022). Besides, in KO-ZP2 rat females only 16.5% of ovulated eggs possessed a ZP (Yan Wang et al., Reprod., 160, 353–365, 2020). Due to these contradictory results, we would like to know the relevance of the ZP2 protein in the hamster as, unlike mice, its ZP does not lack ZP4 and can be a useful four-protein-ZP with a present ZP model. Thus, the current research aimed to assess the role of ZP2 protein on hamster ZP formation and fertility. For that, we produced a novel ZP2-KO hamster model using CRISPR-Cas9 technology as in our previously reported protocols (Zhiqiang Fan et al., PLoS ONE, 9 (10): e109755, 1-9, 2014). A ten-nucleotides deletion mutation localized in the fifth exon of genomic hamster ZP2 has been achieved, which produces a change in the reading frame and triggers a premature STOP codon, leading to the translation of a 140-nucleotides long truncated ZP2. Our results showed that female KO hamster were infertile since no offspring was obtained after the observation of 18 mattings of 6 females. On the other hand, ovarian sections were evaluated by histological analysis, and we observed that folliculogenesis was not affected in the KO animals. Moreover, ovulated oocytes were collected by superovulation from oviducts. Oocytes from ZP2-KO females had thinner ZPs (7.33 ± 0.53 µm of thickness) compared to the WT (12.37 ± 0.91 µm). After isolating all ZPs from oocytes, 2 pools of 50 ZPs per genotype (ZP-KO and WT) were analyzed by proteomic identification (LC/ESI-MS/MS) and no ZP2 peptides were found on the KO samples. For the embryo study, we recovered embryos at 0.5-, 1.5-, and 2.5-days post coitum. Zygotes obtained from ZP2-KO females were fertilized, as two pronuclei and two polar bodies could be seen, but 1.5 day-embryos failed to divide (no cleavage was observed), and all 2.5 day-embryos were degenerated with their ZP broken. Moreover, cracked, isolated ZPs were found in the oviducts of every KO female. In conclusion, our results prove that ZP2 is not mandatory for the ZP formation, folliculogenesis and fertilization in the golden hamster. However, ZP2 would be necessary for fertility in this species due to an impairment of the early-embryonic development that compromises cell-division and leads to embryonic loss.

This research was part of the projects PGC2018 094781 B I00 and PID2021-123091NB-C21 supported by MCIN/ AEI /10.13039/501100011033/ and by FEDER Una manera de hacer Europa.

Sex-specific contours of mitochondrial respiration characteristics at the pre-elongation stage in individual bovine embryos.

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Keywords: embryo metabolism, extracellular FLUX-analyzer, embryo sex

Sexual differences related to metabolism are known in in vitro produced (IVP) bovine embryos (Gómez et al., Theriogenology, 114, 180-184, 2018). Long-term effects of IVP on early preimplantive embryos or advanced developmental stages out of culture are still unclear. In view of partielly occuring reduced female calf ratio after transfer of IVP-derived blastocysts to recipients, we asked whether sex-related differences in metabolism still exist in pre-elongation stage embryos (D13) after transfer to recipients at expanded blastocyst stage (D7). Therefore, the aim of the present study was to compare mitochondrial respiration characteristics of male and female IVP-derived bovine embryos at day 7 (D7) and 6 days after transfer to recipients at day 13 (D13). Therefore, male and female expanded bovine IVP-derived blastocysts were produced by routine IVP procedures (IVM: modified TCM (Sigma); IVF: modified Fert-TALP (Parrish,Theriogenology, 81, 67-73, 2014), IVC: SOFaa (Holm et al., Theriogenology, 52,683-700, 1999) + 0.6 % fatty acid-free BSA, 5 % CO2, 5 % O2, 38.8° C) using male and female sex-sorted semen from the same bull, respectively, for in vitro fertilisation. A subset of these male and female IVP-derived D7 embryos were transferred to synchronised recipients followed by embryo flushing at day 13 of development. Energy metabolism analysis was performed on pools of D7 embryos (male vs. female embryos, pools of 10 embryos, 6 replicates) and individual D13 pre-elongation stage embryos comparing male (n=26) and female (n=28) embryos using an extracellular FLUX analyser (Seahorse XFp). This was supported by the use of a custom designed embryo cage system and the Cell-Mito Stress Test Kit (Agilent) containing three serial injections (Oligomycin 0.5µM; FCCP 4.0µM; Rotenone/AA 0.25µM), which allows the investigation of specific mitochondrial characteristics (Wave Software, Agilent). The results of our study showed that female embryos used a significantly higher (unpaired t-Test, $p<0.05$) proportion of the total oxygen consumed for mitochondrial respiration compared to their male counterparts at day 7 (87.5% vs. 70.5%) as well as at day 13 of development (54.3% vs. 35.71%). Consistently, female embryos show significantly higher ($p<0.05$) maximal respiration rates (1.3-fold at day 7 and 1.7-fold at day 13) compared to male embryos during the Cell-Mito stress test. The total amount of oxygen consumed for ATP-linked respiration was significantly higher in female embryos at both day 7 (1.3-fold) and day 13 (2.4-fold). This was accompanied by a higher mitochondrial coupling efficiency in male embryos compared to female embryos at day 7 (74.5% vs. 64.4%). In conclusion, our study revealed sex-specific implications for several mitochondrial respiration characteristics in IVP-derived bovine D7 embryos. Strikingly, both maximal respiration and ATP-linked respiration remain at significantly higher levels in female embryos for at least 6 days after transfer to recipients, even into the pre-elongation stage. As it is currently unclear how long these differences between male and female embryos persist and whether they are physiological or due to inadequate culture conditions, further studies are required.

TAI/FTET/AI, OPU - IVF and ET

Zinc sulfate attenuates the negative effects of Roundup on in vitro bovine oocyte maturation.

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Keywords: Roundup, embryo, cattle

Roundup (R©) is the most used herbicide; glyphosate, its active ingredient in doses >50μg/ml has anti-reproductive effects acting mainly through promoting endocrine disruptions, oxidative stress and apoptosis. Here, we examined if very low doses of R© (1ppm, equivalent to 0.36μg/ml of glyphosate) affect developmental competence of in vitro matured-fertilized bovine oocytes and if Zn, in the form of ZnSO4, can reverse the negative effects of R© acting as antioxidant. The selected dose of R© was more than 20 times lower than the mean R© levels found in cases of very mild R© intoxications in humans. In 5 replicates, follicles from abattoir derived cow ovaries were aspirated and COCs (n=1324) were allocated in 4 groups. The maturation medium (IVM) was either the standard medium (TCM199, plus 10%FCS and 10ng/ml EGF, group C n=339), or modified with the addition of: 1ppm R© (group R, n=425), 0.82μg/ml of Zn (group Z, n=276), R© and Zn (group RZ, n=284). The COCs were matured for 24 hours at 39oC in 5%CO2 in maximum humidify. Matured oocytes were fertilized with coincubation with frozen-thawed semen (106 sperms/ml), and 20 hours post insemination (pi), the presumptive zygotes were denuded, and the embryos were cultured in 25μl droplets (SOF supplemented with 5% FCS) covered by mineral oil, at 39oC in 5% O2, 5%CO2 and maximum humidity. Cleavage and blastocyst formation rates were evaluated 24 h pi and on days 7,8, and 9, respectively. In each replicate, groups of 5 day7 blastocysts, were snap frozen for molecular studies. The expression of 6 genes related to oxidation (SOD2, GPX1), epigenetic regulation (DNMT1, DNMT3A) and apoptosis (BCL2, BAX) was measured by Real Time PCR. Cleavage and embryo yield were analyzed by ANOVA, while gene expression was analyzed by R Studio using permutation analysis, imbedding in the package lmperm. Cleavage rate in group R (76.7±7.2) was lower ($p<0.01$) than C (87.5±6.6), while in group ZR (77.5±9.7) tended ($p=0.06$) to be lower than C; no other differences were detected among groups. Blastocyst formation rate in group R was steadily lower compared to those of groups C and Z ($p<0.03$). On day 7 blastocyst formation rate in group ZR (17.0±3.2) was lower ($p=0.03$) than in C (23.3±4.1), while on day 8 (21.9±5.3) tended ($p=0.06$) to be higher than that of R (15.4±5.1) and did not differ from C (28.0±8.4). Significant ($p<0.05$) changes were detected in the expression of SOD2, GPX1 and DNMT1. The expressions of antioxidant genes, as well as DNMT3A were the highest ($p<0.05$) in group R, while the combination of Zn with R© (group RZ) mitigated the negative effects, leading to lower expression of the antioxidant genes. These results imply that R©, even at very low doses, induces oxidation disrupting fertilization and embryo development, which can be partly prevented by providing anti-oxidative protection to the maturing oocyte.

Single administration of FSH delivered in hyaluronic acid for ovarian stimulation prior to ovum pick-up in Italian Mediterranean buffalo

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Keywords: buffalo, OPU, FSH

Buffalo is a livestock species that has undergone a massive increase in number of heads and milk production in the last decade; moreover, the Italian Mediterranean buffalo (IMB) is a breed greatly requested around the world because of its high milk production. Ovum pick-up (OPU) and in vitro embryo production (IVEP) undoubtedly is the best tool to rapidly speed up the propagation of the breed and the genetic progress through the maternal lineage. The aim of the work was to evaluate the efficiency of hyaluronic acid (HA) as delivery vehicle for FSH compared to the standard FSH administration protocol (CTRL, Petrovas G. et al., Animals 30;10(11):1997. 2020) to support the growth of a homogeneous population of medium-sized follicles and oocyte developmental competence, in terms of embryo yields. HA is a suitable and safe vehicle for FSH in prepubertal buffalo (Currin L. et al., Theriogenology, 197, 84-93, 2023). Briefly CTRL (40 mg of FSH - Folltropin, Vétoquinol S.A., Magny-Vernois, France - administered intramuscularly 2 times/day for 3 consecutive days after the removal of the dominant follicle, with OPU performed after 28-32 hours of coasting) was compared with HA protocol involving a single intramuscular administration of the entire dose of FSH (240 mg) in 15 ml of 1% HA solution. IMBs (8/group, over 4 replicates) underwent OPU as previously described (Petrovas G. et al. Animals 30;10(11):1997. 2020). Follicular and oocyte population were recorded, good quality oocytes were in vitro matured, fertilized and cultured to the blastocyst stage according to standard procedures (Di Francesco S. Theriogenology 77.1: 148-154. 2012). The CTRL and HA treatments gave similar results (mean±SE) in terms of total number of aspirated follicles per animal (15.2±1.4 vs 14.4±1.1; respectively), as well as that of small (7.2±0.8 vs 6.6±0.9, respectively) medium (3.7±0.7 vs 3.8±0.5, respectively) and large follicles (4.76±0.6 vs 3.8±0.4, respectively). Similarly, the oocyte population, both in terms of total number of oocytes recovered per animal (10.3±1.1 vs 10.0±1.3, respectively in the CTRL and HA) and of oocytes suitable for IVEP (7.9±0.9 vs 7.6±1.0, respectively in CTRL and HA) is similar in the two groups. Finally, also with regard to embryo yields, the two protocols gave similar results (36.1% vs 35.1%, respectively in CTRL and HA). These preliminary results demonstrated that a single administration of FSH delivered in HA is as efficient as the standard FSH treatment on follicular and oocyte population as well as embryo yields, and hence can be used for ovarian stimulation in IMB. The possibility of replacing the six FSH administrations of CTRL protocol with a single administration conveyed by HA allows to minimize animal handling with a reduction of stress for them and risks for operators, especially in contexts of wild and semi-wild breeding in which the animals are often unaccustomed to be handled.

The use of a brief synchronization treatment after weaning, combined with superovulation, affects the gene expression of surviving pig blastocysts

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Keywords: blastocysts, synchronization, pig

The combined use of estrus synchronization and superovulation (SS) treatments affects the expression of genes in the ovaries and endometrium, impairing follicle and oocyte growth, as well fertilization and embryo development. However, the effects of such treatments on embryo transcriptome remain unknown. This study aimed to analyze alterations in gene expression of day 6 blastocysts that survived a brief synchronization treatment followed by superovulation. The sows were assigned to one of the three groups: SS7 sows (N=6), which received altrenogest (ALT) treatment for 7 days starting from the day of weaning, and were superovulated with eCG 24 hours after the end of ALT treatment and hCG at the onset of estrus; SO sows (N=6), consisting of sows not treated with ALT but superovulated with eCG 24 hours after weaning and hCG at the onset of estrus; Control sows (N=6) comprising of weaned sows that showed natural estrus. Surgical embryo collection was carried out on day 6 of the cycle (day 0 = onset of estrus). The number of viable embryos to the total number of embryos and oocytes/degenerated embryos collected was lower ($p<0.05$) in the SS7 sows (140/187; 75%) than in the SO (174/186; 94%) and control (105/114; 92%) sows. Microarray analysis (5 embryos per sow) were conducted on blastocysts with good morphology, following the IETS criteria. To identify differentially expressed genes (DEGs) between groups, ANOVA with an unadjusted P-value < 0.05 and a fold change </> 1.5 was used. Compared to controls, SO treatment had minimal impact on blastocyst gene expression. Only 4 pathways were disturbed, with 4 modified transcripts, which were unrelated to reproductive functions or embryonic development. In contrast, SS7 blastocysts exhibited moderate gene expression alterations, including both DEGs and fold changes, compared to controls. Seven pathways were disrupted, affecting 10 transcripts in total. Upregulation of certain pathways, such as the metabolic pathway, involved two upregulated genes (RDH10 and SPTLC2) related to reproductive functions, which notably may indicate suboptimal embryo quality. The downregulation of other pathways, such as the glutathione metabolism pathway, with downregulated genes (GSTK1 and GSTO1) involved in cellular detoxification of reactive oxygen species, could hinder the embryos' response to oxidative stress, potentially impairing subsequent embryo development. The gene expression changes observed in the present study in SS7 embryos, and the results of previous reports indicating SS7 treatment negatively affect fertilization, embryo production, and reproductive tract gene expression, led us to consider its use in embryo transfer (ET) programs inconvenient. These findings have implications for the swine ET industry, as they prevent the simultaneous use of two hormonal treatments (synchronization and superovulation) that function effectively when applied separately.

Supported by MCIN/AEI/ERDF (RTI2018-093525-B-I00), Spain, Fundacion Seneca (19892/GERM/15), Spain, and the Research Council FORMAS (Project 2019-00288), Sweden.

Birthweight data of IVP calves

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Keywords: IVP, Birth weight, imprinting

Since the introduction of IVP in the 1990s, it has been observed that IVP calves have a higher birthweight, often referred to as large-offspring syndrome (or LOS), as compared to in vivo derived AI and flush (or MOET) calves. This increased birthweight was reduced using serum-free IVC culture media (Wagtendonk de Leeuw *et al.*, Theriogenology. 2000 Jan 15;53(2):575-97), but it was not completely normalized to the level of AI. To improve animal welfare and further increase the use of IVP embryos in cattle breeding, the increased birth weights need to be normalized. However, this problem is difficult to tackle due to the lack of solid data on reported calf weights. In most countries, no or only estimated data on birth weight are available. Therefore, we started collecting real birth weight data of Holstein cattle on 10 different farms in The Netherlands, where all IVP, flush and AI calves were weighed just after birth. In addition, gestation length, parturition ease (scored on a scale from 1-5, with 1 being the easiest), sex of the calf, colostrum intake within 2 h after birth (in liters) and afterbirth were registered. All embryos were collected, cultured and processed in standard CRV media (Mullaart E *et al.*, 37th AETE, vol. 19; 2022: e22223). Data from 171 IVP, 83 flush and 855 AI calves were registered. The results confirmed that IVP calves were significantly heavier than the flush and AI calves (43.7 ± 6.2, 40.2 ± 4.7 and 40.0 ± 5.8 kg, respectively). Within the AI and flush group, the male calves where significantly heavier than the female calves (+4.1 and +3.4 kg, with p=0.0001 and 0.001, respectively, by t-test), which was expected. However, the weight of the male and the female IVP calves did not differ significantly (1.1 kg heavier males, p=0.23 by t-test). This indicates a disproportionate increase in the birth weight of female over male IVP calves, obscuring the sexual dimorphism in body weight that normally occurs in newborn calves. Using a 97th percentile as a cut-off for LOS (i.e., 47 and 52 kg for female and male, respectively, based on birth weight data from AI calves), 10% of IVP males and 24% of female calves would be categorised as displaying LOS. No differences were observed between gestation length, parturition, sex ratio, colostrum intake, and afterbirth for the 3 groups. It is not clear why females are relatively heavier than male IVP calves. Aberrant DNA methylation and genomic imprinting frequently occur during in vitro embryo development and are often discussed as causative for the increased birth weight of male and female IVP calves. In addition, only female embryos carry an imprinted, inactive X chromosome that undergoes transient reactivation and random inactivation at the blastocyst stage. It is possible that the female-specific X-inactivation dynamics is perturbed in IVP embryos, making them more sensitive for imprinting deviations and higher birth weights.

HCG application at the time of embryo transfer in Brangus cattle recipients: Effects on pregnancy rate under tropical heat-stress conditions

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Keywords: HCG, pregnancy rate, heat-stress

The use of Human Chorionic Gonadotropin (HCG) has been carried out occasionally in embryo recipient protocols in *Bos indicus* cattle. It has been suggested that HCG stimulates ovulation, accessory corpora lutea formation, and therefore, a progesterone increase (Cunha, *J. Dairy Sci.*, 105, 8401-8410, 2022). The main aim of the present research was to assess the potential effects of HCG at the time of embryo transfer in Brangus cattle recipients on seasonal pregnancy rates under subtropical heat-stress conditions. A total of 1,810 embryo transfers (blastocyst stage) were carried out in Brangus cattle (*Bos indicus x Bos taurus*; Age: 4-8 y.o.; body condition score: 3.0-3.5) maintained under the same nutritional, management, and environmental conditions [Köppen-Geiger (Aw), tropical savanna climate, Paraguay; Coord.:S~24°6´0”; W~57°04´60”; Prec.:~1,500 mm; R.H.:~90%; M.T.:~23.4°C (summer: ~30°C; winter: ~15°C); Alt.:~60 m.a.s.l.]. Four groups were considered for the study: control winter (CW; n = 430), control summer (CS; n= 750), HCG winter (HW; n= 210), and HCG summer (HS; n= 420). All recipients were synchronized following a conventional protocol: Day 0: intravaginal progesterone (P4) device (CIDR: 1.38 g) + 2.5 mg intramuscular (IM) estradiol benzoate E2B + 50 mg P4 (IM). Day 7 fresh embryos were transferred and at the time of embryo transfer, 2,000 IU of HCG (IM) were administered in HW and HS groups. The diagnosis of pregnancy was confirmed by ultrasonography on day 35 after embryo transfer. The data were analyzed by GLMM/ Chi-square (x2) (SPSS® 25, IBM Corp., USA). Significant differences were observed in pregnancy rates when just seasons were compared [524/1,170 (44.8%) vs. 427/640 (66.7%) for summer and winter, respectively; $P \leq 0.05$)]. Moreover, significant differences were detected regarding CS compared to HS [291/750 (38.8%) vs. 233/420 (55.4%); $P < 0.05$)] and when CW was compared to HW [269/430 (62.5%) vs. 158/210 (75.2%); $P < 0.05$)]. No differences were observed when HS and CW groups were compared ($P > 0.05$). In conclusion, HCG application at the time of embryo transfer improved the pregnancy rate irrespective of the season considered. This effect may be due to the formation of accessory corpora lutea, and, as a consequence, increased levels of progesterone concentration. The differential effects of HCG were more pronounced during the summer. The significant increase in the pregnancy rate observed derived from HCG treatment could be interesting for mitigating the heat-stress-derived detrimental effects during the summer season; however, the use of HCG at the time of embryo transfer would be recommended in winter season as well since the pregnancy rate improvement during this season was demonstrated in Brangus cattle recipients maintained in tropical environments.

This research was partially supported by ANID 21201280 and DIRGI-CP2022-005.

Ovum pick-up (OPU) in Bos indicus dairy cattle breeds: Oocyte recovery rates and in vitro embryo production using unsorted and sex-sorted sperm

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Keywords: OPU, sex-sorted sperm, embryo production

The ovum pick-up (OPU) technique improves the cattle's genetic gain, shortening the generation interval and increasing the genetic progress. The main objective was to assess the efficiency of OPU in *Bos indicus* dairy cattle breeds evaluating the potential oocyte recovery rates and *in vitro* embryo production using unsorted and sex-sorted sperm. A total of 656 OPU sessions (one per cow) were carried out in two *Bos indicus* cattle breeds [Gyr (GY): 537 and Guzerat (GZ): 119; Age: 3-6 y.o.; body condition score: 3.0-3.5]. *In vitro* embryo production procedure was carried out using unsorted and sex-sorted semen (same breed sire for each group; 1 x 10^6 spz/mL in both types). Four experimental groups were randomly performed: Gyr-unsorted (GYU; n = 173), Gyr-sorted (GYS; n= 374), Guzerat-unsorted (GZU; n= 54), and Guzerat-sorted (GZS; n= 65). All cows were super-stimulated as follows: Day 0: intravaginal CIDR (1.38g progesterone/cow) + intramuscular progesterone (100 mg/cow) + intramuscular 17-beta-estradiol (2 mg/cow); Day 1: Intramuscular eCG (2500 IU/cow); Day 4: OPU. The oocyte-derived parameters assessed were: oocyte yield (OY; total number of oocytes) and quality (GI-II and GIII; based on the number/presentation of COCs layers and ooplasm homogeneity), viable oocyte/donor (OD; viable oocytes per donor), matured oocytes (MO; matured oocytes based on COCs expansion, perivitelline space, 1st polar body extrusion, cytoplasmic color, and zona pellucida shape), viable MO/donor (MOD; viable matured oocytes per donor). The embryo-derived parameters evaluated were: cleaved-embryos/MO (CLMO; cleaved embryos obtained from the total number of matured oocytes), cleaved-embryos/donor (CLD; cleaved embryos obtained from the number of viable matured oocytes per donor), total embryos (TE), viable TE/donor (TED; viable embryos per donor), TE/GI-II (TEG; grade I and II embryos), TE/MO (TEMO; embryos obtained from the total number of matured oocytes), and DE/donor (DED; degenerated embryos per donor). The data were analyzed by GLMM (SPSS® 25, IBM Corp., USA). Differences were observed in OD (29.14±3.89 vs. 17.12±0.85), MOD (26.80±3.53 vs. 14.14±0.72), CLMO (18.93±8.69 vs. 7.89±3.72), CLD (18.63±2.90 vs. 7.26±0.55), TE (10.74±3.73 vs. 6.28±1.35), TED (6.49±1.35 vs. 3.59±0.27), TEG (5.20±1.26 vs. 2.97±0.25), and DED (1.28±0.50 vs. 0.61±0.10) when both breeds were compared being greater in GZ ($P \leq 0.05$). Moreover, differences were detected in TED (5.53±0.74 vs. 3.10±0.23) and TEG (4.70±0.68 vs. 2.39±0.20) when both types of semen were compared being greater using unsorted semen ($P < 0.05$). Differences were observed in CLD, TE, TED, TEG, and DED being greater in GZU compared to GYU, GYS, and GZS ($p<0.05$). No differences were observed among GYU, GYS, and GZS regarding the same parameters ($P > 0.05$). In conclusion, Guzerat breed was superior in most of oocyte-derived and embryo-derived parameters. TE and TEG were improved by using unsorted semen. Finally, the differential results obtained in GZU regarding oocyte and embryo production performance per donor suggest that OPU technique showed better results using Guzerat breed and unsorted semen.

This research was partially supported by ANID 21201280 and DIRGI-CP2022-005.

Embryo transfer in Brangus cattle recipients: Effects of transfer side on pregnancy rate and embryonic/fetal loss under subtropical conditions

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Keywords: Embryo transfer, pregnancy rate, uterine horn

Embryo transfer (ET) practice increases the number of offspring per cow; however, the ET efficiency depends on several factors. The main objective of the present study was to evaluate the potential effects of the embryo transfer side (right and left uterine horn) on the pregnancy rate (PR) on Day 30 and 60 post-transfer in Brangus cattle in subtropical conditions. A total of 426 embryo transfers were carried out in Brangus cows (Bos indicus x Bos taurus; Age: 4-8 y.o.; body condition score: 3.0-3.5) maintained under the same nutritional, management, and environmental conditions [Köppen-Geiger (Aw), tropical savanna climate, Paraguay; Coord.:S~24°6´0"; W~57°04´60"; Prec.:~1,500 mm; R.H.:~90%; M.T.:~23.4°C (summer: ~30°C; winter: ~15°C); Alt.:~60 m.a.s.l.]. OPU sessions were carried out in Brangus donors, which were super-stimulated as follows: Day 0: intravaginal CIDR (1.38g/ cow) + intramuscular progesterone (100 mg/cow) + intramuscular 17-beta-estradiol (2 mg/cow); Day 1: Intramuscular eCG (2500 IU/cow); Day 4: OPU. The embryos were produced following a standard IVP protocol. OPU-derived COCs were subjected to standard in vitro maturation and fertilization using conventional unsorted semen (1 x 10^6 spz/mL). The embryos were cultured for 7 days post-fertilization before embryo transfer. The resulting embryos were transferred fresh to estrous synchronized cows. Recipients were synchronized following a conventional protocol: Day 0: intravaginal progesterone (P4) device (CIDR: 1.38 g) + 2.5 mg intramuscular (IM) estradiol benzoate E2B + 50 mg P4 (IM) and subjected to embryo transfer. The transfer was randomly performed to the right (n= 263) or left (n= 163) uterine horn. Pregnancy diagnosis was carried out using ultrasonography on Day 30 and 60 following embryo transfers. The data were analyzed by GLMM/ Chi-square (x2) (SPSS® 25, IBM Corp., USA). No differences were observed in PR when the right and left uterine horns were compared on Day 30 (53.6%, 141/263 vs. 52.1%, 85/163, $p>0.05$). Moreover, no differences were detected in PR between uterine horns on Day 60 (40.7%, 107/263 vs. 36.2%, 59/163 for right and left uterine horns, respectively, $p>0.05$). Finally, differences were observed when PR was diagnosed on Day 30 and Day 60 regarding both right (53.6% vs. 40.7%, $p< 0.05$) and left (52.1% vs. 36.2%, $p< 0.05$) uterine horn. No differences were observed regarding embryo loss rates in the right (13.1%) and in the left (15.9%) uterine horns when Day 30 was compared to Day 60 ($p>0.05$). In conclusion, PR was greater on Day 30 compared to Day 60 post-embryo transfer in Brangus recipients. PR was not associated with the right or left uterine horn after embryo transfer irrespective of the day of pregnancy. Important differences were observed regarding embryo loss rate in the 30-day period between PR diagnoses. The embryo loss rate increased over time regardless of the uterine horn considered.

This research was partially supported by ANID21201280 and DIRGI-CP2022-005.

Are there any differences between blastocysts derived from prepubertal and pubertal heifers in the aspect of mitochondrial function ?

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Keywords: pre- pubertal heifers, cows, mitochondria, blastocysts, transcriptome

In the cattle-breeding industry, there is increasing commercial demand for in vitro embryo production from young and even prepubertal heifers. Therefore, the goal of the present study was to assess the difference between mitochondrial DNA content, oxidative stress, and developmental competence in blastocysts derived from prepubertal vs. pubertal heifers. We also compared transcriptomic profiles of those embryos. Two groups of OPU derived blastocysts were analysed in the study (n=120 from 24 prepubertal and 12 pubertal heifers). Mitochondrial content was determined based on mitochondrial DNA copy number evaluation (qPCR). Oxidative stress was measured by intracellular glutathione concentration, reactive oxygen species levels and mRNA expression of antioxidant-associated genes. The developmental competence of embryos was evaluated by mRNA expression of blastocyst quality markers (OCT4, SOX2, NANOG, PLAC8, IGF1R, IGF2R, 342 PLAU, SSLP1, DSC2, DNMT3A, and AQP3). Total RNA was isolated from examined embryos (n=15 embryos in each group) and RNA-seq was performed. Analysis of the transcriptome profile was determined by DAVID. The rate of oocytes developing to blastocysts in vitro was significantly lower when oocytes originated from prepubertal vs pubertal animals (12% vs 32%, $P<0.05$). Blastocysts from two groups did not differ in terms of morphological quality. Morphologically appropriate blastocysts derived from prepubertal heifers had higher concentrations of reactive oxygen species and glutathione ($P<0.05$) compared to blastocysts from pubertal heifers. In the blastocysts produced from prepubertal heifers we found higher mitochondrial DNA copy number (453667 ± 17243 vs 403667 ± 7371, $P<0.05$) and also alterations in expression of developmental competence gene markers ($P<0.05$). The total of 436 differentially expressed genes (DEGS) were identified in the examined embryos. KEGG pathway analysis confirmed that many DEGS were involved in mitochondrial function via the influence on oxidative phosphorylation, expressed by significant differences of the level of genes such as: ATP synthases (ATP5MF, ATP5PD, ATP12A), NADH dehydrogenases (NDUFS3, NDUFA13, NDUFA3) and cytochrome c oxidase (COX17) ($P<0.05$). The impaired oxidative phosphorylation in blastocysts derived from prepubertal heifers, in addition to higher mtDNA copy number and altered gene expression of developmental competence markers, suggest lower quality of the blastocysts derived from prepubertal animals, despite their unaltered morphology.

Optimization of heterologous IVF with Iberian ibex (Capra pyrenaica) sperm and domestic goat (Capra hircus) oocytes

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Keywords: ibex, goat, heterologous IVF

Assisted reproductive technologies are key to maintain genetic stocks of wild species, such as the Iberian ibex (*Capra pyrenaica*). *In vitro* fertilization (IVF) could be used to test the fertilization ability of stored semen, but optimal IVF conditions remain largely unexplored. Heterologous *in vitro* fertilization (IVF) of domestic goat (*Capra* hircus) oocytes constitutes an excellent platform to optimize IVF procedures for Iberian ibex. Previous studies showed that supplementation of SOF medium with 2% estrus sheep serum (ESS) during heterologous IVF with prepubertal goat oocytes improved blastocyst rates. The aim of this study was to optimize IVF procedures for Iberian ibex and to analyze whether ESS is required for fertilization. Cumulus-oocyte complexes obtained from adult domestic goat ovaries were matured *in vitro* and then incubated with $2x10^6$ frozen-thawed ibex epididymal spermatozoa/ml from the same male in four different media: TALP medium supplemented with 10 µg/ml heparin (TALP, n=141) or synthetic oviductal fluid (SOF) supplemented with 10 µg/ml heparin alone (SOF-0; n=193) or combined with 2 % (SOF-2; n=121) or 20 % (SOF-20; n=131) of estrus sheep serum (ESS), at 38.5 ºC under an atmosphere of 5 % CO_2 with maximum humidity (3 experimental replicates). Presumptive zygotes were cultured in SOF supplemented with 0.4% BSA up to day (D) 4 and in SOF supplemented with FBS from D4, at 38.5 ºC under an atmosphere of 5 % CO_2 and 5% O_2 with maximum humidity. Cleavage rate was recorded at day (D) 2 and blastocyst rates were recorded at D8. Blastocysts were fixed and the development of specific lineages was assessed by immunostaining for SOX2 (epiblast), SOX17 (hypoblast) and CDX2 (trophectoderm). No significant differences were found in cleavage (91.1±2.5 vs. 90.9±0.4 vs. 91.3±3.3 vs. 87.2±3.2 mean±s.e.m. for TALP vs. SOF-0 vs. SOF-2 vs. SOF-20) or blastocyst rates (52.4±4 vs. 49±3.1 vs. 36±8 vs. 37.1±4.9%; mean±s.e.m. for TALP vs. SOF-0 vs. SOF-2 vs. SOF-20). Total, SOX2+ and SOX17+ cell number was similar between groups (Total: 170.9±16.4 vs. 132.2±10.1 vs. 141.1±23 vs. 179±22.2; SOX2+: 17.6±1.4 vs. 24.1±3.5 vs. 13.1±2 vs. 16.4±2.1; SOX17+: 21.9±4.6 vs. 19.7±3.4 vs. 21.1±5.6 vs. 30±7.4; mean±s.e.m. for TALP vs. SOF-0 vs. SOF-2 vs. SOF-20), but CDX2+ cell number was significantly higher in TALP and SOF-20 than in SOF-2 (120.7±15.8 vs. 71.2±11.8 vs. 67.6±19.6 vs. 123.2 ± 22; for TALP vs. SOF-0 vs. SOF-2 vs. SOF-20; ANOVA $p<0.05$). In conclusion, heterologous IVF with Iberian ibex (*Capra pyrenaica*) sperm and domestic goat (*Capra hircus*) oocytes does not require ESS.

Work supported by StG 757886-ELONGAN and PID2021-122153NA-I00.

Correlation between sperm traits and in vitro fertilization outcomes in X-sexed and non-sexed sperm

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Keywords: cattle, sperm motility, cleavage

Cattle sperm characteristics play an important role in the outcomes of *in vitro* fertilization (IVF). Utilization of gender selected semen allows production of male and female offspring to take advantage of sex-influenced characteristics (Kasimanickam R, 2021, Bovine Reproduction, 20, 1000-10). The aim of this study was to investigate the correlation between sperm motility and velocity characteristics with fertilization and cleavage rate in non-sexed and X-sexed sperm. The heterogeneous cattle ovaries of unknown reproductive status were collected at the local abattoir. A total of 75 oocytes were *in vitro* fertilized per treatment with X sexed and non-sexed frozen-thawed semen of proven fertility collected from the same bull (Angus) purchased from American Breeders Service Global Inc Company. Prior to IVF, the sperm motility was analyzed with the aid of a computer assisted sperm analysis (CASA; Microscopic, S.L, Barcelona, Spain). The frozen-thawed X-sexed and non sexed frozen thawed semen with a sperm concentration of 1 x 10^6 sperm/mL (5000 spermatozoa) were analyzed and the experiment was replicated 5 times. The correlation between sperm motility and velocity characteristics with total fertilization and oocyte cleavage rate was analyzed using the analysis of variance (general linear model) and statistical analysis system (SAS®). Treatment means were separated using Fisher's protected t-test and the significant differences were determined by P-value at a significant level of $P < 0.05$. In X-sexed sperm, the results for total sperm motility (52.61±4.25) and total fertilization percentage which was determined by two and more than two pronuclei (24.66± 3.05) were shown to be negatively correlated (r=-0.20; $P < 0.05$). Furthermore, a positive correlation was observed between the total motility of X-sexed sperm (52.61±4.25) and the total cleavage percentage of oocytes which was determined by 2-4 cell of cleaved presumptive zygotes (15.00±5.00; $P < 0.05$), with a correlation coefficient of 0.01. Interestingly, a positive correlation was observed between hyperactive (5.00±3.46) and total cleavage rate (15.00±5.00), with a correlation coefficient of 0.00 ($P < 0.05$). In non-sexed sperm, the results for total sperm motility (61.88±2.73) and total fertilization percentage (29.33±6.10) were shown to be negatively correlated (r=-0.92; $P < 0.05$). Moreover, a negative correlation was observed between non-sexed total sperm motility (61.88±2.73) and the total cleavage rate (42.67±1.15), with a correlation coefficient of -0.74 ($P < 0.05$). Additionally, a strong negative correlation was observed between hyperactive (3.00±3.46) and total cleavage rate (42.66±1.15), with a negative correlation coefficient of -0.50 in non-sexed sperm ($P < 0.05$). The negative correlation between sperm motility and total oocyte fertilization rate in both non-sexed and X-sexed sperm suggests that high total sperm motility is essential for successful fertilization and embryonic development. In conclusion, the sperm motility and velocity characteristics are strongly correlated with fertilization and cleavage rate in both non-sexed and X-sexed sperm, indicating that higher sperm motility and velocity characteristics plays a role in early embryo development.

Effect of embryo transfer application on pregnancy success in repeat breeder cows and heifers

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Keywords: Repeat breeder, embryo transfer, cow, heifer

In Turkey, like other developing countries, embryo transfer (ET) in dairy cattle is not routinely practiced under field conditions. Many factors such as cost, success rate and technical issues are responsible. However, high replacement cost of milking cows with elite heifers or cows makes ET feasible in Turkey in commercial dairy enterprises and recently demand is increasing for ET. Repeat breeding is one of the major factors for culling. In this study, it was aimed to determine the effect of ET applied to repeat breeder cows and heifers on pregnancy success. For this purpose, 11 holstein heifers (the average age: 22 months) and 14 holstein cows (the average days in milk: 329 days) inseminated at least five times but not pregnant were used as recipients. Holstein donor and recipients (cows and heifers) were superovulated and synchronized, respectively. Briefly, superovulation was induced in six donors by intramuscular FSH (Stimufol, Reprobiol, Belgium) at a dose of 10 ml per cow at 12 h intervals for 4 days commencing on 9th day after estrus. Double AI was done at 12 h intervals in donor cows and flushing was done one week later. Only first quality blastocysts were used for ET. Recipient repeat breeder cows and heifers were synchronized with two injections of PGF2α at a dose of 2 ml per animal at 11 days apart. ET was performed 7 days after estrus detection. Pregnancy was detected in 7 heifers (63.6%) and 7 cows (50.0%). Chi-square test was used for statistical evaluation. Even the sample sizes are small, these ratios are very encouraging for repeat breeders animals. Obtaining preliminary results is very useful for the Turkish dairy industry where culling and maintaining the repeat breeder cows and heifers are difficult and very expensive. It is suggested that further investigations should be warranted to evaluate the viability of ET as a therapeutic measure in valuable repeat breeder cows and heifers as an alternative approach to culling.

Acylated ghrelin diminishes the superovulatory response in dairy sheep

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Keywords: ghrelin, antimullerian hormone, superovulation

We have previously shown that the stomach derived ghrelin attenuates gonadotrophins' secretion and suppresses in vitro embryo production in ruminants. As ghrelin secretion is positively associated to fasting and to negative energy balance, here we investigated the effects of long-term infusion of ghrelin on the superovulatory response, embryo yield and anti-mullerian hormone (AMH) secretion in dairy sheep. During the breeding season, twelve ewes were fitted for 28 days with either an osmotic minipump that delivered a truncated form of acylated ghrelin molecule, at a dose of 1.5μg/kg/day (treated -T, 28-day ghrelin infusion), or with an empty pump (non-treated controls -C). Ten days after pump insertion the estrus cycle of all animals was synchronized with intravaginal progestogen sponges. Starting 48 hours before sponge removal, the ewes were superovulated with 6 decreasing doses of porcine FSH (133mg/ewe) given twice daily, and the animals were naturally mated to fertile rams. Blood samples were collected at pump insertion and along with the first and the last FSH injection for Antimullerian Hormone (AMH) determination, using a commercial ELISA kit. Embryos were collected six days after estrous/mating by laparotomy. After uterine flushing, the ovaries were exteriorized, the corpora lutea and all visible follicles were counted; the follicles were aspirated and the follicular fluid of small and large follicles was separately stored for AMH assessment. The data between groups were tested by t-test. The superovulatory response (number of CLs) and the collected embryos (morulae and blastocysts) were higher ($p<0.001$) in group C ewes (CL 8.3±1.3, embryos 5.5±1.8) than in group T (CL 2.8± 1.3, embryos 1.3±0.6). More ($p<0.05$)small follicles were found on the ovaries of group C (7.4± 1.5) compared to group T (5.5± 1.0), while the number of large follicles did not differ between groups. At pump insertion, the serum AMH concentration did not differ between groups ($p=0.8$), but it was higher at the first (C, 3.9±1.2pg/ml vs T 2.1± 1.0, $p=0.06$) and the last FSH dose (C 4.3±1.0 pg/ml vs T 2.0± 1.1pg/ml, $p=0.03$) in control ewes. AMH concentrations were higher ($p<0.03$) in group C small follicles (C, 2.01±0.45 ng/ml; T, 0.96±0.45 ng/ml), but not in large ($p>0.05$). These results imply that ghrelin wanes the pool of small follicles and their responsiveness to FSH, and it confirms findings from other species that the determination of AMH concentrations could be used as potential predictor of ovarian response to superovulation in sheep.

Comparative study of In-vitro embryo production (IVEP) in Gir & Sahiwal (Bos indicus) Indian cattle breeds

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Keywords: Ovum Pick up, In vitro fertilization, Oocyte, Embryo culture, Gir breed, Sahiwal breed

Gir and Sahiwal (*Bos indicus*) are among the major milk-producing Indian cattle breeds. The study aimed to compare the oocyte recovery rate and embryo production using IVP in Gir (n=17) & Sahiwal (n=10) cattle donors. The study was carried out at Central Research Station, BAIF Development Research Foundation, Uruli Kanchan, Pune, Maharashtra, from October 2019 to October 2022. All these experimental donors were maintained under the same management practices. A total of 698 Ovum Pick Up (OPU) sessions (Gir 354 & Sahiwal 344) were performed. All OPUs were done using ultrasound device with intravaginal micro convex transducer with 7.5 MHz frequency (USG Model: Exago by IMV). OPU procedure was carried out about once every 15 days irrespective of season. In all the OPU sessions during the experimental period, 20-gauge OPU needle was used and the vacuum pump pressure was maintained in between 70 to 90 mm of Hg. Temperature range maintained of vacuum pump was in between 37.0 to 38.5 ^{O}C. OPU was performed without using any pre-stimulation protocols for all these non-lactating donors. The donors were ranged between 1st to 4th parity. All the IVP cycles were randomly processed with media available and no specific media was used for any specific breed. All the recovered oocytes after grading, were further processed in laboratory for IVP with a protocol of 20 to 22 hours of maturation period, 16 to 18 hours for fertilization and 7 days post fertilization for culture. For both breeds, grading of oocytes was done between grade 1 to grade 5 based on presence of COC layers surrounding oocyte and cytoplasm content of oocytes. All the steps of IVP were performed in one laboratory. The culture conditions were same for both breeds like 5% CO2, 5% O2, 90% N2 and the incubation at 38.5 °C with high humidity. On day 7th blastocyst rate was observed. Grading of embryos was done as per IETS embryo grading manual. Both the parameters were analyzed using mixed model with animal (donors) as a random effect while breed as a fixed effect while Tukey test was used to identify the critical differences between the breed. The analysis was carried out using R software version 4.2.3. The oocyte recovery rate was not affected by breed, but the embryo production were influenced by breed variable. The average oocyte recovery in Gir breed was 9.76 ± 1.27 and in Sahiwal 6.90 ± 1.20 per OPU session ($P<0.05$). Gir breed produced 2.75 ± 0.32 viable embryos per OPU session vs 1.72 ± 0.33 in Sahiwal ($P<0.05$). In conclusion, oocyte recovery and embryo production by IVP, were higher in Gir cows compared to Sahiwal ones.

In vitro maturation of bovine oocytes using a portable CO2 incubator

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Keywords: maturation, incubator

During in vitro maturation (IVM) oocytes are placed in media supplemented with hormones for 22 to 24 hours to complete the meiotic maturation, using incubators with stable temperature and a CO_2 concentration of 5 %. These incubators in IVP laboratories differ in size and models but most of them are static. In some cases there is no opportunity to use a static incubator at the place of oocyte collection, and the IVP laboratory is too far away. For example in ovum-pick-up (OPU) programs with different teams working at a distance an alternative to transport of oocytes only in warmed media without CO_2 is needed. The aim of this study was to investigate whether IVM of bovine oocytes could be performed successfully in a portable incubator. Tissue-culture-medium 199 (TCM) supplemented with hCG and eCG was used as maturation medium. Cumulus-oocyte-complexes (COCs) were collected from ovaries of slaughtered cattle and divided into 3 different maturation groups: in the first group 60 COCs were matured in 100 µl droplets TCM in a petri-dish with an oil overlay (lab/oil), 20 COCs per droplet. 60 COCs were placed in 250 µl TCM in 0.5 ml tubes without oil in the second group (lab/free), also 20 COCs per tube. For both groups an incubator (Heracell 150i, Thermo Scientific™, Schwerte, Germany) with 38.0 °C and 5 % CO_2 was used. The third group was also matured in tubes without oil, but in a portable CO_2 incubator (CellTrans+, Labotect GmbH, Göttingen, Germany) employing the same maturation conditions (cell/free). After 24 h maturation rates were analyzed via Hoechst staining. Embryos were generated using a standard IVF and IVC protocol up to day 8 (IVF = day 0). At day 7 and 8 cleavage and developmental rates were recorded. Additionally at day 8 blastocysts and expanded blastocysts were stained with Hoechst 33342 and ethidium homodimer to count live and dead cells. Four trials with 240 COCs per group were performed. Data were analyzed with Oneway-Anova followed by a Tukey test. Maturation rates (89.7 ± 1.5 %, 78.6 ± 0.6 %, and 78.1 ± 0.6 %, respectively), cleavage (79.3 ± 8.3 %, 74.7 ± 3.6 %, 74.6 ± 2.3 %) and developmental rates (23.9 ± 6.3 %, 22.3 ± 1.4%, 21.8 ± 1.5%) did not differ significantly ($P > 0.05$) between the oocytes and embryos from the 3 groups, nor did the ratio of live and dead cells of the stained embryos (19.5 ± 6.4, 21.4 ± 8.3, 20.0 ± 5.4). This shows that portable incubators could be used instead of static incubators during maturation of COCs without a loss of oocyte quality and developmental competence. It offers an alternative method especially to OPU-IVP teams working at different stations.

Preliminary results on glycaemic response after oral glucose tolerance test (OGTT) in sows derived from assisted reproductive technologies

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Keywords: Insulin, glucagon, pig.

In human, murine and rabbit species, individuals derived from embryos produced in vitro (IVP) may present, among others, disorders in glucose metabolism (Chen et al. 2014, Diabetes 63:3189–3,98; García-Domínguez et al. 2020, Animals 10:1043-1059). In pigs, available information is very scarce and we have reported in 45-days-old piglets differences in the glycaemic response after an oral glucose tolerance test (OGTT) between IVP-produced animals and those conceived in vivo by artificial insemination (AI) (Paris-Oller et al. 2022, JDOHaD 13:593-605). However, it is unknown if these differences are corrected or maintained during adult life. The objective of the present study was to evaluate the glucose tolerance in the same colony of pigs during their adult life by means of an oral glucose tolerance test (OGTT). The animals were obtained from a previous study (Paris-Oller et al. 2021, J Anim Sci Biotech 12:32-44) that were born after artificial insemination (AI group) and surgical transfer of in vitro-produced embryos (IVP group). All animals were kept under same housing and feeding conditions since birth. The OGTT was performed in AI (n=8) and IVP (n=10) sows with 3.5-3.6 years age, and weighing from 227 to 249 kg. For the OGTT, animals were previously fasting for 24h and 2h without drinking water. Sows ingested 1.75 g/kg body weight of glucose solution (100% glucose carbs, Myprotein) and blood samples were collected via auricular vein before OGTT (t=0) and over 240 minutes (15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min) following glucose administration. Blood glucose concentration was immediately measured by a glucometer (Aposan) using test strips, and blood serum was obtained and freeze (-80ºC) until determination of insulin (immunoturbidimetric method) and glucagon (10-1281-01 Mercodia, Uppsala, Sweden). Data were analysed using an ANOVA test with nested design (animal within reproductive treatment group) and reproductive treatment (AI, ET) and time of sampling and interaction treatment and time as the main factors. Data are expressed as mean ± SEM. Values of $p<0.05$ were considered significant. Glycaemia was influenced by time of sampling ($p=0.019$) and was higher in AI-derived animals than in IVP group (66.95±1.29 *vs.* 60.57±1.31 g/dL, $p<0.001$), while the interaction group and time was not significant ($p=0.401$). On the other hand, the insulin concentration was only influenced by the origin of the animals, with higher values in IVP than AI animals (58.66±4.88 *vs.* 75.79±4.27 µUI/ml, $p=0.013$). As for the glucagon concentration, it was similar for all the times of sampling and between groups (AI: 2.84±0.39 *vs.* IVP: 2.75±0.24 pmol/L, $p=0.664$). These observations suggest that, up to some extend, the differences in the response to OGTT in IVP-produced pigs are maintained during their adult life. Moreover, IVP sows challenged with an OGTT show changes in the insulin response. Increasing the number of animals, and determination of complementary biochemical parameters are needed for a better interpretation of the results.

This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039 /501100011033 and “FEDER Una manera de hacer Europa”.

Laser-Assisted Biopsy is an efficient technique to perform genetic analysis of Bovine Embryos in Selected Breeding Programs

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Keywords: In vivo produced bovine embryos, Laser-Assisted Biopsy, WGA, Embryo genomic selection

Embryo genomic selection is increasingly being used to select the best embryos within cattle breeding programs. However, during the early stages of embryo development, the number of cells, and then the amount of DNA from embryo biopsy, is limited. Whole genome amplification (WGA) is used to amplify this limited amount of DNA for downstream genetic analysis. Although the reliability of this genetic analysis is evaluated by the call rate (CR, proportion of SNPs detected from the chip) to be used in genomic evaluation) and depends for a significant part on the cell sample quality. Since 2021, at Auriva -Elevage, a new laser-assisted biopsy technique has been used to minimize damage to the embryo during the biopsy process. In a recent previous study, we demonstrated that the laser-assisted biopsy method is an efficient and minimally invasive method with a higher in vivo embryo survival rate after 48 h of culture compared with the conventional microblade process. It results in a similar pregnancy rate compared to that obtained for non-biopsied embryos (Gamarra et al., Reprod., Fert. and Develop. 2022, 35(2)). However, with these samples, a significantly lower SNP call rate was observed in the laser method when compared to the microblade technique. The aim of this study was to evaluate on a larger number of embryos, the impact of laser-assisted biopsy of in vivo produced embryos on Single Nucleotide Polymorphism (SNP) call rate in comparison with the conventional microblade method used in Auriva-Elevage breeding programs. For the laser biopsy procedure, a hole in the zona pellucida of Day 7 in vivo produced embryos (n=1486) was created by two laser pulses of 3.7 ms (Octax Laser-Germany), through which a biopsy pipette was inserted to aspirate 3 to 8 trophoblast cells. For microblade biopsy, embryos (n=3028) were immobilized by a holding pipette, and a steel blade was used to cut 3 to 10 trophoblast cells. Collected cells were stored at -20°C until whole genome amplification (WGA). DNA extraction and WGA were performed using the REPLI-g Single Cell Kit (Qiagen, Manchester, UK). WGA-DNA from each group were genotyped on Illumine EUROG MD V4 chip in order to evaluate the CR. Biopsied embryos were slow frozen using Ethylene Glycol (1.5 M) plus sucrose (0.1M) for later direct embryo transfer. Biopsies with a call rate ≥ 80% are considered to allow an accurate estimation of the genomic breeding values. The results show that the percentage of call rate (≥80%) was non-significantly different (Chi-2, p=0.17) for laser-assisted biopsy (89%, 1319/1486) compared to microblade one (90%, 2728/3028). This study confirms that laser-assisted biopsy of in vivo-produced bovine embryos is an efficient and minimally invasive method to obtain genetic material for whole genome amplification. It can be considered as a good alternative to the conventional microblade method to perform genetic analysis of bovine embryos.

Folliculogenesis, Oogenesis, and Superovulation

Recombinant FSH (bscrFSH) vs. pituitary FSH (FSH-p): Ovarian response and in vitro embryo production in superovulated Brahman cattle

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Keywords: recombinant FSH, pituitary FSH, superovulation

The use of recombinant FSH is not frequent in *Bos indicus* cattle superovulation (SOV) protocols. The goal of the present study was to determine potential differences in ovarian response and *in vitro* embryo production between a single chain recombinant FSH (bscrFSH) SOV and pituitary FSH (FSH-p) SOV-derived protocol in Brahman cattle. Twenty healthy Brahman cows (*Bos indicus*; body condition score: 3.0-4.5) were randomly divided into two experimental groups (G): G1 (FSH-p: FSH from purified pig pituitary extract; n = 10) and G2 (bscrFSH: recombinant FSH; n = 10). All cows underwent both superovulation treatments in a cross-over design. Regarding G1 SOV, a conventional protocol was applied (Day 0: intravaginal progesterone (P4) device (CIDR: 0.5 g) + 2.0 mg intramuscular (IM) estradiol benzoate E2B + 50 mg P4 (IM); Day 4: total dose = 200 mg of FSH-p divided in 4 day/12 h intervals/8 decreasing doses: 40/40 + 30/30 + 20/20 + 10/10 mg; Day 6: fifth and sixth FSH-p dose + two PGF2α i.m. doses (500 µg of D-cloprostenol each); Day 7: CIDR removal at the seventh FSH-p dose application); Day 8: estrus detection + 1st AI; Day 9: 2nd AI (semen from the same sire was used for all AIs); Day 15 (embryo collection). For G2 cows, the same protocol was applied with modifications (total dose = 195 µg of bscrFSH divided in 4 day/24 h intervals/4 decreasing doses: 40/40 + 30/30 + 20/20 + 7.5/7.5 µg). Ovarian structures [No. follicles (FL on day 8): FL, No. corpora lutea (CL on day 15), and Non-ovulated follicles (NOFL on day 15)] were monitored by using ultrasonography. Moreover, the number of unfertilized oocytes (UFOs) and the number of viable embryos (E) were assessed according to the IETS guidelines. The data were analyzed by GLMM (SPSS® 25, IBM Corp.). No significant differences were detected regarding FL (13.5±1.7 vs. 11.2±2.1) and CL (9.3±1.4 vs. 6.8±1.3) in G1 and G2, respectively ($P > 0.05$). Significant differences were observed in NOFL between G1 and G2 (1.1±0.3 vs. 5.4±2.4; $P \leq 0.05$). No significant differences were observed between G1 and G2 regarding UFOs (1.0 ± 0.4 vs. 0.6 ± 0.3; $P>0.05$); however, significant differences were observed regarding the number of viable embryos (6.9 ± 1.2 vs. 4.2 ± 0.5; $P = 0.05$) when FSH-p and bscrFSH-derived SOV protocols were compared. In conclusion, no differences were observed in the ovarian structures irrespective of the SOV protocol used except in the number of NOFL. The number of viable embryos was greater using the FSH-p-derived SOV protocol. The significant increase in the number of NOFL observed in the bscrFSH SOV protocol could be related to an overstimulation-derived effect at the dose used. Therefore, bscrFSH-dose adjustments will be necessary to apply it successfully in *Bos indicus* cattle.

This research was partially supported by ANID 21201280 and DIRGI-CP2022-005.

Metabolic stress during the postpartum period in dairy cows might influence follicular growth dynamics at the time of breeding. A retrospective analysis.

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Keywords: negative energy balance, folliculogenesis, fertility

Metabolic and oxidative stress during the early postpartum period is strongly linked with reduced fertility in high-yielding dairy cows. A recent study in our laboratory aimed to examine the associations between blood non-esterified fatty acids (NEFAs) and antioxidant concentrations at week 2 and 8 postpartum (pp) with the granulosa cell (GC) transcriptome in the preovulatory follicles at the time of breeding (at wk 8 pp). For that, estrous synchronization was performed at wk8 by inserting a progesterone releasing intravaginal device (PRID DELTA) for 7 days, and injecting Dinoprost at day 6. Ultrasound-guided transvaginal follicular aspiration of the (largest) dominant follicle (from 27 cows) was performed exactly at 38h after the PRID removal to collect follicular fluid (FF) and granulosa cells as described in Marei *et al*. 2022, JDS 105(8): 6956-6972. The transcriptomic profile of the granulosa cells was determined using RNA sequencing. Based on the expression level of LH surge-responsive genes (*AREG, PTGS2,* SRGN, FST, INHBA, HSPH1, NSDHL, TBC1D8B, PCBD1; Gilbert et al. 2011 Reproduction 141:193-205) we found that 11 cows were already in the post-LH surge phase, while 16 cows were still in the pre-LH surge phase. A significantly higher mean estradiol: progesterone (E2:P4) ratio was confirmed in the FF of the pre-LH compared with the post-LH cohort ($P<0.05$). The aim of the present study was now to perform a retrospective analysis to investigate if the difference in follicular growth dynamics or the timing of LH surge was dependent on the metabolic health of the cow during the early pp period (at wk 2) or at the time of sample collection (at wk 8). We compared wk 2 and wk 8 blood concentrations of NEFAs, antioxidants (ß-Carotene, Vitamin E, Glutathione peroxidase, and oxidative stress index), as well as Vit A, Glucose, and Insulin-like growth factor 1. This was done using independent sample T-tests after checking the data homogeneity of variance. None of the analysed wk 2 nor wk 8 blood parameters were different between the two groups, except w2 blood NEFA concentrations, which were significantly higher in the cows that were still in the pre-LH surge phase at the time of sample collection compared with the post-LH cows (mean ± SD: 623±259 vs 411±127 µmol/L, $P<0.05$). This suggests that an elevated blood NEFA concentration at w2 may be associated with a slower follicular development after estrous synchronisation at the time of breeding. Blood and FF NEFA concentrations at wk 8 were not different between the two groups ($P>0.1$), showing that the observed association could be a persistent long-term effect of elevated NEFAs on follicular cell viability or on response to hormonal regulation. While these data may further elucidate why metabolic stress during transition in dairy cows is associated with reduced fertility, further confirmation on a larger scale is needed.

Beneficial effects of melatonin in hypoxic condition on canine oocyte nuclear maturation through reduction of oxidative stress

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Keywords: Oocyte, Melatonin, Hypoxia

Unlike other domestic animals, in vitro maturation of oocytes in canine results in very low nuclear maturation rate accompanied with high degeneration. High fat content of canine oocytes predisposes them to oxidative stress and production of high levels reactive oxygen species (ROS). Melatonin (MTN) a highly lipophilic hormone with a strong antioxidant effect which acts through its G- protein coupled receptors (MTNR-A1and B1) has been proven beneficial in supporting oocytes during culture.
Canine cumulus oocyte complexes (COCs) were collected after routine ovariohysterectomy and processed by chopping the ovarian cortex, for the following experiments. Each experiment was replicated four times.
Experiment 1: analyzed expression of MTNRs by immunofluorescence staining. MTNR-1 A and 1 B were highly expressed in the oocytes and with lower intensity in the cumulus cells. The distribution of MTNR-A1and B1 signal didn't follow a nuclear or peri-nuclear pattern and both were evenly scattered within the ooplasm.
Experiment 2: The COCs (n=300) with three or more layers of cumulus cells, were cultured in the absence (control) or presence of 100nM, for 72h in two groups; [1] low O2 (5%) and [2] high (20%) in air at 38.5℃. Nuclear stage of the oocytes in meiosis was determined under Nikon fluorescent microscope, after denudation and fixation and Hoechst staining. Melatonin at 100nM concentration had a beneficial effect on the nuclear maturation profile of canine oocytes in both conditions. However, the oocytes cultured in low O2 versus cultured in high O2 for 72h exhibited the lowest percentage of oocytes at GV stage (6.7%±4.2 vs 19.8%±3), highest MII maturation rate (32.3%±6.4 vs 15.81%±8.1), minimum degeneration (20.5% ± 3.2 vs 45.2%±5.15) and maximal meiotic resumption (GVBD-MII; 56.2%±8.6 vs 19%±3), when the basic maturation medium was supplemented with 100nM of melatonin in ($P < 0.05$).
Experiment 3: analyzed the effects of melatonin on production of ROS using DCHFDA staining. Densitometry using ImageJ software showed that the overall intensity of fluorescence was lower in oocytes treated with 100nM melatonin ($p<0.05$).
Experiment 4: analyzed impact of melatonin supplementation on expression level of genes related to ROS repairing enzymes (GPX1, SOD1, SOD2, GSR & CAT). Freshly collected and in vitro matured COCs were snap-frozen (25 COCs per group/repeat) after in vitro maturation and used for RNA extraction and qRTPCR. Melatonin supplementation in high O2 reduced the expression of all ROS repairing enzymes significantly. It also reduced the GPX-1 ($p<0.005$), catalase ($P<0.01$) expression in the COCs cultured in low O2.
Overall, these data suggest that melatonin protection of oocytes from oxidative stress results in reduced degeneration and increased nuclear maturation. The beneficial effect of melatonin supplementation during in vitro maturation of dog oocytes in production of developmentally competent oocytes requires further investigation.

Acknowledgements: This project was funded by Duchenne UK.

Endocrine disrupting chemicals induce alterations in lipid droplets of bovine cumulus cells

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Keywords: endocrine disrupting chemicals, cumulus cells, lipid droplets

Endocrine disrupting chemicals (EDC), omnipresent in the human environment, are potentially harmful to female fertility (Panagopoulos P. et al., Best Pract Res Clin Obstet Gynaecol, 2023). We developed a test strategy for effects of EDCs on female fertility by using the relevant animal model of bovine oocyte in vitro maturation (IVM), which complies with the 3Rs. To identify endpoints sensitive to EDC activity, biological processes within the cumulus oocyte complex (COC) were previously tested upon exposure to known EDCs. Here, we report the effect of EDCs ketoconazole (KTZ; CYP450 enzyme inhibitor) and diethylstilbestrol (DES; estrogen receptor agonist) on lipid droplet (LD) dynamics, considering the importance of lipids in oocyte developmental competence. COCs were matured for 24h (as reported in Asimaki K. et al., Front. Toxicol., 2022) either with vehicle DMSO (0.01% v/v for DES, 0.1% v/v for KTZ), KTZ (10^{-8} M, 10^{-7} M, 10^{-6} M) or DES (10^{-9} M, 10^{-7} M, 10^{-5} M) (Sigma-Aldrich, Missouri, USA). COCs (45 per group) were stained with the specific neutral lipid dye LD540 (courtesy of Spandl J., Traffic., 2009) and cumulus cells (CCs; over 20000 per group) were imaged with confocal microscopy (top-to-bottom, 1µm z-step). LD count per cell, area, and clustering (% of LDs within a cluster) were recorded and analysed with IBM SPSS statistics software to compare groups with one-way ANOVA, followed by Tukey's post hoc test. KTZ treatment resulted in a concentration-independent increase of LD area in CCs at all concentrations (increasing KTZ: 0.40±0.04, 0.40±0.05, 0.41±0.04 µm^2; vs vehicle control 0.37±0.04 µm^2, $p < 0.001$). In addition, LDs were more clustered (70±9%, 66±13%, 73±8%; increasing KTZ) compared to the vehicle-control (56±16%, $p<0.001$). The LD count per cell was comparable between the vehicle-control (9±2) and KTZ-treated groups (9±2, 9±2, 9±2; increasing KTZ). DES treatment of COCs led to an increase of LD area in CCs exposed to 10^{-9} M (0.44±0.15 µm^2) and 10^{-5} M (0.44±0.04 µm^2) DES, compared to the vehicle group (0.39±0.03 µm^2, $p<0.001$), but no such effect was observed at 10^{-7} M (0.4±0.05 µm^2) DES. CCs exposed to 10^{-7} M DES had a slight increase in LD clustering (66±13%) compared to the vehicle group (61±12%, $p<0.005$). Finally, LD count slightly decreased at 10^{-5} M DES (7±2%) when compared to vehicle- and DES-groups (9±2%, $p<0.001$). We have previously shown that KTZ (unpublished) or DES do not affect cell viability but 10^{-8} M KTZ reduces blastocyst rate and 10^{-5} M DES halts oocyte nuclear maturation. Here we report that KTZ treatment possibly resulted in an increase in lipid content, based on the larger LD area, as well as LD clustering, in a concentration-independent manner. DES treatment at all concentrations affected the LD organization dynamics. LD area was increased at 10^{-9} M DES, suggesting a potential increase in lipid content. A similar effect was observed at 10^{-5} M DES, concurrent with a reduction in LD count per cell. Finally, LD clustering was increased only at 10^{-7} M DES. In the future, these observations will be validated by lipidomic analysis of the neutral lipid content of cumulus cells.

Project funded by EU Horizon 2020 Research and Innovation Programme Grant No 825100 (FREIA)

Physiology of Male Reproduction and Semen Technology

Sexually inexperienced photo-stimulated bucks increased plasma testosterone concentrations during first contact with nulliparous and multiparous anestrous goats

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Keywords: goats, naïve male goat, photoperiod

In sheep, social isolation from ewes decreases testosterone secretion in males. Furthermore, sexually inexperienced rams secrete low concentrations of testosterone during their first interaction with ewes. This study aimed to determine whether sexually inexperienced photo-stimulated male goats increase plasma testosterone secretion during first contact with nulliparous and multiparous anestrous females. This study was performed in Torreon, State of Coahuila, Mexico (25°23′ N, 104°47′ W; 1200 m above sea level). Male kids (*n*=6) were born in December and were weaned at 40 days of age. Male goats were housed together in a pen and were totally isolated from any visual, auditory, tactile, and olfactory sensory signal from female goats. At 10 months of age males were subjected to a photoperiodic treatment of artificially long days from November 1 to January 15 (16 h of light and 8 h of darkness per day), from this date onward males perceived the natural photoperiod until the end of the experiment. Nulliparous (*n* = 30) and multiparous (*n* = 30) females were used. Before introduction of the males with females a transrectal ultrasonography was performed to determine the ovulatory state using an Aloka SSD-500 equipped with a 7.5 MHz transducer. An anovulatory state was established in those females in whom a corpora lutea were not detected. All females were anovulatory. In males, blood samples to determine plasma testosterone concentrations were collected by venipuncture of the jugular vein. Blood samples were taken weekly when the photoperiodic treatment ended from January 16 until May when the study ended. Plasma testosterone concentrations were determined using ELISA. In march, when the males were 15 months of age were randomly separated into two groups of 3 each, and were exposed for the first time to nulliparous and multiparous females (1 male × 10 females) for 15 consecutive days. Plasma testosterone concentrations were analyzed using a completely randomized model with repeated measures using generalized estimation equations. Plasma testosterone concentrations changed over time between the two groups of males; interaction week × group of males. P<0.001). Photo-stimulated males increased plasma testosterone concentrations in the first week of March during first contact with nulliparous (10.8 ± 2.6 ng/mL vs first week of January, 1=2.0 ± 0.7; P<0.05) and for multiparous females (9.3 ± 1.9 ng/mL vs first week of January, 1=1.16 ± 0.14; P<0.05). In conclusion, sexually inexperienced photo-stimulated males increased plasma testosterone concentrations during first contact with nulliparous and multiparous anestrous females.

Bicarbonate and caffeine trigger capacitation-like changes (acrosome damage) in boar sperm after 2 h in vitro incubation.

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Keywords: in vitro capacitation, sperm, porcine, BSA, bicarbonate, caffeine

Sperm capacitation is a crucial process within the reproductive female tract. This complex mechanism is associated with biochemical changes that enable spermatozoa to bind, penetrate and fertilize the egg. Hyperpolarisation of the plasma membrane, change of ions concentration levels (including calcium uptake), phosphorylation of sperm proteins, ROS production or changes in motility patterns are involved in sperm capacitation. These modifications rely, at least in part, on different compounds that we could find in the female genital tract, such as bicarbonate. The purpose of this study was to assess, *in vitro*, the effect of the inclusion of different molecules (bicarbonate, bovine serum albumin (BSA), and caffeine) on sperm quality parameters after 2 h incubation at 38.5 °C. To address these effects, we used eighteen boar commercial artificial insemination doses to test, in e*xperiment 1*, three concentrations of bicarbonate (19, 37 and 56 mM; n=6), in *experiment 2*, three concentrations of BSA (1.5, 3 and 4.5 mg/mL; n=6) in a based media (BM) supplemented with 37 mM of bicarbonate. Finally, in *experiment 3, the* absence or presence of caffeine (5.15 mM; n=6) in the BM containing bicarbonate (37 mM) and BSA (3 mg/ml) was analysed. The BM contained sodium chloride (NaCl: 112 mM), potassium chloride (KCl: 4.02 mM), sodium phosphate monobasic monohydrate (Na2HPO4: 0.83 mM), magnesium chloride (MgCl2: 0.52 mM), D-(+)-glucose (13.9 mM), sodium pyruvate (1.25 mM), and calcium chloride (CaCl2: 2.25 mM; 0.25 mg/mL). Total motility (TM), membrane integrity (VIAB) and acrosome damage (ACR; H33342/PI/PNA), capacitation status (chlortetracycline staining CTC), and mitochondrial membrane potential (JC1) were analysed. Experiment 1 revealed that higher concentrations of bicarbonate (37 and 56 mM) decreased ($P<0.01$) TM and VIAB but increased ($P<0.01$) ACR after 2 h of incubation compare to the fresh control. In contrast, in experiment 2, the BSA concentration of 3 mg/mL reduced only the VIAB, whereas all the concentrations tested increased the average membrane potential (JC1) and decreased TM after incubation in comparison to the fresh control. Finally, in experiment 3, the capacitated pattern measured by the CTC technique and ACR increased after 2h of incubation compared to fresh control, although no differences were found in the presence/absence of caffeine. Our results showed that, according to our *in vitro* experimental conditions, bicarbonate is the main compound triggering capacitation-like changes in the boar sperm. This effect could be explained, at least in part by the already described presence of a bicarbonate gradient in the female genital tract that may be linked to the release of sperm from the female sperm reservoirs. Further studies are needed in order to elucidate the specific mechanism by which the boar sperm is capacitated *in vitro*.

This study was supported by the projects PID2019-108320RJI00, and RYC2020- 028615-I, funded by MCIN/AEI/10.13039/501100011033 (Spain) and FEDER funds (EU). PPF was supported by the grant FJC2020-045827-I funded by MCIN/AEI/10.13039/501100011033 and European Union NextGenerationEU/PRTR.

Evaluation of the progesterone inhibitor RU486 in boar sperm cryopreservation

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Keywords: sperm, porcine, RU486, cryopreservation, mifepristone

Boar sperm cryopreservation is a sub-optimal method due to the high sensitivity of sperm to cold shock. In addition, sperm cryopreservation induces capacitation-like changes, such as sperm membrane modifications, thereby affecting calcium channels, which causes variations in the ion exchange. These structural changes and intracellular signalling lead to increased acrosomal reactions or different patterns of movement, called cryo-capacitation. On the other hand, progesterone (P4) is an inductor of capacitation, including acrosomal reaction, mainly by inducing changes in the plasmatic membrane that allow the entry of calcium, which in turn activates the signalling pathway of adenylate cyclase and the production of cAMP. Being said that the use of a specific progesterone inhibitor could revert the P4 action, this preventing capacitation-like changes that happen during sperm cryopreservation. The aim of this study was to evaluate the effect of different concentrations of a progesterone inhibitor (RU486) in the freezing extender, on post-thaw boar sperm quality. Throughout the experiment, commercial artificial insemination semen doses from six fertile boars were centrifuged and diluted (300 x 10^6 sperm/mL) in a freezing extender (LEY: 20% egg yolk and 80% lactose), supplemented with RU486 (A: non-supplemented (control); B: 5 mM; C: 10 mM; D: 20 mM). The samples were cooled to 5 °C, extended (1:3; v:v) in LEYGO (LEY + 9% glycerol and 1.5% Equex) and 0.5 mL straws frozen in LN_2 vapours, and thawed at 37 °C/20 sec. Total motile sperm (%TMS) and other kinetics parameters were evaluated with the IA Station CASA system (SPERMTECH®). Furthermore, membrane integrity and acrosome damage were determined by using triple staining (Hoechst 33342/propidium iodide PI/PNA-FITC) under epifluorescence microscopy. Sperm with undamaged membrane and acrosome (%VIAB) or reacted acrosome (%VIAB/AR) and sperm with damaged membrane and acrosome (%DEAD/AR) were recorded. Moreover, sperm with high membrane potential (%HMP) were analyzed by using double staining (Hoechst 33342/JC1). Data were analyzed using SAS (Version 8.2, SAS Institute Inc., Cary, NC, USA). The GLM procedure evaluated the effects of the freezing extender on the different sperm quality parameters. Results are shown as the mean ± standard deviation, these outcomes from the addition of different concentrations of RU486 failed to improve ($P<0.05$), relative to the control group, any of the parameters evaluated post-thawing. These results expressed in %TMS (A: 31.7 ± 18.0, B: 33 ± 18.3, C: 35.9 ±3.4, D: 24.8 ± 14.3), %VIAB (A: 76.4 ± 10.9, B: 69.03 ± 12.8, C: 67.9 ± 6.6, D: 66.23 ± 9.3), %VIAB/AR (A: 0, B: 0, C: 0, D: 0.12 ± 0.3), %DEAD/AR (A: 3.6 ± 1.9, B: 6.05 ± 4.9, C: 6 ± 3.3, D: 4.9 ± 3.1) and %SHMP (A: 29.93 ± 33.9, B: 24.18 ± 19.4, C: 23.3 ± 21.7, D: 23.7 ± 32). In conclusion, the progesterone inhibitor RU486 does not affect any post-thawing sperm quality parameter analyzed. Further studies are needed to test whether the combined use of RU486 and progesterone could induce a reduction in cryo-capacitation changes.

This study was supported by PID2019-108320RJI00, and RYC2020-028615-I, funded by MCIN/AEI/10.13039/501100011033 (Spain) and FEDER funds (EU).

Bioenergetics changes during in vitro capacitation of ram spermatozoa

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Keywords: sperm capacitation, energy metabolism, hyperactivation

Several biochemical and physical changes take place during sperm capacitation to prepare spermatozoa for oocytes' fertilization. Such changes, especially those related to modifications in the motility pattern (hyperactivation) and the increment of tyrosine phosphorylation, seem to require more energy. In spermatozoa, ATP is the primary source of energy and is mainly produced through oxidative phosphorylation (OXPHOS) and glycolysis. However, the metabolic exigencies during capacitation, including the preferred metabolic pathway, seem species-specific, and little is known about it in ram spermatozoa. The present study investigated metabolic variations during *in vitro* capacitation in ram spermatozoa together with diverse kinematic parameters to better understand this event. After removing the seminal plasma from ejaculates (n=9) with a density gradient (Bovipure®), ram spermatozoa were incubated at 38.5°C from 0 to 180 min under capacitating (CAP) and non-capacitating conditions (NC). The CAP medium was composed of synthetic oviductal fluid (SOF) with 10% of estrous sheep serum, while the NC medium by SOF with 0.1% polyvinyl alcohol. The ATP content and lactate excretion rate (final subproduct of glycolysis) were evaluated using a luminometer. The percentage of capacitated and acrosome-reacted sperm were assessed with a fluorescence microscope using the chlortetracycline fluorescence assay (CTC). Kinematic parameters associated with the acquisition of hyperactivation (curvilinear velocity: VCL; linearity: LIN; and amplitude of the lateral displacement: ALH) were measured by the CASA system. After 180 min, the proportion of capacitated and acrosome-reacted sperm (AR) increased ($p<0.05$) under CAP conditions compared to NC conditions (65.34 ± 2.18% vs. 13.50 ± 2.67% CAP; 28.16 ± 3.22% vs. 16.40 ± 2.75% AR). VCL and ALH increased ($p<0.05$) at the same time that LIN decreased ($p<0.05$) in those sperm incubated under CAP conditions at 180 min compared to NC conditions (VCL: 144.89 ± 4.26µm/s vs. 55.01 ± 4.78 µm/s; ALH: 3.35 ± 0.08 µm vs. 1.19 ± 0.1µm; LIN: 29.02 ± 2.93% vs. 48.12 ± 3.45%), which confirms the presence of the hyperactivated-like pattern. The ATP content of sperm was drastically reduced ($p<0.05$) after 180 min under CAP conditions in comparison to NC conditions (60.63 ± 4.21amol vs. 111.36 ± 3.86amol). Finally, the production of lactate also decreased ($p<0.05$) after 180 min under CAP conditions compared to NC conditions (6.19 ± 2.34nmol vs. 9.91 ± 2.53nmol). Our results revealed that *in vitro* capacitation of ram spermatozoa increases ATP consumption, which seems to promote a shift in the usage ratio of the two metabolic pathways mentioned, from glycolysis to OXPHOS. In addition, the increment of ATP demands during sperm capacitation could be in part attributed to the noted increase in the hyperactivated-like population.

Multidimensional characterization of the pig sperm chromatin by flow cytometry

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Keywords: Boar, semen, chromatin, DNA fragmentation

Artificial insemination (AI) is critical in the modern pig industry. The status of the sperm chromatin is often overlooked but is responsible for many cases of boar subfertility (low prolificacy and economic losses). Classical parameters analyzed, such as DNA fragmentation, only consider one aspect of the complexity of sperm chromatin structure. This study analyzed by flow cytometry 181 AI doses (22 boars, 2 collections, 3 ejaculates/week/collection) from a stud center in León (Spain) after production (day 0, D0) and after 11 days (D11) of storage at 17 °C. The analyzes were the DNA fragmentation index (SD-DFI and %DFI) and chromatin maturity (%HDS) by SCSA (Sperm Chromatin Structure Assay); Disulfide bridges between protamines (disulfide index [DB], median fluorescence intensity [MFI] and cell% of high, moderate and low thiol presence) by monobromobimane (mBBr) staining; Chromatin compaction (MFI and cell% for high, intermediate and low compaction) by chromomycin A3 (CMA3); and DNA oxidative damage by 8-oxo-dG immunostaining. The data were analyzed by Pearson correlations (false discovery rate adjustment), variable clustering (Hoeffding D statistic), and principal component analysis (PCA) with hierarchical clustering in the multidimensional space defined by the parameters and the relationship with the sources of variation (male, collection, and storage). Parameters within the same technique showed moderate to high correlations and significant ones among techniques, especially after storage. Thus, these techniques could identify different and potentially complementary characteristics in the boar sperm chromatin. At D0, 8-oxo-dG positively correlated with SCSA variables and mBBr MFI and negatively with the DB, whereas %HDS positively correlated with %high-mBBr, mBBr MFI, and low CMA3 and negatively with %moderate-mBBr and the DB. At D11, 8-oxo-dG correlated with SD-DFI and %HDS, and %HDS with %high-mBBr. The variable clustering was more similar before and after the storage, tending to associate the mBBr parameters with the SCSA ones and 8-oxo-dG at D0 and clustering out the CMA3 parameters. At D11, the mBBr MFI and the DB clustered with the CMA3 parameters, suggesting a modification in the chromatin structure possibly stemming from the reorganization of the disulfide bridges. The PCA resulted in a first principal component (PC1) influenced mainly by mBBr parameters and a second one (PC2) by SCSA and the 8-oxo-dG. The hierarchical clustering showed the storage day as the factor contributing the most to the variability among observations and along the PC1. PC2 did not result in cluster separation but suggested that boars mostly differed according to SCSA and 8-oxo-dG. This study demonstrates the potentiality of simultaneously using different chromatin analyses for boar sperm characterization and multidimensional techniques to interpret the data emerging from these analyses. Further studies should test if the variability due to the storage and the male explain fertility differences after AI.

Funded by RTI2018-095183-B-I00 (MINECO/AEI/FEDER, EU) and LE023P20, (Junta de Castilla y León/FEDER, EU). We thank Topigs-Norsvin España, AIM (León) and Marta De Prado (INDEGSAL).

A new protocol combining uterine fluid and sperm selection improves porcine in vitro fertilization output

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Keywords: biofluids, fertility, pig, selection.

From the ejaculation to the fertilization place, the sperm cross a dynamic environment within the female reproductive tract taking contact with different biofluids proceeding from male (seminal plasma, SP) and female (uterine fluid, UF; oviductal fluid). These fluids contribute to sperm selection so that the most suitable sperm can reach the oocyte. Thus, our aim was to improve knowledge about the effect of SP and/or UF on the sperm fertilizing ability before and after an in vitro selection, mimicking the in vivo events. For this purpose, we performed the in vitro fertilization (IVF) after contact with SP and/or UF before and after an in vitro selection by density gradients. Ejaculates were collected from 4 boars with proven fertility, and centrifuged (500 g, 5 min) to remove the SP. Four experimental groups were prepared ($20x10^6$ sperm/ml): 1) Control, sperm without biofluids (in PBS); 2) SP, sperm with 20% SP; 3) UF, sperm with 20% UF; 4) SP+UF, sperm with 20% SP and 20% UF. The samples were incubated for 3h at 38°C, then split in two aliquots, one of which was centrifuged (700 g, 5 min) with a capacitation medium, the other one was in vitro selected by Percoll density gradient 45/90% (700 g, 30 min). Then, each group was used to perform the IVF, and the following parameters were evaluated: penetration rate (%), monospermy rate (%), and efficiency (% of monospermic oocytes from total number of penetrated oocytes). A normality test followed by ANOVA test was performed by SAS (2016). When the IVF was performed before the selection, penetration rate was significantly higher in control (57.52±11.36%) than SP (21.08±7.64%), UF (24.20±5.26%), and SP+UF groups (17.40±8.30%) ($p< 0.001$), without differences in monospermy. Then, the efficiency was calculated, resulting in a higher percentage in control (45.60±4.93%) than SP (17.05±4.84%), UF (18.87±2.10%), and SP+UF groups (14.15±6.02%) ($p< 0.001$). Interestingly, after in vitro selection, sperm previously incubated with UF showed a penetration rate significantly higher (UF= 69.88±4.05%; SP+UF= 66.66±6.58%) than control (46.10±8.36%) and SP (48.12±11.86%) ($p< 0.02$), without difference in monospermy. Regarding the efficiency, UF and SP+UF groups were significantly higher (53.43±2.80%, 51.24±3.88%, respectively) than control (39.18±5.71%) and SP groups (31.74±4.64%) ($p= 0.01$). Based on the results obtained, UF incubation combined with a sperm selection induces a marked improvement in IVF. Therefore, further studies are needed to find possible biomarkers in the new combined sperm preparation system (UF and sperm selection) here presented responsible for this improvement.

Funding: Ministry of Science and Innovation (PID2019-106380RB-I00/AEI/10.13039/501100011033).

The reproductive fluids surrounding boar sperm before in vitro selection determines sperm molecular changes

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Keywords: biofluids, molecular markers, reproduction

The events prior to fertilization are characterized by the sperm interaction with different molecules proceeding from the surrounding environment. While crossing the male and female tracts sperm metabolize several substrates from the fluids in which they are immersed (seminal plasma–SP, uterine fluid–UF) and adsorb lipids from them inducing changes in sperm membrane composition (Shan, Int J Mol Sci; 22(16): 8767, 2021; Menezes, BMC Genomics; 20(1):714, 2019). These components may contribute to the selection of sperm suitable for fertilization. Thus, we investigated sperm lipidome after incubation with SP and/or UF and in vitro sperm selected by density gradient. Ejaculates were collected from 5 boars with proven fertility, and centrifuged (500 g, 5 min) to remove the SP. Four experimental groups were prepared ($20x10^6$ sperm/ml): 1) Control, sperm without biofluids (in PBS); 2) SP, sperm with 20% SP; 3) UF, sperm with 20% UF; 4) SP+UF, sperm with 20% SP and 20% UF. The samples were incubated for 3h at 38°C, then in vitro selected by Percoll density gradient 45/90% (700 g, 30 min) was performed. Each sample was centrifuged at 5200 g for 10 min at 4°C and stored at -80°C until analysis. Then, lipidome profiling was assessed by Ultra-Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry. Once the data were obtained, they were processed by LipidMS R-package, grouped by lipid species, and subjected to a univariate T-test analysis. Sperm lipidome results revealed a higher amount of total lipids in UF than Control, SP and SP+UF groups ($p=0.01$). In particular, glycerophosphoserines, glycerophosphoethinolamines, triglycerides, fatty acids, monoglycerides, diglycerides, ceramides and sphingosine were more abundant in UF than the other groups ($p<0.01$). Monoglycerides were also more abundant in SP than control and SP+UF groups ($p<0.04$). Regarding glycerophosphoethinolamines, fatty acids and monoglycerides, they were also more abundant in SP than SP+UF ($p=0.04$). In conclusion, the difference in sperm lipidome profiles depends on the fluid with which they take contact. In particular, the presence of UF increases the sperm lipid concentration, suggesting its involvement in the improvement of the reproductive performance.

Funding: Ministry of Science and Innovation (PID2019-106380RB-I00/AEI/10.13039/501100011033).

Validation of the open source OpenCASA sperm motility analysis software in three wild ruminant species

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Keywords: wild ruminants, semen, motility, CASA, methods agreement

Computer-assisted sperm analysis (CASA) is a technology enabling the assessment of sperm quality by automatic or semi-automatic evaluation of microscopic images or videos, typically for morphology (CASA-morph) or motility (CASA-mot) assessment. Currently, CASA includes analyses of features such as viability or DNA damage. Whereas the development of CASA has been parallel to proprietary software, open-source solutions have been proposed. OpenCASA was presented recently and has been tested in several species (Alquézar-Baeta et al. PLoS Comput. Biol. 15:e1006691, 2019). In this study, OpenCASA v. 2 was validated with ISAS v. 1.019 CASA software (Proiser, Valencia, Spain) for analyzing the motility of several wild ruminant spermatozoa: Red deer (n = 32), roe deer (n = 15), and chamois (n = 36). Image sequences were acquired from epididymal samples on a Makler chamber at 37 °C with a Nikon E600 microscope (10× negative contrast) and a Basler A312fc camera at 53 images/s, 25 image/s for roe deer samples. Total and progressive motility and kinematic parameters were obtained for each sample in both softwares (semi-automatically in ISAS and automatically in OpenCASA), using the same parameters for defining sperm detection (head area and motility features). The concordance correlation coefficient (CCC) and the Bland-Altman method (bias and coefficient of agreement) estimated between-method agreement (R statistical environment). CCC (rho with 95%CI) showed a high or moderately high agreement for total and progressive motility (roe deer: 0.79 and 0.89; red deer: 0.95 and 0.87; chamois: 0.90 and 0.69). Bias (mean ISAS-OpenCASA difference) and coefficient of agreement for total motility were 9.4 and 10.5 for roe deer, -3.0 and 11.7, and 0.35 and 7.4; and for progressive motility were -1.5 and 13.7, -6.5 and 9.9, and -13.2 and 9.4. Kinematic parameters (velocities and linearity estimations) showed CCC rho values around 0.7 and, in general, bias and coefficients of agreement below 10. These results indicate a good agreement between the two methods for these species; differences between methods could be explained by the algorithmic determination of sperm tracks, which are undisclosed in proprietary software. Since OpenCASA is based on open-source software, it could be easily expanded and adapted for other lab needs and analysis systems.

The sperm DNA oxidative damage and fragmentation are early predictive factors of the in vivo fertility of boar

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Keywords: boar, semen, chromatin, DNA oxidation, DNA fragmentation, fertility

Artificial insemination (AI) is the modern industry's standardized pig production system. Therefore, early detection of subfertile boars is essential to minimize economic losses in farming. Subfertility has been linked to DNA instability and fragmentation, and it usually goes unnoticed in field conditions. We hypothesized that DNA oxidative damage (a determinant for DNA fragmentation) could be an early predictive factor of boar subfertility. This work adapted the 8-hydroxy-2'-deoxyguanosine (8-OHdG, a marker of global DNA oxidative damage) immunodetection to study boar spermatozoa and its relationship with DNA fragmentation and boar fertility. AI doses from 18 boars (3 ejaculates/boar, 54) were donated by an insemination center near León (Spain). Fertility data was provided for the boars (parity and prolificacy, results after AI in productive conditions), classifying them in high (H), medium (M), and low (L) in vivo fertility. The AI doses were analyzed by flow cytometry right after production (day 0, D0) and after 11 days of storage at 17 °C (D11). The parameters used in the study were: mean fluorescence intensity (8-OHdG-MFI) and % of positive spermatozoa (%8-OHdG+) after 8-OHdG immunodetection, as measurements of DNA oxidative damage; and DNA fragmentation as %DFI (spermatozoa with increased DNA fragmentation index) and chromatin maturity as %HDS (spermatozoa with elevated DNA stainability) from the Sperm Chromatin Structure Assay (SCSA). The data were analyzed by linear mixed-effect models (effect of the fertility group) and Pearson's correlations (association among chromatin parameters). Group L showed a higher %DFI than M at D0 ($p<0.05$) and higher %HDS, 8-OHdG-MFI, and %8-OHdG+ than M and H both at D0 ($p<0.05$) and D11 ($p<0.001$). The L boars also showed a significantly higher %8-OHdG+ at D11 than at D0 ($p<0.001$). The association analysis indicated that %8-OHdG+ strongly correlated with 8-OHdG-MFI ($r=0.941$, $p<0.001$), and moderately with %HDS ($r=0.688$, $p<0.001$). 8-OHdG-MFI showed a moderate correlation with %HDS ($r=0.673$, $p<0.001$). This study suggests that the sperm DNA oxidation status could indicate boars' potential fertility. 8-OHdG immunodetection could be used at the beginning of the productive life of young boars to optimize the selection criteria of the best individuals for production maximization. Specifically, DNA oxidation status and chromatin immaturity could be valuable for the discrimination of subfertile males. Thus, the classical SCSA and the novel 8-OHdG immunodetection techniques should be considered for integrated use in a new generation of advanced tests in pig farms. Further studies should test the relation of DNA oxidation on other productive parameters and the extrapolation to other mammal species.

Funded by RTI2018-095183-B-I00 (MINECO/AEI/FEDER, EU) and LE023P20, (Junta de Castilla y León/FEDER, EU). We thank Topigs-Norsvin España, AIM (León).

Effect of date palm pollen on ram semen quality parameters during liquid storage at 5°C

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Keywords: ram semen, liquid storage, phoenix dactylifera L. pollen

The *Phoenix dactylifera* L. date palm pollen (DPP) is widely used in the traditional pharmacopeia of Moroccan oasis population. In fact, pure or mixed to medicinal plants, pollen powder is used to stimulate lactation in humans (Baliga et al., 2011; Hassan, 2011) and cattle. DPP and male palm flowers were traditionally claimed to be aphrodisiacs and fertility enhancers. In this context, the present study aimed to determine the phenolic compounds profile of the methanolic (MeOH) extract of DPP and its effects on Sardi ram semen quality parameters during liquid storage at 5 °C for up to 24 hours in skim milk extender (SM). To achieve this, DPP extracts were obtained after maceration in solvents of increasing polarity (hexane, methanol, ethanol, ethyl acetate and water) overnight in the dark at 30°C before undergoing evaporation under low pressure (Rotary evaporator Buchi R-210). The extracts were then tested for their radical scavenging abilities. MeOH, showing the best results, was chosen to continue this study and was analyzed for its phenolic compounds profile using high performance liquid chromatography coupled with a diode array detector (Shimadzu Cooperation, Kyoto, Japan) (Tokul Ölmez et al., 2020). Otherwise, semen samples were collected from six rams and extended with SM supplemented with DPP MeOH extract at 0, 1, 2 and 3 µg/ml to a final concentration of 0.8×10^9 spz/ml before being stored for up to 24 hours at 5 °C. The semen motility parameters were evaluated after 0, 4, 8 and 24 h using a computer-assisted sperm motility analysis (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain), while viability, abnormality and lipid peroxidation were evaluated using nigrosine eosin, Diff-Quik staining (Automatic Diagnostic Systems S.L., Spain) and measurement of thiobarbituric acid reactive species (TBARS) formed (Allai et al., 2016). SM supplemented with 3 µg/ml showed the best results and was then used to further detail this study. Statistical analysis was performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. The data, including motility, viability, abnormality, and lipid peroxidation, were tested for normality and homogeneity using the Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. A single-factor analysis of variance (ANOVA) was conducted to analyze the effects of the extracts at each storage time and the effect of storage duration in each extract. The results showed the presence of nine compounds belonging to the classes of phenolic acids and flavonoids in the pollen sample mainly ellagic acid, rutin and fisetin. Besides, a significant increase ($P < 0.05$) was noted in sperm total and progressive motilities, velocity and viability parameters in semen conserved with MeOH DPP extract added to skim milk compared to the control. Other beneficial effects have been recorded such as a significant decrease ($P < 0.05$) in sperm abnormality and lipid peroxidation. In conclusion, skim milk supplemented with 3 µg/ml of MeOH DPP extract enhanced ram semen quality after liquid storage. Further studies are required to verify these ameliorative effects on fertility rate in Sardi ewes during artificial insemination leading towards its incorporation into sperm extenders.

Comparative study of Windsnyer and Large White boars sperm cryo survival rate

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Keywords: Windsnyer boars, Large White boars, cryopreservation

Cryopreservation allows preservation of genetic variability through biotechnological reproduction programs. However, approximately 40 to 60% of boar sperm do not survive cryopreservation process. The objective of this study was to compare sperm cryo survival rate of Windsnyer and Large White boars. Total of 36 ejaculates (6 replications/boar) were collected from three Windsnyer and Large White boars of proven fertility with the use of hand-gloved technique method, twice per week. Boars semen were pooled and extended with Beltsville Thawing Solution [(BTS) IMV Technologies, France], held at 18˚C for 2 hours and centrifuged. The sperm pellet was re-suspended with Fraction A (20% egg yolk + BTS) and cooled at 5˚C for 1 hour then diluted with Fraction B [3% Glycerol (Laboratory Consumables & Chemicals Supplies cc, Johannesburg, South Africa) + 20% egg yolk + BTS] and loaded into 0.25 mL straws (Embryo Plus, Brits, South Africa). The semen straws were placed on liquid nitrogen (LN_2) vapour for 20 minutes and then transferred to the LN_2 tank. Thawing was accomplished by immersing the semen straws in water at 40˚C for 30 seconds. Sperm motility, viability and morphology characteristics were evaluated following thawing. Sperm motility was evaluated with the use of Sperm Class Analyser® (Microptin, Spain) system. Sperm viability and morphology were evaluated at 100X magnification under the microscope (Olympus, BX 51FT, Tokyo, Japan). The data were analyzed using the analysis of variance (general linear model) and statistical analysis system (SAS®). Treatment means were separated using Fisher's protected t-test the significant differences were determined by P-value at a significant level of $P<0.05$. Greater than 90% sperm total motility was recorded in the fresh semen of Large White and Windsnyer boars ($P>0.05$). Furthermore, highest frozen-thawed sperm total motility (51.1±12.7) and progressive motility (27.1±10.8) percentage was recorded in the semen of Large White boars ($P<0.05$). However, highest post-thawed live normal (31.8±6.7) and dead (67.8±7.2) sperm percentage was recorded in the semen of Windsnyer boars ($P<0.05$). Therefore, sperm cryo survival rate was maintained better in the semen of Large White boars.

The potential of Spirulina platensis as a feed supplement for rams reared in endemic fluorosis areas to enhance semen characteristics and seminal plasma composition

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Keywords: Fluorosis, ram semen, Spirulina platensis

Fluorosis is caused by long-term ingestion of high levels of fluoride and has negative effects on sheep production. Moroccan sheep reared in endemic fluorosis areas are particularly susceptible to this condition which leads to decreased bodyweight, wool production, and fertility. Rams may also experience decreased testosterone levels, lower semen quality, and increased oxidative stress (Rahim et al., Toxicology 465, 153025, 2022). Chronic fluorosis is common in areas of Morocco where livestock production is a vital economic contributor. Thus, there is a need to address the effects of fluorosis on ram reproduction in these areas. The present study aimed to investigate the potential of *Spirulina platensis* as a feed supplement for rams reared in endemic fluorosis areas to enhance semen characteristics and seminal plasma composition. Twenty-one rams aged 5 months were chosen for the present experiment, and they were separated into 4 homogeneous groups on body weight (BW) basis, age, and initial health conditions. Among these groups, G0 (3 rams) and G1 (4 rams) served as controls belonging respectively to fluorosis free (Settat) and endemic fluorosis (El Fokra) areas. The other two groups, G2 (6 rams) and G3 (8 rams) (belonging to El Fokra) were respectively supplemented with 250 mg/kg BW and 500 mg/kg BW. All animals were allowed to be taken to pasture and reared extensively. The experiment was carried out for 13 months (from 5 to 18 months of age). During the last 4 months of the experiment, semen collections (n= 168) and measurements were performed every 15 days. Seminal plasma was tested for total proteins, lipid peroxidation, superoxide dismutase, reduced glutathione, catalase, and vitamin C. Statistical analysis was performed using JMP.SAS.VR 2011 software, including single-factor ANOVA and Dunnett test with a p-value of 0.05. The study showed a significant decline in semen characteristics in G1 compared to G0. However, when *Spirulina platensis* was added as a dietary supplement, there was a noticeable improvement in semen characteristics for the other two groups (G2 and G3). These improvements included an increased semen volume, a better mass motility, and fewer abnormalities. Additionally, these findings indicate that the levels of total proteins, vitamin C, and the antioxidant system in the seminal plasma of fluorotic rams were adversely affected. However, the use of *Spirulina platensis* as a dietary supplement restored these imbalances. In conclusion, this study highlights the negative impact of fluoride on ram semen characteristics and seminal plasma composition. It demonstrates the beneficial effects of *Spirulina platensis* as a dietary supplement as it enhances semen characteristics and balances the antioxidant system in fluorotic rams.

Acknowledgment: The authors express their gratitude and appreciation for the assistance provided by all the organizations (INRA, OCP, CNRST, UM6P) involved in the SHS-ELM-01/2017 project.

IZUMO1 is required for gamete fusion in rabbits

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Keywords: Fertilization, gamete fusion, knock-out, rabbit, CRISPR.

Fertilization requires the fusion of the sperm membrane with the oolema. Such cell fusion event is poorly understood, although a discrete number of proteins have been proved essential for this process by gene ablation experiments (knock-out, KO) in mice. Between them, IZUMO1 was the first sperm protein required for murine fertilization. However, it remains unclear whether IZUMO1 or other of the proteins deemed as essential based on mouse models are also required for fertilization in non-rodent mammals, as only TMEM95 has been proven to be required for fertilization in mice and cattle. The objective of this study has been to determine if IZUMO1, a protein essential for gamete fusion in mice, is also required for fertilization in rabbits, a phylogenetically distant species (*Lagomorpha*). To that aim, we have generated a line of *IZUMO1* KO rabbits by CRISPR technology. A mosaic male carrying wild-type (WT) and edited alleles was crossed with WT females. Within the heterozygous (Hz) offspring a KO allele composed by a deletion of 5 nucleotides at the beginning of the coding region was selected to establish the line (F1 generation). WT and Hz males (F2 generation) were able to father litters, but 9 females mated with 3 *IZUMO1* KO males (3 females/male) did not deliver. As expected, given the sperm exclusive expression of *IZUMO1*, female fertility was unaffected by the ablation. To identify a potential fertilization failure in sperm lacking IZUMO1, oocytes were collected from WT females mated with WT or KO males (3 females/group) at 15 hours post-insemination (hpi) by oviduct flushing. Cumulus cells were almost completely absent in both groups and presumptive zygotes were cultured up to 40 hpi to assess cleavage rate. All oocytes recovered from females mated by *IZUMO1* KO males (n=34) failed to cleave, whereas normal fertilization rates were observed for WT males (27/33). Following fixation and DAPI staining, no pronuclei was detected in the uncleaved oocytes recovered from the cross with KO males, and KO sperm were present their periviteline space, evidencing that sperm lacking IZUMO1 were able to reach the oocyte and traverse cumulus cells and zona pellucida, but were unable to fuse its membrane with that of the oocyte. In conclusion, the essential role of *IZUMO1* in gamete fusion is conserved between mice and rabbits.

Work supported by project PID2020-117501RB-I00.

Impact of ejaculate fractions used during artificial insemination on porcine uterine vascularization

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Keywords: ejaculate, uterine vascularization, pig

The interaction of the components of an ejaculate with the uterus may result in the modification of the uterine environment, including vascular changes of the endometrium that may influence fertilization (O'Leary, S. et al. *Reproduction* 1470–1626, 2004; Álvarez-Rodríguez, M. et al. *Int. J. Mol. Sci.* 21, 5477. 2020; Bogacki, M. et al. *Genes* 11, 1302. 2020). Then, we aimed to study the effect of accumulative ejaculate fractions in semen doses on the vascularity of different uterine regions in the sows. A total of 20 sows were divided into a non-inseminated group (control-C, n=5); and 3 groups of sows inseminated (AI group) with different semen fractions (F): F1, inseminated only with the rich fraction of the ejaculate (n=5); F2, inseminated with F1 + intermediate fraction (n=5); and F3, inseminated with F2 + poor fraction (n=5). At day 6 post-insemination, the sows were sacrificed and the uterus were collected. The 3 uterine areas evaluated were the following: Region 1, which belongs to the uterine horn close to the oviduct; Region 2, which corresponds to the central zone of the uterine horn; and Region 3, the uterine horn next to the uterine body. The uterine samples were routinely fixed and processed for paraffin embedding. The immunohistochemistry with the primary antibody CD31+ was performed to identify the vascular endothelium and the slides were digitized at 0.172 pixel/µm (Pannoramic MIDI II scanner3D Histech®). The entire field was photographed at 10x with the SlideViewer® microscope and the images (5-7 images/slide) were analyzed with ImageJ® to obtain a tissue segmentation to calculate the percentage of the vascular area. Statistical analysis (SPSS®) included an ANOVA, Pearson's R correlation and non-parametric Kruskal-Wallis test comparing C and AI groups, semen fractions (F1 vs. F2 vs. F3) and uterine regions (Region 1 vs. 2 vs. 3). Significant differences were considered when *p-value* <0.05. A total of 302 histological images were analyzed, which corresponds to 8.93 mm^2 of uterine tissue. The interaction of the ejaculate with the uterus represented a significant increase of 5.1 percent in the vascular area of the AI group compared to the C. All ejaculate fractions showed significantly greater percentage of vascular area compared to C, being F2 the one with the highest values (F1: 24.2 percent ± 1.15; F2: 27.5 percent ± 1.05; F3: 23 percent ± 1.03 vs C 19.8 percent ± 1.2, $p<0.05$). Focusing on each anatomical area, the F1 and F2 showed significantly higher percentage of vascular area than F3 in the region 1. However, no statistical differences were found between fractions in the regions 2 and 3. The percentage of vascular area in different areas of the endometrium of pregnant sows 6 days after AI is modified by the semen fraction of the ejaculate used to prepare the semen doses.

Acknowledgments: Séneca Foundation 21656 / 21, Ministry of Science and Innovation PID 2019 106380 RB I 00 / 10 13039 501100011033 and Ministry of Science and Innovation PDC2022-133589-I00

Localization of toll-like receptor 7 (TLR7) in bull, ram and dog spermatozoa and assessment of its use as a sexing target

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Keywords: sexing, spermatozoa, TLR7

The X-linked toll-like receptor 7 (TLR7) seems to be a useful target for immunological sex sorting of spermatozoa in bull, mouse and goat using a TLR7/8 ligand known as Resiquimod (R848) (Umehara *et al.*, *Nature Protocols,* 15, 2645–67, 2020). Identification of a reliable target which can be used to sex spermatozoa would have great potential in both animal breeding and biomedical research on sex-linked diseases. Our objective was to localize TLR7 expression in the spermatozoa of bull, ram and dog and analyze its efficacy in separation of X- and Y-chromosome bearing spermatozoa.
The localization of TLR7 was determined via immunofluorescence staining of spermatozoa fixed on slides with 50:50 acetone:methanol (n=4 replicates). Slides were blocked for 1 hour with 5% normal goat serum in PBS and antibodies were diluted in this blocking solution [anti-TLR7; 1/100; BS-6601R, Bioss Antibodies, Woburn, USA][CY3 conjugated anti-rabbit; 1/400; A10520; Invitrogen, Waltham, USA]. TLR7 expression was categorized into negative or positive. Bull spermatozoa were incubated in a swim-up column of synthetic oviductal fluid (SOFaaci; Marei, Theriogenology, 86, 940-948, 2016) supplemented with 500 µM creatine, 2 mM glucose and 0.4% fatty acid free BSA +/- 0.03 µM R848 [HY-13740; MedChemExpress, South Brunswick Township, USA] following the method for bull sexing described by Umehara *et al*. Contrary to previous reports, TLR7 was found on the equatorial region of the sperm head and/or the tail region in bull sperm. Dog spermatozoa showed the same equatorial band with expression also on the post-acrosomal region of the sperm head; ram spermatozoa showed TLR7 expression homogenously throughout the entire post-acrosomal region of the sperm head. In assorted samples, approximately half (49% [SEM: 9%]) of bull spermatozoa expressed TLR7 indicating that TLR7 expression is restricted to X chromosome-bearing spermatozoa. After sexing, there was no significant change in the sperm concentration in the R848 treated sperm relative to control in either the bottom fraction of medium (7% [13%]) or the top fraction of medium (15% [24%]). After treatment with R848 only 24% [12%] of spermatozoa in the top fraction of medium expressed TLR7, suggesting that R848 treatment may have been effective, although this did not reach statistical significance via a paired T-test (p= 0.1552). No difference was observed in the proportion of TLR7 positive sperm in the bottom fraction of medium or in any of the controls. In contrast to bull spermatozoa, preliminary data indicated that 90% of dog spermatozoa expressed TLR7 (n=1). Similarly, 97% of ram spermatozoa expressed TLR7 (n=1), suggesting that R848 may not be an effective sexing target in these species, although further work is required to validate this. In summary, TLR7 expression appears to be localized to the spermatozoa head in dog, bull and ram, and is also expressed in the tail region of some sperm in bulls. Further work is required to determine the efficacy of utilizing R848 to separate X- and Y chromosome-bearing spermatozoa.

Sperm interaction with oviductal tissue in porcine

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Keywords: biomedicine, decellularization, sperm quality

Decellularization is a process by which cells and nuclear components are removed from a tissue in order to obtain decellularized extracellular matrix scaffolds to be used in a wide range of biomedical applications. One of the methods used for tissue decellularization is through detergents, which are toxic to cells, so they must be eliminated (Afarin Neishabouri, Frontiers in Bioengineering and Biotechnology, 10, 2022). Nevertheless, the effect the sperm interaction with reproductive decellularized tissues remains partially unknown.

Thus, the aim of this research was to study the effect of decellularized oviductal tissue (DOT) on porcine sperm quality over time. For this purpose, 4 experimental groups were prepared: 1) Control: semen sample (SS); 2) UW: SS + unwashed DOT; 3) W24: SS + DOT washed 24 h; 4) W48: SS + DOT washed 48 h. The oviducts were obtained from prepuberal sows, were dissected, and subjected to a decellularization process using the detergents Sodium Dodecyl Sulfate (SDS) (0.1%) and Triton X-100 (1%) (Sigma-Aldrich®, St. Louis, USA) under conditions of agitation at 150 rpm and refrigeration at 4 ºC. For the W24 and W48 groups, the oviducts were washed for an additional 24 and 48 h respectively with Phosphate Buffer Solution (PBS) (Sigma-Aldrich®, Madrid, Spain). On the other hand, semen samples (n = 5) were obtained by gloved hand method from boars (initial criteria: motility ≥ 70% and morpho-anomalies ≤ 25%) and were diluted with ND-10 (IMV Technologies, L'Aigle, France) to obtain 30 × 10^6 spermatozoa/mL. Once prepared, the seminal sample was coincubated with 2 × 2 mm portions of the DOT according to the experimental groups and then, sperm quality was evaluated (motility and kinetic parameters were analysed by CASA system (Proiser R + D, Paterna, Spain) and sperm viability, acrosome integrity and mitochondrial activity by fluorescence microscopy (Leica® DM4000 Led, Wetzlar, Germany) immediately after preparation and after an incubation for 1 and 3 h at 38 ºC. These parameters were compared by a sphericity for repeated measures test and was carried out using SAS University Edition program (SAS, 2016).The results showed that UW group negatively influenced sperm quality, significantly affecting total (71.07 ± 6.01) and progressive motility (35.47 ± 4.41) and viability (70.73 ± 3.33) parameters compared to the control (84,40 ± 2.26; 46.00 ± 3.47; 80.73 ± 2.40, respectively), with $p < 0.01$ in all cases. On the other hand, W48 group had no effect ($p > 0.05$) on viability (79,80 ± 3.08) and damaged acrosome (3.80 ± 1.09) compared to the control (70.73 ± 3.33; 3.07 ± 1.06), whereas the W24 group significantly ($p < 0.05$) affected viability (75.87 ± 2.20) and damaged acrosome (4.60 ± 0.97). In conclusion, W48 group had similar sperm quality to the control, hence, additional washes were required to completely remove residual detergents in the DOT and therefore avoid cytotoxicity and maintain sperm quality. In this way, interaction studies of reproductive decellularized tissue with sperm or other types of cells can be carried out.

Funding: Ministry of Science and Innovation PID2019-106380RB-I00 MCIN/ AEI /10.13039/501100011033 and PID2021-12309NB-C21 MCIN/AEI/10.13039/501100011033.

Identification and quantification of tyrosine phosphorylated proteins induced by FERT medium during rabbit sperm capacitation at different times

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Keywords: rabbit spermatozoa, capacitation and tyrosine phosphorylated proteins

Rabbit sperm capacitation is still a largely unknown process. Thus, the main objetive of this study was to examine the molecular mechanisms that modulate capacitation in this species at different times. Semen from six New Zealand White x California male adult rabbits was collected free from gel and sediments and pooled. Spermatozoa was selected by a swim-up procedure: sample was centrifuged twice at 1000 g for 5 minutes at room temperature and incubated in 2 mL of Tyrode's medium in a tube 45° inclined for 20 min with 5% CO_2 and at 38.5 °C. The top layer (800 µl) was then incubated in FERT medium (Tyrode´s medium with 2 mM sodium bicarbonate, 36 mM sodium lactate, 1 mM sodium pyruvate, 0.2% fatty acid-free BSA, 10 µg/mL heparin and 0.001% (w/v) of phenol red) (Sigma; St. Louis, USA) for 4, 6 or 8 h at same conditions. Capacitation status was evaluated by chlortetracycline (CTC) staining. Identification and distribution of tyrosine phosphorylated proteins (TyrPP) in rabbit spermatozoa was investigated by indirect immunofluorescence (IIF) and western-blot (WB) using the monoclonal anti-phosphotyrosine antibody (4G10, Millipore; Massachusetts, EEUU) and anti-alpha tubulin as loading control in WB (mAB 926-42213; LICOR, Nebraska, EEUU). At least 200 spermatozoa were scored per sample in CTC and IIF assays (n=5). Five different immunotypes were stablished depending on Tyr-PP localization: I) non staining, II) equatorial region, III) equatorial and acrosome region, IV) flagellum, V) equatorial region or/and acrosome and flagellum of the spermatozoa. Differences between experimental groups in CTC staining and TyrPP immunolabeling were compared by means of X^2 test and the quantification of TyrPP-WB by image J analysis were statistically analysed by ANOVA using GraphPad InStat software. Incubation of rabbit sperm selected by swim-up in FERT medium during 8 h increased the percentage of capacitated (51.4 vs. 19.6 %) and reacted (17.9 vs. 7%) sperm patterns by CTC compared with swim-up sample ($p< 0.001$). The localization of Tyr-PP in rabbit sperm by IIF revealed that in swim-up sample the immunotype III increased ($p< 0.001$) in relation to ejaculated sample. After incubation in *in vitro* capacitating conditions, the inmunotypes with labelling in the flagellum, IV and V increased ($p< 0.001$) compared to swim-up sample as in other species (Gimeno-Martos,Anim Reprod Sci.,221:106567,2020; Ruiz-Díaz, *Animals (10)*1467,2020) and could be related to changes in motility to promote oocyte binding. Also, WB analyses revealed an increase in the amount of TyrPP proteins (in the 37 KDa band) of capacitated samples relative to raw and swim-up samples compatible with results in rabbit sperm incubated with other medium during 16h (Saez-Lancelotti;*PLOS ONE*,5(10),e13457,2010). In conclusion, these preliminary results show, for the first time, that capacitation of rabbit spermatozoa can be induced by incubation in FERT medium for 8 h as demonstrated by increased percentage of Tyr-PP localization in the flagellum of spermatozoa and increased TyrPP levels.

This work has been funded by the MINECO (RTI 2018-094404-B-C-21 and 22). Gimeno-Martos S. holds a Margarita Salas Contract by the MINECO and the EU-NextGenerationEU.

Effect of different temperature conditions on sperm quality during storage: a comparison between sperm rich fraction and the whole ejaculate in boars.

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Keywords: storage, porcine, sperm quality

Lately, the application of semiautomatic sperm collection, which involves collecting the entire ejaculate, increased due to its practicality. This stands in contrast with traditional collection method, where only the sperm-rich fraction is used. Also, there has been a shift towards centralizing the distribution of semen doses in the swine industry, resulting in changes in the temperature conditions to which spermatozoa are exposed to during transport. Hence, the objective of the study was to assess sperm quality from the rich fraction and the complete ejaculate in different temperature conditions during storage. A total of 12 ejaculates (including 5 different boars of Pietrain breed) were used to form experimental groups: SR=sperm-rich fraction and BE=including the bulk ejaculate. Seminal doses (diluted in AndroStar® Plus extender; Minitüb, Tiefenbach, Germany) of each group (SR and BE) were kept in four temperature conditions: A) 15˚C for 5 days (15-group); B) 5˚C for 5 days (5-group); C) 5˚C until day 3 then 15˚C until day 5 (5/15-group); D) 15˚C until day 3 then 5˚C until day 5 (15/5-group). 5 ml of each group was stored with a concentration of $30x10^6$ sperm cells per ml. All samples were evaluated on day 1, 3 and 5 for motility and motion parameters by Computer Assisted Sperm Analysis (ISAS® software, PROiSER R+D S.L., Valencia, Spain), as well as for viability (propidium iodide), acrosome integrity (FITC-PNA) and mitochondrial membrane potential (JC-1) by fluorescence microscopy. On day 5 of storage, thermal- and cold-resistance tests were performed keeping aliquots in 38˚C for 5 hours or in 0˚C for 5 min, correspondingly. After that, the same parameters as mentioned above were evaluated. Statistical analysis was done by SAS OnDemand for Academics (2016) software using repeated measures design. To evaluate statistical differences after thermal- and cold-resistance tests, SPSS 28.0 software was used. A normality test followed by one-way ANOVA and a *post hoc* Tukey test was applied. For the variables not normally distributed, the non-parametric Kruskal-Wallis test was used. Significant differences were considered when $p<0.05$. The results showed that sperm from SR group kept at 5˚C for 5 days (81.61±1.08%) or first at 5˚C and then at 15˚C (81.50±1.58%) have lower viability than those stored at 15˚C for 5 days (85.17±1.48%; p=0.024 for 5-group and p=0.020 for 5/15-group) or first at 15˚C and later at 5˚C (85.22±1.47%; p=0.022 for 5-group and p=0.018 for 5/15-group). Similar pattern was observed for BE group in total motility, meaning there were fewer motile sperm when kept for 5 days at 5˚C (85.89±2.52%) than when kept at 15˚C (91.83±0.86%; p=0.002) or first at 15˚C and then at 5˚C (90.67±1.03%; p=0.013). Interestingly, after performing thermal- and cold-resistance test, all the differences between thermal conditions disappeared in both groups (p> 0.05). The findings show that the sperm from both, sperm rich fraction as from the whole ejaculate, can be stored, besides the common temperature at 15˚C, using a combination of 15ºC and 5ºC. However, the reverse combination (5/15-group) should be avoided as it results in decreased sperm quality.

Funding: Ministry of Science and Innovation (PID2019-106380RB-I00/AEI/10.13039/501100011033).

Potential sperm freezability biomarkers in young bucks of Cabra Blanca de Rasquera

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Keywords: sperm, freezability, bucks

Potential sperm freezability biomarkers as tools for early breeder selection for the creation and maintenance of sperm cryobanks are not determined in young bucks. Therefore, a retrospective study was performed to relate thawed sperm quality of 6 male donors of the Cabra Blanca de Rasquera breed at the age of 3 years old with melatonin concentration, scrotal circumference (SC) and body weight (BW) at early ages in order to help in the early selection of young males suitable for semen freezing. SC and BW measurements as SC/BW ratio and blood extraction for melatonin determination were performed every month from June to December, when the six males were 9 months old and finished at the age of 15 months. For melatonin determination, a jugular blood sample was collected (before 08.00 h) and immediately centrifuged at 1500× g for 10 min. The harvested serum was then stored at –20oC until hormone analyses. The concentration of melatonin in serum was measured by commercial Goat Melatonin (MT) ELISA Kit. Cat No. MBS267560. The frozen sperm samples (n=6 replicates/male) for this study belong to semen collections taken in autumn by artificial vagina. Then, all individual fresh ejaculates were centrifuged twice and diluted in the extender (15% powdered egg yolk and 5% of glycerol, final concentration), equilibrated for 4h at 5°C and packed into 0.25 mL straws before freezing in liquid nitrogen vapor. After thawing, sperm viability and morphology were assessed using eosin/nigrosin stain evaluating 200 cells/slide and 2 slides/sample. Statistical analyses were performed using SPSS program to compute the significance levels for Spearman correlations. Although significant variation (mean±SD, $p<0.05$) on melatonin concentration (129.3±33.0, pg/mL) and sperm viability percentage (45.4±11.0, %) between males along the negative photoperiod was observed, no significant correlations were found between thawed sperm quality parameters in adult males and melatonin concentration registered in early ages along the negative photoperiod, only a positive correlation between this hormone concentration and bent tail alive sperm percentage ($r=0.38$, $p=0.021$). Also, BW (39.4±3.4 Kg, mean ± SD) in young males showed a positive relation to dead sperm showing bent tail ($r=0.39$, $p=0.028$), to alive sperm with bent tail ($r=0.48$, $p=0.003$) and to the total percentage of bent tail sperm ($r=0.51$, $p=0.001$). Similarly, but negatively, SC/BW ratio was negatively correlated to alive bent tail sperm ($r=-0.46$, $p=0.005$), dead sperm with bent tail (r=- 0.37, $p=0.027$) and total sperm with bent tails ($r=-0.53$, p><0.001) after sperm thawing. Finally, only one negative correlation was found between SC (23.6±1.6 cm, mean ± SD) in young males and thawed sperm viability ($r=-0.37$, $p=0.027$) in adult sperm samples. In conclusion, further studies are needed to find more reliable correlations between young male parameters and thawed sperm quality in this endangered Catalonian local breed. Nevertheless, our results suggest the importance of considering the SC/BW ratio instead to only consider these parameters independently.> <0.05) for melatonin concentration (129.3±33.0, pg/mL) and sperm viability (45.4±11.0, %) was observed between males throughout the photoperiod, no significant correlations were found between thawed sperm quality parameters in adult males and melatonin concentration at an early age. A positive correlation was seen between melatonin concentration and proportion of bent tail live sperm ($r=0.38$, $p=0.021$). Also, BW (39.4±3.4 Kg, mean ± SD) in young males showed a positive relation to dead sperm with bent tail ($r=0.39$, $p=0.028$), to live sperm with bent tail ($r=0.48$, $p=0.003$) and to the total proportion of bent tail sperm ($r=0.51$, $p=0.001$). Similarly, SC/BW ratio was negatively correlated to live bent tail sperm ($r=-0.46$, $p=0.005$), dead sperm with bent tail (r=- 0.37, $p=0.027$) and total sperm with bent tails ($r=-0.53$, $p<0.001$) after sperm thawing. Finally, only one negative correlation was found between SC (23.6±1.6 cm, mean ± SD) in young males and thawed sperm viability ($r=-0.37$, $p=0.027$) in adult sperm samples. In conclusion, further studies are needed to find more reliable correlations between young male parameters and thawed sperm quality in this endangered Catalonian local breed. Nevertheless, our results suggest the importance of considering the SC/BW ratio instead to only consider these parameters independently.> <0.001) after sperm thawing. 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Embryology, Developmental Biology, and Physiology of Reproduction

Secretome of follicular fluid influences cytokine uptake by equine cumulus-oocyte complexes when added during in vitro maturation

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Keywords: cytokine, oocyte, equine

In vitro matured equine oocytes exhibit poor developmental competence compared to their *in vivo* counterparts, being in part related to a suboptimal composition of commercial maturation media (Fernández-Hernández, P.Animals; 10(5):883.2020). Cytokines present in follicular fluid (FF) play a role in ovulation and oocyte maturation (Liu, X. Front. Cell Dev. Biol; 8:578.2020). Our aim was to assess the presence of cytokines in the secretome obtained from mare preovulatory FF and its effect on equine oocyte *in vitro* maturation (IVM). When a preovulatory follicle was detected, 3000 IU of hCG were administered IV and 32 hours later FF was retrieved by flank aspiration (4 mares) as previously described (Hinrichs, K. Theriogenology; 34(1):107-112.1990). 3 ml of FF from each mare was diluted 1:1 individually in sterile PBS and centrifuged (4000 g,1 h at 4°C) using a 10K Amicon® Ultra-15 Centrifugal Filter Unit. The protein concentration of the retrieved secretome was measured and aliquots were kept at -80°C. Cumulus-oocyte complexes (COCs) were recovered from five mares in eight ovum pick-up sessions and matured in TCM-199 with 10% FBS, FSH (5 mIU/ml) in 5% CO_2/95% air atmosphere at 38.2°C and 100% humidity for 28 hours. COCs were matured (20 µl of IVM medium/COC) in the absence (CTR) or presence of secretome (pooled from two mares) at 40 µg/ml (S40) (Marinaro, F. Biology of Reproduction;100(5):1180–1192.2019). IVM medium was recovered before (pre-IVM) and after maturation (post-IVM) and kept at -80°C. Sixteen cytokines were measured using a MILLIPLEX MAP Equine Cytokine/Chemokine Magnetic Bead in the secretome from individual mares (n=4) and in IVM medium (n=5). Normal distribution was ensured using a Shapiro Wilk test and a repeated measures t-test was used to compare each cytokine in pre-IVM and post-IVM (*$p < .05$); data are presented as the mean ± standard error of the mean (pg/ml). Only FGF, Eotaxin, IP10 and RANTES were detected in secretome, the remaining cytokines laid below the detection range. Secretome cytokine concentrations (pg/ml) were: FGF (78.8±1.7); Eotaxin(49.6±25.2); IP-10(173.1±36.3) and RANTES(1.1± .3). The cytokine concentration pre-IVM vs. post-IVM was: CTR group, FGF (36.9±2.9 vs 37.4± 3.7); Eotaxin (37.4± 3.7 vs 54.2±12.3) and RANTES (1.4± .03 vs2.0± .9); S40 group, FGF (37.4± 3.7vs17.8± 5.6)*, Eotaxin (72.7± 6.3 vs41.9±10.8)*, RANTES (1.3± .06 vs 1.1± .06). FGF and Eotaxin concentrations dropped significantly in the S40 treatment after IVM compared to control ($p<0.05$). Different cytokines were detected in the secretome from preovulatory FF and the addition at 40 µg/ml during IVM seemed to induce uptake of FGF and Eotaxin by COCs. More studies are required to elucidate the role of these cytokines in oocyte maturation and if secretome addition enhances equine oocyte developmental competence.

MCCIN/AEI(PID2020-112723RB-I00;RYC-2017-21545;RYC2020-028915-I) and Junta de Extremadura-FEDER (IB20005). L-C, M is funded by an Acción II grant, UEX (Ref.Beca RC1); M-G, C is funded by the Investigo Program (Ref.PI-152-22), Junta de Extremadura.

Cumulus cell gene expression identifies key mechanisms influencing porcine oocyte developmental competence

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Keywords: cumulus cells, L-carnitine, FAO

Cumulus cells (CCs) can transfer metabolites and small molecules to the oocyte and have pivotal roles during oocyte growth and maturation through supporting e.g. metabolic processes and the capacity to regulate oxidative stress. The aim of this study was to identify key mechanisms within metabolism influencing porcine oocyte developmental competence and distinguish genes whose expression in CCs could predict oocyte quality. Oocytes collected from prepubertal gilts and cycling sows were used to compare oocytes of low and high developmental competence (Silva et al, Molecular Reproduction and Development., 90:323, 2023). Random gilt and sow ovaries were collected after slaughter and follicles with a diameter of 2-6 mm were aspirated. For both gilts and sows, immature cumulus-oocyte complexes (COCs) were randomly placed into the immature or *in vitro* maturation (IVM) groups, where total RNA from CCs was extracted either directly following aspiration or after 44 h IVM in porcine oocyte medium (POM) (6% CO_2, 38.8 °C). For all groups, CCs from triplicate pools of 50-60 COCs were used for RNA extraction and downstream analyses. RT-qPCR was performed employing TaqMan hydrolysis probes (Applied Biosystems, Foster City, California) and relative gene expression was calculated for in total 11 genes by the ΔCq method with efficiency correction after normalisation against *ACTB*, verified as the most stable reference gene by NormFinder. Normally distributed data were analysed using two sample t-test assuming unequal variance. Relative to CCs from sows, gilts showed a 14.8 fold increase (P=0.026) of *BBOX1* transcripts responsible for the last step of the L-carnitine biosynthesis pathway, while transcripts encoding CPT2, an indicator of the rate of fatty acid oxidation (FAO), exhibited a level of 0.48 (P=0.037) before IVM. After maturation there were no significant differences between gilts and sows in the genes involved in FAO, while transcripts encoding key enzymes of the pentose phosphate pathway (PPP) (G6PD), and glycolysis (ALDOA), were present in gilt CCs at levels of 0.64 (P=0.044) and 0.49 (P=0.070), respectively, relative to those in sows. The results suggest that FAO was downregulated in gilt CCs at the time of aspiration, caused at least in part by an insufficiency in L-carnitine as indicated by elevated levels of *BBOX1* transcripts. After IVM there appears to be sufficient L-carnitine to sustain a similar level of FAO in gilt CCs to that seen in sows. This study implies porcine COCs have the molecular machinery to modulate L-carnitine synthesis, and additional supplementation of the media with L-carnitine should be exercised with caution as excessive concentrations could yield adverse effects. Gilt COCs might not have adequate stores of ATP and be less competent in responding to oxidative stress compared to sow COCs at the end of maturation, as demonstrated through delayed FAO and downregulation of glycolysis and the PPP. Higher expression of *CPT2* in CCs before maturation and of both *G6PD* and *ALDOA* after maturation are potential markers of oocyte quality.

Supplementation of Mito-TEMPO during in vitro maturation and its effects on development and cryogenic viability of bovine embryos.

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Keywords: Embryo, Antioxidants, Vitrification

Despite many efforts, bovine embryos produced in vitro are still characterised by lower development rates, lower pregnancy rates and reduced cryogenic viability compared to ex vivo embryos. It has already been shown that the addition of mitochondrially active antioxidants to the maturation medium has a positive effect on ROS levels, development rates and cryogenic fitness of bovine embryos. Therefore, the aim of the present study was to investigate a possible effect of supplementing the maturation medium with Mito-TEMPO (Sigma, St. Louis, USA) on early embryonic development and cryogenic viability of bovine blastocysts. For the study, cumulus-ocyte complexes (COC) were obtained from the ovaries of slaughtered cows by slicing. Subsequent maturation was performed in TCM199 medium supplemented with (treatment) or without (control) 1 µM Mito-TEMPO for 22 hours in 4-well plates (NUNC, 400 µl, no oil overlay) with 50-70 COC per well (39°C, 5% CO2, 20% O2). For fertilisation, frozen semen was purified with SpermFilters® (IVF Bioscience, Falmouth, UK) and added to mature oocytes at a concentration of 2×106 cells/ml (Fert.-TALP. medium, 400 µl, NUNC). At 19 hours post fertilisation, COC were denuded by vortexing and then cultured for 8 days in synthetic oviduct fluid (SOFaa + 0.3% BSA, 400 µl, NUNC, oil overlay, 39°C, 5% O2, 5% CO2). On day 7 of culture, intracellular reactive oxygen species (ROS) levels were quantified in blastocysts from both experimental groups using fluorescence staining with 5 µM DCFDA (Sigma, St. Louis, USA) and comparative analysis was performed using an image analysis tool (ImageJ). In addition, day 7 blastocysts from both experimental groups were individually vitrified using BO-VitriCool™ media (IVF Bioscience) and the Cryotop® vitrification system (Kitazato, Shizuoka, Japan). Warming (BO-VitriWarm™ media, IVF Bioscience) of the vitrified blastocysts was followed by another post-warming culture for 72 hours to determine viability, expansion and hatching rates. GraphPad Prism software (version 9.3.1., Boston, USA) was used for all analyses and ANOVA was used to highlight differences between the two groups. The level of statistical significance was set at $p < 0.05$. The results of our study showed no significant effect of Mito-TEMPO supplementation during maturation on early embryonic development measured as blastocyst and hatching rate during in vitro culture. The same was observed for the measured ROS levels of blastocysts in both experimental groups and for the viability and expansion rate after warming. In contrast, the Mito-TEMPO supplemented group had a significantly ($p < 0.05$) higher hatching rate at 24, 32, 48, 56 and 72 hours after warming than the control group. These results confirm our hypothesis that the antioxidant Mito-TEMPO has a beneficial effect on mitochondrial properties, resulting in a reduction of cryo-induced damage after vitrification.

Description of a new quantitative method for the evaluation of mitochondrial distribution pattern in equine mature oocytes

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Keywords: Mitochondria, oocyte, equine.

In immature equine oocytes mitochondria are homogeneously distributed throughout the oolema (Ambruosi, International Journal of Endocrinology, 6, 1-5, 2011). In equine oocytes undergoing maturation, mitochondria migrate and result in the so known “aggregated patten" in which mitochondria are not evenly distributed within the cytoplasm being associated with cytoplasmic maturation (Alm, J Reprod Fertil Suppl 56, 473–482, 2000). However, no quantitative methods have been optimized to evaluate MDP in equine oocytes and only qualitative methods are currently used (Torner, Reprod Dom Anim, 42,176-183, 2007). Our aim was to design a quantitative method to evaluate the MDP based on the ratio of peripheral to central fluorescence intensity of the oocyte using confocal fluorescence. Equine cumulus-oocytes complexes (COCs, n = 56) were retrieved by ovum pick up (OPU). The oocytes were subjected to *in vitro* maturation (IVM) in TCM-199 medium, with 10% FBS and 5 mU/ml FSH for 26-28 hours in 5% CO2/95% air atmosphere at 38.2 °C. Oocytes were denuded and stained with MitoTracker™ Red CMXRosat 50 nM in TCM-199 with Hank´s and 10% FBS (v/v) for 15 minutes at 38 °C in the dark. Then, the oocytes were fixed with 4% formaldehyde in PBS + 0.01% PVA (w/v) and kept in the dark at 4 °C. The oocytes were also counterstained with 2.5 µg/ml of Hoechst 33342 for 10 minutes at 37 °C, mounted on slides and visualized; only metaphase II (MII) oocytes were considered. Fluorescence measurements were run on an inverted epifluorescence microscope (Axio Observer 7, Carl Zeiss, Germany) using a LD LCI Plan-Apochromat 25×/0.8 multi-immersion objective at a zoom of 1.3× with image acquisition (Axiocam 712 mono, Carl Zeiss, Germany) and analysis system for video-microscopy (ZEN Blue 3.4, Zeiss). Image processing and qualitative and quantitative analyses were performed using Fiji-ImageJ software. Firstly, based on qualitative analysis, MII oocytes were divided into two groups according to the distribution of mitochondrial pattern: homogeneous (HoD, n = 17) and heterogenous (HeD, n = 39). For the quantitative analysis, the background was subtracted and a 1-pixel line along the diameter was drawn; the intensity profile was calculated, and a fitting curve was generated. Next, fluorescence intensities were normalized and ratios of peripheral to central fluorescence intensity was calculated. Statistical analysis was performed using the Shapiro-Wilk test combined with t- test; $p < 0.05$. MDP ratio was (mean (arbitrary units) ± standard error of the mean): 0.8 ± 0.02 for HoD and 0.3 ± 0.02 for HeD; significant differences were observed between groups ($p < 0.001$). We describe a new approach to quantify mitochondrial distribution pattern in mature equine oocytes.

Funding: MCCIN/AEI (PID2020-112723RB-I00; RYC-2017-21545; RYC2020-028915-I) and Junta de Extremadura-FEDER (IB20005); M. L-C, is supported by an “Acción II” grant from UEx (Ref. Beca RC1) C.C. M-G, is supported by the Investigo Program (Ref. PI-152- 22) from Junta de Extremadura.

High concentrations of lipopolysaccharides are associated with decreased progesterone concentrations in equine follicular fluid

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Keywords: follicular fluid LPS, steroids and cytokines, mares

Accumulation of lipopolysaccharides (LPS) in follicular fluid (FF) impairs steroid production and oocyte developmental competence in cows and mice. This has not been investigated previously in mares. This study aimed to assess the FF concentrations of LPS and their association with steroid concentrations (E_2, estradiol and P_4, progesterone) and inflammatory response (IL-6, interleukin-6 and TNF-α, tumor necrosis factor-alpha) in FF of mares. At the slaughterhouse, FF and the follicle wall of the largest viable follicle (>30 mm in diameter) were collected from nonpregnant mares. To assess follicle viability, follicle walls were fixed (Bouin's solution) and stained with hematoxylin and eosin. Viable follicles (*n*= 16) with no degenerative changes were selected for further analysis. For LPS detection in FF, a colorimetric assay (Pierce™ Chromogenic Endotoxin Quant Kit, ThermoFisher Scientific, USA) was used. Immunoassays were used to measure the FF concentrations of E_2 (DRG, Germany) and P_4 (Progesterone III, Roche Diagnostics, Germany). Concentrations of IL-6 and TNF-α in FF were measured using ELISA Nori® kits (Genorise Scientific, USA). Spearman correlation coefficients between all measured variables in FF were calculated. Independent samples t-test was used to compare means of E_2 and P_4 concentrations in mares with high (≥LPS median concentration; 7.18 EU/mL, EU= endotoxin unit) and low (<LPS median concentration; 7.18 EU/mL) LPS values. A P value <0.05 was considered significant. The minimum and maximum concentrations were 5.21-12.08 EU/mL for LPS, 0.08-16.66 µg/mL for E_2, 28.10-79.50 ng/mL for P_4, 101.87-1080.71 pg/mL for IL-6, and 54.02-689.46 pg/mL for TNF-α. There were negative correlations between LPS concentrations and the concentrations of P_4 (r=-0.679, P=0.005), IL-6 (r=-0.556, P=0.025), and TNF-α (r=-0.637, P=0.008). Taken together, LPS is detectable in FF of mares and is negatively associated with progesterone concentrations in FF. There is no clear explanation regarding the negative associations between LPS concentrations and the concentrations of pro-inflammatory cytokines in FF. It is well-known that the IL-6 and TNF-α in FF are not only produced locally by follicular cells, but also transported from the systemic circulation. The crosstalk between maternal health related to Gram-negative bacterial infection and inflammation may alter the oocyte's microenvironment, which may affect oocyte quality. Further studies are ongoing to investigate the effect of LPS on the oocyte's developmental competence and subsequent embryo quality in mares.

Funded by the Ministry of Higher Education of the Arab Republic of Egypt.

Derived-proteome of extracellular vesicles from uterine fluid is modified depending on seminal plasma fraction during early pregnancy

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Keywords: extracellular vesicles, proteomics, seminal plasma, uterine fluid.

In porcine artificial insemination (AI) seminal doses are prepared using only the rich fraction of the ejaculate. However, this method is controversial, as seminal plasma (SP) promotes embryo development via specific signaling pathways within the female genital tract. Extracellular vesicles (EVs) participate in these pathways establishing intercellular communication by transferring their contents (RNA, proteins and lipids) to target cells. We hypothesized that SP from ejaculate fractions differentially affect the protein content of EVs in uterine fluid (UF). Therefore, this study aimed to characterize the uterine EVs (uEVs) proteome of sows, after being inseminated and prior to embryo implantation with different cumulative fractions of the boar ejaculate. A total of 6 fertile boars (Pietrain) were used and 3 different seminal doses were prepared ($30x10^6$ spermatozoa/60 ml): 1) F1=sperm rich fraction, 2) F2=F1+intermediate fraction and 3) F3=F2+poor fraction. Subsequently, 15 crossbred sows (Large-White x Danbred) with similar parity and body condition were inseminated with seminal doses (5 per group) using post-cervical AI method. Five non- inseminated sows, were used as a control group. After 6 days of AI, all sows were sacrificed, their genital tracts were dissected and UF was extracted by flushing the uterus with PBS. Embryos were isolated from the flushes under stereomicroscope to ensure pregnancy. Then, uEVs were isolated by ultracentrifugation and characterized by TEM. Size and protein concentration of uEVs were also analyzed by DLS (range from 142.40±30.53 to 202.92±18.20 nm) and Bradford (range from 0.57±0.27 to 3.35±0.97 µg/µl). Finally, 20 samples were analyzed by HPLC-MS/MS to study their proteome. Bioinformatic analysis was performed by GO analysis and most statistically significant (FDR<5%) GO terms were checked with REVIGO to discard redundancy. The classification of the proteins was assessed using DAVID. A total of 142 proteins were identified in uEVs, of which 16 were common to all groups. Thirty proteins were detected exclusively in uEVs from non-inseminated sows. Whereas 31 proteins were detected only in uEVs from sows inseminated by F1, 6 proteins were detected exclusively in F2 group and other 6 proteins were only detected in F3 group. These proteins exclusive to each group were shown to be involved in protection against oxidative stress, immune tolerance, embryogenesis, blastocyst attachment and angiogenesis during pregnancy. In conclusion, we revealed protein cargo of uEVs depending on the boar ejaculate fraction, however, we must consider that uEVs during pregnancy can be of embryonic and endometrial origin and the presence of embryos could influence uEVs proteome. Therefore, further validation is needed to ensure the presence of specific proteins in uEVs from different experimental groups.

Supported by Séneca Foundation 21656/21, Ministry of Science and Innovation PID2019-106380-RBI00/10130395011000011033 and Ministry of Science and Innovation PDC2022-133589-I00.

Effects of a polycaprolactone and polyethylene glycol diacrylate, 3-D printed scaffolds, on bovine embryo development in vitro

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Keywords: polycaprolactone (PCL), polyethylene glycol diacrylate (PEGDA), bovine, embryo development, bovine embryo assay

Despite the improvements in Assisted Reproductive Technologies (ART), in vitro environment is still far from physiological, causing low blastocyst quality and impaired epigenetic reprogramming. In the last years, 3D printing has been implemented in several research areas and can be used to create an IVF device that mimics the oviduct, the organ where fertilization takes place. However, before creating the device, the feasibility of the materials to support IVF needs to be tested. We aimed to evaluate the suitability of scaffolds printed with polycaprolactone (PCL) (CELLINK, Gothenburg, Sweden) and polyethylene glycol diacrylate 500 (PEGDA500) (CELLINK, Gothenburg) to support IVF and further embryo development. We carried out a bovine embryo assay (N=3 replicates) and the following experimental groups were settled: Rinse PCL (N=148) and rinse PEGDA500 (N=143), where the IVF was performed in IVF medium conditioned by the scaffolds during 24h; Scaffold PCL (N=144) and scaffold PEGDA500 (N=131), where the IVF was performed in the different scaffolds, and control group (CTRL) (N=259), where no scaffold nor conditioned medium were used. Prior IVF, both materials were sterilized in 70% ethanol for 5 min, washed in PBS for 30 min and 24h in IVF medium. For IVF, in vitro matured oocytes were incubated during 22h in Fert-TALP medium (Parrish et al, Theriogenology, 25, 591-600, 1986) with frozen-thawed bull sperm ($1x10^6$spz/ml) selected by Bovipure gradient (Nicadon, Sweden). After IVF, zygotes were washed and cultured in SOF medium supplemented with 0.3% BSA (w/v) covered with paraffin oil (NidOil, Nicadon) on a normal petri dish. To assess IVF outcomes, blastocyst rate at day 7 (BR7) and 8 (BR8) were registered. At day 8, blastocysts were fixed and stained with Hoechst 33342 to assess the cell number per embryo under fluorescence microscopy. Data were analyzed by One-Way ANOVA. Differences were considered significant when $p<0.05$. We found significant differences in blastocyst rate (expressed as BR7±SD, BR8±SD) between CTRL (20±4%, 21±0%) and rinse PEGDA500 (4±3%, 7±5%), and between CTRL and scaffold PEGDA500 (7±6%,10±7%), while we did not find significant differences between CTRL and rinse PCL (18±4%,22±6%) or scaffold PCL (25±10%,29±8%). Results showed similar cell numbers, being 100.65±39.49 for CTRL, 116.59±37.14 for rinse PCL, 83.96±32.87 for scaffold PCL, 103.11±30.28 for rinse PEGDA500 and 77.09±23.08 for scaffold PEGDA500. These data suggest that PEGDA500 has a detrimental effect on bovine embryo development since it promotes a lower blastocyst rate at day 7 and 8. This is an unexpected effect since PEGDA500 has been proposed as a nice candidate to perform studies for embryogenesis and organogenesis (Hribar et al, Lab Chip, 15, 2412-2418, 2015), and could be due to the different cell types or to some leaked compound used to print and stabilize the scaffold. On the other hand, PCL shows great biocompatibility since embryo development was not impaired by the presence of the scaffold during IVF. In conclusion, the current data suggest that PCL could be used to construct an IVF device.

The effect of iloprost on energy status of the in vitro produced bovine blastocysts

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Keywords: bovine, embryos

Oviduct fluid is composed of substances and co-factors that support cleavage and early development *in vivo*. Imitating the oviductal environment has been the challenge for making *in vitro* culture (IVC) successful, but there is still no general consensus defining IVC conditions for *in vitro*-produced (IVP) embryos. None of the attempts to improve this conditions have produced consistent bovine blastocyst production rates above 40-50%. Prostacyclin (PGI2) is a lipid molecule synthesised primarily in endothelial cells as well as oviductal endothelial cells. For this reason, several studies have tested the role of PGI2 in pre-preimplantation period by supplementing the IVC medium with iloprost, a PGI2 analogue. The results of the study performed on pigs demonstrate that the use of iloprost during culture improves the *in vitro* development of porcine embryos. The authors showed that an analogue of PGI2 plays an important role in meiotic progression in porcine oocytes through the regulation of cAMP/PKA activity (Kim et al., 2010). In cows, iloprost positively affected the development of IVP and somatic cell nuclear transfer (SCNT) embryos cultured *in vitro* by stimulating the cAMP response element-binding protein (CREB)-COX2 signalling pathway (Song et al., 2009). Although, in our previous study we found that, treating cumulus oocytes complexes (COCs) with iloprost improved oocyte quality and maintained their developmental capacity during *in vitro* maturation (IVM) the role of iloprost during IVC remains unknown. Therefore, in the present study, we examined the effects of iloprost on blastocyst developmental rates and quality, as well as mitochondrial function in the *in vitro* produced bovine embryos. Ovaries were collected from mature Holstein cows at a local abattoir and cumulus-oocyte complexes (COCs) were isolated by aspiration from ovarian follicles (n=1308). Following 24h of *in vitro* maturation (IVM; TCM 199 Maturation Medium, 19990/0010, Minitube) COCs were *in vitro* fertilized (IVF; TL sperm capacitation medium, 19990/0020, Minitube) and embryos were *in vitro* cultured (IVC; SOF synthetic oviduct fluid medium, 19990/0040, Minitube) for 7 days. Two groups were established to conduct the study: 1) control, and 2) experimental, embryos exposed to an iloprost (50μM). All methodology of IVP are described in Kowalczyk-Zieba et al., (2020). The analyses were performed using the statistical software GraphPad PRISM 6.0. Iloprost had no direct effect on blastocyst rates on Day 7 (control 134±20.4 vs. experimental 147±22.8, $P>0.05$). However the number of expanded (65±48.5 vs. 84±56.4, $P<0.05$) and hatched (9±6.7 vs. 15±10.1, $P<0.05$) blastocysts was higher in iloprost-treated groups. Embryos treatment with iloprost during *in vitro* production resulted in reduction in intracellular reactive oxygen species levels in blastocysts Day 7 ($P<0.05$). Iloprost impoved mitochondrial membrane potential and active mitochondria, using JC-1 fluorescent reaction and MitoTrckerRed CMXRox, respectively in blastocyst Day 7 ($P<0.05$). Furthermore, supplementation the IVC medium with iloprost influenced on mRNA expression of the genes involved in mitochondrial function (Clpp, GLu1, GPx4 and Polg2) and blastocyst quality markers (OCT4, SOX2, NANOG, PLAC8) using RT-qPCR. In the blastocyst produced with iloprost we found higher mitochondrial DNA copy number ($P<0.05$) analysed by qPCR. In conclusion, our results demonstrate that treating bovine embryos with iloprost may improve blastocyst quality and maintain their developmental capacity during IVC. We thus propose iloprost as a new agent for the prevention of developmental loss of bovine embryos in ARTs.

Supported by Polish National Science Centre: 2015/17/B/NZ9/011688.

Stage- and quality grade-dependent expression of developmental competence gene markers in comparison with prostaglandin E2 synthesis in the early- and late-cleaved bovine blastocysts

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Keywords: cow, prostaglandin E2, blastocyst

Prostaglandin (PG) E2 plays a role in oviductal transport of gametes, hatching from the zona pellucida and early embryonic development. The expression profiles of 3 PGE2 synthases were differ between early- and late-cleaved bovine embryos, with higher PTGS2 expression and lower cPGES mRNA level in 16-cell embryos and with higher PTGS2 and cPGES expression in expanded grade C blastocyst from late-cleaved group. The aim of the study was to examine whether the stage- and quality grade-dependent (classified by morphological assessment) expression profile of embryo developmental competence markers is associated with expression of PGE2 synthases in bovine blastocyst from early- and late-cleaved embryos. Ovaries were collected from Holstein cows at a local abattoir and cumulus-oocyte complexes (COCs) were isolated. After 24h of IVM, COCs were fertilized, and according to Lonergan et al. (1999), early-cleaved embryos (good quality) were separated at 30 hpi whereas late-cleaved embryos (bad quality) were isolated at 36 hpi. All IVP methodology and blastocyst classification are described in Boruszewska et al. (2019). For RNA isolation, 5 repetition of 5 embryos/tube from each category of stage and quality within 2 analyzed group were used. The expression of mPGES1, mPGES2, cPGES, OCT4, SOX2, IGF1R, IGF2R, PLAC8 were examined by RT-qPCR. Statistical analyses were conducted using two-way ANOVA (fixed factor: experimental groups; random factor: developmental stage/quality of blastocysts) followed by Tukey's multiple comparison test or a correlation analysis using Pearson correlation coefficient (GraphPad PRISM). We found that PTGS2 mRNA level was higher in hatched and in grade C blastocysts; and cPGES level was higher in early blastocysts, blastocysts, and expanded blastocysts and in grade A, B and C blastocysts from late-cleaved group ($p < 0.05$). The mRNA level of SOX2, OCT4, IGF1R, IGF2R and PLAC8 was higher in early blastocysts in late-cleaved group ($p < 0.05$). The IGF1R and IGF2R mRNA levels were higher in grade A and B blastocysts, OCT4 and PLAC8 mRNA levels were higher in grade B blastocysts, and SOX2 mRNA level was higher in grade B and C blastocysts in late-cleaved group ($p < 0.05$). In early-cleaved embryos, PTGS2 mRNA level correlated positively with mRNA level of all markers in expanded blastocysts from late-cleaved group ($p < 0.05$). The cPGES mRNA level correlated positively with mRNA level of IGF1R, IGF2R, SOX2 and PLAC8 in expanded blastocysts from early-cleaved embryos; and with mRNA level of SOX2, OCT4 and PLAC8 from late-cleaved group ($p < 0.05$). In summary, the mRNA levels of PGE2 synthases and developmental competence genes were affected by the embryonic stage of development and quality classified by morphological assessment. Our research accounts for many correlations between mRNA level of gene markers and PGE2 syntheses (mainly PTGS2 and cPGES), that vary in bovine blastocysts depending on the time of first cleavage.

Sex-specific gene expressions of bovine elongated embryos triggered by the physiological conditions of the recipients

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Keywords: Male, female, elongated, embryo, gene

Sex-biased embryonic losses due to the differential secretion of signalling molecules by male and female embryos in response to the maternal microenvironment are believed to be one of the causes of skewed male to female ratio at birth in cattle and other animals. Therefore, this study was conducted to understand the gene expression patterns in bovine elongated male and female embryos developed in multiparous cows or heifers. For this, *in vitro* produced 4-8 cell stages of male and female embryos were transferred to multiparous cows and heifers. The elongated embryos were then recovered on day 13 of the gestation period and classified according to their origin and sex. Total RNA was isolated from each group of embryos using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). RNA sequencing libraries from 5 elongated embryos per group were prepared using the NEBNext Ultra II RNA library prep kit for Illumina (NEB, Ipswich, MA, USA) and sequenced using Illumina NovaSeq in a 2x150 bp configuration. Adapters were trimmed using Trim Galore (Babraham Bioinformatics) and clean reads were mapped to the bovine reference genome using the bowtie2 alignment tool. Quantitation was performed using the RNA-Seq quantitation pipeline of the Seqmonk tool (Babraham Bioinformatics). Differential expression analysis was done using the EdgeR package (Robinson et al. 2008, Bioinformatics, 26, 139-140) and genes which showed expression differences with p-value < 0.05 and false discovery rate < 0.1 were filtered. Functional enrichment analysis was performed using gprofiler (https://biit.cs.ut.ee/gprofiler/gost). The results indicated that 13948, 13775, 13972 and 13341 genes were expressed in both male and female elongated embryos developed in cows and heifers, respectively. Among these, including *ACTG1, COX1, COX2* and *COX3,* the expression level of 38 genes was highly expressed with > 9000 read counts in all sample groups. These are involved in energy production and metabolism. On the other hand, including *CYP39A1, CYP2R1, CYP27B1, CYBRD, PAG8* and *PAG12,* a total of 197 genes were differentially expressed between the male and female elongated embryos developed in cows. Some of these are involved in steroids and lipid biosynthetic processes. Similarly, including *GSTO1, PRKD1, POU2AF1, NOS2,* and *HSD3B1,* a differential expression of 293 genes was detected between the elongated male and female embryos developed in heifers. Some of these genes are involved in organ development, tissue morphogenesis, and female sex differentiation. Therefore, this study indicates that although genes associated with energy production and metabolism were highly expressed in all embryo groups, the cow's maternal environment could induce differential expression of genes that are potentially associated with steroid and lipid biosynthetic processes in male and female embryos, but the heifer's maternal environment could induce the expression of genes associated with sex differentiation and organogenesis or tissue morphogenesis in male and female embryos differently.

The BMP15 added to IVM medium of prepubertal goat oocytes increases oocyte EGFR expression and cumulus-oocyte communication

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Keywords: goat, BMP15, IVEP

Oocyte competence for embryo development depends on bidirectional communication with cumulus cells (CCs; Gilchrist, Hum Reprod Update, 14(2):159-77, 2008). This is mediated by the transzonal projections (TZP), which are thin cytoplasmatic filaments that project from the CCs and penetrate through the zona pellucida to the oocyte. Oocyte-secreted growth factors (OSFs) such as bone morphogenetic protein 15 (BMP15) induce proliferation and differentiation of CCs and improve embryo development (Sudiman, J Assist Reprod Genet, 31(3):295-306, 2014). OSFs are suggested to promote the epidermal growth factor network, which is associated with oocyte competence (Richani, Hum Reprod Update, 24(1):1-14, 2018). Our aim was to study the effect of adding BMP15 to IVM medium on embryo competence of prepubertal goat oocytes (1-2 months old), the TZP and the epidermal growth factor receptor (EGFR) expression. Cumulus-oocyte complexes (COCs) were collected by ovary slicing and matured in TCM-199 with FSH, LH, estradiol, EGF and cysteamine during 24h at 38.5ºC with 5% CO_2. IVM medium of the experimental group (BMP15 group) was supplemented with 100 ng/ml of BMP15 (R&D systems, USA) . The control group was IVM medium without BMP15. A total of 733 IVM-oocytes (7 replicates) were in vitro fertilized with $4x10^6$ sperm/ml frozen-thawed semen in BO-IVF medium (Bioscience, UK) for 19h. Presumptive zygotes were cultured for 8 days at 38.5ºC with 5% CO_2 and 5% O_2 in BO-IVC medium (Bioscience, UK). A sample of 10 COCs/replicate (3 replicates) were recovered at several time points during IVM (0h, 6h, 12h and 24h) for TZP density assessment. Actin filaments were stained with phalloidin-FITC and TZP density was quantified as phalloidin-FITC average fluorescence intensity in the zona pellucida area. The EGFR protein levels of MII-oocyte were assessed after 24h of IVM. A total of 40 MII-oocytes for BMP15 group and 26 for control group (4 replicates) were quantified by immunofluorescence using an anti-EGFR antibody (Invitrogen, USA). Fluorescence was quantified with ImageJ software. Data were statistically analyzed by two-way ANOVA followed by Tukey's correction. The results of blastocyst development of BMP15 (380 IVF-oocytes) and control (353 IVF-oocytes) groups showed no significant differences (8.8% ± 3.0 and 7.4% ± 2.8, respectively). The TZP density at 6h IVM of BMP15 COCs was higher ($p<0.05$) than control COCs (20.7 ± 1.6 and 11.9 ±1.6 arbitrary units, respectively). There was an increase ($p<0.05$) in the BMP15 group at 6h compared with 0h (14.9 ± 1.2) that was not observed in control group. There were no significant differences between groups at 12h or 24h. The expression of EGFR was higher ($p<0.05$) in MII-oocytes treated with BMP15 than control ones (22.5 ± 0.7 and 18.3 ± 0.8 arbitrary units, respectively). In conclusion, adding BMP15 to IVM medium of oocytes from prepubertal goats promoted EGFR expression and increased TZP density at 6h after follicular recovery, suggesting an enhanced cumulus-oocyte communication. However, it did not affect in vitro development after IVF.

This study was funded by the Spanish Ministry of Science and Innovation (PID2020-113266RB-100).

Effect of a biphasic in vitro maturation system with c-type natriuretic peptide and oestrogens on the quality of prepubertal lamb oocytes

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Keywords: Keywords: IVM, pre-maturation culture phase, lamb

One of the problems presented by oocytes from prepubertal animals in vitro matured is a lack of synchronization in nuclear and cytoplasmic maturation (Kochhar et al., Reprod Dom Anim 37:19-25, 2002). Biphasic maturation with meiotic inhibitors has shown to increase developmental competence of prepubertal goat oocytes (Soto et al., Plos One 23;14(8):e0221663, 2020). The aim of this study was to assess the effect of C-type natriuretic peptide (CNP) and 17ß-estradiol (E2) on lamb oocytes 'quality. Ovaries from lambs (1-5 months old) were recovered at a local slaughterhouse. Cumulus-oocyte complexes (COCs) were collected by ovary slicing and selected using HEPES-buffered (25 mM) TCM-199 medium with the meiotic inhibitor 3-Isobutyl-1-methylxanthine (500µM) and heparin. COCs were cultured in TCM-199 with 200 nM CNP and 10 nM E2 for 6h and then in conventional IVM medium (TCM-199 with FBS, FSH, LH, oestradiol and EGF) for 24 hours (biphasic group) at 38.5ºC with 5% CO2. Oocytes in the control group were cultured in conventional IVM medium for 24 hrs under the same conditions. After 24 hrs (control group) and 30 hrs (biphasic group) of culture, a sample of COCs were denuded and stained for assessing reactive oxygen species (ROS) and glutathione (GSH) levels and with Brilliant Cresyl Blue (BCB) stain for evaluating the growing of oocytes. BCB determines the intracellular activity of G6PDH enzyme which is active in growing oocytes and degrades the dye (BCB-). After BCB staining, all the oocytes classified in BCB+ (fully grown) and BCB- (growing oocytes) were parthenogenically activated (PA) with ionomycin and DMAP and cultured in BO-IVC medium (Bioscience, UK) for 8 days at 38.5ºC with 5% CO2 and 5% O2 .The rates of BCB stained oocytes, oocyte cleavage at 28 hrs and blastocysts at day 8 after PA were statistically analyzed by Chi Square test with Yates correction test. The data from ROS and GSH levels were analyzed using unpaired T-test. The rate of oocytes that had reached their growth phase (BCB+) were higher ($p<0.01$) in biphasic group (91.4%; n=105) than control group (63.5%; n=104), while both ROS (30 oocytes per treatment) and GSH (30 oocytes per treatment) levels were lower ($p<0.01$) in biphasic group than control group. The IVM group showed 1.8 and 1.18 more intensity than biphasic group in ROS and GSH stain, respectively. After PA of 153 oocytes from biphasic group and 163 oocytes from control group, oocyte cleavage rate was higher ($p<0.01$) in biphasic group (52%) compared to control group (35%) although there was no significant difference between both groups ($p=0.1081$) in blastocyst production at day 8 of in vitro embryo culture (12% and 6% of the total oocytes activated from biphasic and control group, respectively). In conclusion, the results from this study show that biphasic maturation increases the quality of oocyte and the rate of embryo produced after parthenogenetic activation in prepubertal lamb, but it does not affect blastocyst production. Further experiments are required to explain these results.

This study was funded by the Spanish Ministry of Science and Innovation (PID2020-113266RB-100).

GATA3 is dispensable for ovine blastocyst formation and first lineage differentiation, but plays a role in epiblast development

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Keywords: ovine, blastocyst, GATA3

The first lineage differentiation in mammalian embryos leads to the formation of the inner cell mass (ICM) and the trophectoderm (TE). In mice, GATA3 drives TE fate and regulates other TE genes such as *Cdx2*, although its ablation does not abrogate blastocyst formation. In bovine, a recent report has described that GATA3 deletion downregulates the core pluripotency factor NANOG. The objective of this study has been to elucidate the role of *GATA3* in ovine embryos by evaluating the developmental potential of *GATA3* KO embryos generated by CRISPR. *In vitro* matured oocytes were microinjected with Cas9 mRNA and a sgRNA against *GATA3* (C+G) or with Cas9 alone as microinjection control (C). Microinjected oocytes were fertilized and cultured *in vitro* up to Day (D) 8 (in SOF medium) or D12 (in N2B27 medium from D6/7), when pictures were taken and embryos were fixed and immunostained to detect GATA3, CDX2 (TE marker), SOX2 and NANOG (ICM/epiblast markers). Embryo genotyping in C+G group was performed by deep sequencing. Blastocyst rate was similar in the group containing *GATA3* KO embryos (C+G) and in the control group (C) (26.3±1.1% vs. 27.2±3.3%, mean ± s.e.m, 5 replicates, t-test; $p>0.05$). In C+G group, 23/55 (41.8%) blastocysts genotyped were *GATA3* KO (containing only frame-shift alleles). GATA3 protein was not detected in KO embryos, which showed normal morphology and expressed CDX2. *GATA3* ablation did not affect CDX2, SOX2 or total cell numbers (CDX2+: 80.9±22.6 vs. 94.5±18.6; SOX2+: 15±3.5 vs. 17.4±2.4; total: 139.2±24.5 vs. 156.5±22.9; mean±s.e.m for KO vs. WT). However, the number of NANOG+ cells was significantly reduced in KO embryos (9.7±6.1 vs. 16.2±3.1; mean±s.e.m for KO vs. WT, Mann-Whitney test $p<0.05$). Embryo survival from D6/7 to D12 in a post-hatching culture system was similar between C+G and C groups (93.03±2.98% vs. 83.93±6.83%, mean ± s.e.m, 4 replicates). No differences were found in embryo area between KO and WT (0.34±0.05 vs. 0.32±0.03 mm^2, mean ± s.e.m for KO vs. WT), but the number of embryos showing surviving epiblast cells (12/30 [40%] vs. 16/20 [80%]; KO vs. WT) and SOX2+ cell number (10.97±3.64 vs. 18.25±4.03; KO vs. WT) were significantly reduced in KO embryos (Chi-square and Mann-Whitney tests; $p<0.05$). No differences were found in the number of embryos developing an embryonic disc (4/12 [33.33%] vs. 5/16 [31.25%]; KO vs. WT). In conclusion, *GATA3* is dispensable for ovine blastocyst formation and TE vs. ICM specification, but its ablation impairs proper NANOG expression and further epiblast development *in vitro*.

Work supported by StG 757886-ELONGAN and PID2021-122153NA-I00.

MEK signalling pathway is required for hypoblast specification in ovine blastocysts in vitro

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Keywords: ovine, embryo, MEK pathway

Proper development of the first cell lineages is critical for embryo survival. The second lineage specification differentiates the inner cell mass cells into epiblast, which will form the proper foetus, and hypoblast, which together with the trophectoderm will form the extraembryonic membranes and the foetal part of the placenta. In the mouse, hypoblast differentiation is induced through the MEK signalling pathway, and MEK inhibition with 1 µM PD0325901 (PD) impairs hypoblast specification. However, whether hypoblast differentiation in ungulates completely depends on MEK pathway remains unknown. Controversial results have been reported in bovine and porcine embryos treated with 0.4 to 10 µM PD and analysed with diverse hypoblast markers. However, the effect of MEK inhibition in ovine embryos remains to be studied. The aim of this work was to analyse whether hypoblast differentiation depends on MEK signalling in ovine, by culturing embryos in different doses of PD. First, to determine the specificity of lineages development markers, the reliability of epiblast (SOX2 and NANOG; n = 14) and hypoblast (SOX17, FOXA2 and GATA6; n = 11) markers was tested by immunofluorescence in day (D) 8 *in vitro*-produced blastocysts. SOX2 signal was consistent with an epiblast marker, as all SOX2+ cells allocated to the ICM region. Only 41.9±6.2 % of the SOX2+ cells were co-labelled by NANOG, indicating that NANOG expression might appear later along epiblast development. SOX17 and FOXA2 labelling was restricted to hypoblast cells, being highly concordant (>94 % of the cells labelled by one of them were co-labelled by the other). In contrast, GATA6 was expressed by both hypoblast and TE cells. Next, D5 *in vitro* embryos were randomly cultured in N2B27 medium supplemented with 1) 0.4 µl/ml DMSO (Control, n = 124); 2) 0.5 µM PD (0.5PD, n = 125); 3) 2.5 µM PD (2.5PD, n = 126) or 4) 10 µM PD (10PD, n = 126) until D8, when blastocyst rates were recorded and lineages development was analysed by immunofluorescence for SOX2 (epiblast), SOX17 and FOXA2 (hypoblast). No significant differences were detected in blastocyst rates at D8 between Control (35.9±2.9%); 0.5PD (30.3±9.1%); 2.5PD (25.9±3.2%) and 10PD (29±4.5%) (mean±s.e.m, ANOVA $p>0.05$). MEK inhibition did not affect either SOX2+ epiblast (27.9±3.6 vs. 31.6±3.9 vs. 23.1±3.3 vs. 16.6±2.2 for control, 0.5PD, 2.5PD and 10PD, respectively) or total cell number (239.5±38.9 vs. 208.5±27.2 vs. 138.3±16.5 vs. 131.6±15 for control, 0.5PD, 2.5PD and 10PD, respectively) (mean±s.e.m; ANOVA $p>0.05$). However, the number of SOX17+ (83.2±19.9 vs. 57.5±15.7 vs. 1±0.7 vs. 0±0 for control, 0.5PD, 2.5PD and 10PD, respectively) and FOXA2+ hypoblast cells (139.5±28.6 vs. 78.4±23.7 vs. 0±0 vs. 0±0 for control, 0.5PD, 2.5PD and 10PD, respectively) was significantly reduced in 2.5PD and 10PD (mean±s.e.m; ANOVA $p<0.05$). In conclusion, MEK signalling pathway is required for hypoblast specification in ovine. Revealing the key signalling pathways involved in early lineages development could help to design strategies to increase preimplantation embryo survival.

Work supported by StG 757886-ELONGAN and PID2021-122153NA-I00.

Characterisation of oviduct epithelium spheroids for the study of embryo-maternal communication in cattle

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Keywords: oviduct epithelium spheroids, spheroid characterization, Bos Taurus

Oviduct epithelial cells (OEC) cultured *in vitro* have been used for a long time to gain insights into early embryo-maternal communications. Most previous *in vitro* models included OEC monolayers grown on plastic dishes or on inserts in air-liquid interphase (ALI) systems (Schoen and Chen, 2018, DOI: 10.21451/1984-3143-AR2018-0012). However, OEC adherent to plastic rapidly dedifferentiate while the ALI system is technically challenging and takes three weeks before OEC differentiation. Hence, easy-to-use and physiological *in vitro* models are still needed. The aims of this study were (i) to characterise bovine oviduct epithelial spheroids (bOES) cultured in suspension under different culture conditions, and (ii) to test the impact of co-cultured embryos on bOES morphology. Isthmic mucosal fragments were isolated from peri-ovulatory oviducts obtained at a local slaughterhouse and were cultured for 3 days in TCM-199 HEPES + 10% FBS (M199) at 38.8°C under 5% CO2 in air. At Day 3, groups of 25 bOES of 100-200 µm in diameter and with ciliary beating were allocated to one of the following condition: 1) 500 µl of M199 (M199/500); 2) 25 µL droplet of M199 under mineral oil (M199/25); 3) 25 µL droplet of SOF + 5% FBS (SOF/25); or 4) 25 µL droplet of SOF + 5% FBS + 25 presumptive IVF zygotes (SOF/25/E). All groups were cultured for 10 days at 38.8°C under 5% CO2 (4 replicates). BOES were evaluated for morphology, movement, cell viability (ethidium homodimer/Hoechst 33342 staining) and immunodetection of cytokeratin and vimentin. Moreover, bOES on Days 3, 6 and 13 in M199 were analysed by qRT-PCR for gene expression of *ESR1, ESR2, PGR, OVGP1, ANXA1, VMAC, HSPA1A,* and *HSC70* as target genes, and *GAPDH, PPIA and YWAHZ* as reference genes. Normalized relative gene expression was calculated using the delta-delta-Ct method. Data were compared between groups by one-way ANOVA or Kruskal-Wallis test followed by Dunn's post-hoc-tests if appropriate. Normal bOES are defined as vesicle-shaped, 100-200 µm diameter and lined by a semi columnar epithelium layer including ciliated cells, with the apical pole and ciliary beating outside. The proportion of viable cells in bOES was not affected by culture conditions or time and remained high (>80%) up to Day 13. Numbers of morphologically normal bOES (with a cavity) and their movement amplitude decreased over time in all culture conditions ($P < 0.0001$), although 100% of morphologically normal bOES were still moving at Day 13. Proportions of normal BOES were higher in M199/500 (43%) and SOF/25 (27%) than in M199/25 (13%) ($P<0.05$). Co-culture with embryos in SOF/25 increased the proportion of normal bOES at Day 13 (47% vs. 27%, $P < 0.05$). OES cells displayed a positive signal for cytokeratin and a negative signal for vimentin. Relative gene expression of *ESR1, ANXA1, HSPA1A* and *HSC70* in bOES remained stable during culture while that of *ESR2, PGR* and *OVGP1* decreased from Day 3 to Day 13 ($P<0.05$) and *VMAC* was below the detection threshold. To conclude, bOES constitute an innovative easy-to-use and physiological model to study embryo-maternal interactions. Developing embryos in the vicinity of bOES supported spheroid morphology by mechanisms that remain to be investigated.

Oviduct epithelium spheroids support embryo development under oxidative stress conditions in cattle

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Keywords: oviduct epithelium spheroids, embryo development, oxidative stress

Monolayers of bovine oviduct epithelial cells (OEC) have been shown to improve development rate and quality of bovine blastocysts co-cultured under stress conditions (20% O_2), although the cells were rapidly dedifferentiating after reaching confluence (*Schmaltz-Panneau et al.*, 2015, https://doi.org/10.1111/rda.12556). Here, we tested bovine oviduct epithelium spheroids (bOES) with more stable OEC differentiation status (Pranomphon et al., Characterisation of oviduct epithelial spheroids for the study of embryo-maternal communications in cattle; submitted AETE abstract number X) as a new co-culture approach to support embryo development under usual (5% O_2) and oxidative stress (20% O_2) culture conditions. We hypothesized that bOES co-culture up to embryo genome activation (5 days post-IVF) would be sufficient to overcome oxidative stress conditions. Ovaries were collected from a local slaughterhouse. Oocytes were aspirated and selected for maturation for 22-23 h at 38.8°C under 5% CO_2 in air. IVF was performed using frozen-thawed Percoll-washed semen from two Holstein bulls of proven fertility at a final concentration of $2x10^6$ spermatozoa/mL for 18 h at 38.8°C under 5% CO_2 in air. After IVF (day 0), groups of 25 presumptive zygotes were allocated to one of the 6 following conditions: in 25 µL droplets of SOF medium + 5% FBS at 38.8°C without bOES for 8 days under 5% CO_2 and 5% O_2 (control-5%) or 20% O_2 (control-20%); under 5% O_2 with 25 bOES up to day 5 (5dBOES-5%) or day 8 (8dBOES-5%); and under 20% O_2 up to day 5 (5dBOES-20%) or day 8 (8dBOES-20%). Cleavage rates were evaluated on Day 2 and blastocyst formation rates on Days 6, 7, and 8. Blastocysts on Days 7 and 8 were fixed and stained with Hoechst 33342 for evaluation of cell number. Data were analysed by one-way or two-way ANOVA followed by Tukey's post-tests if appropriate (significant differences with a p-value < 0.05) using RStudio (R software version 4.2.2). The cleavage rates did not change between treatments, ranging from 71% to 78% (4 replicates). Under 5% O_2, the presence of bOES for 5 or 8 days did not affect the blastocyst rates at Day 7 (19 to 23%) and Day 8 (26 to 31%). However, under 20% O_2, the presence of bOES significantly increased the rate of development at Day 7 (16 vs. 26%) and Day 8 (18 vs. 29%) compared to control without bOES. The presence of bOES during 5 or 8 days had similar positive effects on embryo development. Furthermore, the blastocyst cell numbers were significantly increased compared to controls in 8dBOES-5% (102.6 ± 8.4 vs. 134.7 ± 10.2; $P<0.05$) and in 5dBOES-20% and 8dBOES-20% (82.1 ± 4.5 vs. 112.7 ± 7.8. and 138.1 ± 10.5, respectively; $P<0.0001$). In conclusion, bOES were able to overcome the negative effect of high oxygen level during bovine embryo development *in vitro*. Their presence during the first 5 days of culture, i.e., during major embryo genome activation, was sufficient to produce this positive effect.

Sex steroid determination during periestrous and peridiestrous period in saliva of sows derived from assisted reproductive techniques

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Keywords: sex steroids, saliva, pig

Emerging evidence indicates a long-lasting effect of *in vitro* fertilization on molecular physiology and metabolic dysfunction (Feuer and Rinaudo, J Dev Orig Health Dis 8, 2017). In pigs, clear evidence of phenotypical differences between artificial insemination and in vitro-derived animals have been reported (París-Oller et al., Res Vet Sci 142, 2021; Paris-Oller et al., J Dev Orig Health Dis 13, 2022). These differences might be extended to reproductive phenotype. Therefore, the reproductive stage and the concentrations of 17ß-oestradiol (E2) and progesterone (P4) were monitored in saliva samples of 4-year-old sows obtained from a previous study (Paris-Oller et al., J Anim Sci Biotech 12, 2021). Animals were born after artificial insemination (AI group; n=8) and surgical transfer of in vitro-produced embryos (IVP group; n=12), housed in an open pen and fed under the same conditions. Sows' interest in the male was recorded by a boar station (Compident) that registered the number and duration of visits to the station. Simultaneously, sows were subjected to the back pressure test. Estrus (d1 estrous cycle) was defined as the day of longer residence time at the station together with immobilization response to the back pressure test. Saliva samples were collected using Salivette® tubes containing the polystyrene sponge previously chewed by the sows for 10 seconds. Tubes were centrifuged (1.000 g, 5 min) and samples stored (-80ºC) until hormone analysis by quimioluminescence (Immulite). Concentration of E2 and P4 was determined in samples collected from days 1-3 (periestrous period; PEP) and 13-15 (peridiestrous period, PDP). Data (mean±SD) for number of visits to boar station (n), duration of the visits (sec), E2 (pg/ml) and P4 (ng/ml) were normalized and then analyzed by one-way repeated-measurements ANOVA followed by uncorrected Fisher's to compare variables between groups. $P < 0.05$ was considered significant. During PEP, sows from IVP group visited the boar station more times (10.60±1.91 *vs.* 3.30±1.05) and for longer time (239.77±43.12 secs *vs.* 77.69±26.01 secs) than AI sows. This observation is attributed to the higher hierarchy of IVP animals in this herd. No differences were found in E2 concentration (108.03±2.50 pg/ml and 106.81±4.95 pg/ml, respectively for IVP and AI animals). However, P4 concentration was higher in IVP (0.23±0.03 ng/ml) than AI sows (0.12±0.01 ng/ml). During PDP, only IVP sows visited the boar station (10.80±8.60 times for 265.0± 213.13 secs) and no differences between groups were found in E2 concentration (121.16±5.91 pg/ml and 119.11±3.40 pg/ml) neither P4 (0.21±0.06 ng/ml and 0.32±0.05 ng/ml; respectively for AI and IVP sows). Surprisingly, E2 levels in saliva were higher during PDP than PEP in IVP-derived animals but not in AI-derived animals (P=0.06). As for P4, no differences were found between concentration during PEP and PDP in any group of animals. Further analysis of saliva samples collected from whole estrous cycle will help to interpretation of data.

This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa".

MicroRNA-181d secreted by extracellular vesicles from bovine uterine fluid improve quality of in vitro produced embryos

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Keywords: extracellular vesicles, microRNAs, bovine blastocyst

Extracellular vesicles (EVs) contain proteins, lipids, metabolites, and microRNAs which rule cell-to-cell communication. We have demonstrated that bta-mir-181d was upregulated in uterine fluid EVs, suggesting that this miRNA may be involved in the arrival of the embryo to the uterus. Bta-mir-181d exerts its regulatory effects through modulating several pathways associated with blastocyst formation and embryo lineage segregation as: PI3K/AKT, TGFß, Wnt, and Notch (Leal et al., J Animal Sci Biotechnol, v. 13, p. 116, 2022). We evaluated the effects of miR-181d mimic and its inhibitor on the development and quality of bovine in vitro produced embryos. Presumptive zygotes (n=1512) were cultured in SOF+0.3% BSA (Control) or supplemented with: 1 µM miR-181d mimic (miR181d), or 1 µM miR-181d control mimic (CM), or 1 µM of its inhibitor (Inh181d), or 1 µM of control inhibitor (CInh), in different time frames based on the physiological location of the embryo in the reproductive tract: i) days 1-4 (miR181d-OV or Inh181d-OV: representing miRNA effect in the oviduct), ii) days 4-7 (miR181d-UT or Inh181d-UT: representing miRNA effect in the uterus), or iii) days 1-7 of culture (miR181d or Inh181d or CM or Control). The miR-181d mimic, inhibitor, and their corresponding controls were purchased from Qiagen's miRCURY LNA miRNA line. Embryo developmental rates were recorded on days 4 (≥16-cell) and 7 (blastocysts: BD7) of culture and representative number from both stages were collected to assess their quality (mitochondrial activity by MitoTracker Deep Red; lipid droplet content by Bodipy). One-way ANOVA and Tukey test was used for all analyses. At day 4 of culture the proportion of embryos with ≥16-cell was lower ($p<0.05$) in Inh181d, CInh and Inh181d-OV compared to the other groups. At day 7, blastocyst yield was higher ($p<0.05$) in Control: 25.4±0.9%, CM: 25.0± 0.7%, miR181d: 24.5±0.9%, miR181d-OV: 24.1±1.0% and miR181d-UT: 24.9±0.8%, compared to CInh: 14.7±0.5%, Inh181d: 14.7±0.5%, Inh181d-OV: 14.0±0.4% and Inh181d-UT: 14.5±0.8%. No differences were found in mitochondrial activity and lipid droplets in embryos at ≥16-cell. Mitochondrial activity in BD7 from miR181d and miR181d-UT was increased ($p<0.05$) compared to all other groups. BD7 from miR181d-OV, Control and CM had higher mitochondrial activity than them from CInh and inhibitor groups, while BD7 from CInh had higher mitochondrial activity compared to both inhibitor groups. Lipid droplets content was decreased ($p<0.05$) in BD7 from miR181d and miR181d-UT compared to other groups. While BD7 from miR181d-OV, Control and CM had lower ($p<0.05$) lipid droplets compared to them from CInh and inhibitor groups. In conclusion, supplementation of miR-181d between days 4 to 7 or the entire period of IVC modulates blastocysts mitochondrial activity and lipid content, suggesting that miR-181d may play a role in embryo-uterine interaction.

Funding: KCB by Maria Zambrano & YNC by Margarita Salas, Ministry of Universities; Spanish Ministry of Science and Innovation: PID2019-111641RB-I00

MicroRNA-133b from oviductal extracellular vesicles of pregnant cows improve in vitro embryo quality

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Keywords: extracellular vesicles, microRNA, blastocyst quality

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Extracellular vesicles (EVs) are present in reproductive fluids and play an important role in cell-to-cell communication through their cargoes, especially microRNAs. We have detected mir-133b in oviductal fluid EVs from pregnant cows (Mazzarella et al., Front Vet Sci., v. 8, p. 639752, 2021), which is related to embryo development through the TGFß pathway. We aimed to evaluate the effects of miR-133b mimic and its inhibitor, on the development and quality of bovine in vitro produced embryos. Presumptive zygotes (n=1844) were cultured in SOF+0.3% BSA (Control) or supplemented with: 1 µM miR-133b mimic (miR133b: designed for mimicking mature endogenous miR-133b), or 1 µM Mimic's negative control (CM), or 1 µM of its inhibitor (Inh133b), or 1 µM of control inhibitor (CInh), in different time frames based on the physiological location of the embryo in the reproductive tract: i) days 1-4 (miR133b-OV or Inh133b-OV: representing miRNA effect in the oviduct), ii) days 4-7 (miR133b-UT or Inh133b-UT: representing miRNA effect in the uterus), or iii) days 1-7 of culture (miR133b or Inh133b or CM or Control). The miR-133b mimic (339173), inhibitor (339131), and their corresponding controls (CM: 339136 and CInh: 339131) were purchased from Qiagen's miRCURY LNA miRNA line. Embryo developmental rates were recorded on days 4 (≥16-cell) and 7 (blastocysts: BD7) of culture and representative number from both stages were collected to assess their quality (mitochondrial activity by MitoTracker Deep Red; lipid droplet content by Bodipy). One-way ANOVA was used for all analyses. At day 4 of culture the proportion of embryos with ≥16-cell was lower ($p<0.05$) in Inh133b, CInh and Inh133b-OV compared to the other groups. At day 7 blastocyst yields were significantly higher ($p<0.05$) in Control: 29.2±0.6%, CM: 27.8± 0.6%, miR133b: 26.8±0.6%, miR133b-OV: 26.9±0.7% and miR133b-UT: 27.7±0.5%, when compared to CInh: 15.7±0.4%, Inh133b: 14.5±0.8%, Inh133b-OV: 15.2±0.5%, Inh133b-UT: 15.1±0.7%. MiR133b and miR133b-OV groups showed decreased mitochondrial activity ($p<0.05$) in ≥16-cell embryos and BD7, compared to all groups, while CInh group showed increased mitochondrial activity ($p<0.05$) only in BD7 compared to all other groups. Control, CM and miR133b-UT groups showed lower mitochondrial activity ($p<0.05$) compared to CInh, Inh133b and Inh133b-OV groups both in ≥16-cell and BD7. At BD7, Inh133b-UT increased their mitochondrial activity compared to Control, CM, and mimic groups. Lipid droplets in BD7 from MiR133b and miR133b-OV were significantly reduced ($p<0.05$) compared to the other groups, while them from miR133b-UT, Control and CM had reduced lipid droplets compared to CInh and inhibitor groups. In conclusion, medium supplementation with miR-133b during days 1 to 4 or the entire period of IVC modulates blastocysts mitochondrial activity and lipid content, highlighting the potential role of miR-133b in embryo-oviduct interaction.

Funding: KCB by Maria Zambrano & YNC by Margarita Salas, Ministry of Universities; Spanish Ministry of Science and Innovation: PID2019-111641RB-I00

Nobiletin supplementation to the culture medium increases in vitro porcine embryo development

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Keywords: nobiletin, porcine embryo, quality

Due to its potential as an antioxidant, anti-apoptotic, and free-radical scavenger, Nobiletin (Nob) has been used to promote *in vitro* oocyte maturation and embryo development in bovine (Cajas et al., Int J Mol Sci, v.21, p.5340, 2020; Cajas et al., Biol Reprod, v. 105, p. 1427, 2021). However, the effects of Nob on porcine *in vitro* embryo development are still unknown. This study aimed to evaluate the influence of Nob on the developmental kinetics and quality of *in vitro*-produced porcine embryos. Immature cumulus-oocytes complexes (COCs) were collected from prepubertal gilts and cultured in 500 µL of in vitro maturation (IVM) medium for 44 h. First 22 h, the medium was supplemented with 10 IU/mL eCG and 10 IU/mL hCG, then COCs were transferred to fresh IVM medium without hormone supplementation for an additional 22h. Mature COCs were denuded and co-incubated with thawed sperm (3000 spermatozoa per oocyte) in 100 µL drops of fertilization medium for 5 h. Presumtive zygotes were incubated in 500 µL of in vitro culture (IVC) medium supplemented with 2.5 or 5 µM Nob (MedChemExpress) (N2.5; n=220 and N5; n=234, respectively), or with 0.03% dimethyl sulfoxide (DMSO; vehicle for nobiletin dilution; n=213) or without any supplementation (Control; n=226) for 2 days. Then, embryos were cultured for 5 d in fresh IVC without Nob supplementation. Embryo developmental rates were evaluated at 48, 144, and 168 h of IVC, and their quality was assessed by i) mitochondrial activity (staining with MitoTracker Deep Red) and ii) ROS and glutathione (GSH) content using CellROX Deep Red Reagent and CellTracker fluorescent, respectively. One-way ANOVA and Tukey test was used for all analyses. No differences among groups were found in the cleavage rates at 48 h. However, at 144 and 168 h of IVC, N2.5 increased ($p<0.05$) blastocyst rates (45.0±1.4% and 48.0±1.1%, respectively), in comparison with N5 (32.4±0.6% and 35.2±1.1%, respectively), DMSO and Control groups (33.4±0.7% and 37.9±1.7%; and 34.8±1.0% and 38.2±1.15% respectively). In addition, no differences among groups were found in the mitochondrial activity, ROS and GSH levels of embryos at 48 h of IVC; however, at 144 h of IVC mitochondrial activity was increased ($p<0.05$) in blastocysts from the N2.5 group compared to the N5, DMSO, and Control groups. In addition, N2.5 group induced a significant reduction ($p<0.05$) of ROS and GSH content in blastocysts at 144 h of IVC when compared to all other groups. This result can be attributed to the higher mitochondrial activity observed in the embryos from the N2.5 group, leading to increased electron exchange in the inner mitochondrial membrane. This observation suggests that GSH was consumed to mitigate the detrimental effects of the elevated ROS levels. In conclusion, IVC medium supplementation with 2.5 µM Nob to the embryo culture medium increased porcine blastocysts development rates and enhanced embryo quality, thus improving the efficiency of the system.

KCB by Maria Zambrano & YNC by Margarita Salas, Ministry of Universities; Spanish Ministry of Science and Innovation: PID2019-111641RB-I00

3D-organization and spatial localization of chromatin and epigenetic marks in relation to nucleolar activity in porcine oocytes

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Keywords: Epigenetics, porcine oocytes, chromatin conformation

This study employed porcine oocyte developmental biology as translational model investigating epigenetics in oocyte maturation. Previously, studies in various species confirmed that the chromatin of oocytes is subjected to large-scale modifications correlated to transcriptional silencing during final maturation. These modifications seem essential both for completion of the oocyte's meiosis and subsequent embryonic developmental success. In the present study, we focused on a putative interconnection between nucleolar transcriptional activity and spatial chromatin organization towards completion of oocyte growth. Porcine cumulus-oocyte-complexes (COCs) were aspirated from >3mm follicles of abattoir material. 300 COCs were then divided into 2 groups, pre-categorized by supravital brilliant-cresyl-blue (BCB) staining into fully mature (BCB+) and still maturating (BCB-). Following denuding and fixation, oocytes, in the same ratio of BCB- to BCB+, were processed for 3D-immunofluorescence (n=140) using antibodies against upstream binding factor (UBF; nucleolar activity), H3K9me3 and centromeric protein A and B (CENP) as epigenetic heterochromatin markers, as well as for 3D-DNA-FISH (n=140) with fluorescent oligonucleotides specific for porcine meta- and acrocentric heterochromatin sequences. Finally, the remaining oocytes (n=20) were prepared for TEM according to standard protocol. Analysis by high-resolution microscopy included confocal and TEM. Qualitative assessment of cellular ultrastructure by TEM (as previously described by P. Hyttel, Oocyte Maturation and Fertilization: A Long History for a Short Event, Chapter 1, 2011, pp 1-37) revealed distinct differences in manifestation of the perivitelline space, organelle abundance, shape and localization, as well as chromatin organization and localization between BCB+ and BCB- oocytes, supporting BCB-staining as viable method for rough categorization regarding maturation status. Immunostaining and labeling of chromatin allowed to detect all chromatin conformations (as previously described by Pan et al., Biology of Reproduction, Volume 99, Issue 6, 2018, pp 1149–1158), from non-surrounded nucleolus (NSN) to surrounded nucleolus (SN) and their intermediate conformations (pNSN, pSN) in both BCB groups. However, the BCB+ group contained a higher percentage of oocytes expressing chromatin conformations categorized as mature, whereas the opposite was true for the BCB- group. UBF-activity was only present in NSN and pNSN categorized oocytes and detected significantly more often in the BCB- group. The distribution of centromeric (CENP) and pericentromeric chromatin (H3K9me3) as well as repeated sequences DNA-FISH signal displayed distinct changes in their 3D-organization between NSN an SN conformation, characterized by significant signal-condensation around the nucleolus towards final maturation. Altogether these results demonstrated that there is an evident interconnection between nucleolar transcriptional silencing and the display of specific spatial 3D-chromatin organization patterns of both oligonucleotide sequences and epigenetic marks for constitutive heterochromatin, characterizing mature oocytes with higher competency for embryonic development.

Comparison between liquid and lyophilized media for bovine embryo production

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Keywords: lyophilization, IVC medium, cattle

Each year, more than one million bovine embryos are produced in vitro worldwide. Traditional in vitro culture media consist in either homemade freshly prepared medium or liquid commercial medium. Liquid media formulations involve limited lifespan and high transportation costs. As an alternative, there are ready-to-use lyophilized media in the market offering the advantages of easier transportation and longer lifespan. It has been reported that lyophilization of bovine IVC medium yields the same blastocysts rates as freshly prepared medium (Rubessa et al., RFD 2017). However, no information is available about the quality of the resulting embryos. The aim of the study was to compare the performance of a ready-to-use commercial lyophilized embryo culture medium to the traditional homemade medium, in terms of blastocyst development and quality. Blastocyst quality was evaluated by the total cell number (TCN), the inner cell mass (ICM) ratio and the survival after vitrification. Bovine oocytes were matured in TCM199 supplemented with 20ng/mL of Epidermal Growth Factor (EGF). Presumed zygotes (n=292, 4 replicates) were randomly allocated to either freshly prepared SOFaa supplemented with 0.3% BSA (Liquid group) or to COW-IVC-LYO (EmbryoCloud, Murcia, Spain; LYO group) a semi-defined lyophilized medium supplemented with BSA, and cultured for 8 days in groups of 25 embryos in 50µL drops. Blastocyst evaluation occurred 7- and 8-days post insemination (dpi). Blastocyst rates were calculated as the proportion of blastocysts out of zygotes placed in culture. Hatching rates were measured as the proportion of hatching/hatched blastocysts out of total 8 dpi blastocysts. At 8 dpi, all hatching and hatched blastocysts were fixed in 4% paraformaldehyde for differential staining, consisting in Hoechst and anti-CDX2 antibody labeling TCN and trophectoderm, respectively. The rest of the Q1 and 2 blastocysts were vitrified. Developmental and vitrification data were analyzed using a binary logistic regression model, while data concerning cell numbers were analyzed using an independent samples t-test. Differences at $p<0.05$ were considered significant. The results showed similar blastocyst rates at 7 dpi (Liquid 17.2%, LYO 19.7%) and 8 dpi (Liquid 29%, LYO 21.8%). And no differences were found either in hatching rates (Liquid 19%, LYO 18.2%). Concerning quality, there were no differences in TCN (Liquid 195.88 ± 16.18, LYO 170.33 ± 24.65) or ICM ratio (Liquid 0.29 ± 0.04, LYO 0.30 ± 0.03). Finally, regarding survival after vitrification, no differences were found in re-expansion 24h after warming (Liquid 90.9%, LYO 83.3%), and hatching rates 24h (Liquid 27.3%, LYO 27.8%) and 48h (Liquid 31.8%, LYO 38.9%) after warming. In conclusion, the tested lyophilized embryo culture medium supports embryo development reaching similar blastocyst rates to the freshly prepared traditional medium. Moreover, the blastocyst produced using lyophilized medium were of similar quality to the blastocysts produced in the traditional medium, in terms of TCN, ICM ratio and survival after vitrification.

This work was funded by IJC2019-039404-I, Moving Minds 2022 and Fundación Séneca (21651/PDC/21).

Transforming growth factor beta (TGFß) pathway is required for the proliferation of bovine extra-embryonic membranes following blastocyst hatching

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Keywords: Conceptus elongation, embryonic disc, hypoblast, embryo culture.

Following blastocyst hatching, ungulate embryos undergo a prolonged preimplantation period termed conceptus elongation that is characterized by the proliferation of extraembryonic membranes (hypoblast and trophectoderm) and the formation of an embryonic disc by epiblast cells. This is the most susceptible period for embryonic loss, but the study of the processes occurring during elongation has been limited by the lack of *in vitro* systems. Luckily, a recent culture system based on N2B27 medium allows post-hatching embryo development including complete hypoblast migration and the formation of an embryonic disc (ED)-like structure. Transforming growth factor beta (TGFß) signalling pathway plays a relevant role in epiblast and hypoblast development in mice, but its role in ungulates is not well characterized. The objective of this study has been to test the effect of the inhibition of the TGFß pathway on post-hatching bovine embryo development up to D12 *in vitro*. To that aim, Day 7 (D7) bovine blastocysts produced *in vitro* were transferred to N2B27 medium supplemented with TGFß inhibitor (SB431542, TGFßi) at 200 µM (TGFßi-200, n=30), 20 µM (TGFßi-20, n=79) or 10 µM (TGFßi-10, n=54). As a control group, 68 D7 blastocyst were transferred to N2B27 medium without inhibitor (C). Surviving embryos at D12 were fixed to determine hypoblast migration and epiblast survival rates by immunostaining for *SOX17* and *SOX2*, respectively. Supplementation of TGFßi at 200 µM completely abrogated embryo development to D12 *In vitro*. Survival rates in TGFßi-20 were significantly lower compared to the control group, whereas no significant reduction was observed in TGFßi-10 group (81.2±5.7 % vs. 74.2±7.6 % vs. 58.7±5.6 %, mean±s.e.m. for C vs. TGFßi-10 vs. TGFßi-20, ANOVA $p<0.05$). SB supplementation at 10 or 20 µM significantly reduced the proliferation of extra-extraembryonic membranes as evidenced by a reduced embryo diameter (0.76±0.05 vs. 0.46±0.05 vs. 0.49±0.03 mm, mean±s.e.m. for C vs. TGFßi-10 vs. TGFßi-20, ANOVA $p<0.05$). Hypoblast migration rates were reduced in embryos exposed to TGFßi (32/50 vs. 14/39 vs. 18/44, for C vs. TGFßi-10 vs. TGFßi-20, Chi-square $p<0.05$) but TGFßi had no effect on epiblast survival (30/50 vs. 21/39 vs. 31/44, for C vs. TGFßi-10 vs. TGFßi-20, respectively, Chi-square $p>0.05$). SOX2+ cell number was similar between groups (51.9±10.1 vs. 45.1±8.2 vs. 36.7±7.5 mean±s.e.m. for C vs. TGFßi-10 vs. TGFßi-20, ANOVA $p<0.05$), and ED-like formation rates were significantly reduced in TGFßi-10 compared with C (22/50 vs. 8/35 vs. 13/44, for C vs. TGFßi-10 vs. TGFßi-20, Chi-square $p<0.05$). In conclusion, TGFß inhibition impairs the proliferation of extra-embryonic membranes at early post-hatching bovine embryo development.

Work supported by StG 757886-ELONGAN and PID2020-117501RB-I00

The replacement of N2 and B27 supplements by ITS-X reduces epiblast proliferation during bovine post-hatching development

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Keywords: Conceptus elongation, culture medium, supplement, epiblast.

During early post-hatching embryo development the inner cell mass cells complete cellular differentiation into two lineages: epiblast and hypoblast. Epiblast cells proliferate forming an embryonic disc, whereas hypoblast cells expand and migrate to cover the inner surface of the trophectoderm. These developmental landmarks can be achieved *in vitro* upon culture in N2B27 media, composed by a 1:1 mix of Neurobasal and DMEM/F12 media and N2 and B27 supplements. N2 and B27 supplements are particularly complex, adding 17 compounds to the Neurobasal:DMEM/F12 basal medium (NDF12) and it is unknown which of these components are essential for embryo development. To elucidate if those compounds are required by the embryo, we have evaluated the developmental effects of substituting N2 and B27 supplements by ITS-X, keeping NDF12 as basal medium. ITS-X supplement is composed by 4 of the compounds present in N2+B27 mix (insulin, transferrin, selenium and ethanolamine). To that aim, Day 7 *in vitro* produced blastocysts were produced following conventional pre-hatching culture protocols (SOF medium) and subsequently cultured in NDF12 medium supplemented with N2+B27 (N2B27 group) or with ITS-X at the recommended (ITSX-1X group) or doubled (ITSX-2X group) concentration up to Day 12. No statistically significant differences (ANOVA $p>0.05$) were observed in survival rates from D7 to D12 in 4 independent replicates (82.5±8 vs. 60±7.6 vs. 59.5±9.5 %, mean±s.e.m. for N2B27 vs. ITSX-1X vs. ITSX-2X, 4 replicates). At D12 embryo diameter was measured and embryos were fixed to analyze hypoblast migration and epiblast survival by immunohistochemistry for epiblast (SOX2), hypoblast (SOX17) and trophectoderm (CDX2) markers. Embryo size, complete hypoblast migration and epiblast survival rates did not displayed significant differences between groups (size: 732±57 vs. 521±41 vs. 629±41 mm, mean±s.e.m.; hypoblast migration: 24/48 vs. 12/43 vs. 21/44; epiblast survival: 29/48 vs. 20/43 vs. 30/40 for N2B27 vs. ITSX-1X vs. ITSX-2X; ANOVA or Chi-square $p>0.05$), but the number of epiblast cells was significantly reduced in ITSX groups (47.8±9.3 vs. 21±5.5 vs. 23.4±7.8, mean±s.e.m. for N2B27 vs. ITSX-1X vs. ITSX-2X, ANOVA $p<0.05$). In conclusion, ITS-X can partially substitute N2 and B27 supplements during bovine post-hatching development, but epiblast cells proliferate to a greater extent in N2B27 medium.

Work supported by StG 757886-ELONGAN and PID2021-122153NA-I00.

Profiling the protein cargo of uterine extracellular vesicles isolated from pregnant and cyclic heifers

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Keywords: uterine extracellular vesicles, proteomics, embryo-maternal communication

Embryo-maternal communication is essential for successful pregnancy establishment in cattle. Over the last decade, interest has grown regarding the extracellular vesicles (EVs) role in these interactions. EVs can mediate cell-to-cell communication through the delivery of their cargo to target cells. This study aimed to compare the protein cargo of EVs isolated from the uterine fluid (UF) of pregnant and cyclic heifers to identify potential mediators of maternal-embryonic communication in cattle. Oestrous cycles of crossbred beef heifers were synchronized and artificially inseminated (pregnant; n=17) or not (cyclic; n= 11) and slaughtered 7 days after insemination. The uterine horn ipsilateral to the corpus luteum was flushed, and the UF was examined to confirm the presence of an blastocyst stage embryo in pregnant animals. UF-EVs were isolated by size exclusion chromatography and concentrated by ultrafiltration. Flow cytometry using antibodies for specific EV markers (CD63, CD81, and CD44) were utilized to identify and differentiate EV populations. NanoLC-MS/MS with spectral counting was utilized for the identification and quantification of proteins found in the UF-EVs. Statistical analysis was conducted on five animals per group, using ANOVA for flow cytometry data and T-test for proteomic data, both with a significance level of 5%. Bioinformatic analysis was performed with the DAVID database. Flow cytometry analysis confirmed the presence of EVs and no differences in the EV markers between groups. A total of 1,376 proteins were identified, 297 of which were exclusively detected in UF-EVs from pregnant heifers, and 101 unique to cyclic animals. Among the 978 common proteins, 22 were upregulated and 34 were downregulated in pregnant UF-EVs. The proteins found exclusively in pregnant heifers are associated with 22 pathways which include signal transduction (8), cellular processes (5) including cellular structure, transport, catabolism, and cell motility; endocrine system (3), metabolism (3) and Immune system (3). Among the pathways related to signal transduction, Wnt and RAS signaling are particularly important for their role on early embryo development involved in cell-fate specification, proliferation, survival, growth, and migration. The immune system pathways highlighted Th1 and Th2 cell differentiation. Importantly, Th1-Th2 shift is an important mechanism that prevents the bovine embryo from undergoing maternal rejection during pregnancy establishment. Of note, JAK3 which is exclusively found in UF-EVs of pregnant heifers, also mediates the suppressive effects of interferon tau on neutrophil function. In conclusion, the presence of an embryo modulates the uterine environment, more specifically the protein profile within UF-EVs. Thus, understanding the expression pattern and related function of these proteins in UF-EVs may help unveil the signals exchanged between the embryo and the mother during early embryo development.

Impact of Spirulina platensis as a feed supplement of ewes reared in endemic fluorosis areas from late pregnancy to early lactation

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Keywords: endemic fluorosis, pregnant and lactating ewes, Spirulina platensis

In sheep, chronic exposure to the fluoride excess is harmful to teeth, bones, kidneys, liver and heart (Rahim et al, Toxicology, 465, 153025, 2022). Additionally, it was documented that pregnant and lactating ewes are more susceptible to the chronic fluoride toxicity, as a result of their precarious calcium metabolism (Pradhan et al, Int J Pharm Sci Res, 4, 29-40, 2022). *Spirulina platensis* given its richness in bivalent minerals that could chelate fluoride excess, and in antioxidants, mainly c-phycocyanin, which could correct the oxidative stress generated by this halogen. Therefore, this study aimed to evaluate the impact of S*pirulina platensis*, firstly on plasma fluoride levels and oxidative stress in ewes (during late pregnancy and early lactation) reared in endemic fluorosis areas, and secondly on the average daily weight gain of their offspring. The experiment was conducted in El Fokra commune belonging to Khouribga province Morocco, where fluorosis is endemic, and compared to a group of ewes from Settat region, which is free fluorosis. Forty-eight ewes were divided into four equal groups (G1, G2, G3 and G4), G1 and G2 served as controls belonging respectively to free fluorosis (Settat) and endemic fluorosis (El Fokra) areas, while G3 and G4 received respectively 250 and 500 mg/kg bodyweight (BW)/day of *Spirulina platensis*, during late pregnancy and early lactation. Plasma levels of fluoride, glutathione (GSH), ascorbic acid and lipid peroxidation (MDA) as well as erythrocyte enzyme activity of catalase (CAT) and superoxide dismutase (SOD) were evaluated. In addition, the average daily weight gain of their offspring was determined. Statistical analyzes were performed using the JMP SAS 11.0.0 (SAS Institute Inc. Cory, NC, USA) program. In both late pregnancy and early lactation, the results revealed a significant increase in plasma fluoride, GSH and MDA levels as well as a significant decrease in ascorbic acid and enzymatic activities of CAT and SOD in ewes of G2 compared to those of G1. While in both late pregnancy and early lactation, the 250 and 500 mg/kg BW/day of *Spirulina platensis* (G3 and G4, respectively), significantly reduced plasma fluoride levels in ewes, and significantly improved the antioxidant system with a remarkable effect of the second dose. Furthermore, it showed that the average daily weight gain is significantly lower in the offspring of G2 than that of G1. While, offspring of G4 presented an average daily weight gain significantly higher than those of G2 and significantly lower than those of G1. In conclusion, the incorporation of *Spirulina platensis* as a feed supplement is advocated to minimize blood oxidative stress caused by fluoride toxicity in pregnant and lactating ewes and ameliorate the average daily weight gain of their offspring.

Acknowledgments

The authors express their gratitude and appreciation for the assistance provided by all the organizations (INRA, OCP, CNRST, UM6P) involved in the SHS-ELM- 01/2017 project.

Microbiota of the equine placenta

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Keywords: cervical star, pregnant horn, umbilical cord

The presence of a placental microbiome is considered to be controversial, with some authors isolating bacteria whereas others consider their presence to be due to contamination. The aim of this study was to determine which bacteria are present in the equine placenta. Methods: placental samples were obtained from the cervical star (CS) pregnant horn (PH), and the region near the umbilical cord (UC) of 24 foaling mares, aged 6-21 years old, at a Lipizzaner Stud in Hungary during spontaneous parturition. The DNA was extracted followed by 16S rRNA sequencing using the Ion Torrent method to identify the microbiome. Results: All of the parturitions were uneventful with 24 healthy foals born. Expulsion of the placenta occurred within 3 hours in all the mares, mean (± SD) 67 ± 36 minutes. An abundance of bacteria was found. The most abundant phyla were Proteobacteria (42-46.26%), Actinobacteria (26.91-29.96%), Firmicutes (14.19-17.31%) and Bacteroidetes (8.91-9.87%). The top genera found were *Acinetobacter, Brachybacterium, Brevibacterium, Chryseobacterium, Comamonas, Corynebacterium, Devosia, Flavobacterium, Kurthia, Luteimonas, Paracoccus, Planomicrobium, Pseudomonas, Saccharopolyspora, Sphingomonas,* and *Stenotrophomonas*. The diversity of bacterial microbiota was similar in all placental regions at the phylum level but differed at the genus level and alos between placental regions. Thus, Chlamydiae and Fibrobacteres were present only in PH; Ignavibacteriae was present only in UC. Fusobacteria were found in CS and PH, whereas Lentisphaerae and Nitrospirae were found in PH and UC. The results are similar to those obtained by sampling during human, murine and canine caesarian deliveries, although the bacteria were present in greater abundance, indicating possible contamination, However, the localisation of some bacteria to certain areas of the placenta tends to suggest that the healthy equine placenta may indeed have its own microbiome, which may differ according to placental region.

Acknowledgements: We thank the Director and stud personnel for allowing us to take the samples, and for their invaluable help, SLU Bioinformatics Infrastructure (SLUBI) for management and processing of sequencing data. Funding was received from the Marie Claire Cronstedt Stiftelsen. The National Genomics Infrastructure (NGI)/Uppsala Genome Center provided assistance in massive parallel sequencing and computational infrastructure.

Gonadotropin supplementation improved in vitro developmental capacity of Egyptian goat oocytes by modulating mitochondrial distribution and utilization

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Keywords: Goats, oocytes, hCG, IVM, IVF, cytoplasmic maturation.

Supplementation of gonadotropins to IVM medium has enhanced the developmental potential of oocytes **(Dinopoulou et al., 2016; Bahrami and Cottee, 2022)**. Additionally, cytoplasmic maturation such as mitochondrial distribution impact oocyte competence **(Ghanem *et al*., 2021a; Ghanem *et al*., 2021b; Reader *et al*., 2017; Torner *et al*., 2008)**. However, the mechanism by which gonadotropins improve oocyte quality is not completely defined. Therefore, the goal of this study was to investigate the effect of non-ruminant gonadotropin (hCG) supplementation on IVM, IVF, and mitochondrial activity of goat oocytes. In total, 2356 morphologically good quality COCs **(Wieczorek *et al*. 2020)** were recovered from 476 freshly obtained goat ovaries. Selected COCs were incubated in IVM medium consisting of TCM-199 supplemented with 10% (v/v) FBS, 10 ng/ml epidermal growth factor (EGF), 1 µg/ml estradiol (E2), 0.25 mg/ml Na+ pyruvate and 20 IU pregnant mare serum gonadotropin (PMSG) (Gonaser, 500 IU) according to previous publications (**Soto-Heras *et al*., 2019**; **Jose *et al*., 2021**; **Maksura *et al*., 2021**) for 24 hours at 38.5 ºC, 5% CO2 and 95% humidity. The IVM medium was supplemented with hCG (IBSA, choriomon® 5000IU, Egypt) at two different concentrations according to the experimental design which included three experimental groups. The first group (G1) was allocated as a control group. The second (G2) and third (G3) groups were supplemented with 10 and 20 IU/ml hCG, respectively. The maturation rate was calculated after staining COCs with Hoechst 33342® (Sigma-Aldrich, St. Louis, MI, USA) in addition to the cumulus expansion rate which scored from 1-5 based on degree of cells dispersion (**Maksura *et al*., 2021**) and the first polar body (1st Pb) extrusion rate. According to **Saini *et al*. (2022)**, was evaluated fertilization rate. Moreover, mitochondrial activity was assessed using Mito-Tracker green® dye (Invirogen-M7514, USA) fluorescent staining, according to **Ghanem *et al*. (2021)**. The data obtained in the current experiment were analyzed statistically by SAS Enterprise Guide 4. Moreover, a chi-square test and one-way ANOVA were performed. The results indicated a higher rate ($P \leq 0.05$) of nuclear maturation in G3 (82.5%) than in G2 (65.9%) and the control group (64.8%). Furthermore, the fertilization rate (48 hrs post IVF) was significantly improved in G3 (18.7%) compared to G2 (10.6%) and the control group (9.5%). Notably, the percentage of diffuse pattern of mitochondrial distribution was increased in G3 (73.3%) compared to G2 (13.3%) and the control group (6.7%). This change in oocyte mitochondrial distribution was coupled with enhanced fluorescent mitochondrial intensity in G3 (95%) and G2 (89.2%) compared to the control group (79.9%). Our data indicated that hCG is a good substitute for LH during IVEP **(Zombie *et al*. 2018)**. In conclusion, supplementing the IVM medium with a high concentration of hCG (20 IU/ml) increased the rate of metaphase II stage, mitochondrial activity and fertilization rate of goat COCs.

Validation of sgRNAs to target GATA3 gene in Bovine cell lines using CRISPR/Cas9 gene editing

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Keywords: CRISPR, Knockout, Bovine fetal fibroblasts, cell lineage specification and embryo development

CRISPR/Cas9 based genome editing has recently become a popular and efficient technique to carry out targeted genetic modifications of any host genome. Knockout or knock-in of a gene of interest by targeted gene disruption is an effective approach of deciphering its function. Here we present an optimization of CRISPR/Cas9 in bovine fetal fibroblasts (BFF) cell line for application to bovine embryos with improved efficiency. These KO cell lines serve as an important tool to investigate the protein function by analysing the consequences of a specific gene loss. We have successfully generated *GATA3* knockout (KO) BFF cells using the CRISPR/Cas9 mediated genome editing. The method involves designing the CRISPR gRNAs targeting different regions of *GATA3* gene, CRISPR cloning into px459 plasmid, delivery of this CRISPR clone into bovine fibroblast cells, screening of knockouts and MiSeq analysis to verify successful disruption of *GATA3* gene. We have designed 11 guides spanning in different regions of bovine *GATA3* gene including the functional domains in Exon 4 and 5 regions and the transcription initiation site in Exon 2. Designed guides were optimized and screened by their cutting efficiency using the in vitro cleavage assay. The guides with best cutting efficiencies were then tested in bovine fetal fibroblast (BFFs) cell line. Bovine fetal fibroblasts cells were transfected with px459 plasmid bearing the sgRNA and Cas9, screened using puromycin based selection following the subsequent MiSeq analysis to verify the successful knockouts. We have identified two effective guides targeting the ZnF functional domains of *GATA3* gene (sgRNA#5 and sgRNA#8 cutting in Exon 4 and Exon 5 respectively) and one in Exon 2 (sgRNA#1) targeting the transcription initiating site of *GATA3* gene. The results showed a maximum indel frequency of 36.8% and 18% for sgRNA5 and sgRNA8 respectively for the bovine GATA3 gene. Overall, MiSeq data for all screened gRNAs showed that insertion of a base pair upstream of the PAM site resulted in introduction of premature stop codon TAA in the downstream region. *GATA3* is expressed in the outer cells of the morula stage during embryonic development and considered as a key driver of TE initiation and regulation of early lineage development in human and bovine embryos. We are currently assessing the efficiency of the *GATA3* knockout by microinjecting these selected sgRNAs and Cas9 protein RNP mix into putative bovine zygotes 12h post fertilization. This study is thus a step forward to apply recent advents of CRISPR/Cas9 system for gene editing to carry out the functional studies.

Investigating the roles of interferon tau stimulated genes in sheep embryo implantation

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Keywords: embryo implantation, interferon tau, sheep

Interferon tau (IFNT) serves as the molecular signal that initiates maternal recognition of pregnancy and modification of the maternal immune system to favour a successful pregnancy in ruminants. It binds to the Type-1 interferon alpha receptors (IFNAR) present in the luminal cells of the endometrium to upregulate the expression of interferon stimulated genes (ISGs) to support embryo implantation. This hypothesis is being tested as a prelude to confirming the roles of immune cells (especially uterine natural killer cells) in the endometrium at this stage of pregnancy. Ewes kept in the experimental unit of the Royal Veterinary College were synchronised to a common oestrus, mating done by allocating 10 ewes per ram which were of the same breed and pregnancy was confirmed by the presence dye on the back and non-return to oestrus. Slices of caruncular and inter-caruncular area of the endometrial tissues were cut and snap-frozen from eight pregnant and six non-pregnant ewes on day 17 after natural mating. Pregnancy was confirmed by the presence of an elongating blastocyst when the uterus was opened alongside presence of corpus luteum on the ipsilateral ovary. Through RNA sequencing, this study has compared the expression of IFNT and ISGs and the related signalling pathways during embryo implantation in sheep. RNA sequencing was performed using 600 ng of the total RNA for analysis by Illumina with paired-end 150 bp sequencing (PE150) to reach over 30 million reads per sample, followed by a gene ontology enrichment analysis (GOEA). The cutoff criteria were P (BH) < 0.05 and absolute fold change ≥ 2.0. The result showed a significant upregulation of the ISGs (MX1, MX2, ISG15, ISG20, RSAD2, LGALS15, B2M, IFI6, IFI35, IFIH1, CXCL9, CXCL10, WNT7A, CTSL, IFIT2, IFIT3, IFIT5, IFI44, BST2, PLAC8, PLAC9, ADAR, DDIT4, DDX58, IFI44L, NAMPT, NT5C3A, PML, RTP4, TRIM25), in pregnant ewes' caruncular endometrium ($p<0.0001$) compared to those of the non-pregnant counterparts. STAT1, STAT2, and IRF9 which are critical transcription factors for the induction of the ISGs were also upregulated in the pregnant ewes compared to the non-pregnant ones. Six biological processes (defence response to virus, negative regulation of viral genome replication, immune response, innate immune response, complement activation classical pathway and inflammatory response) were observed to be related to interferon activation, and the DEGs associated with each of them were identified. These include ISGs involved in defence response to virus, e.g., MX2, STAT2, ISG15, RSAD2, IFI6, CXCL9, or in other biological processes like negative regulation of viral genome replication (IFIH1, RSAD2, MX1, ISG15), immune response (CXCL9, CXCL10, B2M), and innate immune response (MX2, MX1, IFI6, IFI35, IFIH1). It has also shown significant upregulation of the previously acclaimed ISGs involved in embryo implantation including LGALS15, ISG15 and CXCL10. These data suggest the critical role of IFNT and the upregulation of ISGs in regulating endometrial receptivity, embryo implantation and uterine immunity in sheep.

Cloning, Transgenesis and Stem Cells

Effective generation of double knock-out TPC1 and TPC2 porcine embryos by CRISPR-Cas9 electroporation of oocytes before insemination

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Keywords: electroporation, knock-out, two pore channel

The production of gene edited porcine embryos has major implications for agriculture and biomedicine. Recently, the modification of oocytes prior to fertilization has gained importance due to the complexity of the cloning technique. One of the most promising methods is electroporation, which enables the delivery of the CRISPR/Cas9 system by applying an electric field that creates pores in the membrane. Two-pore channels (TPCs) are membrane proteins that form cation-permeable channels located on the surface of endolysosomal organelles such as lysosomes and endosomes. TPCs are responsible for regulating the release of Ca^{2+} ions in response to extracellular signals. These proteins are involved in various pathophysiological processes such as embryonic development, cell differentiation, cardiovascular function, autophagy regulation, the acrosome reaction in spermatozoa, and Ebola and SARS-CoV-2 infections, among others. We have previously generated embryos and pigs that are KO for TPC2 and embryos that are KO for TPC1. The double TPC1/2 KO model will provide a valuable experimental model for different biomedical areas, as has been shown previously in mice. The aim of this study is to evaluate the efficacy of electroporation to generate edited gene embryos in which two different genes (TPC1 and TPC2) are targeted in a single step. CRISPR sgRNAs were designed to target exon 9 of the TPC1 gene (Navarro-Serna, Int J Mol Sci 23, 2135, 2022) and exon 3 of the TPC2 gene (Navarro-Serna, CRISPR J 4, 132, 2021). *In vitro* matured oocytes were electroporated (128 oocytes) with Cas9 protein and simultaneously with sgRNAs targeting the TPC1 and TPC2 genes (25 µg/µl of each sgRNA and 100 µg/µl of Cas9 protein), with a negative control with no sgRNAs (control-C, 156 oocytes), followed by *in vitro* fertilization and embryo culture for up to 6 days. Cleavage and blastocyst (blastocyst/oocyte) rates were assessed, and mutation and mosaicism rates were analyzed by fluorescent PCR-capillary gel electrophoresis. The simultaneous application of two sgRNAs and the double concentration of Cas had no negative effect on *in vitro* embryo development, with a cleavage rate of 81.3% higher than control (64.7, p=0.002) and similar blastocyst rate (29.7 vs. 30.1%, p=0.94). When the mutation rates were evaluated separately for each gene, rates of 84.4% for TPC2 and 91.3% for TPC1 were observed. When both genes were evaluated simultaneously, 79% of the embryos had at least one allele of both genes mutated. Only 5% of the blastocysts had no mutations at all, while 79% had the double mutation and smaller percentages had mutations in one gene but not the other (TPC1 11% and TPC2 5%). Electroporation of oocytes prior to IVF is an efficient method for producing gene edited porcine embryos for a variety of models, including multiple modifications in one step. Transfer of these gene edited embryos will result in the generation of TPC1 and TPC2 KO pigs after embryo transfer. This model will be of great interest in the biomedical field.

Acknowledgements: Fundación Séneca 22065/PI/22; MICIN PID2020-113366RB-I00; funded by MCIN/AEI/10.13039/501100011033/ and FEDER Una manera de hacer Europa and Contrato predoctoral Universidad de Murcia R-496/2022

Creating porcine phospholipase C zeta knockout (PLCζ-KO) embryos by CRISPR-Cas9 electroporation of oocytes before insemination

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Keywords: electroporation, knock-out, male fertility

Phospholipase C zeta (PLCζ) is found in the cytosol of mammalian spermatozoa. It plays an important role in mammalian fertilization by entering the oocyte and contributing to oocyte activation through intracytoplasmic Ca^{2+} oscillations. Alterations in this protein have been associated with human infertility (Heytens, Human Reproduction 24, 10, 2417-2428). The availability of animal models with specific genetic alterations in this gene/protein will facilitate the study of infertility and the development of new treatments (Hachem, Development 144, 16, 2914-2924, 2017). The aim of this study was to evaluate the efficacy of electroporation of oocytes before *in vitro* fertilization for the generation of the PLCζ-KO edited porcine embryos and to optimize the efficiency of the system using different sgRNA-Cas9 concentrations. The sgRNA was designed to target exon 3 of the PLCζ gene using Braking-Cas software (BioinfoGP, CNB-CSIC, Madrid, Spain). *In vitro* matured oocytes were electroporated with sgRNA-Cas9 protein at two different concentrations, E-Low (6.25 µg/µl sgRNA and 12.5 µg/µl Cas9 protein) and E-High (12.5 and 25 µg/µl), with an unelectroporated control (control-C), immediately followed by *in vitro* fertilization and embryo culture for up to 6 days (Navarro-Serna, Theriogenology 186, 175, 2022; Piñeiro-Silva, Animals 13, 3, 342, 2023). Cleavage and blastocyst (blastocyst/oocyte) rates were assessed, and mutation and mosaicism rates in blastocysts were analyzed by fluorescent PCR-capillary gel electrophoresis. Oocyte electroporation tended to increase the cleavage rate (C: 63.5; E-Low: 77.4, E-High: 76%, p=0.052), although the blastocyst rate was similar for all groups (C: 23.1, E-Low: 18.3 and E-High 21%, p=0.712), indicating that the presence of mutations in PLCζ did not impair blastocyst formation in our model. The mutation rate was 61.2% and 80% for the low and high concentration groups (p=0.025), with an increase in the number of alleles detected per embryo from 2.49±0.53 in E-Low to 2.69±0.51 in E-High (p=0.038), which is related to the increase in mosaicism (E-Low: 47.8; E-High: 67.3%). This is the first reference to our knowledge about the production of porcine PLCζ KO embryos and this study confirms the effective use of electroporation for the transfer of gene editing material. The high level of mutation detected in blastocysts will facilitate the generation of KO pigs after embryo transfer, although the high rates of mosaicism will make it difficult to obtain a biallelic animal in a single step. PLCζ KO pigs will have the potential to generate data for further understanding of the role of PLCζ in mammalian fertilization and oocyte activation.

Acknowledgements: Fundación Séneca 22065/PI/22; MICIN PID2020-113366RB-I00; funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa" and "Contrato predoctoral Plan de Fomento de la Investigación de la Universidad de Murcia para 2022" (R-496/2022).

OVGP1 KO female hamsters are infertile due to a failure in early preimplantation embryo development

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Keywords: oviduct, reproduction, CRISPR Cas9

Oviductal fluid (OF) is the result of blood plasma filtrate and the specific secretions of non-ciliated cells present in the oviductal epithelium. The most predominant protein secreted by these cells is oviductal glycoprotein 1, which is encoded by *OVGP1* gene. The relevance of *OVGP1* has been controversial since *OVGP1* is dispensable for mouse fertility (as evidenced by Knock-out -KO- experiments) (Araki *et al*. Biochemical Journal, 374:551-557, 2003), and it is absent in other mammals such as rats and some bats (Tian *et al*. Biology of Reproduction, 80:616-621, 2009; Moros-Nicolás *et al*. Journal of Molecular Evolution, 86:655-667, 2018). To determine the role of *OVGP1* in other mammals, we have generated *OVGP1* KO in the hamster model (*Mesocricetus auratus*) by CRISPR-Cas9 technology and compared their reproductive performance to that obtained from heterozygous (Hz) and wild type (WT) animals. Fertility assessment by natural mating revealed that KO and Hz males were fertile, being as prolific as their WT counterparts (litter sizes: KO 8.71±0.68; Hz 9.17±1.11; WT 8.25±0.85). Similarly, Hz and WT females were fertile (litter sizes: Hz 9.64±0.79; WT 8.42±0.62). However, female KO hamsters were infertile since no offspring was obtained after the observation of 40 matings of 6 females. Infertility was not caused by fertilization failure or polyspermy as all recovered zygotes showed two pronuclei (10/10 for embryos obtained from WT and KO females). However, embryos recovered from KO females at ~2.5 days *post coitum* (d.p.c.) and ~3 d.p.c. exhibited a significant reduction in blastomere number (~2.5 d.p.c.: KO 2.67± 0.50; WT 5.25 ± 0.30; ~3 d.p.c.: KO 3.69 ± 0.58; WT 7.08 ± 0.37), along with an obvious asymmetry in blastomere size. These embryos may be suffering cell death since microarray analysis revealed that the expression of several genes involved in autophagy and ubiquitin ligase pathway were altered (either, up-, or downregulated) between embryos obtained from WT and KO females. Further characterization of the phenotype by histochemistry and transmission electron microscopy (TEM) evidenced that oviducts from KO females were morphologically normal. On the other hand, transcriptomic analysis of hamster oviducts by RNA-seq showed that 7 genes were differentially expressed: *OVGP1, ENPEP, C1QTNF4, DERL3, AMY1*, and *WDR95* (downregulated in KO animals), and *GPR18* (upregulated in KO animals). Taken together our results evidence that oviductal glycoprotein 1 is essential for female reproductive function in hamsters and, specifically, for proper *in vivo* early embryo development.

This research is part of the projects PGC2018 094781 B I00 and PID2021-123091NB-C21 supported by MCIN/ AEI /10.13039/501100011033/ and by FEDER Una manera de hacer Europa.

Support Biotechnologies: Cryopreservation and Cryobiology, Diagnosis through Imaging, Molecular Biology, and "OMICS"

Thymus satureioides essential oil and antibiotics: Evaluation of their effect on the quality of frozen thawed Beni Arouss bucks semen

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Keywords: Beni Arous buck semen, cryopreservation, Thymus satureioides

The objective of this study was to examine the effect of different concentrations of *Thymus satureioides* (TS) essential oil (EO) and antibiotics on the quality of post-thawed Beni Arouss buck semen. TS EO is known for its antioxidant and antibacterial properties. When incorporated at 0.01% to skim milk–based extender, TS EO proves beneficial in enhancing the preservation of buck semen at 4°C. Semen collection was conducted using an artificial vagina once a week for ten weeks from eight Beni Arouss bucks. The ejaculates were combined, divided into six equal aliquots, and washed before being diluted to 400 x 10^6 sperm/ml with 7% glycerol. Skim milk-based extender was supplemented with different concentrations of TS EO (0%, 0.01%, and 0.05%) without antibiotics, denoted as (CTR-), (S1-), and (S5-), and with 50 mg of streptomycin and 50,000 IU of penicillin per 100 ml, marked as (CTR+), (S1+), and (S5+), respectively to assess the improvement of sperm preservation efficiency by combining them with TS EO. The aliquots were cooled to 4°C, then frozen in 0.25 ml straws using a programmable freezer and ultimately stored in liquid nitrogen. Thawing was performed at 37°C for 30 seconds. Post-thawed semen quality parameters, including motility, live sperm, membrane integrity, lipid peroxidation, and bacterial growth, were evaluated. Sperm motility was assessed using the computer-assisted sperm analysis system, viability was determined through eosin-nigrosin staining, membrane integrity was evaluated using the hypo-osmotic swelling test, lipid peroxidation was measured by the thiobarbituric acid reactive substance assay, and bacterial growth was quantified by calculating the number of colony-forming units per milliliter in a non-selective culture medium. The obtained data was checked for normality using the Shapiro-Wilk test, and all data were not normally distributed. The non-parametric Kruskal-Wallis test was used to assess the impact of antibiotic or concentration of EO, and the means were compared using the Steel-Dwass-Critchlow-Fligner test in case of a significant effect. A probability value of ($p < 0.05$) was considered statistically significant for all parameters. These statistical analyses were carried out using SAS 9.4. S1+ treatment resulted in a lower bacterial growth and lipid peroxidation compared to CTR+ ($P < 0.05$), but motility, viability, and integrity parameters remained the same ($P > 0.05$). On the other hand, when assessing the impact of antibiotics, it was observed that the CTR+ and S1+ improved all quality and bacterial growth parameters compared to CTR- and S1- respectively, but resulted in a significant increase in malondialdehyde formation. However, 0.05% of TS EO showed a toxic effect, regardless of the presence or absence of antibiotics. Based on the findings, the use S1+ extenders are recommended to enhance the cryopreservation of Beni Arouss buck semen.

In vitro production of bovine embryos modified sex-dimorphisms in fetal liver methylome.

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Keywords: IVP/IVD fetuses, methylome, sex-effect

Environmental perturbations of early mammalian embryo affect adult phenotype in a sex-specific manner. This assumes that sex-specific differences exist even before hormonal impregnation, due to differences in sex chromosomes between males (M) and females (F). Because long term effects of embryo environment are thought to be mediated by alterations of the epigenome, we asked to what extent the epigenome of a fetal organ differs between M and F and whether these differences are altered by *in vitro* production of the embryos. Therefore, we compared liver methylomes of M and F bovine fetuses developed from *in vivo* derived (IVD) and *in vitro* produced (IVP) embryos at Day 40, that is before any hormonal impregnation. Thirteen IVD fetuses were recovered from 22 synchronized females at Day 40 post insemination. IVP fetuses developed from embryos produced from slaughterhouse ovaries. Embryos were cultured in SOF medium supplemented with 1% oestrus cow serum, then transferred fresh at Day7 to 38 synchronized recipient females, fetuses were recovered 33 days later to produce 14 Day 40 IVP fetuses. All IVD and IVP fetuses were recovered from euthanized females and fetal livers were dissected. Genomic DNA was extracted, fetuses were sexed by PCR. A total of 21 fetuses (5 individuals of each sex and development condition, but 6 IVD males) were kept for further analyses. Liver DNA was submitted to Reduced Representative Bisulfite Sequencing (RRBS) to profile genome-wide methylation on a single nucleotide level. RRBS analysis was performed using RRBS-toolkit pipeline (Perrier et al. 2018 BMC Genomics) including Methylkit software to identify differentially methylated cytosines (DMCs). Sites for known Single Nucleotide Polymorphisms were filtrated before differential analysis. Only cytosines with a 10-500 coverage were selected for analyses. A threshold of 25% difference in methylation rate and an adjusted p-value<0.01 were retained to select DMCs. We compared DMCs between male and female fetuses developed either from IVD or from IVP embryos. A total of 1543 DMCs were found between IVD M and IVD F fetal livers, most of which (n=1324) on the X chromosome which was not unexpected after X inactivation. In vitro production increased by a factor of 1.7 the total number of DMCs (n=2734) both on X (n=2407) and on autosomes (n=327). While most (91%) of the X-linked DMCs were hypermethylated, those located on autosomes were equally distributed between hyper and hypomethylation in IVD female livers. Interestingly, this was not the case in IVP female livers where 97% of the X-linked and 76% of the autosomal DMCs were hypermethylated. These results point to an hypermethylation of the most sex-dimorphic DMCs in IVP female fetal livers. DMCs were similarly distributed between genes and repeated sequences in IVP and IVD livers but they were slightly enriched in CpG islands in IVP compared to IVD livers (82.5% and 74.7%, respectively). Work is going on analysing methylomes of fetal gonads, chorion, and brain, and integrating transcriptome and methylome data.

This project was funded by ANR BoSexDim and APIS-GENE.

Astaxanthin during post-warm recovery period improved quality of bovine blastocysts obtained from vitrified oocytes

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Keywords: astaxanthin, oocytes, vitrification

Exogenous antioxidants in maturation and culture media are widely used to mitigate the negative effect of vitrification on oocytes. Effect of astaxanthin (Ax) on bovine oocyte cryosurvival has not been documented. The aim of this study was to evaluate the impact of Ax added during post-warm recovery on the developmental competence of bovine vitrified oocytes. Oocytes aspirated from slaughtered-derived cow ovaries were matured *in vitro* (IVM; M199, 10 % FBS, 0.25 mmol·L^{-1} sodium pyruvate, 50 µg/mL gentamicin, 1 I.U FSH/LH (Pluset)) at 38.5 °C and 5 % CO_2, partially denuded and vitrified in M199 medium with 30 % ethylene glycol, 1 M sucrose and 10 % FBS in minimum volume on the electron microscopy grids by ultra-rapid vitrification technique. Vitrified/warmed oocytes were incubated 3 hours for post-warm recovery in the IVM medium either with (V-Ax-2.5 µM; n = 226) or without (V-Ax-0; n = 258) Ax. Fresh IVM oocytes (n = 201) served as a control. Afterwards, oocytes were fertilized *in vitro* using frozen bull semen. Presumptive zygotes were incubated in a B2 Menezo medium with 10 % FBS, 10 mg/mL BSA, 50 µg/mL gentamicin and 31.25 mM sodium bicarbonate on a monolayer of BRL-1 (Rat epithelial cells; ECACC, UK) cells at 38.5 °C and 5 % CO_2 until the blastocyst stage (6-8 days). Experiments were performed in 5 replicates. Relative fluorescence intensity of post-warmed oocytes showed significant (ANOVA; $p < 0.05$) increase of ROS level (CellROX™) in V-Ax-0 group (3.69 AU) compared to control (1 AU), while Ax significantly decreased ROS level (2.08 AU) in vitrified oocytes. Similar trend was observed in relative fluorescence intensity of lipid peroxidation (BODIPY™). Ax reduced lipid peroxidation in vitrified oocytes (0.66 AU), which was caused by oxidative stress due to vitrification (1.38 AU). Significant decrease (Chi-squared test; $p < 0.05$) was observed in cleavage and blastocyst rates in both V-Ax-0 (58.14 % and 14.34 %) and V-Ax-2.5 (54.87 % and 17.26 %) groups compared to control (66.17 % and 29.85 %). However, blastocysts from V-Ax-2.5 group had significantly (t-test; $p < 0.05$) higher total cell number (103.80 ± 2.81) compared to V-Ax-0 group (94.03 ± 5.08) and was comparable to control (105.28 ± 4.45). RT-qPCR assay showed significantly (t-test; $p < 0.05$) higher expression of the *GJB5* gene in Ax group compared to control, confirming the proliferative effect of Ax. Significantly lower expression of *CAT* and *GPX4* genes was detected in the V-Ax-0 group, while Ax increased expression of these genes compared to control. Expression of the apoptotic *CAS9* gene was increased due to vitrification, while Ax suppressed it to the control level. *CDX2*, *SOD2*, *BAX* and *BCL2* gene expression was not affected by either vitrification or astaxanthin. In conclusion, astaxanthin during post-warm recovery period reduced oxidative stress in bovine vitrified oocytes and improved quality of blastocysts up to those from fresh oocytes.

Grant support: The Slovak Research and Development Agency, Slovak Republic (grant no. APVV-19-0111).

Decellularization of female domestic cat's reproductive organs: ovary, oviduct and uterine horn

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Keywords: decellularization, cat, 3D scaffold

Decellularization is an innovative method to create natural scaffolds by removing all cellular materials but maintaining the composition and three-dimensional (3D) ultrastructure of the extracellular matrix (ECM). Obtention of decellularized reproductive organs in cats might facilitate the development of assisted reproductive technologies in feline species. The objective of our research was to compare the efficiency of three decellularization protocols (P1, P2 and P3) in reproductive organs (ovary, oviduct, and uterine horn) in domestic cats. P1 consists of two cycles of decellularization (Campo et al., Biology of Reproduction, 96(1), 34–45, 2017). P2 was similar to P1, but samples were decellularized by means of three cycles. P3 was similar to P2 but the samples were additionally incubated with deoxyribonuclease for 24h at 37 °C. Reproductive organs from nine cats were separated into two sides. One side was the control (non-decellularized organ) while the contralateral was the treatment (decellularized organ). The treatment group was subdivided into 3 groups (n=3 per each group) for entering P1, P2 or P3. Both control and treatment samples were individually analyzed for DNA content, histology (for evaluating collagen, elastin and glycosaminoglycans (GAGs)), and scanning electron microscopy (SEM). DNA content from different protocols were compared using Kruskal-Wallis test with pairwise comparison. Histology results were compared using unpair t-test or Mann Whitney test. Statistical significance was considered when $P<0.05$. The successful removal of nuclear material was confirmed by DNA quantification. The results of the study showed that DNA concentration between control and treated samples after P1 and P2 had no significant difference. However, there was a significant difference between control and treated sample after P3 of each organ. Regarding to ECM content level in control ovaries and decellularized ovaries from each protocol were not significant different. On the other hand, decellularized oviducts demonstrated a significant decreased level of collagen and GAGs in P3 when compared with the control group. Elastin also significantly decreased in decellularized oviduct from P2 compared with control. Decellularized uterine horns demonstrated a significant decreased level of collagen and GAGs in P3, however elastin level remained constant in both controls and decellularized uterine horns for each protocol. SEM revealed that the microarchitecture of the decellularized samples were maintained when compared with controls. In conclusion, when comparing different decellularization methods, P3 was more efficient than P1 and P2 for removal of nuclear material in reproductive organs. P3 demonstrated its successfulness to decellularize ovarian samples by significantly decreased DNA content, maintained ECM contents and maintained tissue microarchitecture. However, P3 was less effective to maintain ECM contents of decellularized oviducts and uterine horns. This method can be further developed for creating 3D ovarian scaffold for follicle culture in this species.

Molecular alterations in oviductal epithelial cells during heat stress in dairy cows

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Keywords: oviductal epithelial cells, gene expression, dairy cows

Climate change, and especially the rise of temperature leads to heat stress, which is a major threat for dairy cows' fertility due to induced alterations of the endocrine status, increased oxidative stress phenomena, reduced fertilizing capacity of the oocyte and increased embryo deaths. The oviduct and its secretions (the oviductal fluid) play a crucial role in fertility and early embryo development. In this study, the spatiotemporal effect on gene expression during a thermoneutral and a heat stress period was examined. The estrous cycles of ten Holstein cows were synchronized during a thermoneutral and a HS period. On day 3 of the cycle, the cows were slaughtered in groups of 3 or four, the oviducts were immediately removed and transferred in the lab within two hours. In each season, the epithelial cells were collected by scraping the oviductal epithelium with a glass slide and the content collected from the ipsilateral and contralateral to the corpus luteum oviducts was pooled. From 4 replicates in each season, total RNA was extracted and reversed transcribed and Real Time PCR was performed in genes that are involved in response to oxidative and heat stress, under stress regulation of transcription, ER stress response, development, growth and apoptosis. The results were analyzed using a two-way ANOVA with a post-hot Tukey test and a Pearson correlation was calculated for each pair of genes, to detect coordinated patterns of gene expression in each season. Anova test showed that season affected significantly the expression of *EIF2A* in the ipsilateral oviduct and *SOD2* expression was altered independently of location. Both genes exhibited higher expression during the summer. *SOD2* contributes to the antioxidant mechanism of the cells while *EIF2A* is a major target of the unfolded protein response (UPR) and acts as a translation regulator. *HSP90AA1A* and *HSF1* genes' were up - regulated during the summer, though the differences were not statistically significant ($p=0.08$). Concerning the coordinated patterns of gene expression, a group of genes (*ATF6*, *ERN1*, *PPP1R15B*, *EIF2A*) was detected, having noticeably stronger positive correlation in the summer than in the spring. These genes participate in signaling pathways which suppress protein translation under conditions of stress. In conclusion, we provide evidence that HS induces antioxidant mechanism which protect the cells from ROS and maintain cellular integrity. Also, HS response and UPR are tightly regulated during summer. We infer that these regulations facilitate the proper folding of misfolded proteins, which along with the attenuated translation precludes further accumulation of misfolded proteins.

Efficiency of immersion-agitation decellularization method on pig uterus: histological analysis of fresh and frozen-thawed tissue

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Keywords: Decellularization, uterus, histology

Decellularization is a technique used to remove cellular and nuclear material from tissues and organs, resulting in a decellularized extracellular matrix (ECMd) that can serve as a biological scaffold for various applications. These include the regeneration of damaged tissues through recellularization with stem cells (Deepak Choudhury. Acta Biomater. 115:51-59, 2020), improvement of culture *in vitro* embryos (Hannes Campo. Acta Biomater. 89:126-138, 2019), and the creation of hydrogels for three-dimensional printing of tissues and organs (Abaci A. Adv Healthc Mater. 9(24): e2000734, 2020). This study aimed to evaluate the efficiency of the decellularization technique in uterine sections from porcine species, comparing fresh and frozen-thawed samples subjected to different decellularization cycles (0-0h cycle vs. 1-24h cycle vs. cycle 2-48h) by the immersion-agitation method. Histological analysis was performed on the samples to assess the success of the decellularization process. The decellularization procedure was carried out on 5.28 ± 2.82 cm long uterine (horn) sections from prepubertal porcine female (Large white). A total of 12 uteri were used (n=4 for each treatment: control (non-decellularizated tissue), fresh or frozen-thawed). Samples taken at the end of each decellularization cycle (Hannes Campo. Biol Reprod. 96(1):34-45, 2017) were fixed in formalin (4%, 24h). Tissues were embedded in paraffin and histological sections 5 µm-thick were stained with hematoxylin-eosin, Masson's trichrome, orcein, and alcian blue for viable nuclei, collagen, elastic fibers, and glycosaminoglycan (GAG) analysis, respectively. Nucleus counting was carried out on a total of 10 fields at 400x, and for collagen fibers, elastic fibers and GAG determinations, five fields at 200x were analyzed. Representative images were obtained with a Pannoramic MIDI-II automatic slide digitizer (Budapest, Hungary) and for image analysis a freeware image analysis software (ImageJ, NHI, USA) was used. For statistical analysis, the Kruskal-Wallis test or ANOVA One-Way was performed. The histological results demonstrated a significant reduction in the number of nuclei between cycle 0 (620.90 ± 33.11 for the fresh treatment and 551.40 ± 30.65 for the frozen treatment) and cycle 2 in both cases, fresh and frozen organs (8.30 ± 8.30, $p<0.01$ and 3.80 ± 2.17, $p<0.01$, respectively). For the rest of the parameters evaluated only significant differences were observed in collagen content between cycle 0 (53.90 ± 5.01%) - cycle 1 (27.45 ± 7.59%, $p=0.03$) and between cycle 0 - cycle 2 (23.97% ± 12.03, $p=0.02$) of the uteri subjected to fresh treatment. There were no significant differences in the percentage of elastic fibers and GAGs between cycle 0 and cycle 2, with $p>0.05$ in all cases. In conclusion, the decellularization protocol used in this study was effective in both fresh and frozen-thawed porcine uteri. However, frozen-thaw treatment preserved the extracellular matrix components better than fresh treatment.

Funding: Ministry of Science and Innovation (PID2019-106380RB-I00 MCIN/ AEI /10.13039/501100011033) and (PID2021-12309NB-C21 MCIN/AEI/10.13039/501100011033).

Effect of Cryotop simultaneous vitrification of large batches of pig blastocysts on miRNAs expression

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Keywords: pig, blastocyst, vitrification, miRNA

MicroRNAs (miRNAs) are non-coding RNAs of 20-25 nucleotides that regulate post-transcriptional gene expression. The miRNAs are modulated by genetic and environmental factors and play important roles in embryonic development and implantation. Little is known about the effects of vitrification on embryo miRNA expression, and no information is available in pigs. The aim of this study was, first, to evaluate the effects of simultaneous vitrification of 20 porcine blastocysts using the open Cryotop device on the miRNA profile and, second, to link the results to previous gene expression data (Gonzalez-Plaza et al., in press, Theriogenology, 2023). Embryos were surgically collected from 12 donor sows on day 6 of gestation. A total of 120 blastocysts were selected for this experiment. Blastocysts (n=60) were vitrified in groups of 20 using the open Cryotop procedure as described before (Gonzalez-Plaza et al., Front Vet Sci, 9:936753, 2022). Control were non-vitrified blastocysts (n=60). Blastocysts from each donor were equally distributed between both groups. Postwarmed and control blastocysts were cultured in vitro for 24 hours to assess viability. The miRNA profile of some viable embryos (n=40 from each group; 5 different pools of 8 blastocysts each) was studied with the GeneChip miRNA 4.1 array of Affymetrix (ThermoFisher scientific, Madrid, Spain). Transcriptome Console 4.0 and Partek Genomic Suite software were used to analyze array data, the interaction with gene expression and biological interpretation. A threshold of 1.5 foldchange and $p<0.05$ were used to identify differentially expressed (DE) miRNA and genes (DEGs). Analysis of miRNA transcriptome was made using reference mammalian species (*Bos taurus, Equus caballus, Homo sapiens, Monodelphis domestica, Macaca mulatta Macaca nemestrina, Mus musculus, Ornithorhynchus anatinus, Pongo pygmaeus, Pan troglodytes, Rattus norvegicus and Sus scrofa)*. The viability of postwarmed (98.3%) and control (100%) blastocysts was similar at the end of the culture. In vitrified blastocysts, a total of 174 miRNAs exhibited differential expression compared to controls. Among them, ssc-miR-7139-3p from sus scrofa was upregulated, while the remaining 173 miRNAs, including ssc-miR-214 and ssc-miR-885-3p from sus scrofa, were downregulated. Under our knowledge, none of these three-miRNA annotated for sus scrofa have been previously described in pig embryos. The integrative analysis showed that 61 of the previously investigated DEGs in Cryotop-vitrified blastocysts compared to controls were regulated by some of these DE miRNAs. These target genes (TGs) significantly enriched the following KEGG pathways: HIF-1 signaling pathway (ARNT, GADPH, MKNK2; all upregulated TGs), Notch signaling pathway (HES1 and LFNG; both downregulated TGs), Ascorbate and aldarate metabolism (MIOX; upregulated TG), and Glycosphingolipid biosynthesis-ganglio series (SLC33A1; upregulated TG). Among them, Notch signaling pathway has been demonstrated as essential for embryo and fetal development. The biological impact of these results after embryo transfer requires further research. This study has been supported by MCIN/AEI/10.13039/501100011033 & ERDF (RTI2018-093525-B-I00), Madrid, Spain. A G-P is granted by MCIN (PRE2019-090508).

Proteome characterization of endometrium during implantation in the domestic pigs

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Keywords: pig, proteome, endometrium

Characterization of the porcine endometrial proteome is important for understanding the complex embryo-maternal communication required for successful pregnancy outcome. It is during the implantation stage when most porcine embryos from natural or artificial breeding undergo death. Therefore, the aim of the present study was to characterize the proteomic profile of the "pregnant" endometrium at day 18 and 24 post insemination compared to that of the non-pregnant endometrium at the same days of the cycle. For this purpose, sows were inseminated postcervically with 1.5 x 10^9 live spermatozoa (for pregnant sows; PS) or dead spermatozoa (for non-pregnant sows; NPS). Sows were slaughtered to obtain endometrial tissue samples from three different attachment sites on day 18 of gestation (n=4 sows) or day 24 of gestation (n=4 sows). In NPS sows, endometrial samples were randomly collected from three different areas of the uterine horn on day 18 (n=4 sows) or day 24 of the cycle (N=4 sows). All samples were subjected to proteomic analysis to generate a data-dependent spectral library by LC-MS/MS. This analysis allowed the identification of 3254 and 3457 proteins in the endometrium of PS and NPS, respectively; of these, 1753 were common and 1501 and 1704 were unique to PS and NPS, respectively. Analysis of PS-unique proteins by functional GO analysis using the UniProtKB database and Cytoscape ClueGO™ Pathway Enrichment Analysis, revealed an interaction of CCR5, HMOX1, IFI35, ISG15, LBP, MAP2K1, MAPK14, SLA, STXBP2, VAMP7 proteins involved in immune system process in the network of cytokine signaling in immune system and CD14, DNM1, LBP, MAP2K1, MAPK14, S100A9 proteins in the network of Toll Like Receptor 4 cascade pathways. The analysis also revealed the interaction of EMILIN1, HAPLN1, ITGAM, TNC proteins involved in biological adhesion annotation in the extracellular matrix organization pathway. Regarding the identified PS-unique proteins involved in reproductive process annotation, ClueGO showed the interaction of DLG1, MAP2K1, MAPK14 proteins in T cell receptor signaling KEGG pathway and CCT3, CCT7 proteins in Folding of actin by CCT/TriC pathway. ClueGO analysis also revealed the involvement of PS-unique proteins with reproductive process annotation in various GO terms of Biological Process such as placenta development (HECTD1, HSD17B2, MAP2K1, MAPK14, RP56) or reproductive structure and system development (HECTD1, HSD17B2, MAP2K1, MAPK14, RP56, MSH2, DLG1, INHBB). This study provides relevant immune- and binding proteins that may play an important role in endometrial development during weeks 3 to 4 of pregnancy. The identification of these proteins may help to clarify the crosstalk between the endometrium and the conceptus at that critical period of pregnancy, when major conceptus loss occurs. Supported by Fundacion Seneca (19892/GERM/15), Spain, and the Research Council FORMAS (Project 2019-00288), Sweden.

In silico-designed vitrification protocols using propylene glycol for bovine oocytes as an alternative to dimethyl sulfoxide based protocols

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Keywords: permeability, in-silico predictions, mathematical modeling

The cryopreservation of bovine oocytes is a widely employed technique in reproductive biotechnologies. However, it still faces significant limitations. Although several strategies have been implemented to optimize and refine vitrification protocols, there still remain shortcomings. The most extended protocols for bovine oocyte vitrification are based on a combination of ethylene glycol (EG) and dimethyl sulfoxide (Me_2SO), which have been demonstrated to provide acceptable outcomes in terms of post-warming survival, spindle configuration, and embryo development. Nevertheless, Me_2SO is associated with high cytotoxicity that can negatively impact oocyte competence. To address this concern, propylene glycol (PG) has been suggested as an alternative cryoprotectant that can be combined with EG to enhance vitrification outcomes. Therefore, the objective of study was to determine the optimal exposure time of IVM bovine oocytes to the equilibration solution containing EG and PG at two different temperatures (25˚C and 38.5˚C). The permeability values to EG and PG were assessed by using *in vitro* osmotic observations and mathematical modeling. For this purpose, IVM bovine oocytes (10 to 16 per group) were exposed to a concentration of 1,55 M PG or 1,55 M EG in TCM199-Hepes + 20% FBS at either 25˚C or 38,5˚C and incubated for 10 min (3 biological replicates). The volumetric response of the oocyte was recorded every 5 s with a time-lapse video recorder. The permeability parameters hydraulic conductivity (L_p) and CPA permeability (P_s) of the oocyte cell membrane were determined by fitting the experimental data to a two-parameter transport formalism. Then, *in silico*-predictions were obtained from the permeability data as previously described (García-Martínez et al. Theriogenology, 184, 110-123, 2022). *In silico*-predictions accuracy was tested by *in vitro* assessing the time required for the IVM oocytes to reach the osmotic equilibrium volume when exposed to ES composed by 7,5% PG + 7,5% EG in TCM199 medium + 20% FBS at 25 or 38,5ºC. Data were statistically analyzed by Shapiro–Wilk for normality and Levene's test for homogeneity of variance followed by one-way ANOVA and Tukey test ($p<0,05$). Results (mean±SEM) showed that the L_p (mm/atm*min) was significantly higher for PG than EG at both temperatures: 25ºC: EG 1,4±0,1, PG 2,7±0,2 and 38,5ºC: EG 2,3±0,2, PG 5,3±0,3. Also, PG showed higher ($p<0,05$) P_s (mm/s) at 38,5ºc than for EG (2,7±0,2 and 1,9±0,2, respectively) while no differences between CPAs were observed at 25ºC (PG:0,7±0,02; EG:0,8±0,1). In general, both permeability parameters were higher at 38,5˚C than at 25˚C at both CPAs. *In silico* predictions showed that original oocyte cell volume recovery is reached within 1 min 25 sec at 38,5˚C and at 3 min 55 sec at 25˚C. Results suggest that PG combined with EG could be used as a replacement of Me_2SO in bovine oocyte vitrification protocols due to its permeability characteristics and lower toxicity.

This study was supported by the Spanish Ministry of Science and Innovation (PID2020-116531RB-I00 and PRE2021-098675) and the Generalitat de Catalunya (2021 SGR 00900).

Practitioners´and Clinical Reports

Cultivation of bovine oocytes in a medium intended to cultivate human oocytes

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Keywords: cattle, embryos, species-specific protein media

In Latvia, in vitro production of bovine embryos was started in the newly established laboratory. Several commercial media were tested. Different studies have recommended that media consisting of species-specific proteins should be used. This study aimed to check whether the medium intended to cultivate human oocytes is suitable for cultivating bovine oocytes/embryos. The embryo production rate *in vitro* was initially tested using slaughterhouse-derived cow oocytes. Ovaries were transported in plastic bags, without a medium, at 25 °C to the lab within 2 hours. Upon arrival, oocytes were aspirated immediately using OPU medium. The 1st and 2nd quality group oocytes were put together (oocytes A, n=15) and the 3rd and 4th quality oocytes were grouped together (oocytes B, n=58). Subsequently, they were washed in the Washing medium (IVF Bioscience, UK). Initially, the protocol started with a 21 h maturation in HEPES (IVF Bioscience, UK) at air O2, 6% CO_2 at 38.8 ºC, maximum humidity. The BO-IVF Medium (IVF Bioscience, UK) at air O2, 6% CO2, 38.8 ºC and maximal humidity was used to fertilize both oocyte groups within 19 h. The Media BO-Semen Prep (IVF Bioscience, UK) was used to prepare the bull sperm. BO-IVC™ (IVF Bioscience, UK) was used on oocytes A, but GAIN™ medium (FertiPro, Belgium, intended for human oocyte/embryo cultivation, and containing 3.5g/l human serum albumin) was used on oocytes B. The process of IVC lasted for 7-8 days in 5% O_2, 6% CO_2, and maximum humidity at 38.8 ºC. The experiment resulted in 12 cultivated embryos out of 15 oocytes A (80%) and 34 embryos out of 58 oocytes B (58.6 %) being cleaved on the 3rd cultivation day. Two blastocysts were obtained in A (16.7% of all cleaved) and 15 out of 34 (44.1% of cleaved) in B on the 7th-8th cultivation day. The results of these two media use are not comparable because different persons carried out manipulations. In conclusion, it is possible to reach a blastocyst stage using media consisting of proteins of another species. Future investigations are needed to establish the effect of another species' protein-consisting media on cow embryo development quality and viability *in vivo*.

Acknowledgements. The present study was financially supported by the Latvia University of Life Sciences and Technologies and by the European agricultural fund for rural development Project No. 22-00-A01612-000008.

Sire conception rate and incidence of pregnancy loss according to estrus occurrence of dairy and beef cows subjected to timed artificial insemination with sexed semen

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Keywords: Synchronization, sire conception, pregnancy loss

The importance of evaluating multiple milestones of embryonic development when classifying sire fertility is recognized, as individual sires have different phenotypes. The objective of this study was to evaluate influence of oestrus expression and different sires (dairy and beef) with sexed and unsexed semen on sire conception rate and incidence of pregnancy loss following timed artificial insemination (TAI). A total of 225 postpartum lactating or dry cows (dairy; *n* = 129 and beef; *n* = 96) from emerging cattle farmers in South Africa were randomly assigned to receive a 9 day Ovsynch + controlled intravaginal drug release (CIDR®) protocol and TAI. Estrus expression was evaluated prior to TAI using Kamar® heat mount patches. TAI was performed with sexed (X-sexed) or unsexed semen supplied by ABS genetics. Eight sires (4 Holstein Friesian and 4 Angus; 2 sexed and 2 unsexed semen from each cattle type) were used to inseminate dairy cows with dairy sires and beef cows with beef sires. Pregnancy was diagnosed on Days 35, 65 and 95 by transrectal ultrasonography to determine pregnancy loss for first (between Days 35 and 65) and second (between Days 66 and 95) periods of pregnancy. Chi-square test was used to determine significant differences on effects of estrus expression on sire conception and incidence of pregnancy loss. Estrus expression in dairy (112/129; 86%) and beef (62/96; 64%) cows was recorded respectively ($P < 0.05$). Sire conception rate was higher for sexed semen dairy sires (5/7; 71%) on Day 35 when compared with beef sires (9/15; 60%) in cows without estrus expression, whereas on the same day/period sire conception rate was higher for unsexed semen dairy sires (39/59; 66%) when compared to beef sires (14/27; 51%) in cows with expressed estrus ($P < 0.05$). Concurrently, sire conception rate was higher in sexed semen beef sires (7/15; 46%) on Day 95 when compared with dairy sires (3/8; 37%) in cows without estrus expression ($P < 0.05$), whereas on the same day/period sire conception rate was higher for unsexed semen dairy sires (29/59; 49%) when compared with beef sires (10/27; 37%) in cows with expressed estrus ($P < 0.05$). Incidence of pregnancy loss was higher in sexed semen dairy sires (2/5; 40%) between Days 35-65 in cows without estrus expression, whereas, on the same period incidence of pregnancy loss was higher in unsexed semen beef sires (3/10; 30%) in cows without estrus expression ($P < 0.05$). Incidence of pregnancy loss was higher in sexed semen dairy sires (3/24; 12%) between Days 66-95 in cows with expressed estrus. Whereas, on the same period unsexed semen dairy sires (3/32; 9%) had higher incidence of pregnancy loss in cows with expressed estrus ($P < 0.05$). In conclusion, both dairy and beef sexed and unsexed AI sires showed individual variation. Sire conception rate and incidence of pregnancy loss increased with cattle type rather than estrus occurrence.

Acknowledgments: GDARD and ARC-AP (Grant: P13000022-02) for funding the study

Workshop: Animal Welfare in ARTs

Animal welfare in ART's

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Keywords: Animal welfare, embryo technologies

The welfare of animals is becoming increasingly important in our society, particularly in Europe where, according the Eurobarometer of March 2016, 94% of people believe that it is important to protect farm animal welfare. Facing this societal evolution, the number of laws and standards regulating and protecting the welfare of animals is increasing around the world: 336 regulations on animal welfare have been noticed by FAO during the last 25 years and 75% of them came from European region. This trend involves a growing demand from policy-makers for expertise and advice on animal welfare in order to create these new regulations in the image of the European Commission who has committed to revise the following pieces of EU animal welfare legislation by 2023, under the EU Farm to Fork Strategy. Logically, we can observe an increasing number of publications on this topic from 442 in 2000 to more than 2500 in 2022. Embryo technologies sector in not spared by this evolution and practitioners and breeding companies must take into account these aspects in their daily activities. Assisted Reproduction Technologies (ART), including embryo technologies, is an efficient way to improve genetics, making it possible to rapidly breed and multiply genetically superior animals. These techniques include medical and technical procedures resulting in efficient and safe reproduction of mammalian species, but these advantages may be challenged by part of the society particularly from the welfare point of view. In that context, this workshop has 3 main objectives. The 1st is to discuss about the perception of animal welfare by people involved in embryo technologies: view on these evolutions, issues encountered, expectations from the workshop of from the AETE, etc... The 2nd is to present the current situation of regulations and current market requirements about animal welfare in Europe and abroad and how these requirements could impact embryo technologies activities. Finally, the 3rd is to highlight some initiatives including some research studies aiming to evaluate impacts of some ARTs on animal welfare. One of these studies will compare these results to other common breeding practices. Moreover, a French survey has been done in reproduction centers (semen and embryo production centers) in 2022 and the main conclusions will be presented. This survey aimed to evaluate animal welfare levels in these specific herds in order to promote good practices through a training guide. Finally, after a short summary of the main conclusions of these exchanges, an interactive discussion with the audience will highlight the main perspectives and future challenges for the sector.

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