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38th SCIENTIFIC MEETING

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Hilary Dobson

Special Celebration

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Utrecht, The Netherlands, 14th -15th September

2022

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Dr. Hilary Dobson

A.E.T.E. Medalist 2022

Dr. Hilary Dobson

A.E.T.E. Medalist 2022

Professor Hilary Dobson

AETE Pioneer Award 2022

The Association of Embryo Technology in Europe (AETE) has decided to give the AETE Pioneer Award 2020 to Professor Hilary Dobson, University of Liverpool, England. Hilary has devoted her time to uncovering the mechanisms of reproductive biology in ruminants. Much of what we know about the hormones that regulate reproduction is founded on Hilary's work. More importantly, Hilary has always used her academic work to help solve problems affecting fertility.

The AETE Pioneer Award 2020 recognises Hilary's passion for discovery, dedication to research, and enthusiasm for reproductive biology. Hilary has another pioneering characteristic — being in the right place at the right time. This is typified by the chance meeting, at the age of 14, of her future husband, Rob. Hilary was helping her father mark out a football pitch when she bumped into Rob, who was one of the football players. Both passions have lasted, with fifty years of marriage and supporting Liverpool Football Club. Hilary and Rob, as well as Liverpool Football Club, are examples of the world's best teams.

Introduction

Hilary was born in 1948, the second daughter to teacher Peggy and engineer Norman Vaughan. After schooling in North West England, Hilary studied biochemistry at the University of Liverpool. She graduated in 1969 and has remained at the University of Liverpool ever since. The University awarded Hilary her PhD in 1973 and her DSc in 1990. Hilary rose through the academic ranks and was appointed Professor of Veterinary Reproduction in 1994. Hilary was one of the first female professors at a veterinary school in England. Hilary did not stop there, and she chaired the Board of

the Faculty of Veterinary Science from 2001 to 2004. Hilary has been Emeritus Professor of Veterinary Reproduction at the University of Liverpool, since 2009.

Hilary has spent her working life researching and teaching reproductive biology. She has been an author on more than 250 international scientific publications. She has served on the editorial board of several scientific journals, including the Journal of Reproduction and Fertility, Animal Reproduction Science, and Reproduction in Domestic Animals. Hilary has also served on committees for the Society for Study of Animal Breeding, and the Society for Study of Fertility. She was the first General Secretary of the European Society of Domestic Animal Reproduction. Hilary is an Honorary Associate of both the Royal College of Veterinary Surgeons, and the European College of Animal Reproduction.

The early years

Hilary had the good fortune to start her research career just as it became possible to measure steroid hormones and gonadotrophins in cattle and sheep. Under the guidance of Professor Richard Fitzpatrick, Hilary used radioimmunoassay to measure these hormones in ruminants. This required Hilary to move 12 miles from Liverpool to Leahurst. Leahurst is the field station for the Liverpool Veterinary School, which is in beautiful parkland in the English county of Cheshire. Hilary has been there ever since.

In 1973, Hilary successfully defended her PhD thesis on “the reproductive hormones of the non-pregnant cow”. After a series of postdoctoral positions at Leahurst, in 1978, the Liverpool Veterinary School offered Hilary a lectureship. At the time Hilary was 8 months pregnant. Subsequently she became one of the first lactating scientists speaking at an international conference on “Clinical uses of milk progesterone measurements”.

Farm life

Hilary married Rob in 1970. Rob is a dairy farmer, and their pedigree herd currently consists of about a hundred and sixty black and white cows, which are milked by three robots. The farm covers about a hundred hectares of land belonging to Lord Cholmondeley’s Estate in Cheshire. Hilary’s first son, Alistair, was born in 1975, and he now manages the farm. Jonathan was born in 1977, and he is a senior manager for a national water processing company. Raising a family

while continuing to work at a University was only possible with the support of Rob and the family. Alistair and Jonathan kept their four grandparents busy for many years.

Being married to Rob also supported Hilary's career. Rob's thoughts helped Hilary enlighten students and staff at Leahurst about what farmers actually needed and wanted from their veterinarian. This also worked the other way, with Rob often being the first to hear about the latest developments in veterinary science. Unfortunately, it is difficult to convince farmers of some new ideas. Hilary was particularly dismayed when she told Rob about how she helped ICI Animal Health develop a synthetic prostaglandin (*Estrumate*) for controlling the oestrous cycle of cattle. Rob and his father were unimpressed and commented that "it would never catch on – we farmers know how to control our animals". Rob has subsequently had to revise this opinion.

Research

Over the last fifty years, Hilary has made important contributions to reproductive biology. This work has produced many papers on how the brain, the ovary and the genital tract contribute to reproduction and fertility. The common themes across Hilary's papers are understanding the role of hormones in reproduction, and linking this endocrinology to practical applications to improve fertility.

In the 1970s, Hilary's first twenty research papers reported her pioneering use of radioimmunoassay to measure the concentrations of hormones in peripheral plasma and milk. Using these assays, Hilary dissected the changes in oestradiol, progesterone, luteinising hormone and follicle stimulating hormone during the reproductive cycle of cattle and sheep. These data helped form the foundation of our understanding of the physiology of hormones in ruminant reproduction. Hilary was also aware that farmers complained that their animals often had abnormal oestrous cycles. Therefore, she extended her endocrine work to uncover mechanisms of ovarian dysfunction, such as cystic ovarian disease. Hilary then applied this knowledge about the endocrinology to test practical treatments for ovarian dysfunction.

In 1992, Hilary was awarded an Association of Commonwealth Universities Fellowship to work in Australia at CSIRO (Commonwealth Scientific and Industrial Research Organisation). This 6-month sabbatical led to some of the most comprehensive and informative studies on hormones ever conducted in ruminants. Working with Bruce

Campbell, David Baird and Rex Scaramuzzi, Hilary used sheep with an autotransplanted ovary. This sheep model allowed daily monitoring of ovarian follicles and frequent collection of blood for hormone analyses. These beautifully intricate and detailed experiments helped to uncover the importance of follicle-stimulating hormone and luteinising hormone pulses in ovarian follicle selection, growth and dominance.

Since the late 1980's, Hilary has also pioneered the understanding of how the environment and stress affect reproduction and fertility. Hilary used two strategies for this work. First, Hilary studied animals in their normal environment. Often with the help of veterinarians from the Diploma in Bovine Reproduction course, Hilary showed that stress reduces the fertility of dairy cattle. A simple but important example is the work with Don Collick and Bob Ward, where Hilary showed that lameness delayed conception in dairy cows. With another practising veterinarian, Steve Borsberry, Hilary found that a range of other periparturient diseases also delayed conception, even though cows were treated successfully. Later, Hilary worked with Martin Sheldon to show that postpartum uterine disease impaired dairy cattle fertility, and that the mechanisms included ovarian as well as uterine dysfunction.

The second strategy Hilary used was to develop models of stress in sheep, such as the stress of transport. Here, with the help of Rob Smith, Jean Routly, Darren Smart and Chrysanthi Fergani, in a series of elegant experiments, Hilary uncovered the minute details of how stress affects the hormones and neurotransmitters in the hypothalamus and pituitary. Conversely, Hilary has also helped uncover how neurotransmitters regulate reproduction and fertility. This approach to stress typifies Hilary's research. She has provided intricate detail about the endocrine mechanisms linking stress and reproduction, but then translated this knowledge to provide practical advice for veterinarians and farmers.

The Diploma in Bovine Reproduction

In 1980, Hilary helped to establish the Diploma Course in Bovine Reproduction — often just called "the DBR". The two-year course was tailored to meet the needs of practising veterinarians, with a week-long module at Leahurst every three months. Hilary used her connections to bring together distinguished researchers in veterinary reproduction from across Europe to teach the course. The Diploma in Bovine Reproduction course

gives veterinarians an insight into research, as well as developing their clinical skills to help their clients improve cattle fertility. More than a hundred and fifty veterinarians have been awarded the Diploma in Bovine Reproduction, and the course is still running. Most of the graduates from the course have stayed in clinical practice and have become leading specialists in bovine reproduction.

Internationalism

Hilary had the good fortune to be one of the first people to embrace the international nature of veterinary research. A typical example followed Hilary giving a delegate at a conference a lift to the train station to meet an urgent appointment. At the time Hilary was using radioimmunoassays to measure reproductive hormones. Sometime later it turned out that the unknown delegate was a Director of the International Atomic Energy Agency of the United Nations, based in Vienna. The agency needed somebody to undertake a mission to review the financial and scientific worthiness of two laboratories in Egypt that were working on reproductive biology. So began a series of such missions for Hilary. Over the next 30 years, she visited many parts of the world as a technical expert for the International Atomic Energy Agency, and Hilary's laboratory became a recognised training laboratory for radioisotopes and radioimmunoassay.

A similar chance event also brought a long-term relationship with Japan. In 1993, Hilary presented a two-week lecture tour throughout Japan. Amongst the more than three thousand practising veterinarians that Hilary spoke to, Dr Sawamuki asked if he could come and experience veterinary life in England. He spent a month on the farm with Hilary and Rob, whilst he joined the local veterinarian to see clinical practice. After a couple of years, Pfizer Japan set up a competition for Japanese veterinarians. The prize was a week on a Diploma in Bovine Reproduction module, followed by a month with a veterinarian. Over the next five years, about twenty-five Japanese veterinarians came to Leahurst — their English was much improved by these visits but not Hilary's Japanese.

Hilary's international reputation also attracted the attention of governments. She produced a report on the use of bovine somatotropin in dairy cattle for the British Government. This was followed by a report on the use of oestradiol in food-producing animals for the European Union.

In vitro fertilisation

Hilary's work is not limited to animal reproduction. In 1985, an anaesthetist who worked at the Royal Liverpool Hospital, as well as Leahurst, noticed that Hilary was working with bovine oocytes. He asked if Hilary knew what a human egg looked like because a hospital consultant wanted to start an IVF clinic. This fortunate event resulted in Hilary becoming the Scientific Director of the Assisted Conception Unit, at Liverpool Women's Hospital. While strict confidentiality was maintained about the identity of the patients, the hospital consultant often told patients that a member of the Veterinary School staff was helping with procedures. For many years Hilary was often thanked by unknown couples for helping them to have a baby.

Autism

Apart from a busy academic work-life and farming, Hilary has also found time to help others. Hilary had a close friendship with a veterinary school colleague, Dr Keith Benson and his family. Their son was on the autistic spectrum. Appreciating the challenges that autism brought for the family, Hilary offered to help the Benson's. As a consequence of this friendship, in 2001, Hilary became a Trustee of Autism Together. Autism Together is one of England's leading providers of services to people with autism. The organization has about nine hundred employees and provides support for more than a thousand families affected by autism. Hilary's enthusiasm and dedication led to her eventually becoming Chairman. She retired from her role as Chairman of Autism Together in 2019.

In summary, I hope that from the brief comments here, that you can see the good fortune that has followed Hilary Dobson throughout her life. From the chance meeting of her future husband on the football pitch, to helping to pioneer our understanding of reproductive biology and fertility in cattle. Now the AETE has the good fortune to include Professor Hilary Dobson as the recipient of the AETE Pioneer Award 2022..

Professor I Martin Sheldon FRCVS

Swansea University Medical School, England

September 2020

Animal Reproduction

THEMATIC SECTION: 36TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

Understanding the trade-off between the environment and fertility in cows and ewes

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Abstract

The environment contributes to production diseases that in turn badly affect cow performance, fertility and culling. Oestrus intensity is lower in lame cows, and in all cows 26% potential oestrus events are not expressed (to avoid getting pregnant). To understand these trade-offs, we need to know how animals react to their environment and how the environment influences hypothalamus-pituitary-adrenal axis (HPA) interactions with the hypothalamus-pituitary-ovarian axis (HPO). Neurotransmitters control secretion of GnRH into hypophyseal portal blood. GnRH/LH pulse amplitude and frequency drive oestradiol production, culminating in oestrus behaviour and a precisely-timed GnRH/LH surge, all of which are disrupted by poor environments. Responses to peripheral neuronal agents give clues about mechanisms, but do these drugs alter perception of stimuli, or suppress consequent responses? *In vitro* studies confirm some neuronal interactions between the HPA and HPO; and immuno-histochemistry clarifies the location and sequence of inter-neurone activity within the brain. In both species, exogenous corticoids, ACTH and/or CRH act at the pituitary (reduce LH release by GnRH), and hypothalamus (lower GnRH pulse frequency and delay surge release). This requires inter-neurones as GnRH cells do not have receptors for HPA compounds. There are two (simultaneous, therefore fail-safe?) pathways for CRH suppression of GnRH release via CRH-Receptors: one being the regulation of kisspeptin/dynorphin and other cell types in the hypothalamus, and the other being the direct contact between CRH and GnRH cell terminals in the median eminence. When we domesticate animals, we must provide the best possible environment otherwise animals trade-off with lower production, less intense oestrus behaviour, and impaired fertility. Avoiding life-time peri-parturient problems by managing persistent lactations in cows may be a worthy trade-off on both welfare and economic terms – better than the camouflage use of drugs/hormones/feed additives/intricate technologies? In the long term, getting animals and environment in a more harmonious balance is the ultimate strategy.

Keywords: oestrus, adrenal, GnRH, neurotransmitters, behaviour.

The 'environment' referred to in this article encompasses everything that affects the cow or ewe (our main species of interest). 'Trade-offs' are responses to that environment – by both animals and farmers.

'Random' observations to put 'trade-offs' in perspective

First: over a 2–3 week period, more than 80% non-domesticated wildebeest roaming the plains of the Serengeti in east Africa mate and calve with no interference by man (Estes, 1966). The trade-off? Predation of neonates by other animals is limited during synchronised births due to the formation of protective mothering groups and crèches, also aided by the

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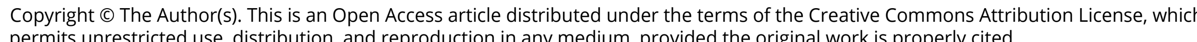
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extraordinary mobility of precocious calves. However, this contrasts with life expectancy as sick animals do not survive to breed.

Second: in dairy cows, production diseases predominate in the first 30 days after calving. Uterine problems represent major detrimental direct effects of the environment on fertility that are long-lasting (Sheldon and Owens, 2017; Piersanti et al., 2019); however, the present review focuses on non-uterine conditions. Such production diseases have an incidence of 6.7% ovarian cysts, 15.2% lameness, 17.2% subclinical ketosis; and treated cows take longer to get pregnant, 64, 100 and 58 days, respectively, (Figure 1a; Borsberry and Dobson, 1989; Collick et al., 1989; Rutherford et al., 2016). Milk fever also extends the calving to pregnancy interval by 12 days (Dobson et al., 2001), and cows with mastitis around the time of the first 'silent' oestrus have delayed luteal activity and late onset of oestrus (Huszenicza et al., 2005). So unsurprisingly, these conditions occur in 60-80% repeat breeders (≥ 3 unsuccessful inseminations; Canu et al., 2010). Indeed, non-uterine inflammatory diseases (mastitis, lameness, digestive and respiratory problems occurring before breeding) reduce rates of oocyte fertilization and development to morulae, and impair both elongation of early conceptuses and secretion of interferon-τ in the uterine lumen. Furthermore, these diseases cause changes in the transcriptome of conceptus cells, increase the risk of pregnancy loss, and reduce pregnancy or calving per breeding (Figure 1b; Ribeiro et al., 2016). Thus, treated production diseases have long-term effects on milking performance, fertility and culling of dairy cows, all of which are detrimental to the sustainability of dairy herds (Carvalho et al., 2019). Is this the price to be paid in the trade-off during domestication for milk production?

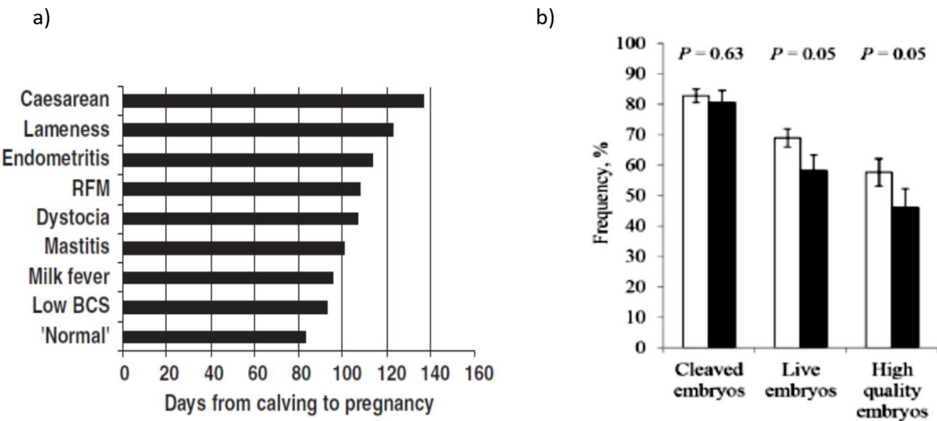


Figure 1. (a) Days from calving to pregnancy after AI in cows with different clinical production diseases (RFM retained fetal membranes; BCS body condition score). Adapted from Dobson et al. (2008); reproduced with permission; (b) Mean percentage of total embryos recovered (±SEM) representing cleaved embryos, live embryos (quality Grades 1-3) and high quality embryos (Grades 1 and 2) obtained from donors without (open bars) or with prior non-uterine disease (black bar). Adapted from Ribeiro et al. (2016); reproduced with permission.

Third: the environment is often compromised during domestication. Adequate housing is important for cow comfort, but while straw-yards increase dairy cow lying time, a trade-off is made against an increase in mastitis (Whitaker et al., 2000). In cubicles with a sand-base, the prevalence of lameness is halved compared to mattresses, and cows spend more time eating (Cook et al., 2004). In addition, cows housed on slippery walk-ways express less intense oestrus with a major impact on pregnancy rates to AI (Britt et al., 1986). Also, high environmental temperatures (>25 ^{0}C, either within housing, or outside without shade) reduce fertility, and this is even more dramatic for high yielding cows that also generate more heat than they can dissipate (Al Katanani et al., 1999). A useful management tool to maintain high pregnancy rates throughout the year would be to produce bovine embryos during the cooler months and use them in embryo transfer during periods of heat stress (Bo et al., 2019). Thus, providing more expensive environments or engaging intricate technologies (e.g., embryo production/transfer)

are used in trade-offs against the deleterious effects of the environment on fertility (review: Dobson et al., 2001; review: Butler, 2003; Canu et al., 2010).

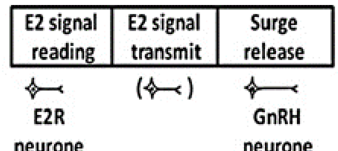
Fourth: for many years dairy farmers have been criticised for increasing milk yield per cow, while not spending enough time to observe oestrus properly, or even resorting to fixed-time inseminations. Also, herd size has increased in an attempt to produce more milk per farm – a trade-off between economies of scale against a reduction in attention-to-detail on an individual cow basis. Would the use of motion sensors (pedometers or neck collars) improve oestrus detection? When progesterone profiles identify possible oestrus events (periods of low milk progesterone with higher values before and after), motion sensors and observations by farm staff detect only 74% potential oestrus events (Holman et al., 2011; Williams et al., 2018). So, why do cows not show signs of oestrus in the remaining 26% situations – a trade-off to avoid getting pregnant again?

To understand these trade-offs, it is necessary to know how animals react to their domesticated environment (i.e., all pressures: production diseases, milk/meat yield, housing, feed, social interactions) and how cows/ewes translate their responses in terms of controlling fertility. In large part, this involves hormonal control of the hypothalamus-pituitary-adrenal axis (HPA) and its interaction with the hypothalamus-pituitary-ovarian axis (HPO). To examine these relationships, all our studies (including clinical field work) are carried out under UK Home Office licenses for work on living animals and with the approval of the University of Liverpool Ethical Review process.

Summary of ovarian follicular phase endocrinology (HPO)

In many of the above 'trade-off' situations, either luteinising hormone (LH) pulse or surge patterns (or both) in dairy cows are disrupted leading to reduced fertility (feeding: Butler, 2003; environmental temperature: Badinga et al., 1994; mastitis: Hockett et al., 2005; lameness: review: Dobson et al., 2008). Greater knowledge about the control of oestrus cycle hormones (including LH) will lead to a better understanding of how animals optimise fertility, and how disruption occurs.

Briefly, in cows and ewes neurotransmitters in the brain (especially hypothalamus) control secretion of gonadotrophin releasing hormone (GnRH) into hypophyseal portal blood, and thus LH release from the pituitary into the peripheral circulation. During the luteal phase, feedback from progesterone and oestradiol restrain small discrete GnRH/LH pulses to approximately one per four hours. As peripheral progesterone concentrations decline, LH pulse amplitude and frequency increase to approximately one per hour further driving ovarian follicular growth and oestradiol production. Towards the end of the follicular phase, when progesterone concentrations are low and oestradiol concentrations are at a maximum (Figure 2; oestradiol-signal reading phase; Battaglia et al., 2000), there is a temporary decrease in GnRH/LH pulse frequency and amplitude at approximately 4–6 h (oestradiol signal transmission phase) before a precisely-timed pre-ovulatory surge release of GnRH/LH. This causes ovulation and formation of a corpus luteum. Alternating exposure to oestradiol and progesterone leads to the expression of sexual behaviour and prepares the uterine environment; and in concert with signals from the conceptus, pregnancy will be established.



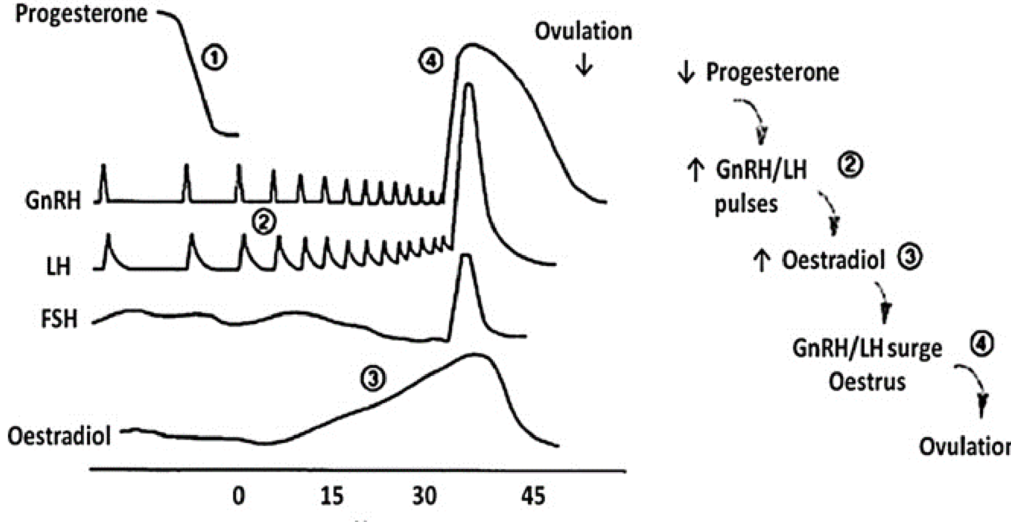


Figure 2. Endocrine events during the follicular phase of the oestrous cycle of ewes. Left, peripheral hormone patterns; right, sequence of regulatory steps; top, theoretical model of neuroendocrine processes involved in generating the GnRH/LH surge (timings as in lower panel). The numbers (1-4) represent steps for which there is evidence of interruption during adverse environmental stimuli (see text). Adapted from Battaglia et al. (1998, 2000) and Dobson et al. (2012); reproduced with permission.

If the environment is not ideal, in cows and ewes GnRH/LH pulsatility is disrupted, as well as the timing and amplitude of the GnRH/LH surge, resulting in failure to initiate a pregnancy. This key disruption is usually temporary so that when prevailing conditions improve, normal hormonal profiles will resume.

The environment - nutrition, milk yield and ovarian cysts in dairy cows

A major difficulty encountered by high-yielding dairy cows is achieving sufficient food dry matter intake (DMI) around calving. Feeding in the dry period has very important consequences for events in the following postpartum period. For example, in the week before calving, feeding duration and DMI are both more than 20% lower in cows that subsequently have mild-or-severe metritis and these same cows produce 6-8 kg milk/day less than healthy herd-mates during the first three weeks after calving (Huzzey et al., 2007). Low DMI is also associated with poor body condition scores and delayed return to normal ovarian cyclicity just after calving; a marked improvement in pregnancy rates occurs once DMI increases resulting in positive energy balance (review: Butler, 2003). Another example of a trade-off – poorly fed animals will switch off reproductive function until their nutritional needs are met.

A further example regarding the consequences of the genetic drive for higher yields: patterns of monthly milk yield and maximum values are not different from normal in those cows that develop ovarian cysts, but weekly analysis reveals shorter durations of peak yield (Nanda et al., 1989a). It is not certain whether this is cause or effect, but some cows trying to meet requirements of sustained milk yields are on a 'knife-edge' and more susceptible to minor environmental changes (such as new social interactions, or changes in diet; Dobson and Smith, 1998). High milk yield may just be one factor leading to formation of ovarian cysts, but there is no correlation between the incidence of cysts with either 3-beta-hydroxy-butyrate (BHB) concentration or body condition scores (Dobson and Nanda, 1992; Tebble et al., 2001). However, these may not be the best indicators: Jackson et al. (2011) provide evidence that high

non-esterified fatty acid (NEFA) values and low urea:BHB ratios can be used before calving as predictors of an increased risk of endometritis, whereas high *Nu* values (multiplication of NEFA and urea values) and low urea:BHB ratios are more useful after calving as predictors of an increased likelihood of cystic ovaries and delayed commencement of luteal activity.

Prolonged anoestrus after calving is characterised by low oestradiol and progesterone values due to suppression of GnRH/LH pulsatility (review: Butler, 2003). Whereas follicular cysts (thin-walled oestradiol-producing structures > 2.5 cm diameter) form after failure of a timely GnRH/LH surge; although several days later sufficient LH may be secreted to produce a luteal cyst (progesterone- producing, > 2.5 cm diameter). Evidence for this suggestion follows:

Dairy cows with naturally-occurring follicular cysts have lower plasma progesterone concentrations than those with luteal cysts (Dobson et al., 1977); but cows with luteal cysts have more additional follicles > 5 mm diameter. Cows with both follicular cysts and other follicles > 5 mm diameter have oestradiol concentrations of ~ 8 pg/ml compared to ~24 pg/ml in cows without other follicles > 5 mm in diameter on either ovary (Douthwaite and Dobson, 2000). It is of note that Nanda et al. (1991a) found that half the cows with follicular cysts, and half the cows with luteal cysts (after prior prostaglandin treatment) respond with an LH surge after an oestradiol injection; therefore, both types of cyst could be due to defects in the LH surge mechanism.

As it is impossible to closely monitor cows at the precise moment ovarian cysts are spontaneously formed, experimental models have been developed. Starting on Day 15 of a 'normal' oestrus cycle, in heifers receiving either:

- high doses of oestradiol and progesterone to mimic high values at the end of pregnancy; 3 out of 8 formed persistent follicles (Ward et al., 2000);
- repeated doses of ACTH to stimulate adrenal secretion; 6 out of 13 formed persistent follicles (Dobson et al., 2000a);
- or prolonged low doses of progesterone to replicate low progesterone values seen after adrenal stimulation; all 13 formed persistent follicles (Noble et al., 2000).

Combined observations from these models reveal that during treatment

- follicle stimulating hormone (FSH) concentrations are normal;
- LH pulse and surge concentrations are lower;
- some prolonged dominant follicles ovulate after 10 days with higher oestradiol concentrations and greater internal diameters than those follicles that persist for > 20 days;
- occasionally persistent follicles luteinise;
- for the second half of the life-span of persistent follicles, oestradiol values are basal, thus the structure can remain for up to 50 days after endocrine function declines, and other smaller follicles appear (Figure 3);
- subsequent treatment with very low dose progesterone or GnRH decreases plasma oestradiol and a new follicular wave emerges.

In short, heifers with experimentally-produced persistent follicles resemble cows with clinically-diagnosed spontaneous cysts. But questions remain – why/how are pulsatile and surge LH secretion disrupted?

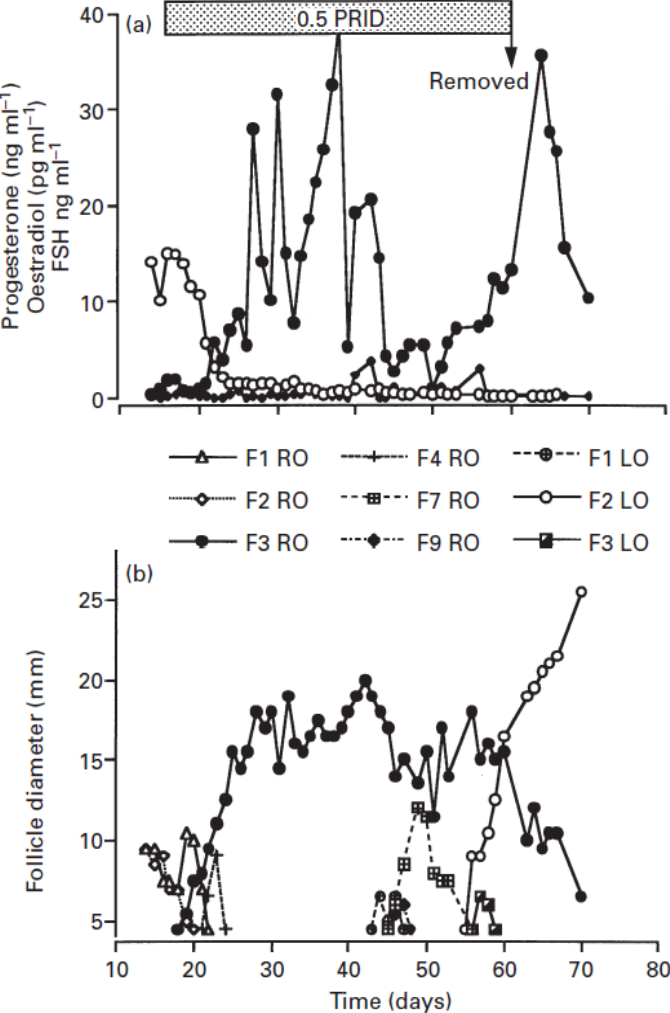


Figure 3. Daily plasma concentrations of (a) progesterone (○) and oestradiol (●), and (b) internal diameters of dominant follicles (●, ○) and subordinate follicles (other symbols) in cows from the last observed oestrus. The horizontal bar indicates presence of half a progesterone releasing intravaginal device (0.5 PRID). RO: right ovary, LO: left ovary. Note the presence of dominant follicle (●) for 50 days, but functionally producing oestradiol for only ~ 25 days; then replaced by a second functional dominant follicle (○) from 55 days onward. Adapted from Noble et al. (2000); reproduced with permission.

Ewe models have also been used to examine in greater detail the functionality, and consequences, of persistent follicles. In ewes, endogenous LH is suppressed by a GnRH antagonist but when replaced with frequent injections of low dose LH for 60 h, normal follicular growth occurs with ovulation after a large LH injection at 60 h, followed by a luteal pattern of plasma progesterone (Campbell et al., 1997). However, if the low dose injections of LH continue for 10 days (with no ovulatory dose of exogenous LH), follicular growth and oestradiol secretion continues for 8 days but then follicular atresia occurs (due to changes within the follicle). Alternatively, stopping the low dose LH injections after 4 days results in an immediate decrease in LH concentrations with a consequent decline in oestradiol secretion but large non-functional follicle structures remain (Figure 4; Dobson et al., 1997). Similarly, if the frequency of low dose LH injections is reduced at 30 h from hourly to as little as every 2 h, there is a marked reduction in oestradiol secretion (review: Dobson and Smith, 1998).

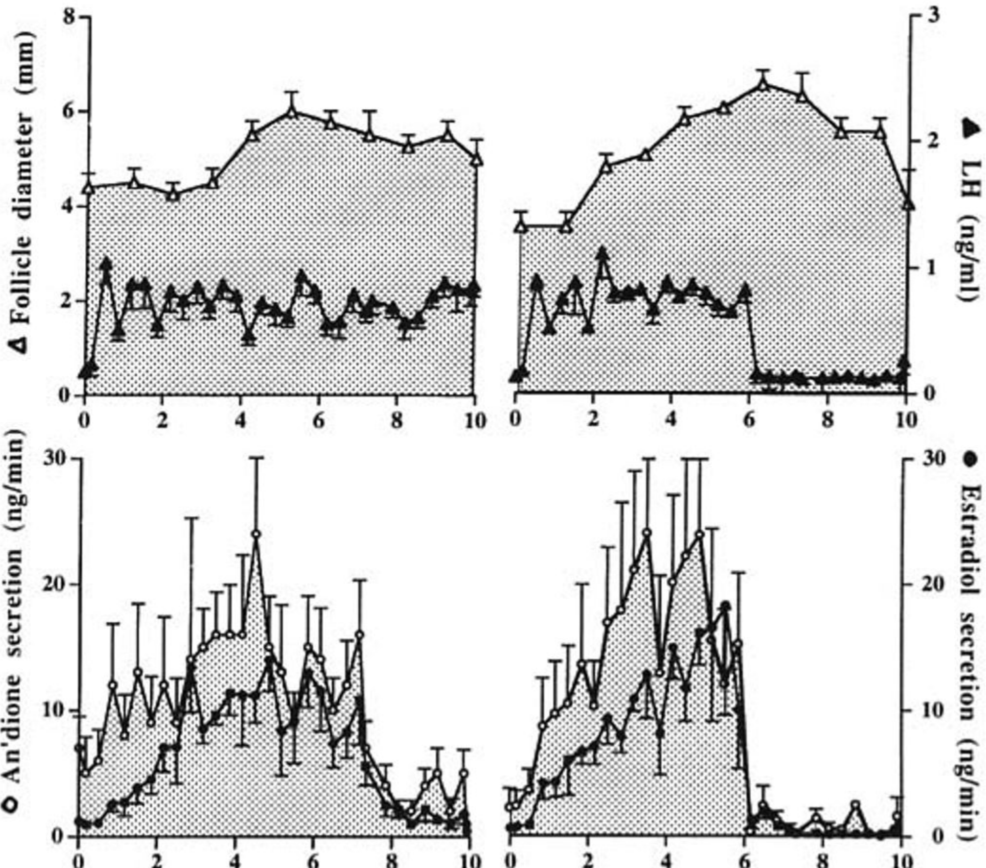


Figure 4. Mean (± SEM) diameter of the largest follicle, peripheral plasma concentrations of LH, and ovarian secretion rates of androstenedione and oestradiol, in ewes treated with GnRH antagonist and hourly injections of LH. The latter were continued for 10 days or stopped on Day 6 (right panels). Adapted from Dobson et al. (1997); reproduced with permission.

Regarding the consequences of prolonged oestradiol exposure, ovariectomised ewes treated with oestradiol implants for 2 to 12 days do not have a normal LH surge after a final challenge with oestradiol (Ozturk et al., 1998). However, LH secretion is provoked by repeated low dose GnRH injections, and, although responses are reduced by 50%, a lowered self-priming effect is still evident indicating inhibitory effects occur at both hypothalamic and pituitary level (Ozturk et al., 1998). By using physiological concentrations, these observations show that it is the duration of oestradiol exposure that causes problems, not excessive concentrations. Furthermore, correction of this oestradiol-induced lesion by administration of physiological does of progesterone for 12 days strengthens the previously empirical choice of similar treatment of clinical bovine cases with follicular cysts (Nanda et al., 1989a; Douthwaite and Dobson, 2000). Progesterone can be directly administered, or endogenous concentrations induced by GnRH treatment in cows (Ribadu et al., 1994).

Clearly, in cows and ewes continuation of LH pulses are required for persistent (cystic) follicular growth and oestradiol production, but why does an LH surge not occur at the end of what appears to be an otherwise normal follicular phase? Cystic follicles in cows are associated with clinical production diseases, including uterine infection (Tsousis et al., 2009). A better understanding of the HPA (and interaction with the HPO) may explain why/how production diseases result in the failure of LH surges during this trade-off.

Summary of adrenal gland endocrinology (HPA)

The hypothalamus-pituitary-adrenal axis (HPA) protects life by monitoring the environment and activating immediate responses to threatening stimuli. Responses are similar in all mammals; during transport, both corticotrophin releasing hormone (CRH) and vasopressin (AVP) are released from the hypothalamus, which in turn cause the anterior pituitary to secrete adreno-corticotrophin hormone (ACTH); that then triggers the adrenal glands to secrete corticoids (predominantly cortisol in cows and ewes; Figure 5; Dobson and Smith 2000b, Smith et al., 2003a, b), as well as transient small amounts of progesterone. Different stimuli differ in intensity as judged by varying concentrations and durations of cortisol and progesterone in peripheral plasma (Smith et al., 2003a; Fergani et al., 2012). Psycho-social stimuli (social isolation, restraint, blindfolding and exposure to predatory cues), transport or rapidly lowered glucose values (after insulin injection), all result in lower cortisol profiles than administration of lipopolysaccharide toxin from *E coli* (LPS). However, there are several important points to note:-

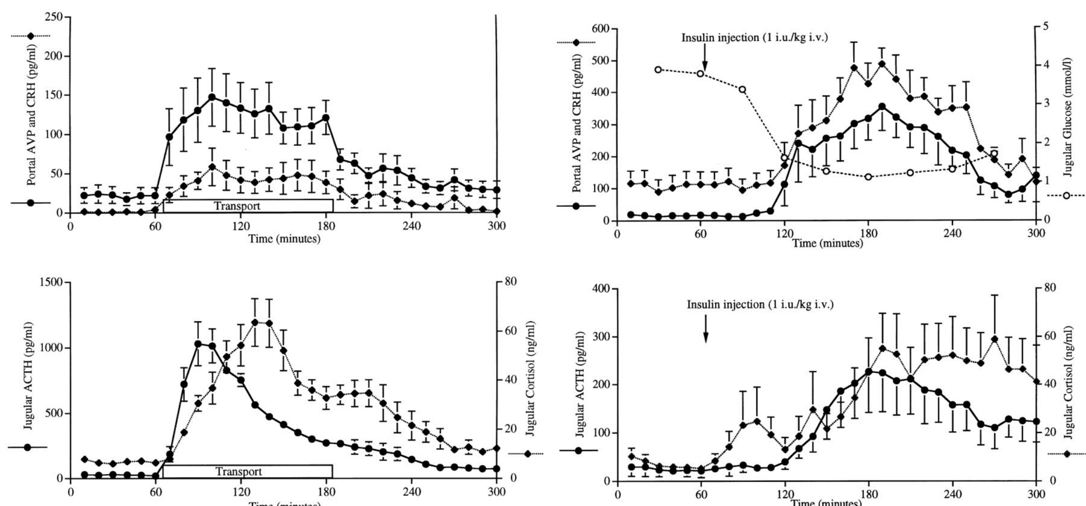


Figure 5. Ewe hypothalamus-pituitary-adrenal responses (mean ± SEM) to 2 h transport (left panels) or insulin injection (right panels). Note different vertical axis between left and right panels; inverse proportions of AVP and CRH after transport or insulin; and decreases in ACTH and cortisol while the stimuli continue. Adapted from Dobson and Smith (2000b); reproduced with permission.

- Maximum ACTH and cortisol values increase rapidly in ewes after the onset of most stimuli but ACTH and cortisol decreases occur very quickly with negative-feedback at both the hypothalamus and pituitary (Figure 5; Smith et al., 2003a, b).
- Chronically lame cows do **not** have continually elevated concentrations of cortisol (Walker et al., 2008a).
- Lower cortisol responses occur during the last of a series of transport stimuli in ewes (Smith and Dobson, 2001), i.e., there is an adaptation of cortisol responses to stimuli, due to individual perceptions, experience and/or responses.
- Acute stimuli immediately release adrenaline from the adrenal glands (Parrott et al., 1994) and pro-opiomelanocortin (POMC; precursor of alpha-melano-stimulating hormone), ACTH and beta-endorphin from the anterior pituitary in ewes (Walsh et al., 1998). These compounds do not readily pass through the blood-brain-barrier to influence hypothalamic function, for the latter they have to be synthesised within the brain (Guillemin et al., 1977; Kastin et al., 1979).
- Each step in HPA axis activation can be mimicked by administering exogenous components: CRH infusion into ewe hypothalamus portal blood increases peripheral ACTH and cortisol (Naylor et al., 1990); ACTH i.v. injections increase cortisol (cows: Alam et al., 1986; ewes:

Phogat et al., 1999a); whereas 10-day treatment of cows with betamethasone (a synthetic corticoid) suppresses plasma cortisol for up to 26 days and delays luteolysis probably by blocking prostaglandin F2-alpha release that is usually stimulated by follicular oestradiol (Dobson et al., 1987).

Models to understand the interaction between HPA and HPO

Many acute stimuli models reveal reduced LH pulse frequency and amplitude as well as delay/block of the LH surge in cows and ewes by affecting difference phases of GnRH surge generation (Figure 2). For example, in rank order of stimulus severity:

- psycho-social models in ewes (social isolation, restraint, blindfolding and/or exposure to predatory cues; review: Ralph et al., 2016)
- transport in cows or ewes (Nanda et al., 1990a; Dobson et al., 1999),
- rapid reduction in plasma glucose by insulin injections (Saifullizam et al., 2010)
- exposure to LPS during the preovulatory period in cows and ewes (Battaglia et al.. 2000; Fergani et al., 2012).

To unravel changes that occur in these models, conventional approaches have been taken by endocrinologists to determine which part(s) of the activated HPA axis are responsible for disrupting normal function at specific levels of the HPO. While some studies investigate endocrine systems in cattle, many more use ewes that are more easily handled, less expensive to maintain, but closely mimic responses in cows. Different components of the HPO (as well as agonists or antagonists) have been administered to assess changes in responses during exposure to a variety of acute or chronic stimuli; and different HPA hormones have been assessed for their impact on HPO function. Moreover, a variety of reproductive states have been involved; for example, intact ewes in the breeding or non-breeding season, in luteal or follicular phases with or without (synthetic) hormonal manipulation, and short- or long-term ovariectomised animals with or without (synthetic) hormone replacement. There are difficulties in interpreting the responses in ovariectomised animals: the HPO itself adapts after ovariectomy, irrespective of any changes in HPA activity; selecting the correct replacement hormone in appropriate doses/patterns/duration affects responses; and the role of the animal's own balancing systems are compromised (or often ignored during interpretation).

Effect of increasing HPA activity on GnRH and LH pulsatile release

Corticoids

In cows and ewes, during transport or treatment with betamethasone, the response to a single low dose of GnRH (to release LH in concentrations equivalent to a pulse) is lower (Dobson, 1987; Dobson et al., 1987). This action at pituitary level concurs with an infusion of cortisol (albeit for 30 h) that acts via glucocorticoid receptors to suppress LH pulse amplitude in ovariectomised ewes (review: Ralph et al., 2016). During transport-induced increases in endogenous cortisol, effects at the hypothalamus must also occur because the frequency of spontaneous LH pulses is lowered in ewes (review: Dobson and Smith, 2000b). Further proof of direct LPS action at the hypothalamus is revealed by a decrease in GnRH pulse frequency and amplitude in ewe portal blood (Battaglia et al., 1997, 2000).

ACTH

In the follicular phase of intact ewes, LH responses to repeated challenges with GnRH are reduced by exogenous ACTH or transport (Phogat et al., 1999a, b). A second exposure to GnRH releases more LH than the first (self-priming effect) by providing more LH in a releasable form

and increasing the number of GnRH receptors on the pituitary cell surface. *In vitro* perifusions of ewe pituitary slices show unequivocally that ACTH reduces the amount of LH released by repeated low dose GnRH; hence, a direct effect occurs at the pituitary and is enhanced with additional oestradiol (Phogat et al., 1997). Oestradiol also induces LH synthesis and GnRH receptor numbers, so any of the above processes may be interfered with to inhibit GnRH self-priming.

CRH

Studies on the role of CRH in interrupting LH release in the ewe are initially confusing. Infusion of CRH peripherally or into the third ventricle of the brain in ovariectomised ewes either does not affect LH secretion, or causes an increase, in contrast to the rat or monkey in which CRH infusion results in prolonged inhibition of LH secretion (Clarke et al., 1990; Naylor et al., 1990). Now it is recognised that intra-cerebro-ventricular (i.c.v.) CRH increases GnRH/LH secretion only during periods of oestradiol negative-feedback (review: Smith et al., 2003a). Whereas, in the follicular positive-feedback phase, i.c.v. CRH suppresses LH pulse frequency (reversible by a CRH antagonist) and decreases both GnRH biosynthesis in the hypothalamus, and the number of GnRH-Receptors in the pituitary (Ciechanowska et al., 2018). There is also, however, direct suppression of CRH on LH released by a second low-dose GnRH challenge from the ewe pituitary *in vitro*, although the effects may have been mediated by *in vitro* pituitary release of ACTH (Smart, 1994). In rats, CRH reduces GnRH secretion *in vivo* or *in vitro* at hypothalamic level via opioid and catecholaminergic pathways (Rivier and Rivest, 1991).

Effect of increasing HPA activity on surge secretion of GnRH and LH

Corticoids

If intact cows or ewes are transported (for 25 min, 2 h or 8 h) just before an expected LH surge, the surge is delayed or totally blocked (Nanda et al., 1989b; Phogat et al., 1999b). The delaying effects on the LH surge are more marked if animals are transported close to the onset of an expected surge (review: Dobson and Smith, 1995). Delays could be due to progesterone from the adrenals because cows or ewes with plasma progesterone concentrations >0.5 ng/ml (endogenous or exogenous) do not have an LH surge in response to oestradiol (Nanda et al., 1988; Phogat et al 1999b). Ralph et al. (2016) conclude that the presence of oestradiol is necessary for cortisol to act at the level of the hypothalamus to interrupt GnRH/LH surges. However, during psychosocial stress, plasma cortisol increases but antagonism of glucocorticoid receptors does not block the effect of cortisol on LH suppression in ewes, indicating that there may be other factors acting in the hypothalamus to suppress GnRH secretion. Any actions of cortisol to inhibit GnRH are likely to be indirect because GnRH neurones in ewes do not contain glucocorticoid receptors, although pituitary LH cells do (Dufourny and Skinner 2002; Breen and Karsch, 2004).

ACTH

If oestradiol is given to ewes in the follicular phase followed by 3 low doses of GnRH, an LH surge occurs within 40 h; if additional ACTH is given along with oestradiol, LH surges are blocked. Negative feedback of oestradiol is necessary to accumulate a readily-releasable LH pool, and it is possible that ACTH acts at pituitary level to prevent the replenishment of enough LH for a surge (Phogat et al., 1999a).

CRH

During the ewe pre-ovulatory period, i.c.v. infusion of CRH suppresses the LH surge causing prolonged oestrus cycles, via effects at the hypothalamus and pituitary (Polkowska and Przekop, 1997; Ciechanowska et al., 2018).

Gonadotrophin inhibiting hormone (GnIH)

This compound, first identified in birds, may be a possible mediator of the effects of increased HPA activity on reproduction. A mammalian homologue of GnIH, RF-amide-related peptide-3 (RFRP-3), suppresses GnRH/LH secretion in ewes but immunohistochemistry and *in situ* hybridisation studies on the hypothalamus of long-term ovariectomised ewes fail to show an increase in GnIH activity after psychosocial stress (review: Ralph et al., 2016).

Opioids

An early series of cow studies using exogenous opioid agonists and antagonists hint that there is opioid suppression of LH release until hour(s) before the LH surge, that is then lifted and the LH surge occurs; however, the suppression is extended by transport (blocking/delaying the LH surge), although the latter is not reversed by one injection of opioid antagonist (Nanda et al., 1989b, 1990b, 1991b, 1992a). Recent work shows that reversal of stimulus-induced LH surge suppression requires several hours of opioid antagonist infusion (Dobson et al., 2020b).

Effect of increasing HPA activity on peripheral FSH concentrations

From the scarce literature available, mid-cycle FSH is increased by HPA stimulation but this is probably due to a reciprocal reduction in follicular oestradiol after suppression of GnRH/LH pulses. Late follicular phase surges of FSH are delayed in parallel with LH surges (Roth et al., 2000; Battaglia et al., 2000).

Models for in-depth studies

With the back-ground of all our above studies, we have recently focused on two main paradigms:

- a chronic spontaneously-occurring model comparing clinically compromised cows (lame, high somatic cell count, low body condition score) with their ‘normal’ herd-mates might reveal ways in which cows utilise coping trade-off strategies, and
- an acute ewe model with or without insulin or LPS treatment that has focused on how these trade-offs may be controlled by neurotransmitters within the brain.

Chronic models of milking cows that are compromised by production diseases

Behavioural observations on-farm reveal that lame cows have lie for longer, spend less time expressing oestrus, have a lower bite rate at pasture and lower body condition scores – all situations that compromise fertility (Walker et al., 2008a). Also, the follicular phase is shorter in cows with high somatic cell count (SCC), and more cows with high SCC and lameness fail to ovulate (Morris et al., 2009, 2013). Further synergistic effects are revealed in cows with 0, 1 or 2 ‘severe’ production diseases that have intervals from calving to the first luteal phase of 31, 44 and 54 days, respectively (Peake et al., 2011).

Spontaneous LH pulse frequency and plasma oestradiol concentrations are lower in lame non-ovulating cows compared to those that do ovulate (Morris et al., 2011). However, the situation is not binary: from 30 to 80 days post-partum, there is a graded effect that ranges

from ~ 30% lame cows with no ovarian activity, another ~ 20% fail to express oestrus or ovulate a low oestrogenic follicle; but in 50% cows, many reproductive parameters are unaffected by lameness (Morris et al., 2011). It is not yet clear how some lame cows cope whereas others shut-down to achieve this graded trade-off.

Turning to oestrus behaviour, continuous visual monitoring for all signs of oestrus in cows (uncompromised by clinical conditions) reveals a precise sequence of events (Dobson et al., 2018). Sniffing other cows is followed by active behaviours of mounting another cow and not accepting a mount, as well as the passive behaviours of being sniffed and standing-to-be-mounted (STBM) by another cow. Chin resting occurs before refusing a mount and STBM. All these behaviours occur in the reverse order after the last STBM. Such distinct behavioural sequences are probably controlled by changes in peripheral progesterone and oestradiol concentrations, as well as by subtle independent mechanisms via pheromones in differing concentrations and/or divergent composition.

Lameness does not affect the above sequence or overall duration of oestrus (although the intensity is reduced; Walker et al., 2008a, 2010). Despite the pain, lame cows are willing to be involved in some sexual activity but the frequency of their own mounting is minimised as well as the duration of being attractive to others (attempts at being mounted). Lame cows also exhibit fewer chin rests – a behaviour that solicits mounting from herd-mates, as well as testing whether it is worth expending energy and/or enduring pain to mount others (another trade-off). So, lame cows may be less attractive to others by emitting poorer quantity/quality of sexual pheromones or even 'stress-related' pheromones, as cows can perceive increased stress in herd-mates by olfactory cues (Boissy et al., 1998). The quantity of the chemical signals may not be as important as the interpretation of chemical messages (i.e., the reward mechanisms).

From a practical stand-point, the efficiency of activity monitoring devices (neck collars and pedometers) is reduced by lameness, low BCS or high milk yield no doubt due to the above effects on oestrus intensity (Holman et al., 2011). As mentioned before, only 74% of all potential oestrus periods (episodes of low progesterone) are identified by combining information from activity monitoring devices and farm staff observations. The endocrine environment is prepared for expressing oestrus but the cows 'choose' not to. Extending these observations, Williams et al. (2018) note that total motor activity detected by the devices is lower in lame cows, and when potential oestrus events (low milk progesterone value between two high values) are not recognised by both devices, progesterone values are slightly higher at the potential oestrus (0.043 *versus* 0.029 ng/ml), contrary to luteal values that are markedly lower in lame cows (1.3 *versus* 0.7 ng/ml; Walker et al., 2008a, 2010).

Overall, studying these chronic conditions on-farm has high-lighted the fact that cows are operating a trade-off between their environment and reproductive efficiency. A delicate knife-edge balance operates – some stimuli do not have a deleterious effect and can be coped with by some animals, but not all. The severity of the environment dictates outcomes, as do combinations of production diseases. A significant way of coping involves modifying oestrus behaviour to avoid getting pregnant, as well as reducing the incidence of ovulation. Interestingly, similarities exist between chronic situations and acute experimental models: LH pulse frequency is decreased, oestradiol concentrations are consequently reduced and subsequent luteal progesterone values are lower. But exactly how do animals modify these vital parts of reproductive function to bring about the trade-off?

Role of neurotransmitters in ewes subjected to transport, insulin or LPS (acute models)

There is a delicate balance between positive and negative influences of steroids and neurotransmitters in various locations in the brain (Figure 6). The ultimate goal is to respond to stimuli in the environment in order to regulate the pattern of GnRH secretion into hypophyseal portal blood.

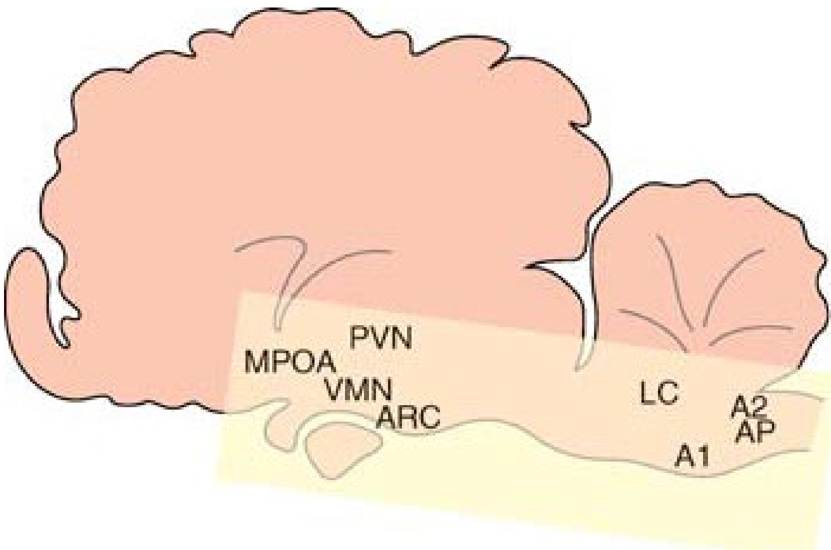


Figure 6. Location of neuronal nuclei in the ewe brain that regulate GnRH and corticotrophin-releasing hormone and arginine vasopressin secretion, indicating the spatial relationship between regions of the hypothalamus (medial preoptic area (mPOA), ventral medial nucleus (VMN) and arcuate nucleus (ARC) of the medial basal hypothalamus)) and the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)). PVN: paraventricular nucleus. Adapted from Dobson et al. (2003); reproduced with permission.

When a stimulus is perceived, noradrenaline-neuropeptide Y neurones are activated in the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC) with on-going synaptic contacts between these cells and CRH/AVP cell bodies in the hypothalamic paraventricular nucleus (PVN), possibly also with links via the medial pre-optic area (mPOA; Dobson et al., 2003). As a result, CRH/AVP are transferred along neurones to the median eminence with subsequent release into the hypophyseal portal system, and consequent stimulation of ACTH secretion from the pituitary. In the PVN, CRH/AVP cells contain glucocorticoid receptors that exert strong and quick negative-feedback, an essential process to prevent prolongation of enhanced glucose metabolism, altered vascular dynamics and dysfunctional immune responses.

Effects of neuronal agents on HPO and HPA activity

Sedation induced by sodium pentobarbitone reduces the magnitude or totally blocks the LH surge in cyclic and oestradiol-treated ewes or cows (Dobson and Ward, 1977; Nanda et al., 1992b). The expected LH surge in late follicular phase ewes or oestradiol-treated anoestrous ewes is also totally suppressed by the alpha-adrenergic blocker, phenoybenzamine, indicating an adrenergic role in initiating the LH surge (Narayana and Dobson, 1979).

Pentobarbitone, diazepam (gamma-amino-butyric acid (GABA) agonist), or xylazine (alpha-2 adrenergic agonist) all immediately decrease basal, as well as transport-induced increases in plasma cortisol, glucose, respiration rate and heart rate (Sanhouri et al., 1991a, c, 1992). However, xylazine does not affect the cortisol response to exogenous CRH, indicating a mechanism mediated through the hypothalamus (Sanhouri et al., 1992). In contrast, an alpha-1 adrenergic antagonist or a beta-adrenergic blocker do not lower cortisol responses to transport (Sanhouri et al., 1991b).

All these systemically administered agents could suppress afferent sensory input to the brain (perception) or interfere with efferent responses within the brain. Concerning the latter, the paraventricular nucleus (PVN) has alpha-1 adrenergic receptors, is well innervated by adrenergic fibres, and administration of an alpha-1 adrenergic agonist directly into the third ventricle of the brain increases peripheral ACTH and cortisol concentrations (Liu et al., 1991; review: Dobson et al., 2003).

<u>In vitro</u> inter-relationships between neurotransmitters, CRH/AVP or GnRH activity in ewes

Perifusions of ewe hypothalamus slices examine <u>direct</u> effects of steroids and neurotransmitters. Exposure to oestradiol increases basal release of AVP (Ghuman et al., 2006), and alpha-1-adrenoreceptor agonists increase AVP secretion (concurring with the above *in vivo* studies) - this response is further enhanced in the presence of oestradiol (Ghuman et al., 2008a). Using the same perifusion system with agonists and antagonists to GABA, Ghuman et al. (2007) conclude that basal AVP release is under GABA-B inhibition, and this negative effect is enhanced by oestradiol.

Concerning *in vitro* GnRH secretion in ewes, oestradiol again has considerable impact via adrenergic and GABA-ergic influence. Basal GnRH increases in the presence of oestradiol (Ghuman et al., 2006) and an adrenergic agonist increases release of GnRH that is prolonged by oestradiol (Ghuman et al., 2008b). In contrast, a GABA-A <u>antagonist</u> results in greater GnRH secretion that is higher in the <u>absence</u> of oestradiol (Ghuman et al., 2008c) concurring with *in vivo* studies (Scott and Clarke, 1993). It is suggested that higher concentrations of oestradiol eventually decrease GABA turnover which in turn facilitates activation of GnRH neurones. This is supported by the marked decrease in GABA tone just before the GnRH/LH surge co-incident with high oestradiol concentrations in ewes (Robinson et al., 1991). Furthermore, in the presence of low oestradiol concentrations, noradrenaline (NA) stimulates GABA to suppress GnRH release, but this effect is decoupled by the high oestradiol values of the pre-ovulatory period (by currently unknown mechanisms).

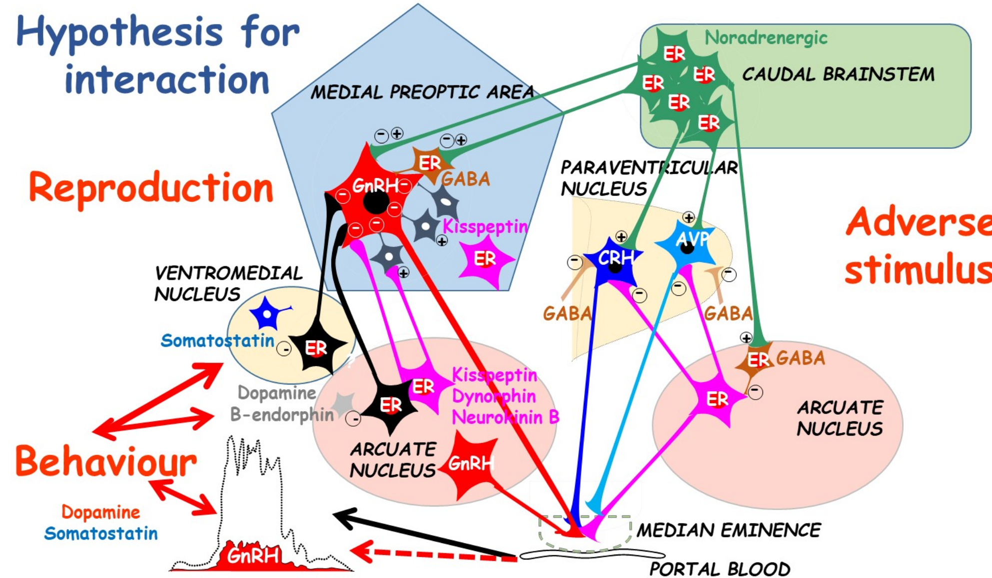


Figure 7. Diagram indicating possible inter-action between neurones involved in GnRH/behaviour disruption following adverse stimuli imposed in the late follicular phase of the ewe. Noradrenergic cells in the brain stem project to both the paraventricular nucleus (PVN) and the medial preoptic area (mPOA). Adverse stimuli activate CRH/AVP neurones in the PVN. Changes in activity of beta-endorphin and dynorphin neurones in the arcuate nucleus (ARC) influence PVN and mPOA output. In the ARC, the activities of oestradiol receptor (ER) neurones (probably kisspeptin/dynorphin/neurokinin B; KNDy cells) are altered by adverse stimuli, as are ER cells in the ventromedial nucleus (VMN). In the median eminence, CRH, but not AVP, terminals and KNDy terminals are in close contact with GnRH terminals providing another site for the disruption of GnRH release. Positive or negative effects at cell bodies are circled. Adapted from original drawing by SPS Ghuman (Dobson et al. (2012); reproduced with permission).

<u>In vivo</u> inter-relationships between neurotransmitters, CRH/AVP and GnRH in ewes

Snapshots of inter-relationships between/within brain and hypothalamic nuclei are obtained by immuno-histochemistry (Figure 7). In the late follicular phase of ewes, noradrenergic terminals are in close contact with many CRH and AVP cell bodies in the PVN but not with beta-endorphin cell bodies in the arcuate nucleus (ARC). Furthermore, GABA terminals

are close to CRH, but not AVP, cell bodies in the PVN, as well as beta-endorphin cells in the ARC (Ghuman et al., 2010, 2011). Although CRH, AVP and beta-endorphin terminals are seen in the mPOA, there are no direct contacts with GnRH cell bodies in this area. Within the median eminence, abundant CRH (but no AVP) terminals are close to GnRH cell terminals in the external zone; whereas, beta-endorphin and dynorphin cell bodies and terminals are in the internal zone (Ghuman et al., 2011; Clarke, 2015). The presence of c-Fos (an early gene activation marker) indicates which cells are currently active: after *in vivo* insulin treatment in ewes, the number of activated noradrenergic neurones in the caudal brainstem increases markedly, along with significant activation of CRH and AVP neurones in the PVN. Despite a general increase in activated neurones in the ARC, the number of activated oestradiol receptor alpha (ER) neurones is reduced by insulin treatment (Ghuman et al., 2011).

All this neuroanatomical evidence supports the hypothesis that brainstem noradrenergic and hypothalamic GABA neurones are important in modulating the activity of CRH and AVP neurones in the PVN, and beta-endorphin neurones in the ARC. These PVN and ARC neurones may also activate inter-neurones to influence GnRH cell bodies in the mPOA, whereas the median eminence is also a major site for direct modulation of GnRH release by CRH terminals (review: Dobson et al., 2003).

Further in vivo studies on regulation of GnRH in intact control ewes

In ewes, when peripheral progesterone concentrations have decreased and oestradiol concentrations reach a threshold value (at least 6–7 h before the expected LH surge onset), complex interactions occur particularly between/within the brain stem and hypothalamus. The hypothalamic mPOA is the main location of GnRH cells and (in response to modulation from the brain stem, ARC, VMN and PVN) the transfer of GnRH proceeds along axons to the median eminence for secretion into portal blood, and onward to the pituitary to release LH (Figure 7).

There are no adrenergic cell bodies in the hypothalamus but axons extending from noradrenergic cells in the brainstem are in close contact with GnRH cell bodies in the ewe mPOA (Clarke et al., 2006). There are also reciprocal links between the ARC and VMN, as well as significant connections to both areas from the PVN (Qi et al., 2008). Regarding neurotransmitters involved in these links, many neurones within the ARC and VMN contain ER, beta-endorphin, dopamine or somatostatin (SST); but existence of any co-localisations in the ewe require more studies (Jansen et al., 1997; Elmquist 2001; Qi et al., 2008; Fergani et al., 2015). Nevertheless, GnRH release is modulated by a specific sequence of interactions between the mPOA, ARC and the VMN. The number of activated ER neurones increases gradually in the mPOA throughout the ewe follicular phase, reaching maximum prior to the surge onset (Figure 8); however, in the ARC, ER activation increases just before the onset of sexual behaviour and remains high throughout the GnRH/LH surge; whereas, in the VMN activation only exceeds base-line during sexual behaviour. Notably, activation of ER cells is maximal during the LH surge in all these areas, substantiating the role of oestradiol-positive feedback in GnRH surge secretion (Fergani et al., 2014b).

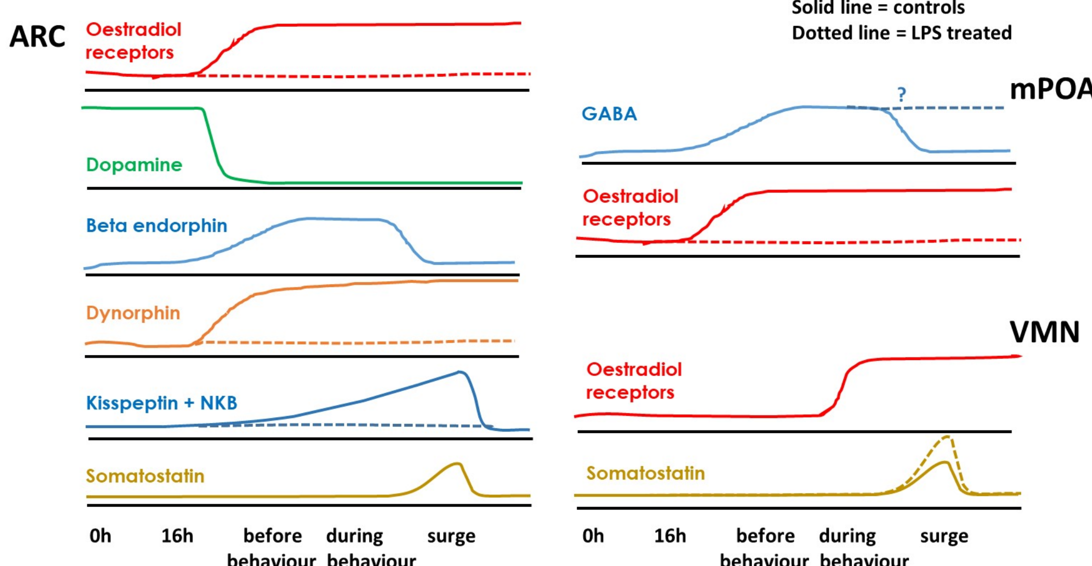


Figure 8. Diagram of neurotransmitter cell influence (i.e., % cells c-Fos activated) in the hypothalamic arcuate nucleus (ARC), medial pro-optic area (mPOA) and ventro-medial nucleus (VMN) at different times during follicular phase of intact ewes with (dotted line) or without treatment with lipopolysaccharide toxin (LPS) at 28 hours; LH surge occurs at 40 hours. No dotted line indicates no difference from controls. Adapted from Fergani et al. (2014b, 2017); and Robinson et al. (1991).

Further cell types within the ARC are also activated at varying times before the GnRH/LH surge in ovary-intact ewes (Figure 8; Fergani et al., 2017). Specifically, activation of dopamine neurones is initially high, but decreases just before behaviour onset, whereas activation of beta-endorphin cells increases in the mid-follicular phase, then decreases a few hours later during the surge.

Also in the ARC, '**KNDy**' cells contain ER and neuropeptides, the latter being both stimulatory (**K**isspeptin, **N**eurokinin B (NKB)) and inhibitory (**Dy**norphin) to keep GnRH secretion tightly regulated. However, the overall balance of different neuropeptides within KNDy cells varies throughout the ewe follicular phase. Initially, there is a shift of the net balance towards inhibitory dynorphin before behaviour starts, followed by a swing towards excitatory kisspeptin and NKB after the influence of increasing oestradiol concentrations during the surge (Fergani et al., 2017). Also, some cells in the ARC and VMN contain SST and these are maximally activated during the LH surge (Figure 8; Fergani et al., 2014b). SST may be involved in GnRH/LH surge termination because it is a very potent inhibitor of electrical excitability of GnRH neurones, but it is also implicated in control of sexual behaviour (see later).

There are claims that the KNDy neuronal network is the true controller of the ewe reproductive system, with the GnRH neurones being the primary output signal from the brain (Scott et al., 2018). This is mainly because KNDy cells constitute the GnRH pulse generator: the signal to stimulate a GnRH pulse is initiated by NKB activity within the KNDy neurone network, while dynorphin stops kisspeptin release from the KNDy neurones thus ending a pulse (Lehman et al., 2010). The majority of KNDy cells are in the ARC and mPOA with a few in the VMN. Approximately 50% KNDy neurones in the mPOA have ER, whereas virtually all kisspeptin cells in the ARC have ER and progesterone receptors. Intriguingly, KNDy cells are inhibited by low doses of progesterone and oestradiol, but stimulated by high doses of oestradiol. KNDy cells also receive synaptic input from neurones that contain glutamate, dopamine, and POMC. Thus, KNDy neurones integrate a lot of information about the internal and external environment of animals, and then act on GnRH cell bodies and dendrites in the ARC and mPOA, as well as the median eminence, to influence the release of GnRH into hypophyseal portal blood (Scott et al., 2018).

Effects of acute LPS treatment on ewe hypothalamic neurotransmitters

Treatment of ewes with LPS increases c-Fos and CRH mRNA within the PVN, increases secretion of CRH into portal blood, and increases CRH-Receptor (CRH-R) activity in the lower part of the ARC and median eminence (Vellucci and Parrott, 1996; Battaglia et al., 1998; Breen and Karsch, 2004; Fergani et al., 2013). At the same time, the LH surge is delayed for as long as the oestradiol signal is disrupted (Fergani et al., 2012). This is accompanied by reduced activation of ER in the mPOA, and ER, dynorphin and kisspeptin cells in the ARC, but possible enhanced SST cell activation in the VMN (Figure 8; Fergani et al., 2013, 2014b, 2017). However, LPS has no effect on the activation of dopamine, β-endorphin or SST cells in the ARC raising the possibility that these cell types are only permissive in the surge induction process in ewes.

Glucocorticoid (cortisol) receptors do not exist on GnRH neurones but are co-localised with progesterone and oestradiol receptors in the mPOA and ARC, possibly in KNDy cells, and it is by this indirect pathway that cortisol signals are transmitted to ewe GnRH neurones (Dufourny and Skinner, 2002; Goodman et al., 2007; review: Lehman et al., 2010; Fergani et al., 2017). There is also an abundance of CRH-Rs in the median eminence (Ghuman et al., 2010). Therefore, there are two possible (simultaneous, therefore fail-safe?) pathways for CRH suppression of GnRH release via CRH-Rs: one being the regulation of kisspeptin/dynorphin and other cell types in the ARC, and the other being the direct association of CRH and GnRH cell terminals in the median eminence.

Effects of acute insulin treatment on ewe hypothalamic neurotransmitters

A CRH antagonist does not prevent the inhibitory effect of insulin on LH pulses in ovariectomised ewes (Clarke et al., 1990). Indeed, contrary to expectation, CRH-R cell activation in the ewe ARC and the median eminence remains unaltered after insulin treatment, and there is immediate increased activation of kisspeptin cells in the ARC but not in the mPOA (Fergani et al., 2014a). This may be a result of insulin activating directly (on kisspeptin neurones) or indirectly (via POMC/beta-endorphin neurone activation). Insulin stimulates SST activation in the ARC of all insulin-treated ewes but this is part of the glucose-sensing mechanism (i.e., stimulus specific), and there is no ER activation in this region. Thus, a reduction in stimulatory kisspeptin cell activation is not part of the GnRH/LH inhibiting mechanism after PVN activation by insulin. However, there is increased SST activation in the VMN along with decreased ER activation in the mPOA: patterns similar to those after LPS indicating a common pathway (Fergani et al., 2015).

Characteristics of oestrus behaviour in cows and ewes

Now that we know more about neurotransmitter and steroid regulation of the HPO, it is worth returning to consider in detail how oestrus behaviour is controlled. Beach (1976) divides ewe pre-copulatory (sexual) behaviour into three components: attractivity, proceptivity and receptivity. Starting with oestrous females being searched for by males (attractivity), the male and female then come near each other, and the male eventually closely noses the female's perineum (proceptivity), transferring both olfactory and gustatory information from the female to the male. A series of courtship behaviours follow including tail fanning (by which the ewe aerially disseminates perineal pheromones), vocalisation, the male resting his chin on the female's back, nudging and pawing of the female (by which the male tests the willingness of the female to be mounted, i.e., in response to male pheromones, the female does not move away). Eventually there is mounting (receptivity), coincident with onset of the LH surge (Fergani et al., 2012). Sight, sound and smell are all important for contacts between oestrous females and males, but smell is imperative to achieve successful mounting in ewes (Fletcher and Lindsay, 1968). The initial sequential build-up of individual but very different behaviours is understandable as females need to be near males for nosing to take place, followed by nudging to test if the female is ready to immobilise, then finally the male mounts the female. However, the reason for the reverse sequential loss of these behaviours, rather than an abrupt cessation

of all behaviours, is less clear. In all female groups, especially cows, a herd-mate will take on the role of male but a similar sequence of behaviours occurs (Walker et al., 2008a)

Effects of chronic or acute stimuli on oestrus behaviour in cows and ewes

Lameness in milking cows does not affect the incidence of oestrus but does reduce oestrus intensity and is associated with lower luteal milk progesterone values prior to oestrus (Walker et al., 2008b). Specifically, the period when herd-mates attempt to mount a lame cow is shorter, and lame cows are mounted less frequently (i.e., are less attractive; Walker et al., 2010). Cows with high SCC or sub-clinical ketosis also have a less intense oestrus with lower oestrus scores (Morris et al., 2013; Rutherford et al., 2016).

In ewes, insulin or LPS treatment in the follicular phase decreases peripheral oestradiol concentrations and delays the LH surge (Saifullizam et al., 2010; Fergani et al., 2012). All behaviours are delayed more or less together as a group, while durations and most frequencies are not affected, indicating that they may all have a common regulating factor, probably oestradiol. However, as they begin and end sequentially, each must also incorporate distinct controlling mechanisms. Furthermore, oestradiol at differing concentrations is able to separate the initiation of behaviour and the preovulatory LH surge (Figure 9; Saifullizam et al., 2010; Fergani et al., 2012).

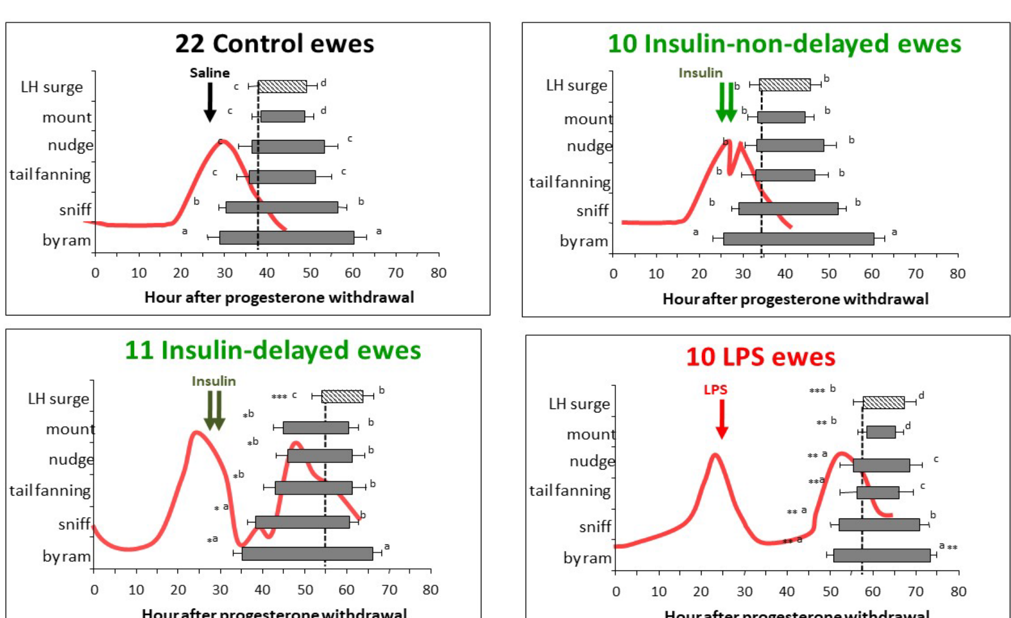


Figure 9. Mean (± SEM) hours from first to last display of different oestrus behaviours after progesterone withdrawal in 22 control ewes, 21 ewes injected with 4 IU/kg insulin (non-delayed n=10, delayed n=11) at 28 and 30 h, and 10 ewes injected with 100 ng/kg LPS at 28 h after progesterone withdrawal. Also shown: mean plasma oestradiol concentrations (red line) and timing of the LH surge (with onset indicated by dashed vertical line). Within each panel, differences between the onsets of each behaviour are indicated by different letters at each end of each bar, respectively ($P<0.05$); differences between the duration of each behaviour are also indicated by the letters at the end of each bar ($P<0.02$). Differences in the timing of onset between panels are indicated with asterisks. Time of treatment is indicated with the arrows. *$P<0.05$ compared to controls and insulin-non-delayed groups, **$P<0.001$ compared to control and both insulin groups, ***$P<0.001$ compared to controls and insulin-non-delayed groups. ****$P<0.05$ compared to controls and insulin subgroups. Adapted from Fergani et al. (2012); reproduced with permission.

In cows and ewes, a period of alternating high/low peripheral progesterone concentrations, followed by oestradiol is the primary ‘trigger’ for the onset of sexual behaviour and the GnRH/LH surge, although the threshold concentration for the induction of sexual behaviour

may be lower than that for the GnRH/LH surge; therefore, the triggering signals may be different in ewes (Caraty et al., 2002; Ben Said et al., 2007). Insulin or LPS treatment of ewes causes a slight increase in progesterone concentrations and this subtle change may be part of the mechanism by which sexual behaviours are disrupted although it is unlikely that the progesterone increment is the sole mediator because the effect is not reversed by the progestin/glucocorticoid receptor antagonist RU486 (Dobson and Smith, 1998; Dobson et al., 2020b).

Role of neurotransmitters in the control of ewe oestrus behaviour

Somatostatin: In the ewe, the ARC and VMN constitute major sites for oestradiol to regulate the induction of sexual behaviour (and the preovulatory GnRH surge; Blache et al., 1991; Caraty et al., 1998). Indeed, there is a positive correlation between activated ER cells in the VMN and peripheral oestradiol concentrations (but not progesterone). Furthermore, there is a distinct temporal pattern of ER cell activation that begins in the ARC and mPOA at least 6–7 h before the onset of ewe sexual behaviour, but only later extends to the VMN during behaviour (Figure 8; Fergani et al., 2014b). Moreover, the ARC and VMN have subpopulations of cells co-localising ER and SST (Scanlan et al., 2003; Herbison, 1995). These SST cells may mediate in the ER control of ewe sexual behaviour (and the preovulatory GnRH surge).

Indeed, intact ewes treated with LPS during the late follicular phase did not express sexual behaviour, and this was accompanied by the failure of ER cell activation in the ARC and changes in SST cell activation in the VMN (Fergani et al., 2014b).

Noradrenaline (NA): Activation of ER neurones in the VMN increases 10-fold during ewe sexual behaviour (Fergani et al., 2014b) co-incident with an increase in noradrenaline (NA) in extracellular fluid of the mediobasal hypothalamus (MBH; containing the ARC and VMN; Fabre-Nys et al., 1997). This NA probably arrives via axons from NA cell bodies in the ewe brain stem where there is co-localisation with ERs (Ghuman et al., 2008a). More detailed investigations to determine whether SST cells receive input from suppressive dopamine and/or excitatory NA cells over this period are awaited to explain the delay in ER cell activation in the VMN.

Dopamine: This neurotransmitter is involved in the control of ewe sexual behaviour (Fabre-Nys and Gelez, 2007). Dopamine neurones in the ARC are maximally activated in the early follicular phase but this decreases markedly just before signs of oestrus begin in the ewe (in a reciprocal pattern to ER activation in the VMN; Fergani et al., 2017). This consistent with initially high extra-cellular concentrations of dopamine in the MBH, followed by a sharp decrease preceding the onset of ewe sexual behaviour (Fabre-Nys and Gelez, 2007).

However, ewes treated with LPS do not exhibit signs of sexual behaviour but dopamine cell activation in the ARC is not affected, indicating that this pathway may be permissive but not indispensable for the initiation of oestrus in ewes (Figure 8; Fergani et al., 2017).

GnRH: Sexual behaviour is enhanced by peripheral or central administration of GnRH in gonadectomised and/or hypophysectomised rodents, eliminating the possibility that GnRH itself mediates behaviour indirectly by increasing levels of gonadal steroids (Schiml and Rissman, 2000). Evidence for exogenous GnRH is not so strong in ewes, but at the end of the follicular phase there is a synchronous surge release of GnRH and LH secretion, with LH returning to baseline after ~10 h, whereas GnRH secretion remains elevated for another 24 h co-incident with the maintenance of receptive behaviour (Figure 10; Caraty et al., 2002). In the ewe, progesterone priming is associated with increases in ER in the ARC/VMN, and greatly increases the magnitude of the GnRH surge; and subsequent sequential actions of oestradiol and GnRH ensure full expression of oestrus behaviour, with extended preovulatory GnRH secretion prolonging receptivity after oestradiol has disappeared from the peripheral circulation. Thus, Caraty et al. (2002) concluded that GnRH has a facilitatory role in the control of sexual behaviour in the ewe.

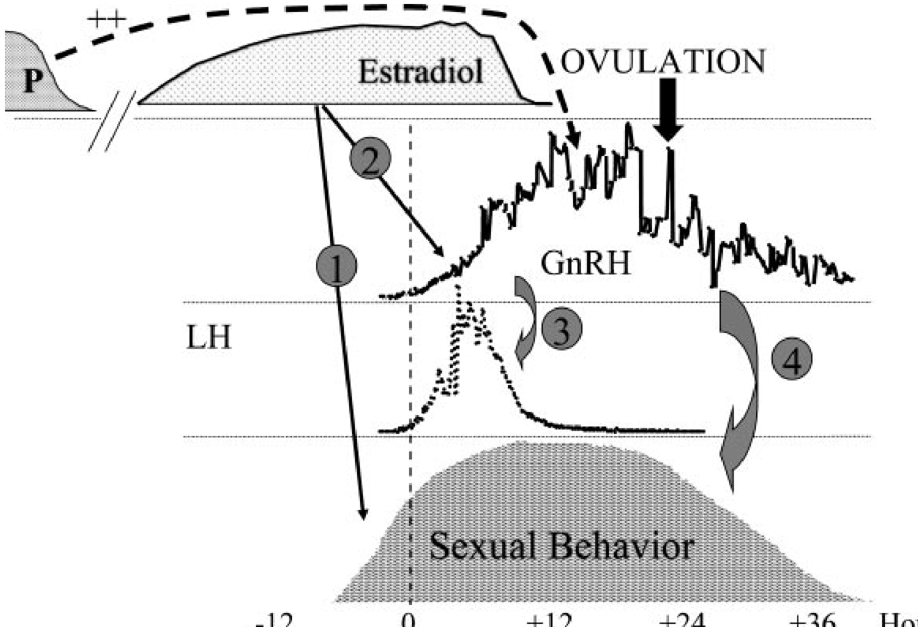


Figure 10. In the ewe at luteolysis, plasma progesterone decreases and oestradiol increases. Oestrus behaviour is initiated (1) when oestradiol reaches a threshold and remains high for 6–10 h. Simultaneously, oestradiol also induces a surge of GnRH in portal blood (2), and LH in the periphery (3). Co-incident with the LH surge, oestradiol declines to basal concentrations while GnRH secretion stays high for an additional 24–36 h, while oestrus behaviour continues (4). One role of progesterone priming (P) is to increase the magnitude of the GnRH surge (broken arrow). Ovulation (black arrow) is precisely timed occurring 22–26 h after the LH surge. Adapted from Caraty et al. (2002); reproduced with permission.

Suppressed intensities of sexual behaviours after LPS are associated with lower oestradiol concentrations in peripheral plasma, partially by directly affecting production in the ovarian follicular granulosa (Price et al., 2013), as well as by decreasing GnRH concentrations in hypophyseal blood (Battaglia et al., 1997; Fergani et al., 2012).

Role of the pheromonal system

Pheromones are used for social communication between animals, male or female. Olfactory substances emitted by an individual induce genetically pre-programmed behavioural and/or physiological responses in recipients (Johnston and Bronson, 1982; Dulac and Wagner, 2006). For example, when vulval skin gland secretions are presented to a bull in the absence of a cow/heifer, positive responses are observed indicating that vulval skin glands may be a specialized site for the production or concentration of pheromones (Rivard and Klemm, 1989). After pheromones have been produced and dispersed, in this other instance by males, the information is received by vomeronasal receptors in the ewe vomeronasal organ (VNO) and then transmitted to the accessory olfactory bulb (AOB) via the vomeronasal nerves, thereby prompting the neuroendocrine system of the recipient ewe to display signs of oestrus (Kratzing, 1971).

However, while there is intense activity of c-Fos and ELOVL5 (a putative pheromone synthetic enzyme; Momozawa et al., 2007) in the vulva of control and LPS treated ewes, a reduction in ram behaviour towards ewes was not accompanied by quantitative changes c-Fos or ELOVL5 in the ewe vulva, but subtle qualitative differences in individual specific compounds (attraction pheromones) remain an option (Dobson et al., 2020a). Furthermore, having been treated with LPS to inhibit sexual behaviour, there were no differences in the ewe vomeronasal organ with respect to cell type or intensity of c-Fos activity, pheromone receptors, or olfactory marker protein, dismissing the vomeronasal organ as a major site involved in the suppression of sexual behaviour (Dobson et al., 2020c). Indeed, along with other hypothalamic nuclei, the bed nucleus of the stria terminalis (BNST) in the ewe is activated just before the expected onset of oestrus and, based on current neuroanatomical data, this activated nucleus is involved in

the transmission of pheromonal signals from the amygdala, as well as transferring information via projections to the mPOA where most GnRH cells are located. However, LPS treatment is not associated with any changes in BNST activation (Fergani et al., 2013), so other parts of the ewe hypothalamus are more likely location(s) of action.

In conclusion

There is still a lot we do not know/understand. The above evidence reviews current information regarding interactions between the environment and fertility via the HPA and HPO, especially focusing on control exerted via KNDy and GnRH cells. However, we still do not know exactly how those cells are governed in any species, particularly co-localisation and/or interdependence of ER, beta-endorphin, dopamine or SST in cells within the mPOA, ARC and VMN. It is also necessary to know more about the expression of oestrus behaviour: is there any direct involvement of GnRH? What is the precise nature of female pheromones, and how/when are they produced? What are the internal mechanisms involved in the 'reward' system that stimulates females to express oestrus?

Returning to the 'random' observations in the opening section outlining examples of trade-offs: Wildebeest migrate large distances to find food and they are very efficient at reproduction – these major trade-offs arise because they are not exploited by humans for meat/milk production. When we domesticate animals for our own use, it is our responsibility to provide the best possible environment (housing, lying/walking areas, etc) otherwise animals trade-off with lower production, less intense oestrus behaviour, and impaired fertility. The incidence of (sub) clinical problems around calving should be minimalised, but why make cows calve so frequently? Lactation persistency is enhanced by increasing milking frequency, feeding more concentrates during declining lactation, and by genetic selection. Pregnancy rates in later lactation are similar to those soon after calving, and later re-breeding of high-yielding cows improves profitability (Dobson et al., 2007). Avoiding life-time peri-parturient problems by managing persistent lactations could be a worthy trade-off on both welfare and economic terms.

Trying to understanding how cows and ewes achieve trade-offs is still on-going. If the environment is not ideal for passing genes on to the next generation, it is essential that animals can temporarily delay conception (often by avoiding expression of oestrus behaviour) until conditions improve. Ultimately, those animals that do develop coping strategies to overcome adverse stimuli, are the ones with the genes that are valuable to achieve trade-offs. From the fore-going discussion, it is clear that survival of the species/individual is so important that the strategy does not depend on a single factor, but is made fail-safe by being multi-factorial, achieved through a complex interplay among excitatory and inhibitory neuronal and hormonal signals that converge on hypothalamic neurones responsible for secretion of GnRH. Understanding the mechanisms of this interplay is of paramount importance for the efficiency of both sexual behaviour and subsequent fertility.

We know that cows and ewes do exert fertility trade-offs, but we need to be sure we are using the right criteria to monitor their impact, e.g., meaningful welfare indices are required, and assessing output per animal/rumen rather than per kg milk/meat may be more appropriate in view of the reverse impact exerted by ruminants upon the environment (green-house gases, waste product disposal, etc.). Domestication needs to be as efficient as possible both economically and environmentally. However, while optimising the inter-relationship between animals and the environment, caution is urged against the camouflage use of drugs/hormones/feed additives/intricate technologies (Higgins et al., 2013). In the long term, a better strategy is to get animals and environment in a more harmonious balance.

Finally, there is one phenomenon that remains totally inexplicable. How/why do some animals display oestrus during pregnancy? In one study, ~ 5% dairy cows displayed oestrus (sniffed and mounted by herd-mates) at least once at varying times throughout pregnancy – some even stood willingly to be mounted and mated by a bull. However,

dried samples of vaginal mucus did not form characteristic fern patterns; and plasma oestradiol concentrations were similar to non-pregnant oestrus herd-mates, whereas plasma progesterone values were all > 1.5 ng/ml when expressing sexual behaviour (Thomas and Dobson, 1989). How/why?

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Author contributions:

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Abbreviations

ACTH: adreno-corticotrophin hormone
AI: artificial insemination
AOB: accessory olfactory bulb
AP: area postrema
ARC: arcuate nucleus
AVP: vasopressin
BCS: body condition score
BHB: 3-beta-hydroxy-butyrate
BNST: bed nucleus of the stria terminalis
CRH: corticotrophin releasing hormone
CRH-R: corticotrophin releasing hormone receptor
DMI: dry matter intake
ELOVL5: Elongation of very long chain fatty acids protein 5
ER: oestradiol receptor alpha
FSH: follicle stimulating hormone
GABA: gamma-amino-butyric acid
GnIH: gonadotrophin inhibiting hormone
GnRH: gonadotrophin releasing hormone
HPA: hypothalamus-pituitary-adrenal axis
HPO: hypothalamus-pituitary-ovarian axis
i.c.v.: Intra-cerebro-ventricular
IU: international units
KNDy: kisspeptin, Neurokinin B and Dynorphin
LC: locus coeruleus
LH: luteinising hormone
LO: left ovary
LPS: lipopolysaccharide toxin from *E coli*
MBH: mediobasal hypothalamus (containing the ARC and VMN)
mPOA: medial pre-optic area
mRNA: messenger ribonucleic acid
NA: noradrenaline
NEFA: non-esterified fatty acid
NKB: neurokinin B
POMC: pro-opiomelanocortin
PRID: progesterone releasing intravaginal device
PVN: paraventricular nucleus
RFM: retained fetal membranes
RFRP-3: RF-amide-related peptide-3
RO: right ovary
SCC: somatic cell count
SEM: standard error of the mean
SST: somatostatin
STBM: standing-to-be-mounted
VNO: vomeronasal organ

Commercial Embryo Transfer Activity

in Europe 2021

Collated by Helene Quinton

National data collectors

Country	Collector	Comment
Austria	Friedrich FÜHRER ; Eva DACHSBERG	
Belarus	Victor MADISON ; Alexander DESHKO	
Belgium (Wallonia)	Isabelle DONNAY	
Belgium (Flanders)	Eric MULLART	
Bosnia Herzegovina		
Croatia		
Denmark	Henrik CALLESEN	
Estonia	Ants KAVAK	
Finland	Marja MIKKOLA	
France	Serge LACAZE	
Germany	Hubert CRAMER	
Great Britain	Peter MAY ; Samantha MARTIN	Data available in september
Greece	Foteini SAMARTZI	
Hungary	Szabolcs SIMAI	No data available this year
Ireland		
Israel	Yoel ZERON	No activity this year
Italy	Giovanna LAZZARI	
Latvia	Ilga SEMATOVICA	
Lithuania	Rasa NAINIENE	
Macedonia		
Norway	Tjerand LUNDE	
Poland	Jedrzej JASKOWSKI	
Portugal	Joao Nestor CHAGAS E SILVA	
Romania	Stefan CIORNEI	
Russian Federation	Victor MADISON ; Denis KNUROW	
Serbia	Aleksandar MILOVANOVIC	
Slovakia	Dalibor POLAK	
Slovenia	Aleksandar PLAVSIC ; Janko MRKUN	No activity this year
Spain	Daniel MARTINEZ BELLO	
Sweden	Renee BÅGE	
Switzerland	Sarah WYCK	
The Netherlands	Anna BEKER VAN WOUNDENBERG; Erik MULLART	
Turkey		
Ukraine	Victor MADISON	No data available this year

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	531	2%	5 316	3 733	6	0%	30	20	537	3 753	7,0
Belarus	295	34%	1681	1097	0		0	0	295	1 097	3,7
Belgium	660	0%	5183	3 425	8 711	0%	54 535	43282	9371	46 707	5,0
Denmark	842	0%	7 808	5 489	27	0%	363	277	869	5 766	6,6
Estonia	0		0	0	12	0%	64	64	12	64	5,3
Finland	463	3%	3 893	2 637	0		0	0	463	2 637	5,7
France	4884	27%	46298	27595	1 463	7%	16 213	9463	6347	37058	5,8
Germany	4045	0%	36 805	25 721	535	0%	5 451	3494	4580	29 215	6,4
Greece	4	100%	0	17	0		0	0	4	17	4,3
Italy	2 250	51%	23 171	15 980	120	0%	1 224	816	2 370	16 796	7,1
Latvia	4	50%	31	18	0		0	0	4	18	4,5
Lithuania	4	0%	6	3	0		0	0	4	3	0,8
Netherlands	2 627	1%	21 823	14 543	0		0	0	2 627	14 543	5,5
Norway	274	1%	1 900	1 850	20	0%	150	98	294	1 948	6,6
Poland	249	44%	2 050	1705	17	0%	200	111	266	1816	6,8
Portugal	78	97%	626	352	22	0%	289	117	100	469	4,7
Romania	17	24%	55	49	0		0	0	17	49	2,9
Russian Federation	316	63%	2647	1 557	572	0%	5 225	4305	888	5862	6,6
Serbia	0		0	0	0		0	0	0	0	
Slovenia	28	25%	135	94	11	36%	40	31	39	125	3,2
Spain	233	63%	2068	1 042	180	9%	2 585	898	413	1940	4,7
Sweden	145	3%	1 349	858	7	0%	0	0	152	858	5,6
Switzerland	425	70%	5 615	3 159	27	0	224	162	452	3 321	7,3
Total	**18 374**	**19%**	**168 460**	**110 924**	**11 730**	**1%**	**86 593**	**63 138**	**30 104**	**174 062**	**5,8**

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
Belarus	83	2%	1 653	481					83	481	5,8
Finland	700	0%	6 791	976					700	976	1,4
France	1 203	1%	12 600	4 076	122	0%	1 756	766	1 325	4 842	3,7
Germany	2 189	0%	26 740	7 235	150	0%	2 425	590	2 339	7 825	3,3
Italy	111	7%	1 214	328					111	328	3,0
Netherlands	5 694	0%	56 981	13 059					5 694	13 059	2,3
Norway	13	0%	174	24					13	24	1,8
Poland	146	6%	2 190	543					146	543	3,7
Romania	10	0%	20	0					10	0	0,0
Russian Federation	13	0%	7	5					13	5	0,4
Serbia	20	6%	256	65					20	65	3,3
Spain	101	4%	1 754	616	16	0%	243	77	117	693	5,9
Switzerland	443	2%	3 936	1 378	40	1%	478	178	483	1 556	3,2
Total	**10 726**	**1%**	**114 316**	**28 786**	**328**	**3%**	**4 902**	**1 611**	**11 054**	**30 397**	**2,7**

Declared bovine *In vitro* embryo production – abattoir

	Dairy			Beef		
Country	Donors	Oocytes	Embryos	Donors	Oocytes	Embryos
Greece	116	1 442	225	0	0	0
Netherlands	1 975	28 143	3 307	0	0	0
Slovenia	134	934	42	65	345	23
Spain	0	0	0	520	3 851	1 720
Total	**2 225**	**30 519**	**3 574**	**585**	**4 196**	**1 743**

Declared bovine embryo technologies – embryo genotyping

	Sexed embryos		Genotyped embryos	
Country	In Vivo	In Vitro	In Vivo	In Vitro
France	1 387	8	1 170	8
Germany	0	533	0	1
Netherlands	0	0	0	5 610
Spain	0	12	0	5
Total	**1 387**	**553**	**1 170**	**5 624**

Declared bovine embryo transfers and exports– *In vivo*

	Dairy				Beef				Total embryos transferred
	Fresh embryos transferred	Frozen embryos			Fresh embryos transferred	Frozen embryos			
Country		Domestic transferred	Foreign transferred	Exported		Domestic transferred	Foreign transferred	Exported	
Austria	1 451	1 863	24	0	6	6	12	0	3 362
Belarus	791	390	0	0	0	0	87	0	1 268
Belgium	675	2 361	364	16	8 156	34 887	264	36	46 707
Denmark	3 155	1 566	0	33	41	139	0	3	4 901
Estonia	0	0	0	0	0	0	7	10	7
Finland	773	1 359	95	543	13	10	53	0	2 303
France	13 704	11 068	1 113	176	2 924	3 911	525	369	33 245
Germany	11 476	14 349	0	184	896	1 930	0	0	28 651
Italy	6 580	1 860	0	0	120	0	0	0	8 560
Latvia	7	7	4	0	0	0	0	0	18
Netherlands	3 055	9 301	0	0	0	0	0	0	12 356
Norway	20	1 737	50	0	20	98	30	20	1 955
Poland	1 029	357	55	0	43	0	19	0	1 503
Portugal	110	146	4	0	38	31	24	0	353
Romania	46	3	0	0	0	0	6	0	55
Russian Federation	113	1 434	259	0	0	4 501	38	0	6 345
Serbia	0	0	0	0	0	0	72	0	72
Slovenia	30	55	9	0	1	13	0	0	108
Spain	475	463	103	55	140	984	201	480	2 366
Sweden	648	1 093	0	0	0	0	0	0	1 741
Switzerland	773	2 650	450	81	34	25	0	0	3 932
Grand Total	**44 911**	**52 062**	**2 530**	**1 088**	**12 432**	**46 535**	**1 338**	**918**	**159 808**

Declared bovine embryo transfers and exports – *In vitro*

	OPU				Abattoir		
Country	Fresh embryos transferred	Domestic frozen embryos transferred	Foreign frozen embryos transferred	Embryos exported	Fresh embryos transferred	Domestic frozen embryos transferred	Total embryos transferred
Belarus	211	34	0	0	4	9	258
Belgium	0	24	0	0	0	0	1 035
Finland	0	765	0	273	0	0	1 909
France	1 967	1 575	151	53	0	0	8 330
Germany	2 658	4 265	0	0	0	0	368
Netherlands	4 462	3 477	0	0	0	0	7 268
Norway	0	110	30	0	0	0	198
Poland	112	145	0	0	0	0	39
Portugal	0	0	44	0	0	0	
Russian Federation	0	24	0	0	0	0	
Serbia	0	7	0	0	0	0	
Spain	183	244	0	0	7	194	23
Switzerland	0	0	0	909	0	0	517
Grand total	**9 593**	**10 670**	**225**	**1 235**	**11**	**203**	**19 945**

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

Species	Country	Embryo collection		Embryo transfer			
		Collections	Viable embryos	Fresh embryos	Frozen domestic	Frozen foreign	Exported embryos
Sheep	Greece	3	13	0	0	0	0
	Romania	2	27	27	0	0	0
	Russian Federation	0	0	0	0	1 878	0
	Serbia	4	32	0	6	0	0
	Spain	33	320	0	60	0	80
	Total	**42**	**392**	**27**	**66**	**1 878**	**80**
Goat	France	0	0	0	3	0	0
	Spain	48	648	0	200	0	200
	Total	**48**	**648**	**0**	**203**	**0**	**200**
Horse	Estonia	10	8	8	0	0	0
	France	1 156	624	846	0	0	0
	Poland	0	0	54	0	0	0
	Portugal	216	124	0	0	0	0
	Russian Federation	16	12	11	0	0	0
	Spain	4	4	0	0	0	0
	Sweden	33	0	0	0	0	0
	Total	**1 435**	**772**	**919**	**0**	**0**	**0**
Rabbit	Romania	37	148	148	0	0	0

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

Species	Country	Oocyte collection				IVP embryo transfer			Exported embryos
		OPU conv	OPU sexed	Oocytes	Embryos	Fresh embryos	Frozen domestic	Frozen foreign	
Sheep	Spain	15	3	384	180	0	0	0	0
Goat	Spain	20	5	504	268	0	0	0	0
Horse	Estonia	0	0	0	0	0	0	7	0
	France	0	0	0	0	0	45	0	0
	Italy	3 440	0	45 836	6 764	7	727	0	3 498
	Poland	0	0	0	0	0	0	34	0
	Portugal	6	0	45	0	0	0	0	0
	Sweden	0	0	0	0	23	0	0	0
	Switzerland	41	0	116	11	0	0	0	3
	Total	**3 487**	**0**	**45 997**	**6 775**	**30**	**772**	**41**	**3 501**

INVITED LECTURES

Thematic Section: 36th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Erasing gametes to write blastocysts: metabolism as the new player in epigenetic reprogramming

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Abstract

Understanding preimplantation embryonic development is crucial for the improvement of assisted reproductive technologies and animal production. To achieve this goal, it is important to consider that gametes and embryos are highly susceptible to environmental changes. Beyond the metabolic adaptation, the dynamic status imposed during follicular growth and early embryogenesis may create marks that will guide the molecular regulation during prenatal development, and consequently impact the offspring phenotype. In this context, metaboloepigenetics has gained attention, as it investigates the crosstalk between metabolism and molecular control, i.e., how substrates generated by metabolic pathways may also act as players of epigenetic modifications. In this review, we present the main metabolic and epigenetic events of pre-implantation development, and how these systems connect to open possibilities for targeted manipulation of reproductive technologies and animal production systems.

Keywords: embryo, metabolism, epigenetic, metaboloepigenetic.

Introduction

The pre-implantation embryo must drive a set of organized events since the earliest stages of development to ensure the generation of totipotent and, subsequently, pluripotent blastomeres that will establish initial cellular lineages. These events include morphophysiological, metabolic and molecular regulation that will lead to pro-nuclei formation, activation of the embryonic genome, cell differentiation, morulae compaction and blastocoel formation. The decisions for each of those events are taken in a dynamic environment and lead to broad-spectrum consequences to embryo metabolism, molecular control, epigenetic reprogramming and developmental capacity. With the growing knowledge of the embryonic response to the environment in several cellular aspects (including *in vivo* and *in vitro* models), the biggest challenge is to unravel the delicate relationship that allows the embryos to modulate the molecular machinery using metabolic tools. In this sense, the term metaboloepigenetics (Donohoe and Bultman, 2012) that defines the relationship between energy metabolism and epigenetic and molecular control, has gained space in studies based on stem cells and embryonic development.

Epigenetics is defined as heritable modifications in nucleic acids and associated proteins that do not involve changes in DNA sequence but might impact the modulation of gene

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expression. It means that in addition to the primary DNA sequence, much of the information on when and where to start transcription is stored in the form of covalent modifications of DNA, RNAs and chromatin associated proteins. More than 100 covalent modifications have already been identified (reviewed by Mach, 2018 and Michalak et al., 2019). Among them, the methylation and hydroxymethylation of cytosine in DNA, and the acetylation, phosphorylation, SUMOylation or ubiquitination of histones residues [specially lysine (K) and / or arginine (R)] are the most studied, precisely because they are related to the accessibility of the genome to the transcriptional machinery. Epigenetic mechanisms are also controlled by non-coding RNA molecules which are not translated into proteins but exert a significant role in the control of gene expression. These include short-chain nc-RNAs (siRNA, miRNA and piRNAs) as well as long non-coding RNAs (lnc-RNAs) (reviewed by Riddle, 2014).

The concept that the metabolism of a cell is integrated in the regulation of epigenetics and transcription is reinforced by the ability of cells to adapt their metabolic and molecular status in response to extracellular environment and nutrient availability (reviewed by Vander Heiden et al., 2009). Since the metabolites are substrates used to generate chromatin modifications, there is an intriguing but rather complex mechanism that connects energy metabolism and epigenetics. Several enzymes have already been characterized as responsible for inserting or removing epigenetic modifications. The activity of these enzymes is regulated, at least in part, by the presence and quantity of energy substrates (Lu and Thompson, 2012).

In this review, we present a brief statement of changes in mammalian pre-implantation metabolism highlighting how the embryos take stage-specific decisions and how these are critical to successfully initiate the developmental program. We also describe how epigenetic reprogramming can act as controllers of development and cellular fate. We follow presenting the communication between these two cellular events, the metaboloepigenetics. At the end, we conclude with the current limitations encountered in the *in vitro* production system, its consequences to the offspring, and future directions to improve embryo quality and viability.

The metabolism of the pre-implantation embryo – all in good time

Mammalian embryonic cells present a very characteristic metabolism, slow during the first cleavages, followed by an acceleration to support intense cell proliferation and differentiation over the next stages (Leese et al., 2007). During the first cell divisions, gene transcription is diminished, and the initial metabolism is mainly sustained by transcripts and proteins that were produced and stored during oocyte maturation in several species (Zhang and Smith, 2015).

This quiescence phase is followed by the major activation of the embryonic genome that, in the bovine embryo, occurs between 8 and 16 cells stage and marks a turning point for the developing embryo (Graf et al., 2014). The initiation of transcription requires a lot from the cellular machinery that is responsible for controlling the compaction of the morulae and the development of the blastocyst (Hamatani et al., 2006).

The success of embryo development is dictated by its ability to activate specific energy production pathways (Figure 1). Differential concentrations of glucose, pyruvate, lactate and amino acids are observed in the fluids of bovine oviduct and uterus, reinforcing the idea that pre-implantation embryos need different energy substrates according to their stage of development (Hugentobler et al., 2007; Hugentobler et al., 2008). Considering that, the supplementation of culture media with these fluids has been proposed to improve bovine embryo metabolism and viability (Lopera et al., 2015; Hamdi et al., 2018).

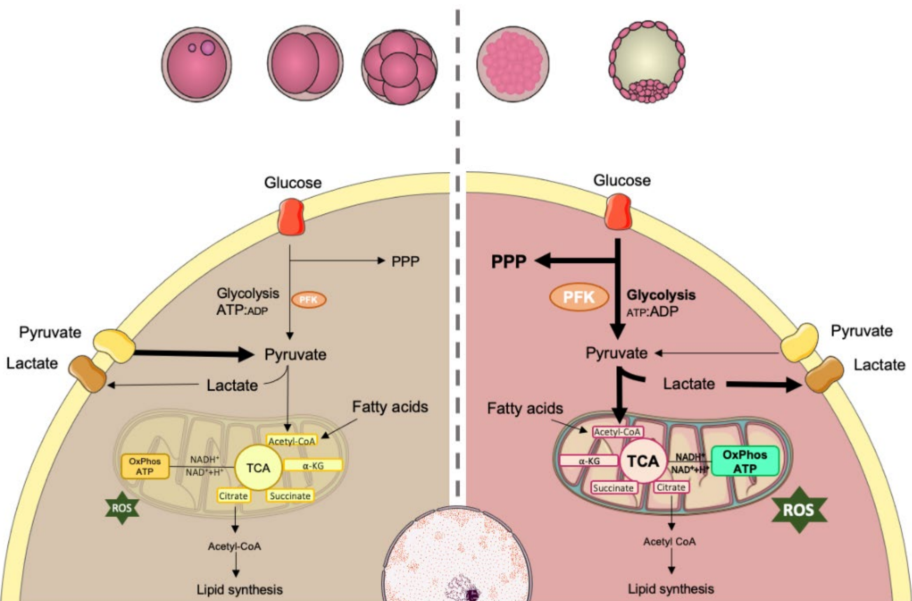

Figure 1. Embryo development requires the activation of specific pathways to produce energy. Prior to compactation, embryo metabolism is mainly supported by pyruvate and amino acids, metabolized through tricarboxylic acid (TCA) cycle and oxidative phosphorylation. At the time of compactation, embryo increases its energetic demand especially for increasing biosynthesis and cell proliferation, but also for the formation and expansion of blastocoel, and hatching. At this time, glucose is metabolized with greater efficiency by two main pathways: the pentose phosphate pathway (PPP) (important for biomass and nucleotide generation) and the glycolytic pathway (that increases ATP production and pyruvate synthesis). At the end of the glycolytic pathway, pyruvate can also be converted to lactate, even in the presence of oxygen. This process is called "aerobic glycolysis" or "Warburg effect". PFK-phosphofructokinase; ATP-adenosine triphosphate; ADP-adenosine diphosphate; ROS-reactive oxygen species. Adapted from SMART Servier Medical Art image bank (SMART, 2020).

During the first cleavages, bovine embryos, as other mammalian embryos, use pyruvate as the main substrate for energy generation as they have a limited ability to metabolize glucose for this purpose (Guerif et al., 2013). The high ATP: ADP ratio allosterically inhibits the enzyme phosphofructokinase (PKF), the key enzyme in glycolysis, decreasing its affinity to fructose-6-phosphate and limiting the glycolytic pathway, as described in mouse embryos (Barbehenn et al., 1974). At this point, oxidative metabolism and oxygen consumption are also low, probably as a consequence of the quiescent state of the bovine oocyte, reinforcing the importance of oocyte quality and the follicular environment to ensure the proper embryo development (Alves et al., 2019). Amino acids such as glutamine and aspartate are also used for energy generation at this stage via the malate-aspartate transport pathway (MAS) (Lane and Gardner, 2005).

At the time of major embryonic genome activation, the embryo requires more energy to increase biosynthesis and cell proliferation, and also to support the formation and expansion of the blastocoel as well as embryo hatching. This higher energy demand modifies the ATP:ADP ratio, allowing glucose to be metabolized more efficiently, as reported for human and bovine embryos (Devreker, 2007; Guerif et al., 2013). After internalization to the cytoplasm, glucose can follow two main pathways: the pentose phosphate pathway (PPP) or the glycolytic pathway.

In the PPP, ribose chains are generated and later used in the synthesis of DNA and RNA. In addition, the restitution of NADPH from NAD^+ is required for the reduction of intracellular glutathione, an important antioxidant for the embryos (Wales and Du, 1993; Stincone et al., 2015). Glutathione reduces the levels of intracellular reactive oxygen species, that are generated as a byproduct of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Burton et al., 2003). In this context, directing glucose to the PPP can be beneficial for the

embryo, as it will inhibit the overflow of substrates into the TCA cycle, thus creating a more suitable redox state for the cells (Harvey et al., 2002).

As previously mentioned, the ATP:ADP ratio is a limiting factor in the glycolytic pathway. Therefore, a reduction in this ratio leads to the activation of PKF and consequently to an increase in the levels of aerobic glycolysis. The pyruvate that is generated through this process is transported to the mitochondria where it is converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDC), thereby connecting glycolysis, which occurs in the cytoplasm, to the tricarboxylic acid cycle (TCA) that occurs inside of the mitochondria. The first reaction in the TCA cycle consists of the transfer of an acetyl group from acetyl-CoA to oxaloacetate, originating the six-carbon compound citrate, which is converted to isocitrate. After oxidation and decarboxylation, isocitrate releases CO_2 and forms α-ketoglutarate, which also loses CO_2 and forms succinyl-CoA. The coenzyme is then released producing succinate, which is oxidized to fumarate, which in turn undergoes hydration to form malate. Malate is oxidized to form oxaloacetate and subsequently citrate (Berg et al., 2002). Citrate can also leave mitochondria and be converted to acetyl-CoA in the cytoplasm by ATP citrate lyase. This enzyme is key to the connection of carbohydrate metabolism and lipid metabolism, since the latter requires acetyl-CoA for synthesis (Zaidi et al., 2012).

In the TCA cycle, one molecule of acetyl-CoA is oxidized releasing two molecules of CO_2, three molecules of NADH, one of FADH2, producing one ATP. NADH and FAD^+ are metabolic coenzymes that play a critical role in the generation of ATP through oxidative phosphorylation. Within mitochondria, oxidation occurs from NADH to NAD^+ and FADH2 to FAD^+ in complexes I and II of the electron transport chain, which leads to the donation of electrons to molecular oxygen. The redox ratio (FAD^+ / NADH) can be a measure of the cells redox state and has been used *in vitro* and *in vivo* to track metabolic changes during cell differentiation and malignant transformation (Yanes et al., 2010). Changes in a cells redox state can be interpreted as a relative change in the rate of glucose catabolism to oxidative phosphorylation as well.

At the end of the glycolytic pathway, the enzyme lactate dehydrogenase (LDH) may also promote the conversion of pyruvate into lactate, even in the presence of oxygen. This process is called "aerobic glycolysis" or "Warburg effect" and is mainly observed in highly proliferating cells, such as tumoral cells, which to a certain level, are metabolically similar to embryonic cells (Warburg et al., 1927 and reviewed by Krisher and Prather, 2012). Lactate produced by the blastocyst through this pathway may play an important role in key events related to the implantation process, facilitating invasion, proliferation, angiogenesis and modulation of the immune response at the site of implantation (proposed by Gardner, 2015).

Lipids are another important substrate for energy production in mammalian oocytes and embryos. Despite the fact that after fertilization the lipid density is barely altered, intracellular lipids can be considered as a potential and more economical source of energy through ß-oxidation, altering mitochondrial activity and ATP production (Sturmey et al., 2009; Dunning et al., 2010; Dunning et al., 2014).

With so many different cards to play, embryos are known for their so-called plasticity, i.e., their high capacity to adapt when facing environmental stress and changes in the availability of substrates. But not surprisingly, this adaptation has a cost and often modifications in the culture system can compromise metabolic and molecular processes, leading to altered viability.

Metabolism is certainly an indicator of viability; however, many questions remain unanswered, such as: what are the impacts of metabolic changes on the molecular and epigenetic control of cells? Are these impacts reversible?

Epigenetic reprogramming in pre-implantation embryos – holding the cards

Each event during initial embryo development requires changes in metabolism and molecular control and it includes reprogramming of epigenetic settings. This reprogramming occurs to ensure that gametes (highly repressive marks) generate totipotent blastomeres (highly permissive marks) and, subsequently, pluripotent blastomeres of internal cell mass and

trophectoderm (more specific marks). The molecular basis for this modification is not yet fully understood but the main events are described below (Figure 2).

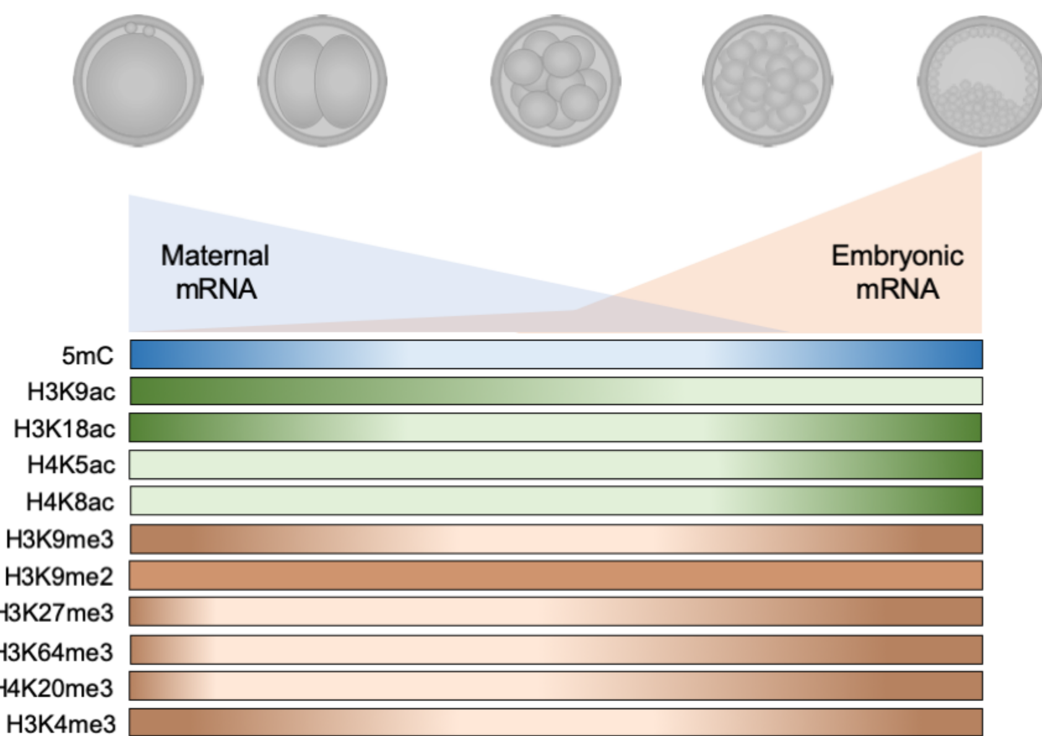

Figure 2. During the pre-implantation development of bovine embryos, the paternal and maternal genome are reprogrammed after fertilization. During this period, the maternal stock of mRNAs is consumed until the embryo is capable of producing its set of transcripts, a critical step known as the major embryonic genome activation. Up to this point, most of the epigenetic marks that were present during the first cleavages were substantially erased (5mC, H3K9ac, H3K18ac, H3K9me3, H3K27me3, H3K64me3, H4K20me3 and H3K4me3). After the major embryonic genome activation all epigenetic marks described for bovine embryos increase, even H4K5ac and H4K8ac that were observed in lower levels since zygote stage. The only exception is H3K9me2 that presents a unique pattern during throughout development. This reprogramming is how the developing embryo 'writes' its own profile of epigenetic marks.

DNA methylation and demethylation

DNA methylation is a chemical alteration promoted by the addition of a methyl group (CH_3) to the cytosines of DNA molecules (Wu and Zhang, 2014), leading to the formation of 5-methylcytosines (5mC). This modification occurs more frequently in CpG dinucleotides in genomic areas, also known as CpG islands (regions longer than 200 base pairs containing more than 50% CpG dinucleotides), that are located mainly in gene promoter regions (Ioshikhes and Zhang, 2000). In general, DNA methylations are associated with transcription repression, genomic imprinting and post-translational histone modifications (Ioshikhes and Zhang, 2000; Fuks, 2005). In genomic imprinting, one of the gene alleles is silenced by the presence of 5mC, depending on the origin of this allele (maternal or paternal imprinting may be observed, depending on the gene and species) (Barlow and Bartolomei, 2014).

The presence of a methyl group can strongly suppress gene transcription by steric hindrance, inhibiting transcription factors binding and increasing the affinity of Methyl Binding Proteins (MBP) to these gene regions (Lazarovici et al., 2013; Dantas Machado et al., 2015). The MBP are proteins that act to make the access of transcription factors more difficult and can, additionally, lead to the activation of other epigenetic mechanisms, such as histone methylation and deacetylation (Cheng, 2014). However, the effectiveness of DNA methylation in blocking the transcription also depends on the amount, region and size of the region where these methylations are present (Messerschmidt et al., 2014). Generally, high methylation level in promoter regions, especially those with intermediate or high density of CpG dinucleotides,

is associated with transcription repression and, therefore, gene silencing (Dupont et al., 2009; González-Recio, 2012; Messerschmidt et al., 2014). On the other hand, promoter regions with low density of CpGs remain transcriptionally active even when hypermethylated (Kulis et al., 2013; Messerschmidt et al., 2014).

The enzymes that catalyze the transfer of a methyl group (CH_3) from S-adenosylmethionine (SAM) to DNA are called DNA methyltransferases (DNMTs). The DNMTs are responsible for maintaining methylations during DNA replication (DNMT1); inserting methylations into DNA regions without the presence of prior 5mC (DNMT3a and DNMT3b); and regulating DNMT3a or DNMT3b activity (DNMT3l) (Neri et al., 2013; Wu and Zhang, 2014; Messerschmidt et al., 2014; Hervouet et al., 2018). The addition of methylations in DNA without the presence of previous marks, promoted by DNMT3A and DNMT3B, is driven by the interaction between them and some transcription factors that guide methyl groups to specific sites of the genome (Pacaud et al., 2014).

The removal of DNA methyl group, or demethylation, may occur passively or actively and allows epigenetic marks to be erased during development or in response to environmental factors (Messerschmidt et al., 2014; Urrego et al., 2014). Passive demethylation occurs when DNMTs are absent or reduced during DNA replication, which results in the synthesis of a new strand of DNA without methylation and, ultimately, leading to dilution of 5mC (Messerschmidt et al., 2014; Urrego et al., 2014). The active demethylation is initially promoted by enzymes capable to oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hC), which can be converted to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). These enzymes are known as Ten-eleven-translocation (TET 1-3). The 5fC or 5caC, products of TET activity, are targets of DNA thymidine glycosylase (TDG) and can be processed by the base excision repair (BER) mechanism. Another mechanism responsible for the active DNA demethylation is started by the activation-induced deamination (AID) or apolipoprotein B mRNA catalytic polypeptide (APOBEC1), which converts 5mC and 5hmC into thymidine and 5-hydroxymethyluracil, respectively, leading to the activation of BER. BER mechanism results in the addiction of an unmethylated cytosine to the specific site where the 5mC was present before the beginning of the demethylation process (Delatte et al., 2014; Messerschmidt et al., 2014).

The DNA methylation profile in somatic cells is mostly stable and hereditary during replication. Nevertheless, during gametogenesis and initial embryonic development, the genome of germ cells is epigenetically reprogrammed. This reprogramming consists in erasing 5mC by demethylation processes (either passive or active) and add 'de novo' methylations (addition of 5mC in new and specific regions of the genome) among other epigenetic modifications such as histone post-translational alterations. During embryonic development, this reprogramming is initially driven by the stock of maternal DNMTs and TETs present in the oocyte and later, by the expression of specific enzymes after the major embryonic genome activation (Messerschmidt et al., 2014; Atlasi and Stunnenberg, 2017).

DNA methylation/demethylation in bovine embryos

Mammalian gametes have two different waves of reprogramming, the first one occurring during gametogenesis and the second one during the first days of embryonic development (Haaf, 2006; Seisenberger et al., 2012; Saadeh and Schulz, 2014). The reprogramming cycle of primordial germ cells is barely described in bovine, however, in mouse, it is known that the genome of primordial germ cells is demethylated and a new profile is established with specific patterns for male or female gametes (Seisenberger et al., 2012; Saadeh and Schulz, 2014). The resulting sperm cells and oocytes have higher levels of DNA methylations than somatic cells and, for bovines, male gametes present even greater number of 5mC than the female ones (Zhang et al., 2016; Duan et al., 2019).

After fertilization of gametes, a second cycle of demethylation begins and the highly methylated paternal and maternal genomes are actively and passively demethylated, except for some genes and regions (approximately 100-200 genes and some retrotransposons) (Zhang et al., 2016). Demethylation at this point is mandatory, since the highly methylated

genome from gametes must be erased in order to produce totipotent blastomeres, capable of being responsive to specific marks for cell differentiation. The embryonic genome is actively demethylated during the first cleavages through the enzymatic activity of the TETs, and passively demethylated due to the low presence of DNMT1 enzyme (Messerschmidt et al., 2014; Urrego et al., 2014). In the absence of DNMT1, the DNMT3a and DNMT3b enzymes act to guarantee the maintenance of some DNA methylations, preserving the imprinting of some genes (Okano et al., 1999). This demethylation process occurs until a large number of 5mC were removed and the embryos reaches its lowest level of DNA methylation. After that, 'de novo' methylation begins to result in the formation of a blastocyst with its own particular profile of DNA methylation e.g. the DNA methylation profiles stabilized for POU5F1, SOX2, NANOG and CDX2 (Urrego et al., 2014; Zhang et al., 2016).

In bovine, the timing of the 'de novo' methylation is not consensus. Some reports demonstrated that the 'de novo' methylation starts after the embryo reaches the 8-16 cells stage, while others verified a later activation in the remethylation process (Dean et al., 2001; Hou et al., 2007; Dobbs et al., 2013; Zhang et al., 2016). This variation can either be species-specific or a consequence of non-optimized culture conditions. For bovines, we even have variations in DNA methylation profiles due to different sub-species or crossbreed animals used as model (Dobbs et al., 2013; Salilew-Wondim et al., 2015; Urrego et al., 2017), although there is no report in literature directly comparing its variations between *Bos taurus* versus *Bos indicus*. However, it is important to highlight that, even with variations, the process of demethylation followed by remethylation was observed in all studies. Furthermore, 'de novo' DNA methylation seems to occur differently between blastomeres, and there are also controversial reports whether the cells with higher levels of DNA methylation are in the inner cell mass or in the trophectoderm (Dean et al., 2001; Hou et al., 2007; Dobbs et al., 2013).

These variations in DNA methylation levels are necessary during embryonic development and can be affected by environmental factors. The use of assisted reproductive techniques, such as superovulation protocols, *in vitro* production (IVP) of embryos and cryopreservation, may affect the DNA methylation pattern in gametes, embryos and offspring. In cattle, the use of assisted reproductive techniques can result in epigenetic reprogramming failures and incapacity to maintain parental imprinting, resulting in problematic phenotypes such as the Large Offspring Syndrome. During bovine embryonic development *in vitro*, some genes that must remain imprinted in one of the alleles are abnormally demethylated (Chen et al., 2017). The longer the *in vitro* culture period of these embryos is, higher is the degree of DNA methylation deregulation in promoters and other gene regions. In addition, if blastocysts are produced using entirely *in vitro* process, they present increased levels of DNA methylation than their *in vivo* counterparts, particularly in gene and promoter regions (Salilew-Wondim et al., 2015).

Mitochondrial DNA methylation/demethylation

Intense investigation of epigenetic mechanisms is still shaping our understanding on the dynamics that regulates nuclear genome. Direct or indirectly, mitochondria activity has been implicated in these events because it controls numerous epigenetic enzymes. However, the existence of an autonomous mitochondrial epigenetic regulation has been the subject of a long debate over the years with controversial reports stating the presence or absence of mtDNA methylation (Patil et al., 2019). With the improvement of detection techniques, recent studies proposed that similarly to nuclear DNA, mtDNA is also subjected to epigenetic modifications that can influence mitochondrial biogenesis, gene expression and function (Zhang et al., 2019a). A recent study demonstrated non-random patterns of mtDNA methylation, predominantly in a non-CpG context when comparing normal versus liver and breast cancer cells (Patil et al., 2019). In mice, mtDNA methylation profiles also show dynamic local and global changes during development and aging (Dou et al., 2019).

In the bovine model, previous data demonstrated that mtDNA methylation shows particular signatures between oocytes obtained from distinct follicular environments (OPU vs. abattoir ovaries); those signatures are also reflected in the blastocysts produced with these oocytes

(Sirard, 2019). Interestingly, the mtDNA methylation negatively correlates with mitochondrial gene expression profile, which may have important consequences to mitochondrial function during preimplantation development. Finally, in the porcine model, mtDNA sequences, including the D- loop control region, have been found to be hypermethylated in oocytes obtained from gilts with polycystic ovaries, contributing substantially to mitochondrial malfunction and decreased oocyte quality (Jia et al., 2016).

Recently, an isoform of DNMT1 was identified to target mitochondria and methylate mtDNA influencing mitochondrial activity (Saini et al., 2017). More specifically, there is evidence showing an upregulation of DNMT1 combined with hypermethylation of mtDNA and altered gene expression in porcine oocytes. However, more evidences are necessary to confirm such connection and to understand the full potential of this type of epigenetic control in the phenotype of the cells.

Post-translational histone modifications

Histones, composed of a globular C-terminal domain and a flexible N-terminal tail, are the basic components of the nucleosomes, which in turn constitute the chromatin. Each nucleosome consists of a histone octamer containing pairs of each histone (H2A, H2B, H3 and H4) surrounded by 146-147 base pairs of DNA (Luger et al., 1997; Ettig et al., 2011). The N-terminal tail, which protrudes from the surface of the nucleosomes, is composed of a variety of amino and is subject to extensive post-translational modifications that impacts transcriptional activation or inactivation, chromatin constitution and DNA replication (Iwasaki et al., 2013). Histone modifications include acetylation of lysine residues, methylation of lysine and arginine residues, ubiquitination of lysine residues, phosphorylation of serine and threonine residues, among others.

The acetylation and methylation of histones H3 and H4 are the most abundant post-translational histone modifications and, therefore, the most studied. Acetylation of lysine, for example, is associated with transcription status of active genes. On the other hand, the methylation of lysines and arginines, although generally associated with gene silencing, may also lead to the activation of gene transcription, depending on the region of insertion in the N-terminal histone and the number of methylations present (one, two or three) (Iwasaki et al., 2013).

There are two mechanisms through which histone acetylation regulates gene transcription. The first one is by reducing the positive charge of the histone proteins, thus decreasing their affinity to the DNA molecule (negatively charged). Consequently, there is a greater DNA exposure to transcription factors (Bannister and Kouzarides, 2011). The second is by creating, stabilizing or breaking regions of interaction between chromatin and regulatory proteins, such as transcription factors or proteins that act on chromatin condensation (Feinberg, 2001; Santos-Rosa and Caldas, 2005). Proteins with bromodomains, for example, are capable of recognizing the acetyl group in histones, making chromatin more accessible to remodelers and transcription factors (Kouzarides, 2007). The literature describes the lysines K5, K8, K12, K16 of histone H4 and the lysines K9, K14, K18, K23 of histone H3 as the best regions for the insertion of acetylation marks (Huynh et al., 2017).

Histone acetyl transferase (HATs) and histone deacetylase (HDACs) have been shown to regulate gene transcription by promoting the addition or removal, respectively, of the acetyl group to the N-terminal histone pool (Grunstein, 1997). There are 17 HAT enzymes, which are divided into two types, A and B. Type A HATs are found in the nucleus and regulate gene expression, working mainly as co-activators of transcription. Type B HATs are located in the cytoplasm, where they acetylate newly formed histones (Guo, 2009). The insertion of the acetyl group is done by specific HATs for each N-terminal region, i.e., the acetyl group present in acetyl-coenzyme A (Acetyl-CoA) is transferred to the ε-amino region of the target lysine (Galdieri et al., 2014). Removal of this group is done by the HDACs or KDACs, releasing acetate anions. There are 18 HDACs divided into class I (HDAC1-3 and 8), class II (HDAC4-7 and 9), class III or sirtuins (SIRT1-7), and class IV (HDAC11) (Vogelauer et al., 2012).

Another important post-translational modification of histones is the methylation of lysine and arginine amino acid residues. Most histone amino acid methylations are related to gene silencing. Unlike acetylation, histone methylation does not cause charge changes, but may cause conformational changes of the proteins, forming a specific binding site for other proteins (Bannister and Kouzarides, 2011).

The role of histone methylation in controlling gene expression depends on the location of the amino acid residue, where it is inserted, and the amount of methyl groups added. For example, the trimethylation of lysine 4 of histone 3 (H3K4me3) is associated with increased gene transcription and formation of euchromatin (decompressed form of chromatin). On the other hand, the triple methylation of lysines 9 or 27 in histone H3 (H3K9me3 and H3K27me3, respectively) is related to the reduction of transcription and the formation of heterochromatin (compact form of chromatin) (Park et al., 2007; Bártová et al., 2008). The presence of H3K9me3 can promote chromatin rearrangement. Heterochromatin 1 (HP1) protein binds to a histone methyltransferase (HMT) and promotes the trimethylation of other nearby H3K9. Consequently there is propagation of the heterochromatin structure along the chromosome until a delimiter is found (Stewart et al., 2005).

The enzymes responsible for the histone methylation are called histone methyltransferases (HMTs) and those responsible for the removal are called histone demethylases (HMDs). There are 3 classes of HMTs enzymes: lysine methyltransferases having SET domain, lysine methyltransferases lacking SET domain, and arginine methyltransferases (Teperino et al., 2010). The identification of the first histone lysine demethylase (KDM1A) is relatively recent (Shi et al., 2004). Other histone demethylase enzymes were then identified and are currently classified as belonging to the KDM1 family (KDM1A and KDM1B) and to the family of demethylases containing Jumonji C domain (JmJC - the largest group of histone demethylases) (D'Oto et al., 2016). The discovery of these enzymes demonstrates that histone methylations is a much more dynamic process than previously estimated, and the modifications can be inserted or removed according to the cell needs (Agger et al., 2008).

Post-translational histone modification in bovine embryos

Post-translational modifications of histones play a crucial role in bovine embryonic development by allowing changes in the gene expression at specific times/regions. For example, soon after fertilization, in paternal origin chromosomes, the protamines are replaced by histones that present high levels of acetylation and low levels of methylation (Messerschmidt et al., 2014; Urrego et al., 2014). On the other hand, the maternal genome undergoes few changes, maintaining the same pattern of lysine acetylation and arginine methylation in H3 and H4 (Rodriguez-Osorio et al., 2011).

Soon after the first cleavages there is a reduction in the acetylation of lysine 9 and 18 in histone H3 (H3K9ac and H3K18ac, respectively); the lowest levels are observed in the 8-16 cells stage. After that, there is an increase in the acetylation of these lysines until the blastocyst stage when the acetylation levels of K18 are higher in trophectoderm cells than in the inner cell mass. Acetylation of lysines 5 and 8 of histone 4 (H4K5ac and H4K8ac, respectively), however, keep stable levels from the first cleavages up to the 8-16 cells stage, only showing an increase after this stage and with no difference between the acetylation profile between cells of the trophectoderm and the inner cell mass (Wu et al., 2011).

Regarding the presence of HATs and HDACs, there are few reports detailing the expression of these enzymes throughout bovine embryonic development. In one study, HDAC1, HDAC3, HDAC7, HAT1 and HAT2 mRNA have been observed in oocytes, 2-cell, 8-cell and blastocysts (McGraw et al., 2003). The same study observed that the expression of HAT1, but not of HAT2, differed throughout the development, being overexpressed at the blastocyst stage. Regarding the expression of HDAC1 and HDAC2, they were found to be increased in blastocysts when compared to 2 cells and 8-16 cells embryos. Meanwhile, HDAC3 and HDAC7 did not differ between the different stages of embryonic development (McGraw et al., 2003). These findings suggest that, in the blastocyst stage, simultaneous acetylation and deacetylation events are

occurring, probably to ensure that these embryos are able to add and remove specific acetylation marks and activate gene expression as needed.

For histone methylation levels in bovine embryos, it has been reported that shortly after the first cleavages, there is a reduction in H3K9me3 until the 8-16 cells stage, while the dimethylation (H3K9me2) levels in this lysine remain constant. This mark is associated with Xist and long terminal repeats (LTRs) silencing in mouse embryos (Fukuda et al., 2014; Wang et al., 2018). After the 8-16 cell stage, there is an increase in di and trimethylations of this lysine up to the blastocyst stage (Fukuda et al., 2014; Wang et al., 2018). The methylation of H3K9 is particularly important because it appears to follow the same profile observed for DNA methylation, both epigenetic mechanisms capable of reducing gene transcription (Santos et al., 2003).

Other important histone repressive marks that have already been identified in bovine embryos are the trimethylation of lysine 27 and 64 of histone H3 and lysine 20 of histone H4 (H3K27me3, H3K64me3 and H4K20me3, respectively). Theses marks present the same reprogramming profile during the development, being remarkably reduced after fertilization and during the first cleavages, then increasing to result in blastocysts with abundance of these histone mark (Ross et al., 2008; Daujat et al., 2009; Wongtawan et al., 2011; Ross and Sampaio, 2018). Opposed to the other methylations previously described, the methylation of lysine 4 of histone H3 (H3K9me), a histone post-translational modification associated with increased gene transcription presents its higher levels at the beginning of development (first cleavages) and at later stages of development (blastocyst), showing its lower intensity in embryos around the 8-16 cells stage (Wu et al., 2011).

Among the enzymes that control histone methylation, some have already been studied in bovine embryos. SETDB1, a specific methyltransferase for H3K9, is abundant in oocytes and embryos up to the 8-cells stage, when it begins to decline and reaches the lowest levels in blastocysts (Golding et al., 2015). Meanwhile, SUV39H1 and SUV39H2, that also act on H3K9 methylation, exhibit high levels of expression only at the 2-cell and 4-cell stages, respectively (Zhang et al., 2012). However, SMYD3, an H3K4-specific methyltransferase is observed in lower levels throughout initial development until the morulae compactation, when a peak is observed in the amount of transcripts for this enzyme (Bai et al., 2016).

Regarding the enzymes that act in the histone demethylation processes, the presence of HDM3A, HDM4A and HDM4C during the embryonic development of bovine has been described, being observed in lower quantity only in embryos with 4 or 8 cells. HDM5B, another histone demethylase, is absent in the early stages of development, only being observed after the embryo reaches 8 cells (Sharp et al., 2018). Meanwhile, HDM4D and HDM4E (specific for H3K9me3 and H3K9me2), although detected at all stages of embryonic development, have increased expression in embryos at the 8-16 cells stage (Liu et al., 2018). JMJD3 presents high levels in oocytes, then it is reduced at the early embryo and increases again in blastocysts (Canovas et al., 2012). JMJD1C, another JmjC-domain-containing demethylase for H3K9me1 and H3K9me2 (Li et al., 2015), presents high levels throughout all the embryo development in bovine (Li et al., 2015).

The reports showing the association between post-translational histone modifications and the enzymes responsible for these modifications throughout bovine embryonic development show that the vast majority of these marks undergo drastic changes around the time of the major genome activation (approximately 8-16 cells). This demonstrates the intense reprogramming that these embryos are going through and suggests that this is a critical decision point that impacts the survival and correct development of the embryo.

Covalent modifications in RNAs

Another well-known epigenetic phenomenon is the modification of N6-methyladenosine (m^6A), a type of RNA methylation. Among more than 150 RNA modifications currently described, m^6A is the most abundant in mammalian messenger RNAs, representing about 0.1–0.4% of adenosine residues in total cellular mRNA (Meyer et al., 2012). Although this modification has

been known for some years, only recently its biological relevance began to be unveiled. Similarly to changes in DNA, the m^6A is reversible and its presence is evolutionarily conserved in many species. This modification can modulate the flow of genetic information and the response to environmental challenges (Schaefer et al., 2017). Therefore, deciphering exactly how this modulation is done represents the breaking of a new frontier in reproductive biology. Overall, the m^6A is found in long internal exons and is preferably enriched within 3 'UTR regions, around stop codons. In addition to mRNAs, m^6A is also present in long non-coding RNAs, such as XIST, small nuclear RNAs and ribosomal RNAs (Meyer et al., 2012).

This epigenetic mark is established by a protein complex called "writer" that includes three well-characterized components: METTL3, METTL14 and WTAP. The METTL3 acts as a binding subunit to the protein S-adenosyl methionine (SAM), while the METTL14 is responsible for the structural maintenance of the complex (Bokar et al., 1997). This complex preferably binds to RNA oligonucleotides containing the GGACU sequence (Harper et al., 1990). The reverse process of RNA demethylation is carried out by "erasers", such as the enzymes FTO and ALKBH5.

The m^6A is related to a series of metabolic processes that involve RNA such as the regulation of gene expression, mRNA stability, translation efficiency, alternative splicing and cytoplasmic turnover (Kasowitz et al., 2018). The functional roles of m^6A are being gradually discovered mainly through experiments in which METTL3 was inactivated; such studies have shown that the loss of m^6A compromises the fate of stem cells and pluripotency (Batista et al., 2014; Geula et al., 2015). In addition, this modification has also been described as being important in the extensive regulation of gene expression for fertility and development (Meyer and Jaffrey, 2014), such as observed for MAT2A mRNA methylation (SAM production) to mediate the downregulation of this mRNA under high-SAM conditions, in mouse embryos (Mendel et al., 2018). The functions of the m^6A are mediated by a group of proteins of the YTH family (YTHDF1, YTHDF2, YTHDF3 and YTHDC1) that specifically recognize the methylated adenosines in the RNA. These binding proteins are called "m^6A readers". So, while methyltransferases (writers) and demethylases (erasers) establish a complex mechanism for regulating the location of the changes along the RNA, the readers mediate their biological functions. Thus, post-transcriptional RNA modifications allow for additional control of gene expression, serving as a powerful mechanism that directs the fate of groups of transcripts to be processed, exported to the cytoplasm, translated and degraded, eventually affecting protein synthesis (Hsu et al., 2017).

As far as we know, all data available in literature for epigenetic modification for bovine embryos were generated using *in vitro* produced embryos. The literature lacks reports about the reprogramming profile of *in vivo* produced embryos, considered the gold standard embryos, along the development. Also, it has been already reported that even slight changes in the conditions of *in vitro* can lead to alteration in molecular control (Leite et al., 2017). Taken together, that information demonstrates the importance of more studies to elucidate the changes occurring during the initial embryonic development in bovine embryos and, ultimately, what can be changed in in vitro conditions to guarantee an accurate epigenetic reprogramming.

Metaboloepigenetics – more than meets the eyes

The concept of the crosstalk between metabolism and molecular control of cells is not recent. For more than two decades, there were reports associating changes in cell culture system with altered metabolism and gene expression profile. However, the mechanisms involved in this response began to be elucidated in other cell types only a few years ago.

Cellular metabolites have been described as enzyme co-factors, responsible for epigenetic changes or even as substrates for chemical modifications in nucleic acids or structural chromatin proteins, once again reinforcing the relationship between metabolism and the molecular profile of the cells (Figure 3). The characterization of these phenomena in embryos

is even more recent (Ispada et al., 2018, 2020; Zhang et al., 2019b). Some of these mechanisms are better described below.

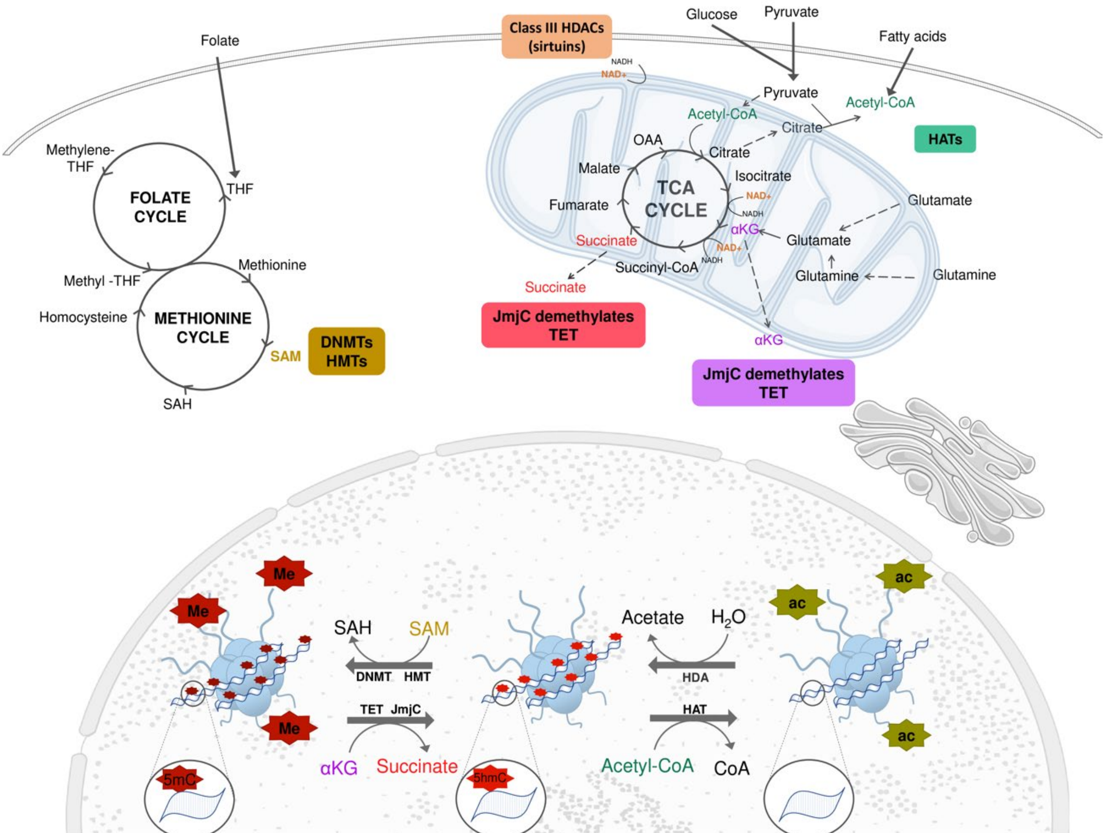

Figure 3. The folate cycle starts with the production of THF (tetrahydrofolate) from folate. THF is then converted into Methylene-THF and Methyl-THF, which is required for the production of methionine. The methionine can be converted to S-Adenosylmethionine (SAM) that acts as a methyl-group donor both DNA and histone methylation through the respective enzymes (DNMTs and HMTs). The consumption of the methyl group from SAM results in SAH (S-Adenosylhomocysteine), which can be converted to homocysteine and, again, to methionine. Glucose and pyruvate can produce acetyl-CoA, either by entering the mitochondria and being converted to citrate, or directly in the cytoplasm. Another source of Acetyl-CoA is the ß-oxidation of fatty acids. The acetyl-CoA generated can be used as an acetyl group-donor for histone acetylation by HATs (histone acetyl transferase enzymes). The removal of histone acetylation is promoted by HDAs enzymes and results in acetate production. Following in the tricarboxylic acid (TCA) cycle, the α-Ketoglutarate (α-KG) can be produced and transported to the cytosol and, later be used as substrate by TET or JmjC domains containing enzymes that promote, respectively, DNA and histone demethylation. Another source of α-KG are glutamine and glutamate. The α-KG that remains in the TCA cycle is converted to succinate. Succinate might continue in TCA cycle by and ultimately lead to the production of oxaloacetate. However, the succinate can leave the mitochondria and prevent DNA and histone demethylations by blocking TET or JmjC domain containing enzymes activity, respectively. At different steps of the tricarboxylic acid (TCA) cycle the consumption of NAD^+ is necessary. NAD^+ modulate sirtuin activity, a specific group of HAT. DNMT-DNA methyltransferase; HMT-histone methyltransferase; TET-ten-eleven translocation methylcytosine dioxygenase; HDAC-histone deacetylase, HAT-histone acetyltransferase. Adapted from SMART Servier Medical Art image bank (SMART, 2020).

Metabolic regulation of Histone acetylation and deacetylation

Acetyl-CoA is a central metabolic intermediate, which is generated in mitochondria as a byproduct of both glucose and lipid metabolism (β-oxidation). In mammalian cells, most cytosolic acetyl-CoA comes from citrate exported from mitochondria by an ATP-citrate lyase (ACL) catalyzed reaction. Increased glycolysis boosts the synthesis of citrate and thus the generation of cytosolic acetyl-CoA. Most of the acetyl group present in the histones is derived from the acetyl-CoA generated in this process (Fan et al., 2015).

The levels of intracellular acetyl-coA are influenced by several factors, such as oxygen tension and energy substrate availability. High glucose content in culture media of tumor cells, for example, leads to increasing in acetyl-CoA, with consequences in histone acetylation and gene expression (Lee et al., 2014). Inversely, quiescent fibroblasts present lower acetyl transfer to histones when compared to proliferating ones (Evertts et al., 2013). This ability of cells to respond to environmental conditions by changing the molecular framework is particularly important for appropriate decision making as cell survival, proliferation or differentiation, according to the metabolic status (Wellen and Thompson, 2012). Despite the fact that acetyl-CoA level is determinant for histone acetylation, it still remains controversial if this is a specific or a global response. The decrease of histone acetyltransferase activity as a consequence of mtDNA depletion interfere in histone acetylation of locus-specific gene expression that respond to this phenotype (Lozoya et al., 2019). However, decreased glucose availability in tumor cell lines diminished acetyl-CoA levels and histone acetylation in a global manner (Lee et al., 2014).

Histone acetylation is regulated by a balance between the activities of HATs and HDACs. In this sense, histone deacetylation, mediated by histone deacetylases (HDACs), is also a crucial event to transcriptional control and cell differentiation. Sirtuins belongs to Class III HDACs and are proteins with different cell functions, including metabolic regulation and deacetylation of histones at specific regions, as gene promoters, and thus controlling several specific cellular events. These class of enzymes respond to the levels of cellular NAD^+, been related to the metabolic and redox status of the cell since it links TCA cycle and oxidative phosphorylation in mitochondria. In this sense, the deacetylase activity of sirtuins are regulated, at least in part, at three different levels: NAD^+ biosynthesis, modulation of sirtuin activity by NAD^+ and competitive use of NAD^+ by other cellular processes (Imai and Guarente, 2016). Other small molecules may also regulate sirtuin activity, such as some specific fatty acids. In muscle cells, oleic acid stimulates the phosphorylation of SIRT1 Ser-434, increasing its catalytic deacetylase activity with consequences in fatty acid oxidation (Lim et al., 2013).

Distinct from class III, classes I and II HDACs are NAD^+-independent but also affected by cellular metabolism. The administration of exogenous ketone body β-hydroxybutyrate to mouse lead to increased global histone acetylation in several organs, but mostly the kidneys, due to HDAC inhibition (Shimazu et al., 2013). The same effect was observed after somatic cell nuclear transfer zygotes exposure to β-hydroxybutyrate, in which the hyperacetylation state of H3K9 remained until blastocyst stage (Sangalli et al., 2018).

The significance of metabolic changes in acetylation patterns is better described in embryonic stem cells than gametes and embryos. In mouse ESC the glycolytic acetyl-CoA production promotes histone acetylation maintaining the pluripotency state. The modulation of glycolysis-derived acetyl-CoA leads to changes in histone acetylation and deacetylation levels, impacting cell differentiation (Moussaieff et al., 2015). The same pattern was recently evidenced in bovine embryos (Ispada et al., 2020). In this case, changes in histone acetylation levels were related to differences induced by pharmacological modulation of pyruvate metabolism in a dose-dependent manner. In the same work, the authors discuss that non-induced differences in blastocyst metabolism also lead histone acetylation changes probably by the translocation of PDC complex to the nucleus promoted by cellular stress conditions (Ispada et al., 2020; Zhou et al., 2020).

Metabolic regulation of DNA/histone methylation and demethylation

Several chromatin-modifying enzymes, such as the DNMTs, TETs and histone demethylases use metabolic intermediates as cofactors or inhibitors, demonstrating a direct interaction between epigenetic regulation and metabolism. A good example of such interaction is SAM, a product of the one-carbon (1C) metabolism, that releases the methyl-group used by DNMTs enzymes to promote DNA methylation. Some nutrients are used as a substrate for one-carbon metabolic pathway, making methylation highly dependent on their availability. Methionine, for example, is the precursor of SAM and a key nutritional factor limiting its synthesis (Serefidou et al., 2019). Thus, fluctuations of methionine can influence DNA methylation and gene expression. Besides, as the establishment of epigenome is particularly vulnerable to

metabolic dysfunction, especially during the prenatal stages, the maternal supplementation with methionine and folate can prevent abnormal fetus development and neural tubal defects (Kalhan, 2016). Alcohol ingestion during pregnancy also affect 1C cycle, leading to changes in DNA methylation pattern and teratogenic effects (Sharp et al., 2018).

Still in this context, the factors involved in one carbon metabolism also act as critical cofactors or inhibitors of histone modifiers, making the histone methylation status also tightly connected to the cells' metabolic state (Serefidou et al., 2019). Recent studies demonstrated that the reduction of SAM induced by defects in methionine metabolism alter the dynamics of histone methylation. It was observed that the depletion of methionine in mouse ESCs leads to a decrease in H3K4 markers and reduces the expression of the pluripotency factor *NANOG*, inducing a more differentiated state (Tran et al., 2019b). Besides, in mice, a restriction in methionine intake rapidly triggered a decrease in H3K4 methylation modulated by SAM, thus reinforcing the sensitive relationship between intracellular levels of SAM and the activity of histone methylase enzymes (Mentch et al., 2015).

Another strong example that highlights the metabolic regulation of epigenetic mechanisms is α-ketoglutarate (αKG), that acts as a limiting factor in the tricarboxylic acid (TCA) cycle (Wu and Zhang, 2014; Tran et al., 2019a). This metabolite can be generated by glutamate deamination by the enzyme glutamate dehydrogenase or, in the TCA cycle, by decarboxylation of the isocitrate by the enzyme isocitrate dehydrogenase. In the TCA cycle, α-ketoglutarate is decarboxylated to succinyl-CoA and CO_2 by α-ketoglutarate dehydrogenase, which in turn is converted to succinate (Berg et al., 2002).

Multiple studies demonstrated that the αKG: succinate ratio can affect the pluripotency status in both murine and human embryonic stem cells (Carey et al., 2015; TeSlaa et al., 2016). The accumulation of succinate and fumarate inhibits TET protein enzymatic activity, leading to higher levels of DNA methylation and consequently, the maintenance of a more differentiated state. On the contrary, pluripotent cells have high αKG: succinate ratio, higher TETs activity and reduction of DNA methylation (Carey et al., 2015).

Other reports also demonstrate that not only diet, but the *in vitro* environment affects DNA methylation levels (Urrego et al., 2014; Salilew-Wondim et al., 2015). In this sense, our group showed that alterations in the α-ketoglutarate: succinate ratio or their precursors in culture media influences DNA methylation in bovine embryos and they fail to perform DNA demethylation during the early stages of development (Ispada et al., 2018). Therefore, reprogramming leading to aberrant patterns of gene expression that could affect embryo viability and possibly offspring phenotype.

In a similar process as DNA, histone demethylases enzymes also use αKG as the cofactor to remove methyl groups on histones and to release succinate and formaldehyde. While αKG is crucial for histone demethylation, it was demonstrated that accumulation of succinate inside the cell can antagonize the activity of the histone demethylases and promote cellular differentiation in mouse and human embryonic stem cells (Carey et al., 2015; TeSlaa et al., 2016).

Final remarks

For many years, embryo production systems were primarily aimed at increasing blastocyst rates and pregnancy, with most of the changes being suggested and implemented empirically. Advances in omics technologies associated with the solid development of bioinformatics tools represent a new era of possibilities for personalized culture systems targeting the breed, age of the donors, semen quality among other specific aspects. These interventions might not only increase quality and viability of embryos, but also help avoiding medium- and long-term consequences to the offspring.

The culture system is capable of imprinting marks on the embryos that remain throughout the development, causing consequences in their adult life and possibly to their future generation (Li et al., 2015). A classic example is the large offspring syndrome (LOS) that has been linked to the use of serum during embryo culture *in vitro*. This syndrome is characterized

in cattle as derived from the misregulation of non-imprinted genes and loss-of-imprinting in specific genes during the early stages of development as consequence of non-optimized ART conditions (Chen et al., 2017). Despite that, it opens the possibility of inserting positive signatures in animals epigenomic when designing new culture media and promoting changes to the in vitro culture system (Figure 4).

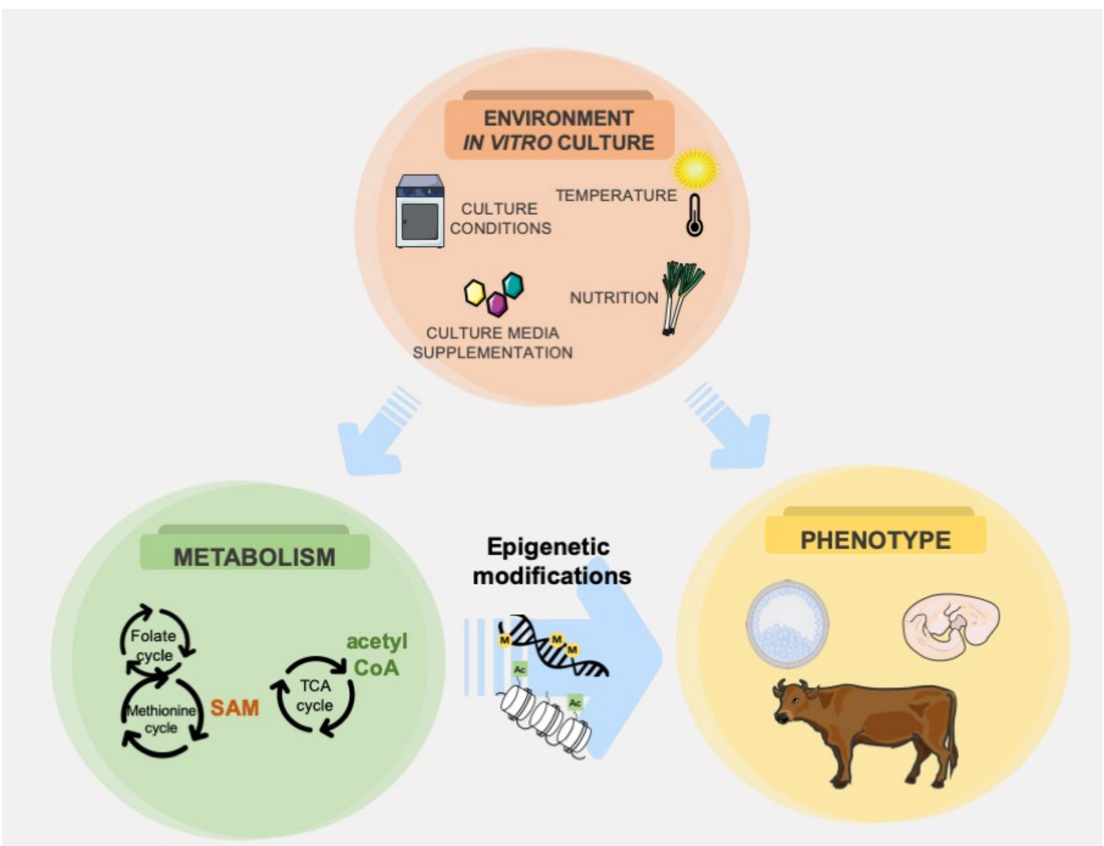

Figure 4. *In vivo* and *in vitro* environmental conditions are capable of influencing the phenotype both directly and indirectly. Indirectly, the environment may lead to changes in cell metabolism and affect the production of co-factors for epigenetic modifications. In this sense, the modifications of culture and production systems based on metabolic characteristics is a promising tool to improve embryo viability and animal production. SAM-S-adenosylmethionine. Adapted from SMART Servier Medical Art image bank (SMART, 2020).

To achieve this goal, metabolic and environmental conditions can be manipulated both in animal production and *in vitro*. Manipulation of the food intake and thermal control are some parameters that can improve the quality of gametes *in vivo*. *In vitro*, distinct supplementation (as the amount of amino acids, metabolites and lipids), physical conditions (as oxygen tension, culture media viscosity) allows the modification of morphophysiological parameters of the cells, as well as epigenomic marks in the nucleus (Leite et al., 2017; Ispada et al., 2020). Furthermore, mitochondrial DNA methylation, recently described in oocytes and embryos may also be a promising target for increasing embryo viability and animal production (Sirard, 2019).

In terms of embryo selection, metabolomics is proven to better predict quality than genomic marks (as SNPs), since metabolites are the result of genome/epigenome and environment interactions (Nicholson and Lindon, 2008; dos Santos et al., 2016). In this sense, mastering the metabolic regulation of epigenetic events is not only an intellectual pursuit, but also a powerful tool to drive specific changes on *in vitro* culture systems to generate more viable embryos and a healthier offspring.

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Defining the male contribution to embryo quality and offspring health in assisted reproduction in farm animals

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Abstract

Assisted reproductive technologies such as artificial insemination have delivered significant benefits for farm animal reproduction. However, as with humans, assisted reproduction in livestock requires the manipulation of the gametes and preimplantation embryo. The significance of this 'periconception' period is that it represents the transition from parental genome regulation to that of the newly formed embryo. Environmental perturbations during these early developmental stages can result in persistent changes in embryonic gene expression, fetal organ development and ultimately the long-term health of the offspring. While associations between maternal health and offspring wellbeing are well-defined, the significance of paternal health for the quality of his semen and the post-conception development of his offspring have largely been overlooked. Human and animal model studies have identified sperm epigenetic status (DNA methylation levels, histone modifications and RNA profiles) and seminal plasma-mediated maternal uterine immunological, inflammatory and vascular responses as the two central mechanisms capable of linking paternal health and post-fertilisation development. However, there is a significant knowledge gap about the father's contribution to the long-term health of his offspring, especially with regard to farm animals. Such insights are essential to ensure the safety of widely used assisted reproductive practices and to gain better understanding of the role of paternal health for the well-being of his offspring. In this article, we will outline the impact of male health on semen quality (both sperm and seminal plasma), reproductive fitness and post-fertilisation offspring development and explore the mechanisms underlying the paternal programming of offspring health in farm animals.

Keywords: assisted reproductive technologies, fetal programming, semen quality.

Introduction

The development of efficient assisted reproductive technologies (ART) such as artificial insemination in cattle has increased the genetic gain in livestock dramatically (Pellegrino et al., 2016). This has resulted in enhanced productivity and health in multiple cattle, swine, poultry and equine species. The success and benefits of practices such as artificial insemination have stemmed from the fact that they can yield pregnancy rates similar to that of natural conception (Buckley et al., 2003) without the need for a male to be maintained on the farm or breeding facility. Like similar ART procedures in humans, routine ART in farm animals bypasses the natural modes of reproduction. Typically artificial insemination and/or IVF involve procedures such as oestrous manipulation (either to stimulate large numbers of follicles to mature and be ovulated or to synchronise females ahead of insemination/embryo transfer), the collection and

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preparation of sperm for insemination or storage, IVF and/or the transplantation of embryos into a surrogate. However, as in many other mammalian species, questions over potential long-term effects on the health of the developing fetus and offspring following ART in farm animals (gamete manipulation, IVF, ICSI, embryo culture/transfer), have been raised. In part, these stem from the phenomenon of Large Offspring Syndrome (LOS). Supplementation of the embryo culture media with serum, embryo culture under atmospheric (20%) rather than physiological (~5%) levels of oxygen and supra-physiological hormonal stimulation in the recipient (Ealy et al., 2019) have all been connected with significant changes in patterns of fetal growth, organ development and elevated incidences of postnatal mortality in ruminants. Routine ART practices in humans (IVF, ICSI, embryo culture/transfer) have also been associated with both increased and decreased patterns of fetal growth and altered cardiovascular and metabolic health in the children (Roseboom, 2018). In both farm animals and humans, altered expression of key growth-regulatory imprinted genes has been identified as one mechanism underlying these phenotypic changes (DeAngelis et al., 2018). These observations highlight the sensitivity of the periconception period to sub-optimal environmental conditions, either *in vitro* (e.g. embryo culture media composition) or *in vivo* (parental diet). Such associations underlie the Developmental Origins of Health and Disease (DOHaD) hypothesis (Velazquez et al., 2019). Here, the maturing gametes and preimplantation embryo respond to changes in their immediate environment, resulting in abnormal profiles of epigenetic (DNA methylation, histone modifications, RNA populations) marks being established onto the parental genomes. Post-fertilisation, rates of embryo development, metabolic homeostasis, blastocyst lineage allocation and epigenetic remodelling have all been shown to be altered by sub-optimal environmental conditions (both *in vitro* and *in vivo*), affecting long-term offspring development and health (Fleming et al., 2018) (See Figure 1).

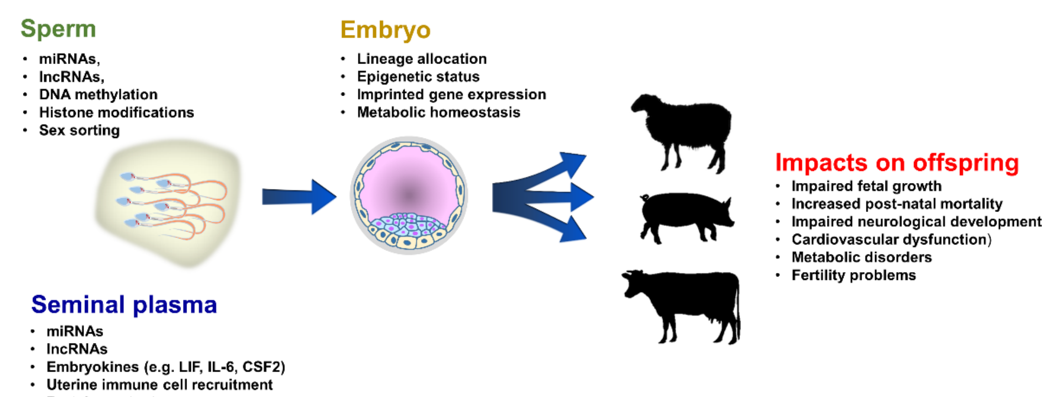

Figure 1. The paternal influence on semen quality, embryo development and offspring health. A range of male factors including nutritional status, age and stress and sperm manipuation can impact on sperm factors including miRNA (microRNA), lncRNA (long-non coding RNA) and DNA integrity. Male health can also affect seminal plasma composition including proteins, cytokines and exosomes. Both sperm and seminal plasma can influence embryo development and long-term offspring development and well-being.

Since the original observations by David Barker, which formed the foundation of the current DOHaD hypothesis (Barker et al., 1989), much of the subsequent epidemiological, clinical and fundamental research has centred on the significance of maternal nutrition and well-being during defined periods of periconception development, gestation and neonatal life. This has been conducted in a range of human, large and small animal models. However, a new focus into the role of the father, the impact his health has on semen quality and how this may affect the long-term health of his offspring has emerged. Previously, the general consensus was that sperm were simply a vehicle carrying the paternal genetic material into the egg, and that the seminal plasma was just a medium to support and transport the sperm. However, we are beginning to understand the epigenetic complexity of the mature sperm, the interaction of the

seminal plasma with the maternal uterine environment and the impact that paternal health has on these fundamental reproductive and developmental processes.

While there is a significant drive to use semen from males of high fertility or desirable physical characteristics, a clear understanding of the short-and long-term effects of using sub-optimal semen in a range of farm animals is still lacking. In contrast, studies in humans and mice have allowed for detailed analysis of how poor paternal health at the time of conception modifies the sperm epigenetic landscape, the development of the preimplantation embryo, growth of the fetus and offspring health. In this article, we will focus on the current knowledge base around the connection between male health, semen quality and reproductive fitness in farm animals. First, we will explore how male fertility can be affected by a range of physiological and environmental conditions. In response to these effects, we will then discuss how sperm epigenetic status and seminal plasma composition appear likely mechanisms mediating the paternal programing of offspring health. Finally, we will outline the long-term effects of poor paternal health on offspring well-being.

Male health and reproductive fitness

In many mammalian species, there are significant differences in fertility between individual males. Studies have shown that in cattle, a general lack of breeding soundness examinations allows for sub-fertile males to be retained within the herd, contributing significantly to failures in herd fertility when compared to bulls used in dairy herds (Flowers, 2013). For many domestic and commercially important farm species, the fertility of an individual male can have a bigger economic impact than that of the females, as breeding males are often used to service multiple females or provide semen for the use in artificial insemination. Furthermore, the use of sub-fertile males can have significant economic and sustainability consequence with increased times to conception, reduced rates of ongoing pregnancy, prolonged seasons of calving and reduced weights of offspring (Thundathil et al., 2016). As such, understanding how environmental factors impact on semen quality and defining the potential post-fertilisation consequences of using semen from males of 'poor' reproductive fitness is important for ensuring commercial viability, the effectiveness of conservation programmes and the long-term health and well-being of offspring.

In many large animal species, sperm abnormalities are categorised into 'compensable' and 'uncompensable'. Here, compensable abnormalities can be overcome through the increase in the number of sperm used during artificial insemination. Such abnormalities are associated with an inability for the sperm to reach and fertilise the oocyte, and so are generally considered to be defects in sperm motility. In contrast, uncompensable defects are associated with an inability to maintain pregnancy and the ongoing development of the embryo/fetus. Such defects are likely attributable to chromosomal defects, increased DNA fragmentation and impaired epigenetic status. Interestingly, some studies indicate that routine semen processing procedures such as the cryopreservation or sex-sorting of sperm by flow cytometry may increase the rates of uncompensable defects (Inaba et al., 2016). The cryopreservation of sperm has been of fundamental benefit to the ability to increase genetic diversity within populations and the spread of favourable/superior traits around the world. However, during the cooling and freezing process, sperm can be exposed to several detrimental factors which can negatively affect genomic integrity, membrane composition and metabolic stability (Ugur et al., 2019). During cryopreservation, sperm are susceptible to changes in membrane protein localisation due to lipid phase separation (De Leeuw et al., 1990). Furthermore, sperm shrinkage during cryopreservation may result in elevated levels of reactive oxygen species being released from the mitochondria (McCarthy et al., 2010) which may detrimentally affect DNA integrity. Indeed, sperm freezing and thawing has been linked to increased levels of DNA damage (Lewis and Aitken, 2005), resulting in reduced rates embryo development in both boar (Fraser and Strzezek, 2007) and chickens (Gliozzi et al., 2011). Separate to cryopreservation, the ability to sex-sort sperm to a high degree (over 90%) using flow cytometry has resulted in its use on a commercial level (Garner and Seidel, 2008). However, numerous studies have questioned the impact of the sorting process on sperm and the embryos they generate (Seidel,

2012). Some studies report reduced rates of embryo development with sex-sorted sperm (Inaba et al., 2016; Steele et al., 2020) which may occur in a bull-dependent manner (Barcelo-Fimbres et al., 2011). Additionally, some studies report changes in the number and structure of organelles like mitochondria, rough endoplasmic reticulum and the nuclear envelope in blastocysts derived from sex sorted sperm (Palma et al., 2008).

As in many mammalian species, scrotal temperature is a critical factor in regulating spermatogenesis (Rahman et al., 2018). Elevated scrotal temperature has been associated with increased amounts of abnormal sperm (Skinner and Louw, 1966), increased amounts of sperm cytoplasmic droplets (Koivisto et al., 2009), lipid peroxidation (Balic et al., 2012) and levels of reactive oxygen species (Rhoads et al., 2013). Recent studies have shown that sperm collected from bulls in the spring display higher indices of sperm quality (intact acrosome status, lower reactive oxygen species production, intact mitochondrial membrane potential) then when collected at other times of the year (Sabes-Alsina et al., 2017). Underlying these effects may be seasonal-dependent changes in semen lipid composition. Sperm collected during the summer possessed higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids and cholesterol than in the winter (Argov-Argaman et al., 2013) associating with decreased proportions of morphologically normal sperm. Additionally, exposure of bulls to a high temperature-humidity index increased rates of sperm death and decreased rates of blastocyst development (Llamas Luceno et al., 2020). Furthermore, elevated scrotal temperature in the bull has also been linked to poor fertilisation capacity of the sperm and perturbed paternal genome DNA demethylation in the zygote (Rahman et al., 2014). Diet can also play a significant role on testicular temperature via its influence on the levels of scrotal fat. Bulls receiving high amounts of dietary energy intake between 6 and 12 or 24 months of age displayed increased amounts of scrotal fat associated with increased scrotal temperature (Kastelic et al., 1997). Daily sperm production and epididymal sperm reserves in these males were significantly reduced when compared to males receiving a control diet (Kastelic et al., 1997).

Separate to the effects in scrotal temperature, nutritional status during calfhood (between 6 and 30 weeks of age) has also been shown to affect the onset of puberty in bulls. Low nutritional intake in calves associated with increased pulsitility of luteinizing hormone and greater testicular development (Barth et al., 2008). Interestingly, these changes were not reversed when males were subsequently given additional feed indicating that these changes, once established, were permanent. On a macro nutrient scale, many studies in ruminants have explored the role of dietary fatty acid supplementation for semen quality. As motility, capacitation and ability to penetrate the oocyte zona pellucida are all influenced by the lipid composition of the sperm plasma membrane, dietary levels of n-3 and n-6 polyunsaturated fatty acids (PUFAs) are important for male reproductive activity (Van Tran et al., 2017). Studies in a range of species including boar (Liu et al., 2015), rabbit (Castellini et al., 2003), chicken (Surai et al., 2000) and bulls (Kaka et al., 2015) have all shown that dietary supplementation with appropriate quantities and ratios of PUFAs can have beneficial effects of sperm lipid composition and male fertility. Interestingly, male age at the time of semen collection/mating appears important for sperm PUFA content. Lower levels of PUFAs (namely Docosahexaenoic acid; DHA) have been reported in semen from older bulls (Argov-Argaman et al., 2013). These observations suggest that as males age, testicular fatty acid metabolism may alter, reducing sperm membrane fluidity and ultimately the capability of the sperm to undergo cryopreservation and/or fertilisation.

In rodents and humans, similar effects on testicular function and sperm quality have been identified in response to a range of dietary and environmental factors such as over- or under-nutrition (McPherson et al., 2014; Watkins et al., 2018), endocrine disruptors (Barakat et al., 2019), stress (Rodgers et al., 2013) and deficiencies in specific nutrients (Lambrot et al., 2013). Here, poor paternal health is associated with defects including altered hormonal profiles, sperm morphological abnormalities and increased DNA fragmentation. Such defects are subsequently linked to reduced fertilisation capacity, lower rates of blastocyst development and reduced rates of ongoing pregnancy and live birth (Colaco & Sakkas, 2018; Ilacqua et al., 2018). While these connections have been widely studied in humans and rodent models, such detailed associations in cattle and large animals are still largely lacking.

Due to the high commercial importance of many breeding males, and with the advances in sequencing technologies, detailed molecular and epigenetic profiling of sperm from males of differing fertility is now being conducted in livestock. Unlike the "biological determinism" paradigm (in which phenotypic characteristics are determined purely by genes), epigenetics provides a molecular mechanism to better interpret the long-term effects the environment has on the emergence of certain phenotypes (Guerrero-Bosagna & Skinner, 2012). Epigenetic mechanisms regulate patterns of gene expression in response to environmental factors and thus act as a link between the environment and an organism's physiology. Comparison of sperm DNA methylation levels in high fertility and subfertile buffalo/bulls revealed differential methylation at genes for transcription, spermatogenesis, sperm maturation, capacitation and embryo development (Kropp et al., 2017; Verma et al., 2014). In boars, lower levels of DNA methylation at the imprinted *GNAS* complex locus has been identified in sperm from males of lower fertility (Congras et al., 2014). Interestingly, the *GNAS* locus is positioned close to quantitative trait loci for fetal growth and body mass (Thomsen et al., 2004) while gene dosing of *GNAS* is associated with postnatal growth and metabolism (Eaton et al., 2013). Similar imprinting of this locus, and regulation by non-coding transcripts of the several genes it contains, has also been reported in mice and humans (Bastepe, 2007). The role of sperm non-coding RNAs in regulating paternal reproductive fitness is highlighted further by recent studies in mice. Injection of specific tRNA-derived small non-coding RNAs from sperm of high fat diet fed male mice into control zygotes results in impaired glucose metabolism and insulin secretion in the resultant offspring when compared to sperm RNAs from control diet fed males (Chen et al., 2016). Sperm have also been shown to transfer both mRNA molecules (Sharma, 2019) and histones (van der Heijden et al., 2008) to the oocyte, believed to influence early zygotic gene expression. In human and mouse sperm, histones have been identified at key developmental and pluripotency genes such as *Oct4*, *Nanog* and *Sox2* (Hammoud et al., 2011). In cattle, over 6000 transcripts have been identified in sperm with over 60% of them being full length including transcripts for developmentally important transcripts such as *PLCZ1* and *CRISP2* (Card et al., 2013). Analyses of sperm transcript levels between bulls of low or high fertility have shown deficits in genes related to gene transcriptional and translational regulation (Feugang et al., 2010). In addition to sperm transcript levels, studies have identified levels of sperm histone methylation (H3K27me3) and acetylation (H3K27ac) as markers of male fertility in Holstein bulls (Kutchy et al., 2018). Furthermore, sperm from bulls with low fertility have been shown to display less DNA condensation, perturbed protamine exchange and increased DNA damage relative to sperm from higher fertility bulls (de Oliveira et al., 2013; Dogan et al., 2015).

As with other aspects of semen quality, the age at which semen is collected from a bull may also affect the epigenetic status of the sperm. DNA methylation profiles have been shown to differ in sperm collected from early pubertal, late pubertal, and pubertal bulls (Lambert et al., 2018). Separately, semen samples collected from bulls younger than 1 year of age have been shown to have lower sperm motility profiles than bulls older than 1 year (Murphy et al., 2018). However, post-thaw viability of sperm from the young bulls was comparable to that of the older bulls. Despite multiple studies demonstrating altered sperm epigenetic status in association with male age or fertility, the potential long-term impact(s) of using these sperm is still to be defined.

The importance of seminal plasma

While the impact of sperm epigenetic status on embryo development and offspring health has received detailed investigation, the significance of the seminal plasma has been overlooked. Typically, seminal plasma has been viewed as medium for supporting and transporting the sperm through the female reproductive tract. However, studies in mice have shown that following insemination, significant influxes of leucocytes are observed within the female reproductive tract for up to 72 hours accompanied by significant increases in the expression of numerous inflammatory mediators (Robertson et al., 1996). Specifically, studies have shown that seminal plasma Tgfb1 and granulocyte colony macrophage stimulating factor

(Csf2) are significant mediators of post-fertilisation uterine responses (Robertson et al., 2018). In humans, similar changes in cervical immune responses to the presence of seminal plasma have been identified, which are absent following intercourse with the use of condoms (Sharkey et al., 2012). Interestingly, as in humans, embryo implantation and fetal development can occur in the absence of seminal plasma in cattle (Faulkner et al., 1968). However, some studies indicate TGFβ infusion of at the time of insemination can improve pregnancy rates in cows, especially in low fertility herds (Odhiambo et al., 2009). In ruminants, proteins which can bind to sperm and can both stimulate and inhibit sperm function have been identified in seminal plasma (Maxwell et al., 2007). Additionally, aspects of sperm quality including motility and chromatin integrity have been shown to be altered in response to differential compositions of bovine seminal plasma (Garner et al., 2001; Maxwell et al., 1996), while analysis of seminal plasma composition between bulls of high fertility and bulls of low fertility have identified differences in the profiles of specific proteins (D'Amours et al., 2010; Killian et al., 1993). Seminal plasma in the pig has similarly been shown to improve sperm survival and motility (Chutia et al., 2014). Here, unwashed boar sperm held at 15º C for 24 to 72 hours in commercial GEPS extender showed significantly higher motility, survival and integrity of the acrosome prior to preservation when compared to washed sperm held in GEPS alone (Chutia et al., 2014). Interestingly, uterine seminal plasma deposition in the pig has been shown to not only influence uterine prostaglandin synthesis gene (*PTGS2*) expression but also the expression of multiple maturation promotion factors within the oocyte, cumulus and granulosa cells within the ovary (Waberski et al., 2018). Furthermore, uterine inflammatory responses persist for up to 8 days post seminal plasma infusion (O'Leary et al., 2004) and significant increases in the number of viable embryos being collected post insemination have been reported in the pig (O'Leary et al., 2004). In equine species, including the horse and donkey, seminal plasma has been shown to have a role in uterine priming by directing the expression of pro- and anti-inflammatory cytokines (e.g. *IL-8, IL-1B*, *TNF* and *COX2*) in uterine endometrial tissue (Fedorka et al., 2017; Vilés et al., 2013). There is also evidence that, in horses, sperm have the ability to initiate an inflammatory-response in the uterus by recruitment of neutrophils (Kotilainen et al., 1994). In the absence of seminal plasma however, endometrial neutrophils have been found to phagocytose stallion spermatozoa, yet in the presence of seminal plasma viable sperm are protected (Asbury & Hansen, 1987; Troedsson et al., 2001). Furthermore, conception rates in mares are associated with seminal plasma availability, with a conception rate of just 5% reported in cases of artificial insemination without seminal plasma, verses 77% when conducted in the presence of seminal plasma (Alghamdi et al., 2004).

Changes in seminal plasma composition have also been linked with fertility in men. When compared to seminal plasma from fertile men, lower levels of prostaglandin-D synthase (PGDS) have been identified in men with azoospermia (Heshmat et al., 2008). Conversely, azoospermic men display elevated levels of seminal prolactin-inducible protein (PIP), galectin-3-binding protein (LGALS3BP) and prostatic acid phosphatase (PAP) (Davalieva et al., 2012) when compared to fertile men. Additionally, proteins such as human cationic antimicrobial protein (hCAP18), lactoferrin and Semenogelin I and II have been identified as being important for fertility in men (Milardi et al., 2012).

In addition to the protein composition of the seminal plasma, there is now a large interest in the role exosomes may have in regulating male fertility. Epididymal exosomes (epididymosomes) contain a range of proteins, microRNAs, tRNA-derived small RNAs (tsRNAs) and fluid and are able to interact with the mature sperm. Analysis of epididymosomes from mice have identified over 350 miRNAs with approximately 60% of them being detectable in the sperm (Reilly et al., 2016). Furthermore, male mice fed a high fat diet display altered miRNA profiles in their sperm, potentially originating from the epididymosomes, which have then been shown to affect offspring development and health (Grandjean et al., 2015). Similarly in humans, seminal plasma also contains a range of tsRNAs which may act to regulate immune responses within the female reproductive tract (Vojtech et al., 2014).

Paternal effects on offspring health

The continued development and use of assisted reproduction technologies amongst farm animals has vastly improved the economic burden of reproductive inefficiency and the productivity of livestock. However, whilst these technologies have enhanced rates of conception and the number of offspring produced, considerations must still be given to the health of the offspring generated. A multitude of paternal factors that can influence offspring health have now been identified, including age, environmental exposures and nutritional status (Fullston et al., 2017). In cattle, a recent study identified 25 paternal candidate genes and differential profiles of sperm DNA methylation that associated with maternal gestation length (Fang et al., 2019). Such effects could conceivably affect fetal growth and weight at birth, factors known to influence adult risk for cardio-metabolic diseases. As discussed earlier, there is evidence that the phenomenon of LOS in cattle is influenced by epigenetic changes that result in alterations of imprinted genes, including *H19/IGF2* regions and the paternally expressed long non-coding RNA *KCNQ1OT1* (Hori et al., 2010; Robbins et al., 2012). It would be of interest to study whether using sperm from low versus high fertility males (known to display differential epigenetic status) affects the incidences and severity of LOS in their offspring. Furthermore, one of the few porcine studies investigating the impact of paternal diet observed that supplementing paternal diet with methyl donors (dietary factors used for the methylation of DNA and histones) lowered fat percentage in F2 offspring coinciding with significant differences in liver DNA methylation, suggesting paternal diet epigenetically programed offspring fat metabolism pathways over multiple generations (Braunschweig et al., 2012). In contrast, methyl donor supplementation in male mice results in significant sperm DNA hypermethylation of genes linked to olfaction and impairments in adult offspring cognitive performance (Ryan et al., 2018).

In mice, transgenerational effects of paternal diet have also been demonstrated. Here, suboptimal paternal low protein diet impaired F1 and F2 mouse offspring cardiovascular function and the rennin-angiotensin system (RAS) activity through both sperm and seminal plasma mediated pathways (Morgan et al., 2020). Underlying these effects on offspring health were significant changes in sperm DNA methylation, testicular expression of central epigenetic regulators and maternal preimplantation uterine immunological mediated responses (Watkins et al., 2018). Other nutritional impairments in the father, such as obesity, have been linked to metabolic dysregulation and sub-fertility in offspring and grand-offspring in mice, implicating poor paternal nutritional status with the development of 2 subsequent generations (Fullston et al., 2015). Paternal obesity in mice was found to result in growth-restricted fetuses with abnormal limb development and placental insufficiency (Binder et al., 2015; Binder et al., 2012). Fetal growth restriction, where a fetus fails to reach its genetic growth potential, is associated with poorer neonatal survival, as well as impaired cardiovascular and metabolic functions in adulthood (Barker et al., 2002; Felicioni et al., 2020; Wallace et al., 2020). Paternal obesity has also been found to impair fertility in female offspring (Fullston et al., 2015), thus impacting the production of future generations. Furthermore, absence of the seminal plasma at the time of conception in mice has been shown to impair embryo development and cell number as well as the adiposity and metabolic health of adult offspring (Bromfield et al., 2014).

Discussion

As with human reproduction, ART in farm animals has revolutionised fertility management on a global scale. While the application of such technologies has enhanced our capacity to treat human infertility and make agricultural practices more efficient, we must be mindful of the potential implications for offspring health. Both human and animal ART are associated with significant implications for the resultant offspring. Here, both increases and decreases in fetal growth and weight at birth have been reported, resulting in complications with postnatal offspring wellbeing and development. Underlying such offspring effects are factors including a failure to recapitulate the natural maternal *in vivo* environment, manipulation of the gametes and the use of sub-quality gametes. The latter can be a direct consequence of the health of the

parents at the time of conception. Poor nutritional status in a range of animal models has been shown to negatively impact gamete quality, fertility and post-fertilisation development. While a significant focus has been to define and improve maternal reproductive fitness, sperm quality and an understanding of the potential long-term consequences of poor paternal health have remained overlooked. From a large animal model perspective, our understanding of the long-term paternal effects is still very limited. The majority of studies so far have been conducted in rodents and humans. While these have provided detailed mechanistic insight into the potential molecular and epigenetic mechanisms through which paternal health links to sperm quality and offspring well-being, data from mice and humans cannot always be extrapolated directly to other mammalian species. Furthermore, a greater understanding of the role of the whole semen, and not just the sperm in isolation, is needed to understand male reproductive fitness effects. In both agricultural and human ART, procedures are routinely conducted in a predominantly seminal plasma free environment followed by the transfer of an embryo into a non seminal-primed uterus. Data from rodent (Bromfield et al., 2014; Morgan et al., 2020; Watkins et al., 2018) and human (Coulam & Stern, 1995; Marconi et al., 1989; Tremellen et al., 2000) studies both show potential reproductive benefits of uterine exposure to seminal plasma around the time of conception and embryo implantation. Therefore, it is imperative that we develop a better understanding of the impacts male health has not only for the benefits of his own health, but also for the health of the mother and ultimately, the health of his offspring.

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Animal Reproduction

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Boar seminal plasma: current insights on its potential role for assisted reproductive technologies in swine

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Abstract

Seminal plasma (SP) supports not only sperm function but also the ability of spermatozoa to withstand biotechnological procedures as artificial insemination, freezing or sex sorting. Moreover, evidence has been provided that SP contains identifiable molecules which can act as fertility biomarkers, and even improve the output of assisted reproductive technologies by acting as modulators of endometrial and embryonic changes of gene expression, thus affecting embryo development and fertility beyond the sperm horizon. In this overview, we discuss current knowledge of the composition of SP, mainly proteins and cytokines, and their influence on semen basic procedures, such as liquid storage or cryopreservation. The role of SP as modulator of endometrial and embryonic molecular changes that lead to successful pregnancy will also be discussed.

Keywords: protein, cytokine, sperm, embryo, pig.

Introduction

The OECD-FAO predicts a 15% increase in global meat production, including pig meat, for 2027 (FAO, 2018). Such increase largely depends of a successful reproductive management of the herds by correctly implementing the reproductive biotechnologies available today (Choudhary et al., 2016), such as artificial insemination (AI) or embryo transfer (ET) to ensure a sustained production of large litters. Therefore, it is mandatory to develop new strategies that help to reach such outputs, alongside higher economical and, potentially, environmental benefits by keeping the number of breeders at optimal levels, e.g. decreasing the number of low-effective breeders (Bromfield, 2016).

The early detection of boars with compromised fertility is then a logical priority for AI programs (Roca et al., 2015). However, it is generally accepted that conventional semen evaluation methods offer only a rough assessment of the real fertility potential of a boar (Dyck et al., 2011). In an attempt to overcome this drawback, special attention has been given to the study of the composition of seminal plasma (SP) as a potential source of biomarkers that

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could help to identify sub-fertile AI-boars that escape conventional semen screening yet providing sub-fertile semen (Pérez-Patiño et al., 2019). Sperm physiology and fertilizing ability are highly influenced by SP composition, which promotes sperm function and survival (Schjenken and Robertson, 2014). Moreover, the use of boar SP as an additive to optimize the quality of functional characteristics of biotechnologically treated spermatozoa has also been described (Parrilla et al., 2009; Caballero et al., 2012). Very recent reports of studies performed at a molecular level indicate that the SP effectively modulates the uterine environment in gilts and sows at different stages of the estrous cycle (pre-, peri-, post-ovulatory, and early pregnancy stages; Waberski et al., 2018; Alvarez-Rodriguez et al., 2019; Martinez et al., 2019c), potentially establishing best conditions for embryonic development and implantation. These findings are extremely relevant, especially in the field of reproductive technologies in pigs, since their implementation could result in important improvements in reproductive efficiency when using AI or ET largely impacting the production sector.

The present review summarizes current knowledge about boar SP, focusing mainly on those aspects related with the preservation of sperm function, as well as a source of potential biomarkers of boar fertility. In addition, the role of SP among the signaling mechanisms to help establish a successful pregnancy will also be discussed.

Seminal plasma characteristics and composition

SP is a complex fluid in which spermatozoa are sequentially suspended during the ejaculation process and it is composed of a mixture of secretions from the testis, epididymis, but mainly from the sexual accessory glands (Mann and Lutwak-Mann, 1981). In boar, the SP represents the major portion of the ejaculate volume (approximately 95%) being physiologically emitted in fractions, which can be collected and analyzed separately; these fractions are different in terms of origin and composition (Rodriguez-Martinez et al., 2009).

The most relevant components of boar SP are listed in Table 1. Below, we will discuss the influence of some of these SP constituents on sperm function. The SP proteins and cytokines will be reviewed in separate sections due to their major role in regulating sperm function and fertility outcomes.

Table 1. Most relevant components of boar seminal plasma.

Component	Source	Suggested functions	Reference
Ions			
Na Cl	Testes; Cauda Epididymis; Vesicular Glands; Bulbouretral Glands	Osmotic balance; sperm motility; sperm morphology; sperm metabolism	López-Rodriguez et al. (2013)
Ca	Testes; Cauda Epididymis; Vesicular Glands	Acrosome reaction; sperm motility	López-Rodriguez et al. (2013)
K	Testes; Cauda Epididymis; Vesicular Glands; Bulbouretral Glands	Sperm motility	Juyena and Stelletta (2012), Johnson et al. (2000)
Bicarbonate	Prostate; Vesicular Glands	Sperm motility; induction of plasma membrane destabilization	Rodriguez-Martinez et al. (2009)
Mg	Cauda Epididymis; Vesicular Glands; Bulbouretral Glands	Enzymatic systems; sperm motility integrity of sperm membrane	Juyena and Stelletta (2012), López-Rodriguez et al. (2013)
Se	Epididymis; Prostate; Vesicular Glands	Sperm motility; sperm morphology; sperm viability and integrity of DNA; structural component of glutathione peroxidase	López-Rodriguez et al. (2013), Pipan et al. (2017), Barranco et al. (2016), Qazi et al. (2019)

Table 1. Continued...

Component	Source	Suggested functions	Reference
Zn	Vesicular Glands	Mitochondrial function; protection against oxidative stress; antibacterial activity of SP; stabilization of DNA nucleoproteins; control of energy for sperm motility; *in vivo* fertility	Boursnell et al. (1972) Guthrie et al. (2008), López-Rodriguez et al. (2013)
Enzymes			
ALP	Epididymis	spermatozoa quiescence Modulation of fertilizing capability acquisition	López-Rodriguez et al. (2013), Bucci et al. (2014)
AST	Epididymis Vesicular Glands	Indicator of sperm cell damage	López-Rodriguez et al. (2013), Ciereszko et al. (1992)
AP	Epididymis Vesicular Glands	Sperm plasma membrane integrity Control of sperm metabolism	Wysocki and Strzezek (2000)
GGT		Protective effect of sperm against oxidative stress	López-Rodriguez et al. (2013)
LDH (isoenzyme LDH 4)	Testes; Epididymis	Necessary for energy metabolism of sperm Maintenance of sperm motility Involved in sperm capacitation	Sopkova et al. (2015), Mann and Lutwak-Mann (1981)
SOD	Epididymis; Vesicular Glands; Prostate	Sperm protection against reactive oxygen species	Roca et al. (2005)
PON-1	Testes; Epididymis	Prevent oxidation of low-density lipoprotein cholesterol	Barranco et al. (2015a)
GPX-5	Testis; Epididymis Vesicular Glands; Prostate; Bulbourethral Glands	Neutralizing H_2O_2 Sperm protection against reactive oxygen species	Barranco et al. (2016)
Energy Substrates			
Glucose, fructose, sorbitol	Vesicular Glands	Energy source and modulators of sperm function	Rodríguez-Gil (2013)
Other relevant components			
Citric Acid	Cauda Epididymis; Vesicular Glands	pH control in boar semen; Zn, Mg and Ca chelator	Setchell and Brooks (1988), Kamp and Lauterwein (1995)
Inositol	Vesicular Glands	Maintenance of osmotic balance	Mann and Lutwak-Mann (1981), Setchell and Brooks, 1988
Phosphate	Testes; Cauda Epididymis; Vesicular glands; Bulbouethral Glands	Sperm motility	Setchell and Brooks (1988), López-Rodriguez et al. (2013)
Glycero-phosphocholine	Cauda Epididymis; Vesicular Glands	Reserve of substrate Reduce sperm motility in-vitro Regulation of osmotic pressure	Mann and Lutwak-Mann (1981), Cooper (1986)
Ergothioneine	Cauda Epididymis; Vesicular Glands	Prevention of lipid peroxidation Maintenance of intracellular SH-groups in a physiologically active condition	Mann and Leone (1953), Nikodemus et al. (2011)
Hypotaurine	Cauda Epididymis	Osmoregulation and reducing agent	Johnson et al. (1972)

Ions

Macroelements such as sodium, calcium, potassium, magnesium and chlorine greatly influence sperm functions (Hamamah and Gatti, 1998). In boar, sodium and chlorine are the most abundant ions and, among other functions, they influence metabolism and hence sperm motility and membrane stability, which can modify the morphology of spermatozoa (López-Rodriguez et al., 2013). Potassium, a metabolic inhibitor, decreases sperm metabolism and hence lowers sperm motility (Juyena and Stelletta, 2012; Johnson et al., 2000). Calcium, of utmost relevance for sperm motility variations, is pivotal when triggering the acrosome reaction (Juyena and Stelletta, 2012). Magnesium is involved in almost all enzymatic reactions, and thus related to sperm motility and sperm membrane preservation (Juyena and Stelletta, 2012; López-Rodriguez et al., 2013). Other ions present in boar SP, such as copper, selenium and zinc, also influence sperm quality (Pipan et al., 2017). Selenium is a component of glutathione peroxidase, an enzyme with antioxidant properties whose presence in the SP has been associated with sperm survival and *in vivo* fertility (Barranco et al., 2016). Moreover, recent studies have shown that selenium in the SP is related to sperm motility, morphology, and viability and the integrity of DNA (Pipan et al., 2017). Zinc is essential for chromatin intactness, preserves mitochondrial function and acts as a protective agent against oxidative stress (Guthrie et al., 2008) and contributes to the antibacterial activity of SP (Juyena and Stelletta, 2012). Finally, higher levels of other ions, including iron and copper, have been correlated with a higher number of functional sperm after storage (Pipan et al., 2017).

Enzymes

There are a wide variety of enzymes in the SP that have different functions. Very recently, it has been observed that more than 3% of the pig SP proteome are enzymes (Roca et al., 2020). Among them, there are several with antioxidant properties, whose main action is to reduce lipid peroxidation to protect spermatozoa from excessive levels of reactive oxygen species (ROS), particularly relevant in pigs due to the high sensitivity of their spermatozoa to oxidative stress (Radomil et al., 2011; Li et al., 2018). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, glutathione S-transferase, phospholipid hydroperoxide glutathione peroxidase, gamma-glutamyl transferase (GGT) and paraoxonase type 1 (PON-1) are among the antioxidant enzymes present in boar SP (López-Rodriguez et al., 2013; Koziorowska-Gilun et al., 2011; Barranco et al., 2015a, c, 2016).

Many of these enzymes have positive effects on the function of frozen-thawed (SOD; Roca et al., 2005) and refrigerated (PON-1 and GPx5; Barranco et al., 2016) spermatozoa, on the sperm concentration in the ejaculate and progressive motility (GGT; López-Rodriguez et al., 2013). Moreover, SOD, PON-1 and GPx-5 have been positively associated with the sperm response to cryopreservation, as they minimize oxidative stress associated with this procedure (Li et al., 2018).

Other enzymes present in boar SP are those related to preserving the stability of the sperm plasma membrane and sperm metabolic function. For instance, lactate dehydrogenase (LDH) and especially its isoenzyme LDH-C4 are important indicators of fertility, displaying higher concentrations among normospermic boars with high sperm motility (Sopkova et al., 2015). Aspartate amino transferase (AAT) activity in SP indicates sperm damage (Forejtek and Navratil, 1984) being negatively correlated to normal mophology, intact acrosomes and normal fertility (Juyena and Stelletta, 2012; López-Rodriguez et al., 2013). High activity of acid phosphatase has been correlated with sperm concentration, motility, and integrity of the acrosome membrane (Wysocki and Strzezek, 2000), while alkaline phosphatase activity might play a role in preventing premature capacitation and, therefore, preserving fertilizing ability (Bucci et al., 2014).

Energy substrates

To maintain their functionality and especially to ensure adequate motility, boar spermatozoa require energy that is usually obtained from exogenous substrates present in SP. Monosaccharides, such as glucose and fructose, and polyols, such as sorbitol, are the main energy sources for sperm present in boar SP, and glycolysis the main pathway of glucose utilization, producing pyruvate/lactate (Mann and Lutwak-Mann, 1981; Setchell and Brooks, 1988; Marin et al., 2003). However, boar spermatozoa can, in the absence of monosaccharides, also use other substrates, such as glycerol, lactate, pyruvate and citrate (Rodríguez-Gil, 2013). It is worth noting that knowledge of energy regulation sperm mechanisms is fundamental to design strategies for adequate handling and storage conditions for best preservation of sperm quality.

There are many other constituents of SP that modulate sperm functions, and some details about them have been included in Table 1.

Seminal plasma and semen technologies

The addition of SP to boar sperm subjected to different biotechnological treatments, such as sperm sex sorting or cryopreservation, has been proposed as a strategy to reduce negative effects (Maxwell and Johnson, 1999; Parrilla et al., 2009). However, contradictory results have been described (Novak et al., 2010; Yeste et al., 2017), probably mainly attributed to variations in the protein composition of SP (Druart et al., 2013; Pérez-Patiño et al., 2016).

Seminal plasma proteins

Proteins are one of the most important components of boar SP, with concentrations ranging from 30 to 60 g/L (Rodriguez-Martinez et al., 2009). Many SP proteins bind to the surface of spermatozoa to modulate their functional capacity (Caballero et al., 2012; Parrilla et al., 2019). These proteins also protect spermatozoa during their transit through the sow genital tract, contributing to the regulation of the temporal kinetics of ovulation and subsequent corpus luteum development and facilitating, in combination with other SP components, early pregnancy success (Waberski et al., 1997; Troedsson et al., 2005; Bromfield, 2016). A direct relationship between several SP proteins and fertility has been demonstrated in several species (Mogielnicka-Brzozowska and Kordan, 2011).

In boar, most SP proteins are spermadhesins (75-90% of total SP protein content; Rodriguez-Martinez et al., 2009), a highly multifunctional family of glycoproteins classified according their ability to bind (AQN-1, AQN-3 and AWN) or not (PSP-I and PSP-II), heparin. Within a boar ejaculate, the relative protein concentrations are low in the pre-sperm fraction and the first portion of the sperm rich fraction (SRF) and increase in latter fractions of the ejaculate (Rodriguez-Martinez et al., 2011) (Figure 1).

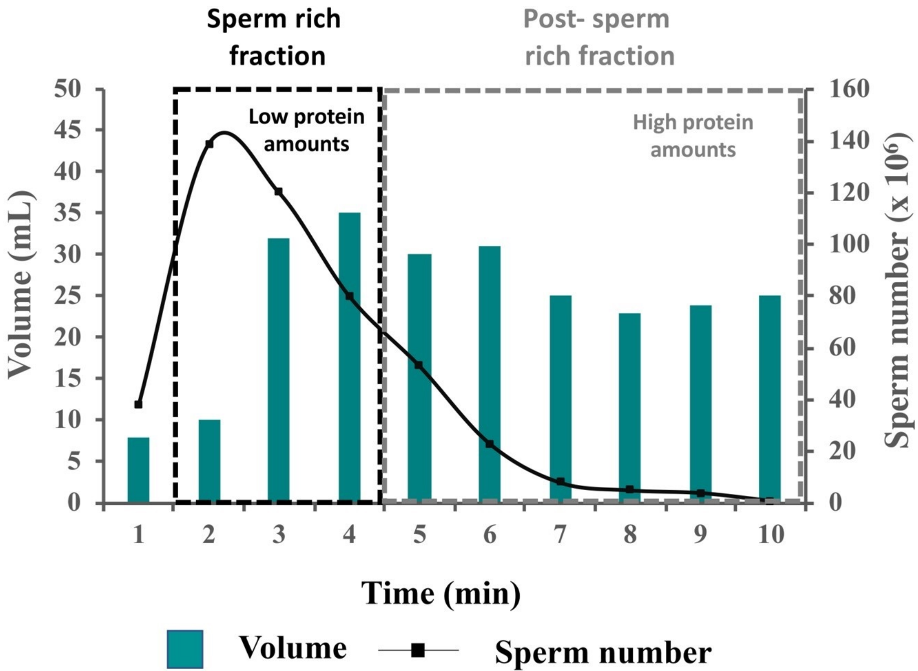

Figure 1. Boar ejaculate: Sperm concentration, ejaculate volume and relative amount of proteins in the consecutive portions of boar ejaculate namely, sperm rich fraction and post- sperm rich fraction. Modified from: Rodriguez-Martinez et al. (2009, 2011).

In vivo, several spermadhesins have been associated with sperm fertilization capability (Caballero et al., 2012). Moreover, numerous studies have focused on determining their potential use as biomarkers of male fertility and as additives for the improvement of biotechnologically treated spermatozoa (Dyck et al., 2011; Caballero et al., 2012; Vilagran et al., 2014). These studies have provided valuable information regarding their effects on sperm functional parameters and, more importantly, their potential relationship with boar sperm fertilization capability *in vitro* and *in vivo* (Garcia et al., 2007; Novak et al., 2010; Dyck et al., 2011; Caballero et al., 2012). However, although these findings were promising, they were focused on the identification of a single protein, while effective fertilization is the result of multiple interactions between different proteins. Therefore, more research is required to ensure the usefulness of these and other proteins as fertility markers.

To identify such proteins useable as fertility biomarkers, we performed proteomic analysis of the entire ejaculate and its fractions (first 10 mL of SRF, the rest of SRF and post-SRF) (Pérez-Patiño et al., 2016). We identified a total of 536 proteins in the entire ejaculate, 374 of which represented in the *Sus scrofa* taxonomy. Despite the high number of proteins identified, just 20 were directly related to reproductive processes, a striking finding when considering that the main effects of SP proteins are on sperm function and attainment of pregnancy (Caballero et al., 2012; Bromfield, 2016). Most likely, this finding was due to the incomplete association of the proteins identified with specific terms in the Gene Ontology knowledge base rather than to an actual deficiency among proteins related to reproductive functions (Pérez-Patiño et al., 2016). Despite this, the bioinformatic analysis showed that many of other identified proteins were related to important functions directly involved in reproductive processes, such as ion-and calcium-binding properties, glycosylation, immune responses and antioxidant activity, among others. In that study, we also showed quantitative rather than qualitative differences in protein SP-composition from the different ejaculate portions analyzed. There were no differences in protein levels between the first 10 mL of SRF and the rest of the SRF. In contrast, we found 34 proteins that were differentially expressed between the SRF and post-SRF. Sixteen of these proteins were represented in the *Sus Scrofa* taxonomy, and eight of them were overexpressed in SRF compared to their levels in the post-SRF. Some of the overexpressed proteins have been previously related to sperm capacitation, the acrosome reaction, zona pellucida binding, membrane stability and

permeability (HEXB, GP2, ARSA, GLB1L3; Pérez-Patiño et al., 2016). Proteins overexpressed in the post-SRF were related to sperm maturation, sperm motility and bactericide activity. The remaining 18 differentially expressed proteins, which were not present in the *Sus Scrofa* taxonomy, were found to be involved in spermatogenesis, sperm maturation or mature sperm functionality in other species of mammals (Pérez-Patiño et al., 2016). These results seem to indicate that quantitative variations in SP-proteins are most likely responsible for the different effects of SP from different fractions on boar spermatozoa (Garcia et al., 2007; Saravia et al., 2009; Novak et al., 2010; Alkmin et al., 2014).

In a subsequent study, Pérez-Patiño, et al. (2019) attempted to increase the number of less abundant SP-proteins, very relevant for biological processes, that could be detected by including a prefractionation step by solid phase extraction (Brewis and Gadella, 2010). A total of 872 proteins were identified, 336 more than in our previous study (Pérez-Patiño et al., 2016). Of these, 37 proteins related to reproductive functions. Notably, among them were the low-molecular weight and highly abundant spermadhesins (PSP-I, PSP-II, AWN, AQN-1, and AQN-3), which, as mentioned above, exert important effects on boar sperm functions (Caballero et al., 2012), ensuring the maintenance of the proper immune environment in the uterus for embryonic development (Rodriguez-Martinez et al., 2011). More importantly, we detected proteins differentially expressed in SP samples from boars with different farrowing rates and litter sizes (data recorded from 10,526 sows; Pérez-Patiño et al., 2019). Eleven proteins were differentially expressed in boars with high- and low- farrowing rates (8 overexpressed and 3 underexpressed in boars with high farrowing rates). Among the overexpressed proteins, 4 proteins (furin, UBA1, SPAM-1, and AKR1B1) showed a direct implication in male reproductive success, such as sperm maturation, capacitation, motility, and fertilizing ability (Pérez-Patiño et al., 2019). We speculate that a higher expression of these proteins would contribute to successful fertilization, as reflected by the higher farrowing rates obtained when these boars were used for AI. Regarding the differentially expressed SP proteins in boars with larger and smaller litter sizes, we identified 4 other proteins; two of them (DSC-1 and CAT) overexpressed in those boars with larger litter sizes. The DSC-1 is involved in the proper functioning of spermatogenesis, while CAT is a well-known antioxidant enzyme, protecting against ROS (Awda et al., 2009). The PN-1 was one of the underexpressed SP proteins, which has been related to seminal vesicles dysfunction in humans when present in excess and with infertility when lacking (Murer et al., 2001). The other underexpressed protein was THBS1, whose presence in SP during AI alters maternal-conceptus communication in early pregnancy stages (Edwards et al., 2011). The low expression of these SP-proteins in boars with larger litter sizes suggests they could be used as potential fertility biomarkers.

Seminal plasma cytokines

Cytokines are a family of proteins of low molecular weight (between 5 and 20 kDa) mainly known for being involved in the immune response as regulatory factors (Jiang et al., 2016; Syriou et al., 2018). The role of cytokines in different reproductive events, including ovarian and testis functionality, embryonic development, endometrial immune responses and proper placental function and parturition, has been described by several authors (Robertson and Moldenhauer, 2014). In humans, cytokines are directly related to semen quality and functionality and play an important role in fertility regulation (Fraczek and Kurpisz, 2015). However, the potential influence of these cytokines on boar fertility profile is far from clear. To increase the understanding of SP cytokines in boars, we used a multiplex assay approach to identify and quantify different cytokines in SP from the SRF and post-SRF (Barranco et al., 2015b). Our results demonstrated that the SP of all analyzed boars contained a variety of measurable cytokines with pro- and anti-inflammatory activity. This fact suggests that, similar to that in humans and mice, that pig-SP could modulate uterine immune mechanisms to facilitate the transition from a primary anti-inflammatory response after AI to a more immunotolerant environment prior to embryo implantation (Robertson, 2005; Bromfield, 2014). More interestingly, our study found that cytokine concentrations varied among boars and ejaculated fractions, as they were more abundant in the post-SRF. Several authors have

described the different tolerances of spermatozoa retrieved from the post-SRF to biotechnological procedures such as liquid storage or cryopreservation (Saravia et al., 2009; Alkmin et al., 2014). The possibility that SP cytokines, by interacting with spermatozoa, are partly responsible for these differences deserves to be thoroughly studied. In this line, we have shown how specific boar SP cytokines could modulate sperm changes at different levels during preservation (Barranco et al., 2019; Table 2).

Table 2. Predictive value of seminal plasma cytokines for sperm quality and functionality parameters in liquid-stored and cryopreserved boar semen samples.

Seminal plasma cytokine	Sperm sample	Total motility	Progressive motility	Viability	H_2O_2 generation	Total O_2 generation	Lipid peroxidation*
TGF-β1	Liquid						
	Cryopreserved	-	-				+
TGF-β2	Liquid	-	-				
	Cryopreserved						
TGF-β1	Liquid	+	+		+		
	Cryopreserved						
GM-CSF	Liquid						
	Cryopreserved				+		
IFN-γ	Liquid				+		
	Cryopreserved	+	+	+	+	-	-
IL-1α	Liquid						
	Cryopreserved			+	-		
IL-1Ra	Liquid	-	-				
	Cryopreserved	-	-	-			-
IL-2	Liquid						
	Cryopreserved	-					
IL-4	Liquid	-	-				
	Cryopreserved		-	-	-		
IL-6	Liquid						
	Cryopreserved					+	
IL-8	Liquid	+	+				
	Cryopreserved	-	-	-		+	+
IL-10	Liquid	+					
	Cryopreserved					+	
IL-12	Liquid		-		-	-	
	Cryopreserved						
IL-18	Liquid	+	+				
	Cryopreserved					-	

+ /-: Positive and negative relationship. *Viable sperm showing lipid peroxidation. Modified from Barranco et al. (2019). TGF: Transforming Growth Factor; GM-CSF: Granulocyte-Macrophage Colony Stimulation Factor; IFN: Interferon; IL: Interleukyn.

Seminal plasma and embryo technologies

Currently, it is widely recognized that SP not only plays a key role as a nutrient and vehicle for spermatozoa but also exerts important functions on the tissues of the female genital tract, impacting subsequent events such as fertilization, implantation and pregnancy (Robertson, 2005, 2007). Numerous studies in human, rodents and domestic species indicate that the SP contains specific constituents with potential to induce modifications at the molecular, biochemical and cellular levels in the female genital tract (Robertson, 2005, 2010; Rodriguez-Martinez et al.,

2011). Thus, the infusion of SP prior to AI alters the expression of many genes related to maternal immunity in the reproductive tract of peri-ovulating sows (Waberski et al., 2018; Alvarez-Rodriguez et al., 2019). The SP promotes the release of factors related to the development of preimplantation embryos during attachment (Schjenken and Robertson, 2014). However, the most substantial information regarding the effects of SP on embryonic developmental competency is derived from studies in rodents. In these species, the infusion of SP during estrus supports not only embryo development but also implantation (Pang et al., 1979; Queen et al., 1981). In the absence of SP, the rates of fertilization and preimplantation embryo development are reduced, and postimplantation pregnancy losses are increased (Peitz and Olds Clarke, 1986; O et al., 1988). Furthermore, in these species, surrogate females are usually treated with SP infusions during estrus in embryo transfer (ET) programs to increase embryo survival and implantation rates post-ET (Watson et al., 1983; Carp et al., 1984; Bromfield et al., 2004). Overall, these findings indicate that the effects of very early signaling of the infusions of SP during estrus remain influential over time and affect later processes related to preimplantation embryo development and implantation, at least in rodents. Despite this evidence, studies of the molecular changes in the preimplantation porcine endometrium and embryos in response to SP, which would be of enormous importance for porcine ET technology, have been limited.

Seminal plasma and the transcriptional pattern of the preimplantation endometrium

Infusion of SP at the onset of estrus interacts with the endometrium and induces the modification of certain cytokines, such as granulocyte macrophage colony-stimulating factor (O'Leary et al., 2004), which is a promoter of the development and viability of mammalian preimplantation embryos (Sjoblom et al., 2002). Furthermore, these authors indicated that endometrial cytokine changes induced by SP infusions lasted for at least the first 9 days of pregnancy and were accompanied by an increase in embryo viability and changes in the kinetics of embryos, delaying their development.

We recently examined the effects of SP on the development and viability of porcine preimplantation embryos and the changes of the global transcriptome of the endometrium (Martinez et al., 2019c). In this study, post-weaning estrus sows received intrauterine infusions of SP or Beltsville Thawing Solution (BTS; Pursel and Johnson, 1975) 30 minutes before each insemination. Embryos and endometrium samples were removed during laparotomy 6 days after the infusions to morphologically evaluate the embryos and analyze the endometrial transcriptome, at Day 6 of the cycle when embryo collection and transfer are usually performed in pig ET programs. The endometrial morphology was affected by the infusion of SP, showing accentuated inflammatory changes compared to endometria from the BTS group. The changes included congestion, leukocyte margination, edema, hemorrhages and infiltrates of immune cells in the mucosal connective tissue and the uterine glands. These results support previous findings in humans and pigs, indicating that the effects of SP infusions during estrus can be observed throughout the preimplantation period in pigs (Aumüller and Riva, 1992; Maegawa et al., 2002; O'Leary et al., 2004).

On the other hand, all studies of the endometrial transcriptome during the porcine peri-implantation period have revealed alterations in the expression of genes associated with the maternal immune response (Samborski et al., 2013; Kiewisz et al., 2014; Lin et al., 2015). However, endometrial receptivity during the preimplantation period, which is greatly influenced by alterations in cytokines and other compounds secreted into the uterine fluid (Morris and Diskin, 2008; Bazer and Johnson, 2014), is also critical for the development of embryos and the appropriate progression of pregnancy. Surprisingly, we identified more than 1,600 expressed transcripts with differential abundance in the endometria of the SP and BTS groups. The endometria from SP sows showed an overrepresentation of genes associated with immune pathways, including genes such as *DLG1, FAS, LGALS1, STAT5A* and *IRF1*. Regulatory T (Treg) cells, which are a subset of CD4+ T cells, are efficient immune suppressors that play important roles in the cell-mediated immune response (Rudensky, 2011) and, therefore, are key to preventing immune rejection of the developing hemi-allogeneic embryo/fetus (Shevach,

2002; Aluvihare et al., 2004). Treg cells play a pivotal role in the progression of pregnancy by suppressing the proliferation of T and B cells (Shevach, 2002; Lim et al., 2006), inhibiting the maturation and activation of dendritic cells (DCs) and macrophages (Taams and Akbar, 2005) and preventing the cytotoxicity of natural killer cells (Ghiringhelli et al., 2005). In this regard, SP-treated sows showed evidence of the overactivation of the transforming growth factor-ß (TGF-ß) signaling pathway, which is a pathway that supports the proliferation of Treg cells by regulating DC function (Ghiringhelli et al., 2005). Altogether, these findings indicated that SP infusions prior to AI increase the development of Treg cells and control the immune response of the female in response to the presence of hemi-allogeneic embryos as early as Day 6 of pregnancy. The implications of these findings could help us reduce the immune response of subrogate females to allogeneic transferred embryos, thus decreasing the high embryonic death characteristic of current ET.

It is known that the development of embryos during the preimplantation period is severely influenced by cytokines present in uterine fluids, which either promote or limit embryonic development (Hardy and Spanos, 2002; Sjoblom et al., 2005; O'Neill, 2008). In our study, numerous genes associated with the cytokine-cytokine receptor signaling pathway were either over- or under-expressed in uterine samples exposed to SP. For example, the CD27 and CD70 genes were downregulated. The proteins encoded by these genes are costimulators of an embryotoxic cytokine (TNF-α) that increases apoptosis and inhibits embryonic development and implantation (Chaouat et al., 1990).

The development of embryos from the zygote stage to the implantation stage is controlled by many hormones, which adjust the maternal physiology to support pregnancy (Waclawik et al., 2017). Pre-AI SP infusions induce hormonal changes in the uterine environment via the overexpression of genes related to steroid and estrogen signaling.

SP-infusions also noticeably altered other pathways implicated in embryonic development and implantation. For instance, we found upregulation of the PI3K/AKT, MAPK/ERK and Wnt signaling pathways, which seem to play a fundamental role in regulating not only cell functions, including proliferation, differentiation, mitogenesis, and cell survival (Songyang et al., 1997; Nayeem et al., 2016), but also sperm functions, such as the capacitation process (Almog and Naor, 2010), and embryonic development and cytoskeletal remodeling of preimplantation trophoblast cells (Qiu et al., 2004; Bazer et al., 2010). Moreover, deficiencies in MAPK/ERK proteins result in embryonic loss by altering the trophoblast proliferation process (Saba-El-Leil et al., 2003; Jeong et al., 2013).

SP infusions induced the overexpression of many other genes of interest in the endometrium, such as *HOXB4, GRHL2, RAB14, MGAT1* and *ACVR2A*. All these genes have been associated with normal early embryonic development (Scott and Carroll, 1987; Blitek et al., 2011; Grasa et al., 2012; Petrof et al., 2014; Yong et al., 2018; Ming et al., 2018). Although the adhesion of embryos to the endometrium occurs on Days 12-16 of pregnancy, SP infusions at estrus altered, on Day 6 of pregnancy, genes that are particularly involved in cell adhesion pathways that can be responsible for increasing implantations success. Altogether, these results show that SP infusions during estrus affect the transcriptional expression profile of porcine endometrium during early pregnancy.

Seminal plasma and preimplantation embryos

SP-infusions during estrus result in a higher percentage of advanced stage embryos as early as 6 days post-AI compared to that in controls (BTS infusions) without affecting neither fertilization rate nor embryo viability (Martinez et al., 2019c). These changes in embryonic development might be associated with variations in ovulation time in response to SP treatment. The SP alters the endocrine-immune-cytokine system in preovulatory follicles (Einer-Jensen and Hunter, 2005; O'Leary et al., 2006) by decreasing the LH peak ovulation interval and, therefore, hastening the time of ovulation (Schuberth et al., 2008). Accordingly, embryos collected from sows treated with SP should have reached a more advanced developmental stage than those collected from non-treated sows. However, SP could also

directly affect embryo development because, as discussed above, the effects of SP extend beyond the time immediately after exposure. Our findings, which indicated that SP did not affect embryo viability but advanced the developmental stage of the embryos, are in contrast with previous results reported by O'Leary and coworkers (O'Leary et al., 2004), who observed that SP increased embryo viability and delayed embryonic development at Day 9 of pregnancy. The discrepancies between these studies could be attributed to differences in the methodology used for evaluating embryonic development. While we used the classical morphological evaluation method to determine the stage of the embryos, the authors of the other study evaluated the developmental stage by measuring the diameter of hatched Day 9 blastocysts, when a number of them collapsed or presented irregular morphology (Sun et al., 2015). In addition, similar to previous studies on ET (Martinez et al., 2014, 2015, 2019a, b, d), we had a high embryo viability rate on day 6 of pregnancy (>90%), which complicated the detection of significant differences between the SP and control groups.

Interestingly, recent studies from our laboratory indicate that SP infusions during estrus also modify the gene expression of Day 6 blastocysts. Transcriptome analysis of these embryos revealed 210 annotated transcripts that were differentially expressed in blastocysts derived from SP sows relative to those found in blastocysts derived from BTS sows (93 upregulated and 117 downregulated). Most of these genes were associated with biological, cellular, metabolic and developmental processes. When we analyzed the differentially expressed genes to identify the significant KEGG pathways, a total of 3 and 13 pathways were enriched in the down- and upregulated gene lists, respectively. Three pathways involved in mineral absorption, regulation of lipolysis in adipocytes and p53 signaling were enriched in the downregulated gene list and included genes, such as *MT-2B, PTGS1, ADORA1, CDK2* and *SERPINE1*, with no evident association with embryonic development or implantation. The pathways enriched among the upregulated genes included pathways related to signal transduction (apelin signaling, FoxO signaling and mTOR signaling), cellular processes (cell cycle, p53 signaling, cellular senescence, adherents junction and signaling pathways regulating pluripotency of stem cells), and the endocrine system (insulin signaling, progesterone-mediated oocyte maturation and relaxin signaling). These pathways contain genes with potential roles in embryonic development, implantation, or progression of pregnancy, such as *MAPK1, SMAD2, CDK1, ApoA-I, PRKAA1* and *RICTOR*. Our results demonstrate that SP infusions upregulated the expression of these genes, which may favor embryonic developmental capability.

The *MAPK1* gene encodes a member of the MAP kinase family that is involved in several cellular processes, such as proliferation, differentiation, transcription regulation and development. Studies in several species have shown that deficits in MAPK proteins cause early embryonic mortality due to the lack of signal transduction for proliferation and invasion of trophoblasts (Saba-El-Leil et al., 2003; Jeong et al., 2013). It has been shown in mice that *MAPK1* is essential not only for embryonic development but also for placental development (Hatano et al., 2003) and development of the mesoderm (Yao et al, 2003).

The protein encoded by the SMAD2 gene mediates the TGF-ß signaling pathway, which is an essential pathway controlling the initial developmental steps, such as epiblast development and patterning of the three germ layers (Liu et al., 2016). In addition, as mentioned above, the TGF-ß superfamily supports the proliferation of Treg cells, which are crucial for preventing immune rejection and thus tolerating the fetal allograft.

Another upregulated gene in SP blastocysts was *CDK1*, a gene indispensable not only for the mitotic cell cycle (Santamaría et al., 2007; Gavet and Pines, 2010) but also for the resumption of meiosis in oocytes (Adhikari et al., 2012), which supports the conclusion that *CDK1* may contribute to embryo viability.

Other interesting genes were *ApoA1, PRKAA1* and *RICTOR*. The *ApoA1* gene encodes apolipoprotein A-I, a principal constituent of high-density lipoprotein, which has been shown to be upregulated in the murine endometrium during implantation (Gou et al., 2015). Moreover, it has been suggested that *ApoA1* plays important roles in embryo implantation by inhibiting lipid peroxidation (Jia et al., 2016). Furthermore, a role for ApoA1 in early embryonic development has also been suggested, as this protein is produced by human preimplantation embryos, and increased *ApoA1* levels are present in spent culture media containing blastocysts

of high morphologic grade (Mains et al., 2011). Sensor systems for cellular metabolism and energy are essential during early embryonic development (McBride et al., 2009). The protein encoded by the *PRKAA*1 gene is the catalytic subunit of AMPK, which is a cellular energy metabolism sensor conserved in all mammalian cells. The metabolic sensor *PRKAA1* seems to be important for embryonic development in sustaining cell polarity and advancing the cell cycle (Lee et al., 2007; Jansen et al., 2009). Finally, another upregulated gene that plays an important role in embryonic growth and development is *RICTOR*, a protein-encoding gene essential for the development of both embryonic and extraembryonic tissues, as its deficiency causes embryonic lethality in mice (Shiota et al., 2006).

Final comments

Achieving a successful pregnancy is the main goal of all reproductive biotechnologies. Boar SP has been demonstrated to be a determinant factor for basic sperm function. Moreover, boar SP acts as a protective factor for spermatozoa in its transit through the uterus and, more importantly, as a factor that prepares the uterine environment to receive the embryo, promoting its proper development and implantation.

Therefore, broadening the knowledge of the molecular mechanisms by which SP exerts its effects at the sperm, embryo and uterine levels is prerequisite for optimal design of adequate protocols that allow improved fertility results for procedures such as AI, cryopreservation and/or embryo transfer.

Currently, due to the use of ´omics techniques, the identification of reliable biomarkers to determine sperm function and fertility seems to be more likely in the near future. The economic impact of an increase in farrowing rates but particularly in live litter size that could result from selecting boars according to the expression of certain proteins, would have a great impact on pig productivity. Similarly, these potential biomarkers could be used as tools for improving current sperm preservation procedures. Future studies based on the recent and continuous advances in ´omics techniques will contribute to the identification of reliable biomarkers that can be used as fertility indicators and additives to improve the yields derived from the application of different assisted reproductive technologies in pigs.

Regarding embryo technologies, the possibilities of an increase in the productive and reproductive parameters derived from ET procedures, by applying strategies based in molecular indicators, would hugely contribute to its broad implementation. This would, in fact, accelerate genetic improvement and improve animal welfare. A greater knowledge of the impact of the different components of SP on the creation of a favorable uterine environment for pregnancy establishment is critical to improve the performance of embryonic technologies in pigs. In this review, we showed that SP infusions during estrus affected the transcriptional expression profile of the endometrium and preimplantation embryos during early pregnancy, by positively influencing the expression of genes and pathways associated with embryonic early development and facilitating achievement of the state of tolerance by the maternal immune system. Obviously, potential effects of SP infusions on the outcomes of ET programs need to be further explored.

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Author contributions

IP: Conceptualization, Writing – original draft, Supervision, Visualization, Validation, Writing – review & editing; EAM: Conceptualization, Writing – original draft, Supervision, Visualization, Validation, Writing – review & editing; MAG: Validation; CC: Validation; JR: Validation; HRM: Visualization, Validation, Writing – review & editing; CAM: Conceptualization, Writing – original draft, Supervision, Visualization, Validation, Writing – review & editing.

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A review of simulation analyses of economics and genetics for the use of in-vitro produced embryos and artificial insemination in dairy herds

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Abstract

The use of in-vitro produced (IVP) embryo transfer (ET) in dairy herds is growing fast. Much of this growth is on dairy farms where the focus is on milk production and not on selling breeding stock. The value of implementing IVP-ET in a dairy herd arises from a higher genetic merit of the IVP-embryo, but the cost to produce a pregnancy with an IVP embryo is greater than the cost of artificial insemination (AI). The first objective of this study was to review estimates of the net benefit of using IVP-ET over AI in dairy herds using existing literature. Another objective was to show how much IVP-ET use in a herd is optimal. Most of the literature is based on simulation modeling, including our own work that focuses on the dairy industry in the USA. We found that the most profitable use of AI and IVP-ET is often a combination of the two. More IVP-ET should be used when the value of surplus calves is greater and the cost of IVP-ET is lower, among many other factors. In the future, use of IVP-ET will be further improved by more accurately identifying superior donors and recipients, reducing the generation interval, and achieving greater efficiency in embryo production.

Keywords: embryo transfer, dairy, profitability, genetic lag.

Introduction

Artificial insemination (AI) and in-vitro produced (IVP)-embryos for embryo transfer (ET) are two reproductive technologies that result in genetic gain by propagating offspring from animals with greater genetic merit. The International Embryo Technology Society reported that more than 1 million embryos were produced in-vitro in countries reporting for 2018 (Viana, 2019). Of these, 49% were in North America, 44% in South America, and 6% in Europe. One report states that the combination of IVP-ET with sexed semen and genomic selection is now being successfully and widely used in North America, South America and Europe (Ferré et al., 2020). We will focus here on the USA because of our greater familiarity with its dairy industry.

In the USA, the National Association of Animal Breeders (NAAB, 2020) reported that 22,026,290 units of domestic and imported dairy semen were sold in the U.S. during 2018. Natural service accounts for approximately a quarter of all dairy breedings. Natural service accounts for approximately a quarter of all dairy breedings. The number of transferable IVP embryos of dairy breeds produced in North America during 2018 was 311,458, of which approximately 59% were actually transferred (Viana, 2019). Therefore, approximately 0.5% of

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dairy breedings were with IVP embryos in the USA during 2018. Use of IVP-ET is growing fast in North America, however. The number of IVP embryos doubled between 2013 and 2017.

Genetic gain has been accelerating since 2010 when genomic testing became widely used to select service sires. The 5-yr moving average rate of genetic gain in predicted transmitting ability (PTA) for the economic selection index Lifetime Net Merit (NM$) is now greater than $70 per year for sires born between 2013 and 2017 (CDCB, 2019). This rate of genetic gain was just $28 per year for sires born between 2003 and 2007. Dairy farms that use only AI make genetic gain in their herds because of genetic gain in marketed AI sires. The Council on Dairy Cattle Breeding (CDCB) data also show that the genetic merit of cows is less than that of service sires. The difference is constant as long as the rate of genetic gain in service sires is constant. Genetic merit of cows lags behind the genetic merit of service sires.

Value of the level of genetic merit in a dairy herd should be based on the difference (genetic lag) in genetic merit between the average cow in the herd and the best available sires (the genetic nucleus; Dechow and Rogers, 2018). This genetic lag is an opportunity cost: each cow consists of "old" sire genetics. For example, when only AI is used and no selection occurs within the herd, the average cow in the herd may be 3.5 yr old. If we assume an annual increase of $50 per year in PTA for NM$, then service sires 3.5 yr ago had a $175 lesser PTA than today's service sires. The genetic merit of a cow, however, can be thought to consist of 50% her sire + 25% her dam's sire + 12.5% her grand dam's sire + 6.25% of her great grand dam's sire, etc. If the generation interval stays the same between generations, then the genetic lag of the average cow in the herd with the genetic nucleus would be $350 PTA of NM$ (200% × 3.5 yr × $50). This is a doubling (200%) of the genetic lag of the first generation. The genetic lag increases with a greater rate of genetic gain in service sires. If the annual increase in PTA of NM$ is $70 per year, then the genetic lag between the average cow and the genetic nucleus is $490 PTA of NM$ (200% × 3.5 × $70). This math is a simplification of reality, but illustrates the important principle of genetic lag. Results are also herd-dependent.

Selection of superior females in the herd reduces the genetic lag with service sires. For example, use of female sexed semen in younger animals or selection of surplus heifer calves based on genomic test results, produces dairy calves that are on average better than the average unselected dairy calf from the herd. The result is a decrease in the genetic lag with the best available service sires. Use of IVP-ET can greatly decrease this genetic lag as will be illustrated later. Use of technologies such as AI, sexed semen, IVP-ET, and selection of surplus animals all contribute to a reduction in genetic lag.

A greater rate of genetic gain means differences in genetic merit resulting from age become greater. In other words, the difference in genetic merit of the best heifers in the herd compared with the genetic merit of the average cow in the herd is becoming greater when the rate of genetic gain is greater. As a result, capturing and propagating the best genetics in the herd is becoming more valuable.

From the perspective of a typical herd, the genetic merit of available service sires is a given factor that cannot be controlled. When the rate of genetic gain of service sires is constant over time, and the reproduction and selection program for females in the herd are constant over time, it follows that use of technologies like IVP-ET in a herd does not accelerate the rate of genetic gain. They do not increase the annual change as is often thought. It does reduce, however, genetic lag with service sires compared with use of AI.

What is the opportunity cost of genetic lag? Again using simple math, a genetic lag of $350 PTA of NM$ is equivalent to a genetic lag of $700 estimated breeding value (EBV) of NM$ (2 × $350 because EBV = 2 × PTA). We use EBV to express the genetic merit of the female herself, whereas PTA is the genetic merit transmitted to her offspring). The $700 is expressed per lifetime, which is 2.8 lactations, or approximately 3 yr (VanRaden et al., 2018). Thus, the opportunity cost of this genetic lag of $350 PTA of NM$ is $700 ÷ 3 = $233 per cow per year. Using a program that would reduce the genetic lag by $50 is worth approximately 2 × $50 ÷ 3 = $33 per cow per year. One dollar reduction in genetic lag is worth $0.67 per cow per year (simplified). This math does not include any discounting for time value of money, differences in actual lifespan, phenotypic response to selection, and assumes that NM$ is the ideal measure of profitability.

The toolbox of technologies such as AI, sexed semen, beef semen, IVP-ET, genetic evaluations, genomic testing, and fertility programs all affect genetic lag. In addition, these technologies have various direct costs and may affect the phenotypic performance of the herd, such as conception rate. For example, the cost to produce a pregnancy with an IVP embryo is much greater than the cost to produce a pregnancy with AI, but the genetic lag using IVP-ET is smaller. The first objective of this study was to estimate the net benefit of using IVP-ET over AI, which is not immediately clear. Another objective was to show how much IVP-ET use in a herd is optimal, if not to create 100% of pregnancies. The goal of this paper is to provide some insight into these questions.

Transfer of IVP embryos can also improve conception rates in herds with low fertility due to heat stress (Stewart et al., 2011). This type of IVP-ET typically uses oocytes from culled cows. Such oocytes are of average genetic merit because every cow is eventually culled, independent of genetic merit. The average culled cow is approximately 5 years old, so the genetic lag is actually a little greater than the genetic lag with the average cow in the herd. The use of IVP-ET to increase conception rates is not a means to increase genetic merit and we will not further discuss this application here.

General principles of an IVP-ET program

An IVP-ET program consists of three components: 1) selection of an appropriate ovum pick-up (OPU) protocol; 2) selection of donors; and 3) selection of recipients. Ovum pick up (egg or oocyte collection) is the transvaginal retrieval of oocytes from ovaries of donor females (Hansen, 2017) often after a hormonal treatment. These oocytes are then matured and fertilized in the laboratory resulting in the production of in-vitro embryos. Approximately 1-wk-old embryos are then transferred into nonpregnant recipients and this procedure may result in pregnancies. Typically, donors have reached puberty, but commercial interest in oocyte collection from prepubertal animals is increasing (Moore and Hasler, 2017). Oocytes also can be collected from animals that are up to 4 mo pregnant (Hansen, 2017). The efficiencies of IVP-ET programs vary, but a reasonable number is four transferable embryos per one OPU occurring every 14 d.

Candidate recipients are non-pregnant animals that have a high likelihood of bringing the transferred embryo to term and produce a live calf. Recipients must be approximately on d 7 of their estrous cycle when an embryo is transferred. High fertility, low risk of abortion, and stillbirth are important selection criteria for recipients because of the high cost of IVP embryos. On the other hand, recipients should be animals of relatively lesser genetic merit because they forego the gestation of their own calf. Foregoing the production of their own calf is an opportunity cost. An opportunity cost is the loss of potential gain from other alternatives when one alternative is chosen. Genomic testing also helps more accurately identify recipients.

Donors are those animals eligible for OPU and those that will generate embryos with the greatest genetic merit. Donors should also be free of transmittable disease. To identify such donors, it is useful to rank candidates using a genetic selection index, such as the PTA for NM$. Reliability of PTA based on traditional parent averages (dam and sire of the candidate donor) is low, especially for young animals (≤ 35%; Weigel, 2011). Low reliabilities imply that the difference between PTA (what we know) and true transmitting ability (what it is) of genetic merit of a trait can be large, which might result in selection of donors of low genetic merit. Therefore, genomic testing with much greater reliability (≥ 70%) is routinely used to identify candidate donors and give more certainty that donors with high true transmitting abilities are selected.

Figure 1 shows genomic PTA of NM$ for 1,247 animals at the University of Florida Dairy Unit. The genetic evaluation was made in 2017. Animals range from a few weeks after birth to more than 2,800 d after birth. The animals were impregnated by conventional and sexed semen, but not IVP-ET. Figure 1 shows a typical distribution of PTA of NM$ as can be found in many herds. Younger animals have greater genomic PTA of NM$ than older cows, but variation exists within the same age. The top young heifers have genomic PTA of close to $800, whereas the average genomic PTA of cows that are 2,500 d old is approximately $0. The genetic trend in these data is approximately $70 PTA per year. This is a greater rate of genetic gain than that in the sires

of these females at the time when the females were conceived. The greater rate occurs because of more emphasis on sire selection in the last 4 yr. Therefore, the genetic lag is being reduced. If IVP-ET was to be used in this herd in 2017, 1-yr-old donors would have genomic PTA of approximately $600.

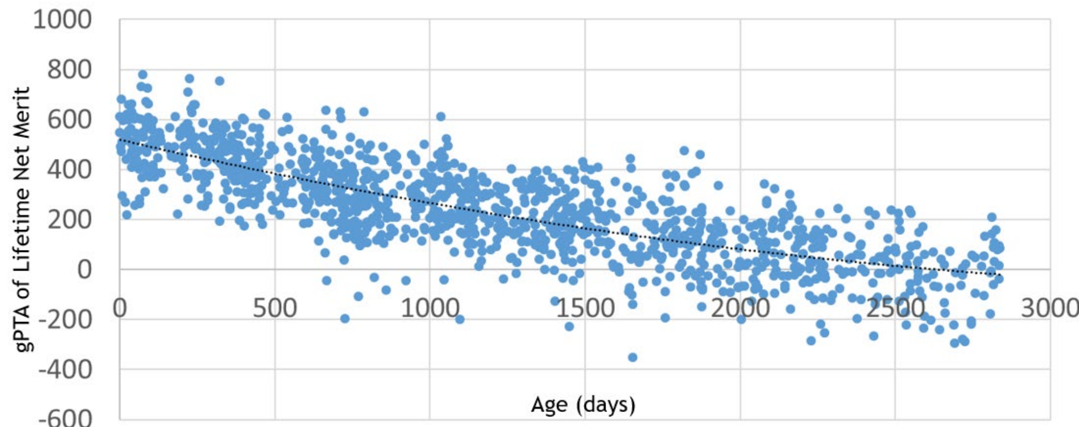

Figure 1. Genomic predicted transmitting abilities (gPTA) of Lifetime Net Merit by age for 1,247 animals at the University of Florida Dairy Unit (2017).

The breeder's equation (Lush, 1937) predicts change in a trait resulting from selection using a simple statistical model. The four factors that determine genetic change per unit of time are genetic variation, selection intensity, accuracy of selection, and generation interval. An IVP-ET program has a high selection intensity because a small number of genetically superior animals provide many calves for the next generation. It also has a short generation interval because donors are typically young (heifers). Use of genomic testing for both the selection of donors and recipients increases the accuracy (square root of reliability).

In vitro-produced embryos for ET allows for rapid multiplication of the best genetics in the herd, but is also more expensive than AI. There is often an economically optimal amount of IVP-ET to be used, depending on, for example, the value of the calves, cost of the IVP-ET procedure, accuracy of identifying the best dams, and alternative options such as sexed and beef semen. Only a few studies are available that looked at the economics of the use of IVP-ET in dairy herds.

Economics and Genetic Lag of IVP-ET vs. AI Programs

Ribeiro et al. (2012) calculated a difference in the cost of a female pregnancy to be $329 more for IVP-ET than for AI using sexed semen. This study did not include the value of differences in genetic merit, however. In Denmark, Thomasen et al. (2016) reported that the greatest increase in economic value of genetic gain in a closed population was obtained when juvenile IVP-ET was used along with genomic selection in the bull-dam part of the population. Combining IVP-ET with genomic testing was profitable in almost all evaluated scenarios when the cost of producing a calf (future sire) by IVP-ET ranged from $500 to $1,500. This study therefore looked at the whole population, including the production of service sires. These authors did not study the cost of IVP-ET to improve the female performance in a closed herd. Recently, Sanches et al. (2019) concluded that IVF is becoming an economically viable practice after they reviewed the current use of IVF by large-scale dairy programs.

Several years ago, we built and validated a detailed simulation model that mimics the genetic, technical, and financial performance of a dairy herd over time (Kaniyamattam et al., 2016). The purpose was to investigate how a herd would respond over time to the use of

various assisted reproductive technologies such as AI and IVP-ET and genetic selection strategies. We wanted to do this as realistically as possible.

In our model, a dairy herd consisted of individual cows and heifers. Each animal had 12 genetically correlated traits that were present in the 2014 NM$ index, such as milk yield, daughter pregnancy rate (DPR), and productive life. An animal's performance (milk yield, fertility, risk of forced culling etc.) was the result of her true breeding value (TBV) for each trait, and permanent and environmental effects. Animals also had EBV that were correlated with the TBV for each trait, depending on the reliabilities of the EBV.

Service sires were not part of the herd and followed a genetic trend of $76 PTA of NM$ per year. Therefore, matings with eligible heifers and cows resulted in calves that had PTA depending on those of the dam and the sire and Mendelian sampling (random variation). Heifer calves that were raised likely became cows. Cows already in the herd had a daily risk of culling. Over time, the herd improved genetically as matings with genetically improved sires produced superior dairy calves. The herd, consisting of individual animals, was followed daily and technical results (such as conception rate, milk yield, average TBV, etc.) and financial results (such as milk sales, profitability) were collected for 20 yr into future?

The following general settings were used to study the economics and genetic performance of various AI and IVP-ET strategies: Annual cow cull rate was set at 34% and the herd had 1,000 milking cows. All dairy heifer calves were genomically tested, which gave high reliabilities and therefore high correlations between EBV and TBV. When more dairy heifer calves were born than were needed to replace culled cows, young heifers were ranked based on the EBV of the trait of interest (often NM$) and heifers with the least desirable EBV were sold. Consequently, retained dairy heifers had more desirable TBV on average than unselected dairy heifer calves (similar to analytic results in Weigel et al., 2012) and the genetic lag with the service sires was decreased. This also resulted in greater profitability.

The herd started with using only AI for the first 5 yr. The first two inseminations in the top 50% of heifers were done with sexed semen. All other inseminations were done with conventional semen. After 5 yr, the IVP-ET program was implemented (Kaniyamattam et al., 2017, 2018) and all or some pregnancies were made with IVP embryos. Next, the herd was followed for another 15 yr.

The performance of an IVP-ET system depends on many factors. We assumed that 4.25 transferable embryos were produced per OPU, independent of the age of the donor. Donors for OPU were selected based on rankings for the desirable EBV (e.g., high NM$). The time between OPU of the same donor was 2 wk. Heifer donors could be collected for a maximum of 4 times between 11 mo of age and start of the breeding period. Once a heifer was confirmed pregnant (from AI), she was eligible for 3 more collections. Cows were eligible for a maximum of five collections. Embryos harvested at d 7 after conception were transferred to recipients on d 6, 7 or 8 of the estrous cycle. Recipients were selected based on reverse ranking for the trait of interest (e.g., low NM$), so that the lowest ranked animals had the first chance to receive a randomly chosen IVP embryo.

We assumed that the conception rates was similar for AI and IVP-ET. Conception rates depended on TBV and environmental effects for the traits, DPR and cow conception rate, as well as parity and breeding number. Risk of abortion and stillbirth was at least twice as high in calves made by IVP as from AI. A recent review on post-transfer consequences of IVP embryos in cattle revealed lower conception rates compared with AI (Ealy et al., 2019).

First study: exclusive Use of IVP-ET or AI

In the first study (Kaniyamattam et al., 2017), we compared four scenarios with exclusive AI use with four scenarios with exclusive IVP-ET use (100% of pregnancies from IVP-ET). Selections of donors and surplus heifer calves were based on one of four selection criteria: EBV of either milk yield, DPR, or NM$, or random selection. Both AI and IVP-ET scenarios produced surplus dairy heifer calves. The lowest ranking surplus calves based on EBV were sold after genomic testing at an age of approximately 3.5 mo. Surplus calves were either sold at $500 each

(3 to 4 mo old), or in case of IVP calves at a higher price that included a premium based on the EBV of NM$. The idea here was that surplus IVP calves had greater genetic merit and may be worth more than surplus calves from AI when sold. Cost of production and transfer of one IVP embryo was set at $165. For the IVP-ET scenarios, the top 2% of females were selected as donors. Half of the donors produced oocytes in 1 wk, whereas the other half was not involved in oocyte collection that week. Oocytes were fertilized with female sexed semen.

Figure 2 shows the average TBV (2 × PTA) of NM$ in sires and cows from year -4 to +15 after implementation of the 8 scenarios in year 1. The genetic lag between sires and the average cow in the herd before year 1 was approximately $500 PTA of NM$ when no selection among females occurred and only AI was used. The genetic lag started to decrease after year 3 when the first cows started to produce that were conceived after selection criteria were implemented.

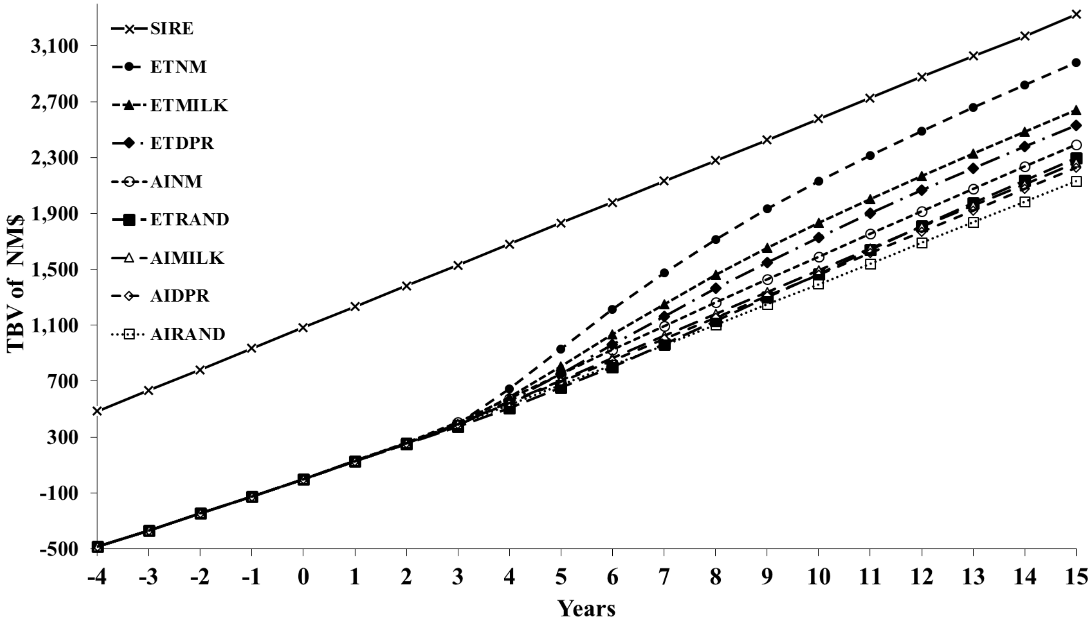

Figure 2. Average true breeding values (TBV) of Lifetime Net Merit (NM$) in sires (SIRE) and cows from year -4 to +15 for 8 scenarios. Name of the scenarios for cows: AI = exclusive artificial insemination program, ET = exclusive in-vitro produced embryo transfer program. Eligible animals were ranked either randomly (RAND) or based on their estimated breeding value of NM$, milk yield (MILK) or daughter pregnancy rate (DPR; Kaniyamattam et al., 2017). Each scenario for cows is a combination of program (AI or ET) and ranking method (NM, MILK, DPR, RAND).

The scenarios using IVP-ET and selection of females based on NM$, milk, and DPR all reduced the genetic lag more than the scenario based on AI with selection on NM$. The IVP-ET scenario based on NM$ reduced the genetic lag to $150 PTA of NM$ (= $300 TBV in Figure 2). This constant genetic lag with the service sires was reached approximately in year 13 after the first use of IVP-ET. Thus, from year 3 to year 13 the genetic gain in the females was greater than that in the service sires, but this was the result of moving from the old genetic lag of $500 PTA of NM$ to the new genetic lag of $150 PTA of NM$. In year 15, the AI scenarios produced approximately 30% surplus dairy heifer calves and the IVP-ET scenarios approximately 54% surplus after years of genetic improvement in reproductive traits. This was only 8% in year 0.

Figure 3 shows profit per cow per year. Change in profitability over time is the combined result of increases in genetic merit and cost of implementing the IVP program from year 1 on. We assumed that there is no inflation. Profitability of the IVP-ET scenarios decreased immediately after year 0 because of the high cost of making IVP embryos. The increased genetic merit of these embryos did not start to pay back until these embryos had become cows (and a little bit as better young stock with improved heifer conception rate).

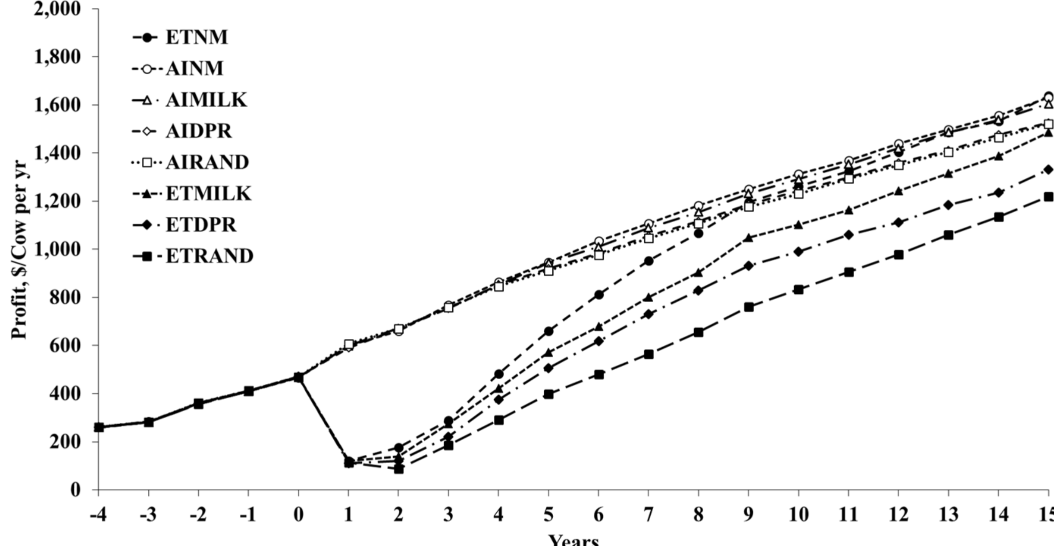

Figure 3. Profit per cow per year from year -4 to +15 for 8 scenarios. Premium pricing of surplus heifer calves is assumed. Name of the scenarios: AI = exclusive artificial insemination program, ET = exclusive in-vitro produced embryo transfer (IVP-ET) program. Eligible animals were ranked either randomly (RAND) or based on their estimated breeding value of Lifetime Net Merit (NM$), milk yield (MILK) or daughter pregnancy rate (DPR). The profit of the 4 IVP-ET scenarios decreases rapidly after the start of the IVP-ET program in year 1 because the embryo transfer cost are greater than the AI cost (Kaniyamattam et al., 2017). Each scenario for cows is a combination of program (AI or ET) and ranking method (NM, MILK, DPR, RAND).

By year 9, the AI and IVP scenarios with selection based on NM$ started to have similar profitability and by year 15 they differed only by $8 per cow per year (Figure 3; advantage IVP scenario) when the greater surplus calf prices for IVP calves were included. In year 15, the break-even price for an IVP embryo was $168 per transfer, so it was very similar to the input price of $165 (Kaniyamattam et al., 2017).

The AI scenarios were more profitable than the IVP-ET scenarios when the surplus calves were sold for the same price, independent of their genetic merit. With selection on NM$, the break-even price for an IVP embryo was $89. This low break-even price is below current market prices for IVP-ET. The advantage of the AI scenario was $185 per cow per year. The 3 other AI scenarios with selection only on milk yield, DPR, or random selection resulted in greater advantages of AI over the IVP scenarios.

The large decrease in profit per cow in year 1 for IVP-ET program was the result of an immediate transition from AI to IVP-ET where costs were assigned as soon as embryos were transferred. A more gradual use of IVP-ET (< 100%) would avoid this large sudden decrease in profitability, but also delay the reduction in genetic lag and delay in future profitability. This first study showed that 100% IVP-ET programs were typically less profitable than 100% AI programs, even though the genetic lag with service sires was much reduced by the IVP-ET programs.

Second study: mixed use of IVP-ET and AI

In the second study (Kaniyamattam et al., 2018), we varied the fraction of pregnancies made with IVP-ET from 0% to 100% with intervals of approximately 20%. The best amount of IVP-ET could be less than 100% of pregnancies because the donors would be more superior (fewer are needed) and genetically good animals (that are not donors) would carry their own calves instead of carrying slightly superior but much more expensive calves from IVP-ET. In addition, avoiding recipients that have low conception rates after embryo transfer might be beneficial.

As expected, Figure 4 shows that the genetic lag with the service sires decreases with greater use of IVP-ET. The rate of decrease in the lag was greatest when IVP-ET use is small. In other words, the more IVP-ET was used, the less the genetic lag changed. It took

approximately 10 yr to transition from the old constant genetic lag based on AI only to the new constant genetic lag based on some use of IVP-ET.

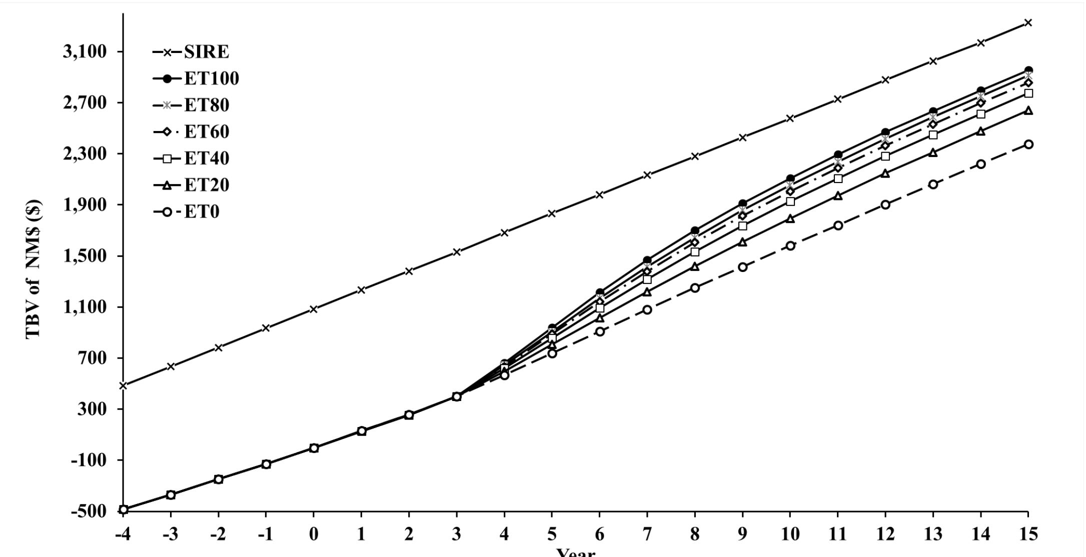

Figure 4. Average true breeding values (TBV) of Lifetime Net Merit (NM$) in sires (SIRE) and cows in year -4 to +15, in scenarios which used in-vitro produced embryo transfer (IVP-ET) to obtain varying proportions of conceptions from IVP-ET: 0% (ET0), 20% (ET20), 40% (ET40), 60% (ET60), 80% (ET80) and 100% (ET100) (Kaniyamattam et al., 2018).

Table 1 shows the results for combinations in: (1) surplus base heifer calf price; (2) premium paid for genetically better surplus heifer calves; (3) IVP-ET price; and (4) the fraction pregnancies from IVP-ET (3 × 2 × 4 × 6 = 144 combinations). As expected, more IVP-ET use was optimal with a greater surplus base heifer calf price, a premium paid for surplus heifer calves, and a lower IVP-ET price. For 6 of the 24 combinations in prices, the 100% IVP-ET program was optimal. All had embryo prices of $100 or less and required a premium paid for genetically better surplus heifer calves. Differences between 0% IVP and 100% IVP could be hundreds of dollars per cow per year when embryo transfer prices were low. When the use of IVP-ET was somewhere in the middle, profitability increased by tens of dollars per cow per year compared with no IVP-ET use or 100% IVP-ET use for the same price assumptions. Figure 5 shows the trend in profitability over time for four scenarios.

Table 1. Sensitivity analysis for 6 surplus dairy heifer calf prices and 4 embryo prices and the optimal proportion of conceptions to be achieved by in-vitro produced embryo transfer (IVP-ET) such that profit per cow is maximized (Kaniyamattam et al., 2018).

Dairy heifer calf sale price[1]		Embryo Price	ET Conceptions (%)[2]						Optimal ET[4%]	Max. Add. Profit[5] ($)
Base price	Premium		0%	21%	42%	63%	82%	100%		
			Additional profit per cow in year 15 ($)[3]							
300	NO	50	0	64	90	80	61	47	46%	91
300	NO	100	0	39	42	7	-36	-75	33%	45
300	NO	150	0	13	-6	-66	-133	-197	19%	14
300	NO	200	0	-12	-55	-139	-231	-319	3%	0
300	YES	50	0	91	155	187	209	241	100%	241
300	YES	100	0	66	107	114	112	119	100%	119
300	YES	150	0	41	58	41	15	-3	42%	58
300	YES	200	0	16	10	-32	-82	-124	28%	18
500	NO	50	35	107	148	150	144	142	69%	158
500	NO	100	35	82	99	77	47	21	41%	99

Table 1. Continued...

Dairy heifer calf sale price[1]		Embryo Price	ET Conceptions (%)[2]						Optimal ET[4%]	Max. Add. Profit[5] ($)
Base price	Premium		0%	21%	42%	63%	82%	100%		
500	NO	150	35	57	51	4	-50	-101	28%	59
500	NO	200	35	32	3	-69	-147	-223	8%	37
500	YES	50	35	135	213	258	293	337	100%	337
500	YES	100	35	110	164	185	196	215	100%	215
500	YES	150	35	84	116	112	99	93	62%	116
500	YES	200	35	59	67	39	2	-29	36%	69
700	NO	50	70	150	205	221	228	238	84%	238
700	NO	100	70	125	157	187	190	116	73%	199
700	NO	150	70	100	108	75	33	-5	36%	110
700	NO	200	70	75	60	2	-64	-127	12%	77
700	YES	50	70	178	270	328	376	432	100%	432
700	YES	100	70	153	222	255	279	311	100%	311
700	YES	150	70	128	173	182	182	189	79%	191
700	YES	200	70	103	125	109	85	67	25%	129

[1]Base female calf sale prices of $300, $500 or $700 at 105 days of age. The dairy heifer calf rearing cost since birth at 105 days was $375; [2]Scenario and actual proportion of pregnancies from IVP-ET: ET0 (0%), ET20 (21%), ET40 (42%), ET60 (63%), ET80 (82%), ET100 (100%); [3]Additional profit per cow in year 15 for varying proportions of conceptions from IVP-ET compared to the scenario with no conceptions from IVP-ET (ET0); [4]The economically optimal proportion of conceptions obtained from IVP-ET; [5]The maximum additional profit per cow per year at the optimal proportion of conceptions from IVP-ET compared to the scenario with no conceptions from IVP-ET.

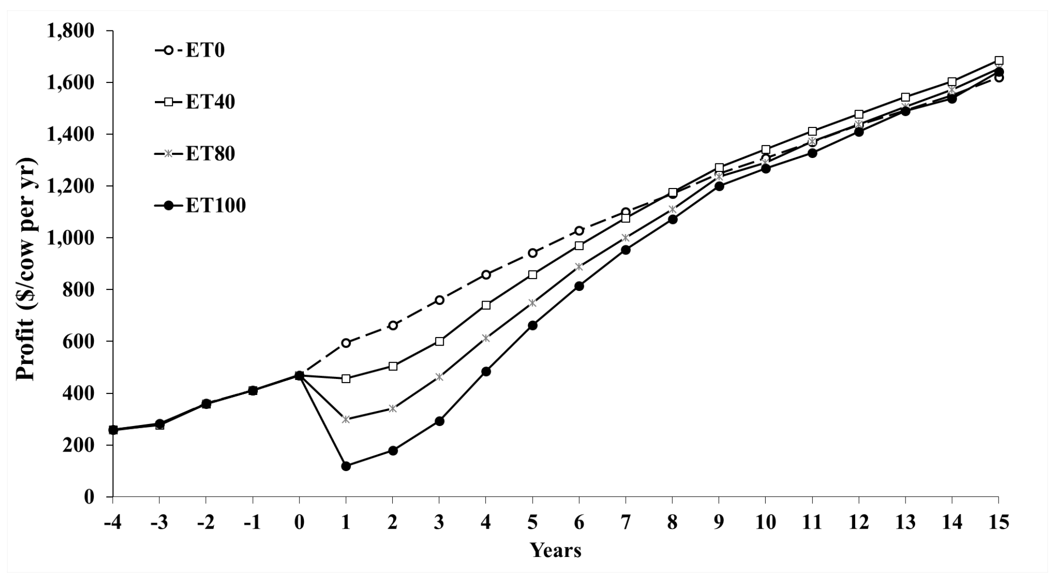

Figure 5. Profit per cow per year in year -4 to +15, in scenarios that used in-vitro produced embryo transfer (IVP-ET) to obtain varying proportions of conceptions from IVP-ET: 0% (ET0), 40% (ET40), 80% (ET80) and 100% (ET100) The cost of the fresh embryo was $165 and the sale price of a 3.5-mo old surplus dairy heifer calf was $500 in addition to a premium price calculated based on the difference of the estimated breeding value of Lifetime Net Merit of sold dairy heifer calves from the IVP-ET scenario compared to the ET0 scenario (Kaniyamattam et al., 2018).

In this second study, the selection of donors was all based on rankings for PTA for NM$ after genomic testing. We did not assume any prior knowledge about the ability of donors to produce transferable embryos. Various factors that determine the production of transferable embryos for an animal are heritable. In one study, heritability estimates for IVP factors in a sample 628 IVP-ET records ranged from 1% to 21%, but were not significantly different from zero (Parker Gaddis et al., 2017). Better understanding of factors that affect the production of transferable embryos should lead to fine-tuning of donor selection.

Further, in this second study we prioritized non-pregnant, non-donor heifers as first eligible to receive IVP embryos. The rationale was that these heifers had greater conception rates than candidate cow recipients and that this was important because of the high IVP-ET prices ($50 to $200). On the other hand, recipient heifers pregnant after IVP-ET have greater opportunity costs of not carrying their own calf compared with recipient cows. Among recipient cows, we gave the highest priority to cows with high PTA for DPR and high PTA for cow conception rate. These cows were expected to have the greatest conception rates, but might not have the lowest PTA for NM$. Again, opportunity cost for the value of their own calf was not considered in selection of cow recipients.

Recipients were selected on the same day the donors were selected. We also ranked candidate recipients independently of their stage in the estrous cycle and looked for estrus daily in the simulation model. If estrus was observed in a selected recipient, the animal was scheduled to receive an IVP embryo on day 6, 7, or 8 after estrus, depending on availability of a fresh embryo. Use of estrus detection instead of estrus synchronization resulted likely in a less than ideal use of candidate recipients, but also at lower direct costs. All eligible animals which were not selected as recipients received AI.

We also assumed that the expected phenotypic performance of calves born from IVP or AI was on average the same if they had the same genetic merit. This may not be the case in practice. For example, in one study, mortality of IVP calves produced by reverse female-sorted semen was greater than in calves produced by AI (Siqueira et al., 2017). Calves born from IVP-ET also have greater risk of large offspring syndrome, which may increase incidences of dystocia and retained placenta (Bonilla et al., 2014). Stillbirths and calf deaths also may increase in IVP calves (Bonilla et al., 2014).

In summary, selection of recipients could be improved by better integration of all factors that determine the profitability of an IVP-ET program. These factors include conception rate, abortion, still birth, value of the IVP-ET calf once born, and the foregone value of the recipient's own calf. An index that integrates these factors is not too difficult to put together.

Market prices for calves and the value of increased genetic merit fluctuates unpredictably over time. Therefore, the decisions regarding the use of IVP-ET may turn out to be not optimal. A risk analysis with variations in prices over time may result in a policy that is most robust under uncertainty in future prices.

Outlook

In these two studies by Kaniyamattam et al. (2017, 2018), we assumed that all IVP embryos were made with female sexed semen, and some sexed semen was used in AI for heifers. Consequently, we had a surplus of dairy heifer calves being born and we used genomic testing to help select and sell surplus dairy heifers calves. All dairy bull calves were sold too.

Alternatively, an economically better strategy may be to use AI with beef semen so that crossbred calves are made. Market prices for crossbred calves are approximately $100 greater than those for marketed dairy calves. In contrast, this strategy would lead to fewer surplus dairy heifer calves, and would limit the genetic gain in retained dairy calves because the heifer selection intensity would be lower. A good strategy might involve a combination of IVP-ET, and AI with sexed semen, beef semen, and even conventional semen (Weigel, 2019). We are currently working to identify such promising strategies.

The USDA's NM$ is a general economic selection index that is useful for a wide range of herds. Other economic selection indexes may be more appropriate in certain markets, such as the Fluid Merit, Cheese Merit and Grazing Merit (VanRaden et al., 2018). A reformulation of the components of the NM$ index using financial investments methods has led to two new economic selection indexes that cause some reranking of service sires (Schmitt et al., 2019). In theory, these new indexes are better at identifying most profitable donors and recipients too.

Further reduction in the generation interval will increase the rate of genetic gain in a nucleus population, for example, in the production of service sires. In-vitro breeding is an emerging technique that greatly reduces the generation interval. It also combines genomic selection with

derivation of embryonic stem cells and in-vitro differentiation of germ cells from pluripotent stem cells (Goszczynski et al., 2019). With this technique, the generation interval can be reduced to 3 to 4 mo. This technique may be soon within reach (Goszczynski et al., 2019).

Individual dairy farms that rely on marketed service sires to produce IVP embryos will continue to have a rate of genetic gain that in steady state will be the same as that of the service sires. Improvements in the ranking of donors and recipients, as outlined above, and improved efficiencies and reduced costs will strengthen the economic viability of IVP-ET programs. IVP-ET programs will become more economically competitive with AI programs and eventually they might become clearly more profitable. The best use of IVP-ET on commercial dairy farms remains an interesting puzzle with many variable factors. The modeling approach could also be extended to include in-vivo production of embryos acknowledging differences in costs, fertility, embryonic deaths and production of embryos.

Acknowledgements

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Author contributions

ADV: Conceptualization, Funding acquisition, Formal analysis, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing; KK: Data curation, Formal analysis, Methodology, Software, Validation, Writing – review & editing.

SHORT COMMUNICATIONS

Short Oral Communications Competition

HH5 double-carrier bovine embryos show impaired development through elongation

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Keywords: Embryo development, conceptus elongation, haplotype, dairy cows, mitochondria.

Genomics analyses in dairy cows have uncovered deleterious haplotypes (alleles) never found in homozygosity at birth and thereby causing pre-term mortality. Between these deleterious haplotypes, Holstein Haplotype 5 (HH5) consists of a large 138 kb deletion causing the ablation of the gene *TFB1M* (Transcription Factor B1, Mitochondrial). Unfortunately, the developmental period when double carrier embryos (DC, i.e., *TFB1M*-null embryos, homozygous for HH5) arrest their development is unknown. Given that the impact of pregnancy loss varies greatly from early losses (before maternal recognition of pregnancy) to later miscarriages, such information is crucial to evaluate the economic losses associated to the inadvertent cross of single carrier (SC, heterozygous) individuals. To solve that question, we have analysed the development of Day 14 conceptuses collected from 3 superovulated SC cows inseminated with a SC bull. Conceptus were recovered by uterine flushing, fixed in 4 % paraformaldehyde and subjected to immunohistochemistry to analyse trophectoderm (CDX2), hypoblast (GATA6) and epiblast (SOX2) development. Following fluorescence microscopy analysis, samples were genotyped by PCR. Mendelian inheritance of the allele was observed in the 25 conceptuses retrieved (5:16:7 for WT:SC:DC). Hypoblast migration was observed in all conceptuses, but a significant impairment in the development of the extra-embryonic membranes (hypoblast and trophectoderm) was evident in DC embryos. The development of such membranes is the main responsible of the increase in conceptus length and the change in shape during elongation. DC conceptuses remained spherical, in contrast to the ovoid or elongated WT (non-carrier) or SC conceptuses, and were significantly smaller (26.2±8.5 vs. 30.5±4.9 vs. 0.7±0.1 mm for WT, SC and DC, respectively, mean±s.e.m., ANOVA $p<0.05$). Embryonic disc was formed in all DC embryos, but its diameter was also significantly reduced compared to WT or SC embryos (319±40 vs. 396±27 vs. 223±13 µm for WT, SC and DC, respectively, mean±s.e.m. ANOVA $p<0.05$). In conclusion, the bovine HH5 DC embryos analyzed arrested their development prior to early conceptus elongation and maternal recognition of pregnancy. Supported by StG-757886 from ERC and PID2020-117501RB-I00 from MINECO.

Preimplantation genetic testing for aneuploidy (PGT-A) reveals a high incidence of chromosomal errors in in vivo and in vitro pig embryos

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Keywords: Ploidy, porcine, cytogenetics

Chromosome errors in embryos can lead to implantation failure, spontaneous abortions, and birth defects; as such, characterising their incidence is of interest not just in humans but also in domestic animals where embryo production is employed for breeding. Recent studies have found an aneuploidy incidence between 14-24% in cattle embryos; however, information on other domestic species is lacking. Here, we present for the first time a characterisation of chromosome errors in both *in vivo* derived (IVD) and *in vitro* produced (IVP) porcine embryos, using single nucleotide polymorphism based PGT-A. Five sows were inseminated and culled at day 4, 5 or 6 of the oestrous cycle (D0 = onset of oestrous) to collect IVD embryos at different development stages by flushing of the distal portion of the uterine horn. Additionally, IVP blastocysts were produced from 10 sows during three fertilisation rounds. For all embryos, the zona pellucida was removed using 0.5% pronase and the samples were stored at -80 °C. Whole genome amplification was performed by using the REPLI-g Advanced DNA Single Cell Kit (Qiagen, Oslo, Norway), and genotyping was completed on a custom Illumina GeneSeek 25K SNP chip (Lincoln, NE, United States). PGT-A diagnosis were obtained by combining Log R ratio (LRR) and B-allele frequency (BAF) graphs to detect copy number variations, and Karyomapping to trace the parental origin of chromosomal errors (maternal or paternal), and to detect triploidy and uniparental disomy. Proportions between groups were analysed by Fisher's exact test and the threshold for statistical significance was set as $p \leq 0.05$. In IVD embryos, the overall incidence of chromosomal errors was 32% (32/101). Although not significantly different ($p > 0.05$), fewer errors were detected at the blastocyst stage as compared to earlier stages: 40% in 4 cell (10/25), 35% in 6-12 cells and morulae (7/20 and 12/34) and 14% in blastocysts (3/22). Conversely, IVP blastocysts showed an 80% incidence for chromosomal errors (n = 51/64). Even with the low sample size achieved, this provides the indication that IVP blastocysts suffer from a higher incidence of chromosomal errors as compared to IVD blastocysts ($p < 0.001$). Triploidy was the most common chromosomal error in IVD embryos (16%, 16/101), followed by whole chromosome errors (10%, 10/101). Surprisingly, two parthenogenetic embryos and one androgenetic embryo were also identified in the IVD embryos. In IVP blastocysts, parthenogenesis affected one in three embryos (21/64). The parthenogenetic embryos arose from just three sows, across two different IVP rounds, suggesting a possible individual effect. The incidence of triploidy in IVP blastocysts was 25% (16/64), and errors arising from either polyspermy (12/16) or meiotic non-disjunction in the oocyte (4/16) were both detected. Errors with a maternal origin were prevalent in IVP embryos (41/57, $p < 0.01$), whereas IVD embryos presented a similar incidence of errors from either parent (17/36 maternal, $p > 0.05$). In conclusion, PGT-A discovered a high incidence of aneuploidy and triploidy in IVD and IVP embryos, suggesting that the future application of this technology might improve embryo transfer success in the pig.

Bovine embryos lacking progesterone receptor (PGR) develop normally through early elongation

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Keywords: CRISPR, progesterone, embryo development, conceptus elongation

Endogenous maternal progesterone levels and exogenous progesterone supplementation have been positively linked with conceptus length and embryo survival in cattle. However, the mechanism by which progesterone enhances conceptus development is unknown. Progesterone may act directly on the embryo or indirectly, by promoting changes in uterine fluid composition favoring conceptus growth. To discriminate between both possibilities, we have analyzed the development of bovine embryos lacking progesterone receptor (PGR) generated by CRISPR technology. To that aim, we have microinjected *in vitro* matured oocytes with mRNA encoding for Cas9 alone (control group, C, solely composed by WT embryos) or combined with sgRNA against *PGR* (C+G group, partially composed by KO embryos). Following fertilization and culture up to Day 7 (D7) blastocyst, subsequent development was analyzed *in vitro* and *in vivo*, assessing lineage development by immunostaining for CDX2 (trophectoderm), SOX17 (hypoblast) and SOX2 (epiblast), and conducting genotyping by miSeq. *In vitro* development from D7 to D12 was similar between groups (85.4±5.7 vs. 81.3±6.3, mean±s.e.m. for C and C+G, respectively, t-test $p>0.05$). In C+G group, 22/45 D12 embryos analyzed were KO (i.e., contained only KO alleles). Embryo diameter at D12 was not affected by embryo genotype (772±74 vs. 648±64 vs. 731±44 µm, mean±s.e.m. for WT, edited non-KO and KO, respectively, ANOVA $p>0.05$), and the proportion of embryos attaining complete hypoblast migration was similar in WT (23/32, 72 %), edited non-KO (12/20, 60 %) and KO (19/22, 86 %) embryos (Chi-square $p>0.05$). No differences were noted either on embryonic disc (ED) formation rate (20/32 63 % vs. 6/20 30 % vs. 10/22 45 % for WT, edited non-KO and KO, respectively Chi-square $p>0.05$). To assess *in vivo* development, 40 blastocysts from C+G group were transferred to two synchronized recipient ewes. Pregnancy was supported by exogenous progesterone (CIDR-Ovis) and conceptuses were recovered 9 days after embryo transfer at a developmental stage equivalent to day 14 (E14). All intact conceptuses recovered from recipient ewes were edited by CRISPR (23/23) and 10/23 were KO. Conceptus growth was not affected by PGR ablation (1.3±0.3 vs. 3.7±1.3 cm, mean±s.e.m. for edited non-KO and KO, respectively, Two-Way ANOVA $p>0.05$) and all conceptuses showed hypoblast migration. Embryonic disc was present in 7/10 (70 %) KO and 12/13 (92 %) edited non-KO conceptuses and embryonic disc size was not affected by the ablation (333±72 vs. 234±43 µm, mean±s.e.m. for KO and edited non-KO, respectively, Two-Way ANOVA $p>0.05$). In conclusion, the ablation of PGR does not impair embryo development up to E14, suggesting that progesterone-mediated conceptus growth enhancement is indirectly mediated by triggering changes in the uterus. Supported by StG-757886 from ERC and PID2020-117501RB-I00 from MINECO.

First evidence of nanoplastic uptake by the maturing oocyte

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Keywords: micro- and nanoplastics, oocyte, bovine embryo model

Plastic pollution has become a growing environmental problem as the result of its widespread use and improper treatment of plastic waste. Over time, plastics that leaked into nature will degrade into smaller particles including micro- and nano plastics (MNPs). MNPs can enter the human body through ingestion, inhalation, and dermal contact (Parta et al., Sci Total Environ, 702:134455, 2020). Previous studies have shown that MNPs can induce an immune response and can cause a neurologic, oxidative, and toxicity response in somatic cells, including those of the reproductive tract in both mammals and aquatic organisms (Xie et al., Ecotoxicol Environ Saf, 190:110133, 2020; Liu et al., J Hazard Mater, 424:127629, 2022; Nie et al., Nanotoxicology, 15;885, 2021). However, the impact of MNPs on oocyte and embryonic development remains largely unknown. Furthermore, it is unknown whether oocytes take up MNPs from the environment.
In this study, the uptake of MNPs by developing bovine cumulus-oocyte-complexes (COCs) and their effects on development have been studied. Given the large similarities in reproduction and early development during early embryonic development between human and cow, the bovine model is an excellent model to study human oocyte and early embryo development (Sirard, In: Animal Models and Human Reproduction, 127-144, 2017; Eds: Constantinescu & Schatten; Wiley & Sons Inc.; ISBN:9781118881606). COCs, collected from slaughterhouse ovaries, were exposed to our standard maturation condition without, control condition (NaHCO3-buffered M199 supplemented, with 100 IU/ml Penicillin-streptomycin, 0.05 IU/mL FSH, 0.1 μM cysteamine, and 10 ng/mL EGF) or with green-fluorescent polystyrene (PS) nanoplastics (Polysciences, Inc., Hirschberg an der Bergstrasse, Germany) of 50nm or 200nm (10 μg/mL) during the 23 h *in vitro* maturation (39˚C, 5% CO2 in air). After maturation, COCs were stained with Hoechst (DNA) and phalloidin (actin), to score the nuclear stage of matured oocytes (one-way ANOVA), and uptake of MNPs, respectively.
Confocal microscopy showed that MNPs of 200nm were only taken up by some cumulus cells, while MNPs of 50nm were taken up by cumulus cells and oocytes. In total, 351 oocytes in 3 replicates were analysed, there was no difference between the nuclear maturation status after exposure to MNPs of 200nm (59.9%) and 50nm (46.5%) during maturation, in comparison to control oocytes (60.8%). In conclusion, bovine oocytes are able to take up 50nm PS particles while cumulus cells are able to take up both 50 and 200 nm PS particles. However, the current experiment did not show an effect of the uptake of plastics on the nuclear stage of the oocyte after maturation. Future studies need to unravel whether the uptake of MNPs may impact oocyte competence.

Microplastics have a negative effect on sperm and oocytes in vitro

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Keywords: microplastics, infertility, reproduction, polystyrene

An annual increase in global plastic production, in addition to poor waste management, represents a massive contamination of the environment. These plastics pollutants can break down to small particles, and when fragmented to a size smaller than 5 mm, are called microplastics (MPs). A decline in fertility was already pointed out by WHO as a major health issue and, considering the lack of studies relating MPs and reproduction, for better understanding of its effects, more studies are necessary. Therefore, the present study aimed to investigate if MPs can affect gametes in vitro. Bovine frozen semen (N = 4 bulls) were thawed and submitted to swim-up separation. 5x106 sperms/mL were then incubated with a range of sizes of polystyrene (PS) beads (SURF-CAL™ particle size standards): 0.05, 0.1, 0.3, and 1.1 µm (1.2 million beads/mL), and CellRox (2 µL/mL) using FERT-TALP for 2 hours at 38.5ºC, 5% CO2, 95% O2. Every 30 minutes an aliquot was collected, checked for motility (N=9 replicates), spread in a SuperFrost slide and fixed with 4% paraformaldehyde (PFA). Fixed samples were stained with Hoechst33342 and FITC-PNA, to check acrosome integrity (FITC-PNA, N=4), bead attachment (N=3 replicates) and oxidative stress (CellRox; N=2 replicates) by fluorescence microscopy. Oocytes were isolated from bovine ovaries by follicle aspiration and only those with a homogeneous cytoplasm and at least three layers of cumulus cells were selected. Oocytes were randomly assigned to 3 groups: (1) 0.3 µm PS beads (1.2 million beads/mL; N=46); (2) 1.1 µm PS beads (1.2 million beads/mL; N=64), and (3) control (N=52) no beads, and matured for 24h at 38.5ºC, 5% CO2, 95% O2; three replicates were performed. COCs were then washed, denuded by pipetting, fixed in 4% PFA, and stained with Hoechst33342 (5 µg/mL) for nuclear stage checking. Oocytes were classified as mature (MT), degenerating (DG), or broken zona pellucida (BZP). All data was analyzed for normality using the Shapiro-Wilk test, a two-way ANOVA was used and the differences checked using Tukey HSD. Both 0.3 and 1.1 µm beads attached to the sperm surface in 6.9±4.8 and 2.1±18% of sperm counted, respectively. Even though a small attachment of beads was identified, they did not affect sperm motility ($p>0.05$). However, sperm incubated with 1.1 µm beads had reduced acrosome integrity at 2h compared to the control (32.3±6.5 vs 59.1±20.5, respectively, $p=0.001$). The results for oxidative stress indicate a small increase of ROS production on sperm incubated with MPs, but no significant differences were detected and more replicates need to be done. Oocytes matured in the presence of MPs had a reduced maturation rate (67.5±12.5%, 41.9 ± 18.1 and 37.6 ±20.0%, for control, 0.3 and 1.1 µm, respectively). It was also shown that MPs promoted an increase in oocytes with BZP (2.6±4.4%, 17.8 ± 3.4 and 23.4 ±11.4%, for control, 0.3 and 1.1 µm, respectively; $p=0.03$). Proteomics of oocytes is currently in progress to better understand the molecular mechanisms by which MPs are damaging oocytes. We have shown, for the first time, that PS MPs exert a negative effect on both male and female gametes in vitro, demonstrating that MPs should be treated as concerning environment toxicants.

TAI/FTET/AI

Alpha-linolenic acid alleviates the detrimental effect of lipopolysaccharides during in vitro ovine oocyte development

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Keywords: lipopolysaccharide, alpha-linolenic acid, oocyte

Administration of LPS in the culture medium detrimentally affected oocyte maturation and reduced cleavage, and embryo formation by orchestrating an inflammatory response (DOI: 10.1016/j.theriogenology.2020.07.034. Epub 2020 Aug 1). On the other hand, certain types of fatty acids such as n-3 fatty acids showed beneficial effects on oocyte developmental competence (DOI: 10.1007/s10815-015-0439-9. Epub 2015 Feb 26). It has been reported that alpha-linolenic acid (ALA) reverses the LPS-induced effects on developmental competence. Hence, the current study was designed to evaluate the impacts of the addition of ALA to the maturation medium to modulate the detrimental effect of LPS on ovine oocyte developmental competence in vitro.

Cumulus-oocyte complexes were matured in vitro in presence of BSA and different concentrations of ALA (0, 10, 50, 100, and 200 µmol $ml^{-1;}$ diluted using ethanol) either in the absence (Negative Control) or presence (Positive Control) of 1 µg ml^{-1} LPS. The concentration of LPS was the lowest level of LPS that negatively affected oocyte developmental competence; as revealed from our previous study. Following maturation in vitro, the oocytes were fertilized, and the presumptive zygotes were cultured in vitro. Rates of cleavage and blastocyst formation were recorded out of cultured oocytes and compared between the ALA-treated and the Control groups (10 replications were performed during the experiment). The logistic regression analysis was conducted to determine the association between the dependent variable. The strength of the association was estimated by an odds ratio measure.

We observed an improvement ($P<0.05$) in the proportion of cleaved oocytes in 10 (81.8 %; n=117/143), 50 (77.9 %; n=116/149), and 100 (79.2 %; n=118/149) µmol ml^{-1} ALA groups as compared to the Positive Control (57.8 %; n=85/147), however, such an improvement was not significant when 200 (68.5 %; n=85/124) µmol ml^{-1} ALA group compared with the Positive Control ($P \geq 0.05$), as discussed in the above refrence/study from our group. The Negative Control produced more cleaved oocytes (77.1 %; n=121/157) as compared with the Positive Control ($P<0.05$).

Our data showed ALA can improve the blastocyst formation in presence of LPS. We observed that 10 (37.8 %; n=54/143), 50 (35.6 %; n=53/149), and 100 (36.9 %; n=55/149) µmol ml^{-1} of ALA produced more blastocyst ($P<0.05$) as compared with the Positive Control (21.8 %; n=32/147); no differences ($P \geq 0.05$) were observed when ALA groups compared with the Negative Control (35.7 %; n=56/157). Nevertheless, the addition of 200 µmol ml^{-1} ALA in presence of (1 µg ml^{-1}) LPS reduced the rate of blastocyst formation (15.3 %; n=19/124) as compared to both Negative and Positive Control groups ($P<0.05$).

ALA potential for preventing LPS-induced effects seems to be presented at concentrations up to 100 µmol ml^{-1} and further enhancement of ALA concentration wouldn't be beneficial. The results from the current study demonstrate that ALA has the potential to mitigate the LPS deleterious effects on oocyte developmental competence.

Can the hyaluronan-binding assay be used to evaluate the fertilizing capacity of dromedary camel spermatozoa?

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Keywords: dromedary camel spermatozoa, hyaluronan binding assay, goat oocyte penetration test

A practical technique to predict dromedary camel sperm fertilizing capability is needed. A hyaluronan binding assay (HBA) is available to predict *in vivo* fertility of human sperm samples and for sperm selection for intracytoplasmic sperm injection. However, it is not known if dromedary spermatozoa can bind to hyaluronan. The objective of this study was to assess the potential of the HBA or computer assisted sperm analysis (CASA) kinematics to predict the fertilizing capacity of dromedary camel spermatozoa. Two semen samples were collected from each of six males at the Camel Reproduction Center, Dubai, and were prepared for cryopreservation (Malo et al., Cryobiology 2017;74:141-147). All animal procedures were approved by the Animal Care and Use Committee (ACUC) of the Camel Reproduction Centre, United Arab Emirates. Aliquots of prepared semen were used for HBA using the Sperm Hyaluronan Binding Assay kit (Cooper Surgical, Denmark) before freezing and after thawing; CASA was made at the same time using a CEROS II® analyser (Hamilton Thorne; MA; USA). The fertilizing ability of thawed spermatozoa was determined using a sperm penetration assay (SPA) with goat oocytes, harvested from ovaries obtained from a local slaughterhouse (Malo et al., Reproduction in Domestic Animals 2017; 52: 1097-1103). Penetration rate (PEN), male pro-nucleus formation (PN) and number of spermatozoa penetrated per oocyte (SP/OC) were evaluated. Note: goat oocytes were used in a zona-free binding assay because of the scarcity of camel oocytes. This is a routine procedure in our laboratory for assessing sperm functionality. Dromedary camel spermatozoa bound to hyaluronan with no differences between males; mean PEN 46.07 ± 4.7%, mean PN 22.81 ± 5.3%, and mean SP/OC 1.70 ± 0.4. Of the CASA parameters in fresh sperm samples, only progressive motility and Straightness correlated with HBA ($r = 0.65$, $P = 0.02$; $r = 0.69$, $p=0.01$ respectively). There was no correlation between CASA parameters and HBA for post-thaw samples. In the SPA, dromedary camel sperm bound, penetrated, decondensed, and formed a pro-nucleus in goat oocytes. There was no correlation between HBA for fresh spermatozoa and SP/OC post-thaw ($r = 0.65$; $p = 0.11$), although there was a correlation between the fresh sperm HBA result and post-thaw PEN ($r = 0.81$, $p = 0.03$) as well as with PN ($r = 0.8$, $p = 0.03$). In conclusion, the HBA score for fresh dromedary camel spermatozoa may predict post-thaw IVF performance, but further investigation is needed, such as expanding to study to include sperm samples of known quality (poor, medium and good).

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Niacin supplementation during oocyte maturation improves bovine in vitro fertilization rate after ICSI

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Keywords: niacin, ICSI, bovine

Intracytoplasmic sperm injection (ICSI) has effectively been used in both human and animal fertility laboratories. Reactive oxygen species production above its critical levels has been shown to deterimentally affect the oocyte competence. Optimal condition for the in vitro oocyte maturation is one critical step for the occurrence of normal fertilization. We hypothesized that the addition of nicotinic acid (niacin), a potent antioxidant, to the oocyte maturation media can improve the oocyte fertilization during ICSI. Therefore, the present experiment was designed to assess the effects of niacin treatment during in vitro maturation on the fertilization rates of the bovine oocytes after ICSI procedure. Bovine ovaries were collected from the local abattoir and then transported to the IVF laboratory in less than 2 hours. Then, good and excellent quality cumulus-oocytes complexes (COCs) were recovered and randomly divided in four groups: in group 1 (control), the COCs were matured and then fertilized (n=168) in a standard maturation medium (Azari et al., Veterinary Research Communication. 41, 49–56, 2017). In group 2, the COCs were matured in a standard maturation medium with no niacin and then fertilized (n=85) using a standard ICSI procedure (Ashibe et al., Theriogenology, 133, 71-78, 2019) under an inverted microscope (Olympus, IX71, Japan). In group 3, the COCs were matured in a standard maturation medium supplemented with 1mM niacin and then were fertilized (n=88) using a standard ICSI procedure as performed in group 2. A semen with high fertility was used in all replicates of IVF and ICSI (n=5). The motile spermatozoa were separated using a swim-up method. In group 4, the COCs were matured in a standard maturation medium with no niacin and then were chemically activated using calcium ionophore A23187 and ethanol 7% with no sperm injection (n=38) for the parthenogenetic division (Bevacqua et al., Theriogenology, 74, 922-931, 2010). Oocytes maturation protocol was basically similar in all groups by using TCM-199 medium supplemented with 10% FCS, 5 IU/mL hCG (Karma, Germany), 10 ng/ml EGF (Sigma, USA), and 0.1 IU/ml human FSH (Follitrope, South Korea). In all maturation media, 50µg/ml Gentamicin (Sigma, USA) was also added. Groups of 30–50 COCs were cultured for 24 h in a 500 µl culture media at 38.5°C in 5% CO_2. Assessment of oocyte fertilizzation was performed using aceto-orcein staining method. The percentage of normal fertilization rates among the groups were compared using chi-square test. The percentage of normally fertilized oocytes in group 1 (control IVF group) was higher than that of the group 2 (ICSI with no niacin) (59.6 vs. 39.0%, $p<0.05$). However, there was no difference in the percentage of normally fertilized oocytes when the ICSI procedure was performed on the matured oocytes supplemented with niacin (group 3) as compared to group 1 (50.9 vs. 59.6%, $p>0.05$). The percentage of fertilized oocytes in group 4 (the chemically activated oocytes) was lower (8.%) than those of the other experimental groups ($p<0.05$). The results of present study demonstrated that the addition of niacin to the maturation culture media can increase fertilization rate of bovine oocytes after ICSI.

Pregnancy rates and parameters of an in vitro embryo production program between buffaloes and cattle in Colombia and Argentina

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Keywords: IVPE, buffaloes, comparison, baseline

Buffalo produce high quality milk and beef, most breeders use maternal models for breeding, implementing embryo transfer programs is a need due to the nature of the production system,. Establishing a baseline to set improvement objectives in the technique is necessary. This research compares the parameters and pregnancy rates in an *in-vitro* production program (IVEP) from cattle and buffaloes in two different Latin American countries. Data from an IVEP from 2012-2019 from 261 buffaloes (crossbred buffaloes, Murrah and Mediterranean) and 61 cattle (Brahman) were included in the analysis. The number of follicles, viable oocytes, cleaved oocytes, and blastocysts was recorded. Data from Colombia and Argentina were registered. Comparisons were performed using the Mann-Whitney Test. P< 0.05 was considered statistically significant, data were expressed as mean±s.d. Cattle embryos were considered as controls for buffalo. There are no statiscal differences (*P= 0,212)* in the number of total antral follicles observed between cattle 9.69±5.03 and buffaloes 8.70±6.62. It has been observed that the presence of a Corpus Luteum is significative for the number of viable oocytes (*P*< 0,001) compared to the presence of a dominant follicle (p=0.333) at the OPU. The number of viable oocytes, cleavage, blastocyst and pregnancy rate from buffalo and cattle was 8.00±5.91 vs 17.98±15,24, 4.12±3.89 vs 11.68±11.66, 1.31±1.79 vs 4.66±4.86, 26% vs 34% respectively (*P* < 0,05). Other authors have reported the differences between cattle and buffaloes in embryo production, low embryo yield and oocyte quality but very few try to discuss about the differences. The good results obtained in cattle as controls and the results from buffaloes show that the buffalo embryo production needs a careful review, of other aspects mainly related to oocyte quality, competence and the clinical conditions (health, ciclicity) of the animals used for the programs. Despite showing the feasibility of the implementation of the IVP-ET in buffaloes, the observed differences between buffalo and cattle demonstrate the need to gain a better understanding of the reproductive biology of the buffalo.

Follicular and systemic levels of IL-6, lipid metabolites, and oxidative stress index during the non-breeding season in mares

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Keywords: follicular fluid metabolism, maternal health, OPU/ICSI

The application of trans-vaginal OPU and ICSI is well est ablished for the commercial equine IVP. These assisted reproductive techniques are especially applied during the non-breeding season of the mare. Interestingly, no research data are available concerning the biochemical composition of the follicular fluid (FF) in small and medium-sized follicles routinely aspirated during OPU and how this is correlated with the serum composition. This study aimed to measure the FF concentrations of interleukin-6 (IL-6), total cholesterol (CHOL), triglycerides (TG), non-esterified fatty acids (NEFA), reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), and oxidative stress index (OSI) in relation to follicle size, and to investigate a possible association with their systemic concentrations during the OPU-ICSI (non-breeding) season in mares. These parameters are representing the maternal inflammatory, metabolic, and oxidative stress status. At slaughterhouse, serum (n=12) and FF of small (SF; 5-10 mm in diameter, n=10), medium (MF; >10-20 mm in diameter, n=11), and large (LF; >20-30 mm in diameter, n=4) follicles were sampled from 12 apparently healthy mares. An enzymatic-colorimetric assay was used (CHOL2 and TRIGL kits; Roche Diagnostics, and NEFA F5 Kit; DiSys Diagnostic Systems, Germany) to assess the concentrations of CHOL, NEFA, and TG. For IL-6 estimation, equine kit (Nori® , Genorise Scientific, USA) was used. Concentrations of d-ROMs and BAP were identified using photometric Diacron® kits (Diacron International, Italy). One-way ANOVA was used to compare means between the different follicle categories, while t-test was conducted to compare between the systemic and follicular concentrations of each variable per follicle category. Pearson correlation coefficients were tested. P value <0.05 was considered significant. Concentrations of all variables did not show significant differences between follicle classes. Concentration of IL-6 did not differ significantly between serum (60.1±1.9 pg/mL), SF (72.8±9.5 pg/mL), MF (65.4±3.5 pg/mL), and LF (60.2±0.6 pg/mL). Concentrations of CHOL, TG, and NEFA, respectively were higher in serum (96.0±5.9 mg/dL, 45.2±6.1 mg/dL and 12.1±2.1 mg/dL) compared to SF (67.0±7.9 mg/dL, 19.3±1.9 mg/dL and 5.3±0.4) and MF (46.3±2.6 mg/dL, 15.5±1.3 mg/dL and 6.1±0.5). Concentrations of CHOL were higher in serum compared to LF (44.7±4.2 mg/dL, 16.7±1.6 mg/dL and 6.8±1.2 mg/dL). Concentrations of d-ROMs in serum (138.7±8.8 Carratelli units; UCARR) were markedly higher than SF (57.6±7.1 UCARR), MF (67.2±9.0 UCARR), and LF (42.0±11.2 UCARR). The higher concentration of BAP in serum (5571.6±619.9 µmol/L) was only significant when compared to MF (2521.8±464.4 µmol/L), but not to SF (4658.0±1116.1 µmol/L) and LF (2244.6±563.9 µmol/L). There was a strong positive association (r=0.8, P <0.01) between levels of IL-6 in serum and MF. Also, values of CHOL, d-ROMs, and OSI in serum were positively correlated with those measured in MF (P <0.05). Taken together, follicular size is not associated with alterations in the studied biochemical components during the non-breeding season of mares. There is a crosstalk between serum and follicular fluid composition. In addition, changes in the blood composition associated with maternal health (IL-6 and OSI) or diet (CHOL and NEFA) during the commercial OPU-ICSI season in mares may lead to an altered oocytes microenvironment, which may affect oocyte quality. More studies are recommended to check the influence of maternal health on the oocyte developmental capacity and subsequent embryo quality in mares.
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Oocyte recovery and relationship between quality of oocytes and embryo production in zebu and crossbred dairy cattle

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Keywords: oocytes grade, blastocyst, cattle breed

This study conceived to compare oocyte recovery within breeds of cattle [Gir (Zebu), Sahiwal (Zebu) and Holstein-Friesian Crossbred (HFCB)] and to investigate the relationship between quality of oocytes and embryo production in respective breeds. The study was conceived to reveal the outcome of ovum pick-up and *in vitro* embryo production (OPU-IVEP) in these breeds which is yet sparse. A total of 34 OPU sessions (11, 11 and 12, respectively) were performed in 4 Gir, 4 Sahiwal, and 8 HFCB donors, without hormonal stimulation. OPU-IVEP was performed according to Patel (2020). Before IVM, oocytes were graded as graded according to presence of layers of cumulus cells and homogeneity of ooplasm as described by Viana *et al.* (2004). Blastocyst rate was calculated by dividing the total no. of blastocysts (produced on day 7 & 8) by total no. of oocytes in IVC. The evaluation of stage and grade of embryos were performed as per IETS guidelines (Manual of the IETS, 4th Edition). All the media used for OPU-IVEP were from IVF Bioscience, UK. Sperm separation medium was from FUJIFILM Irvine Scientific, USA. Descriptive statistics were used to calculate the oocyte recovery per OPU, cleavage rate and blastocyst rate in different breeds and overall means were represented as Mean±SEM. Means were compared between different breeds using one-way ANOVA using GLM. All the analyses were performed using SigmaPlot 11. During the 34 OPU sessions, a total of 148, 248 and 301 oocytes (n=697) were recovered from Gir, Sahiwal and HFCB donors, respectively. Overall, significantly ($p<0.05$) higher percentage of Grade 3 (27.7±4.5) and Grade 4 (38.8±5.6) oocytes were recovered than Grade 1 (6.5±1.4) and Grade 2 (19.3±3.9) oocytes. A total no. of embryos produced after IVEP (n=222, blastocysts rate across breed-35.5%) were 48, 60 and 114 in Gir, Sahiwal and HFCB, respectively. The average no. of oocytes recovered per OPU (overall average 20.5±1.9) in Gir, Sahiwal and HFCB were 13.5±2.3, 22.5±3.2 and 25.1±3.3, respectively. The corresponding cleavage rates observed were 66.5±5.1, 53.0±4.5 and 83.4±2.2%. The average no. of blastocysts per OPU in Gir, Sahiwal and HFCB were 4.4±1.2, 5.4±0.8 and 9.5±1.6, respectively. In all three parameters, values were significantly higher in HFCB compared to Gir ($p<0.05$). Combined no. of oocytes in Grade 3 and Grade 4 had positive correlation with cleavage rate ($r=0.3$) and blastocyst rate ($r=0.4$, $p<0.05$). **In conclusion,** significantly higher no. of oocytes recovered per OPU, cleavage rate and no. of blastocysts per OPU were attained in HFCB than in Gir. If the oocytes of grades 3 and 4 are processed together with the oocytes of higher grades, there is a chance to produce blastocysts also from the oocytes of lower grades. This is important in the zebu donors in which the total no. of oocytes harvested is less than in the HFCB donors.

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Optimizations of an ovum pick up protocol in Donkey.

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Keywords: Donkey. OPU, oocyte maturation

The Donkey is an animal classified as an endangered species, so the cryopreservation of oocytes would allow the creation of a germplasm bank that allows the preservation of its genetic material and the preservation of the species. The main process for obtaining oocytes in live females is Ovum pick up (OPU). This technique is widely used and established in horses, however, in Donkey, it is little used, so it is necessary to standardize an OPU protocol to obtain viable oocytes for cryopreservation. For this reason, the objective of our work is to standardize an OPU protocol in Donkey. To do this, first, a suitable sedation protocol was established during follicular aspiration. 4 different protocols were tested: Acepromazine 0.04 mg/kg + Xylazine 1 mg/kg; Detomidine 0.5mg/kg + Butorphanol 0.5mg/kg; Medetomidine 0.0035 mg/kg + Butorphanol 0.025 mg/kg and Acepromazine 0.02 mg/kg + Xylazine 0.44 mg/kg + Butorphanol 0.02 mg/kg. To check the best protocol, different stress hormones were measured before and after the OPUs, cortisol, C-reactive protein, lactate dehydrogenase (LDH) and creatinine kinase, choosing the most appropriate protocol that produced less stress and greater animal welfare. Before and after the OPUs, the levels of progesterone (P4) and estrogen (E2) were determined to check the effect of aspiration on follicular activity. Subsequently, the OPU (N=15 aspirations) were performed on a total of 9 donkeys, determining the number of follicles aspirated, number of oocytes retrieved and the number of mature follicles. To optimize the maturation process, two different culture media were tested; 1) DMEN/F-12 and 2) TCM-199; both enriched with 10% FBS + 25μg/ml gentamicin and 5mIU/ml FSH + 50ng/ml IGF-1. The oocytes obtained from each aspirated animal were divided between the 2 treatment groups. The degree of maturation was evaluated by the presence of the polar body after decumulation with hyaluronidase and the expansion of cumulus cells. In addition, viability was evaluated by staining with propidium iodide, as well as DNA integrity, cytoskeleton and mitotic spindle, using the anti-α-tubulin antibody linked to the fluorochrome Alexa fluor-488. Analyses were performed by fluorescence microscopy. The statistical analysis used to compare the different sedation protocols was a two-way ANOVA using the Bonferroni test. The statistical analysis used to compare the values of E2 and P4 in the different conditions was a one-way ANOVA using Tukey's multiple comparison test, in all statistical analysis $p<0.05$ was considered a significant difference. The results obtained indicated that the best sedation protocol was that of 0.5mg/kg detomidine + 0.5mg/kg Butorphanol, since it was the one that least increased stress hormones, this decrease being more significant in the case of cortisol ($p<0.01$) and creatinine kinase ($p<0.05$) with respect to the other treatments. Regarding the levels of P4 and E2, these increased after the OPUs, this increase being much greater in the case of P4 ($p<0.001$) compared to the baseline values. Of all the aspirations performed, a total of 259 follicles were aspirated, of which 178 oocytes (67.93%) were recovered and distributed equally for the two treatments, of all these, 127 matured (68.77%) after 34h of incubation. Of all these mature oocytes, 49 (38.6%) matured with TCM-199 medium and 78 (61.4%) with DMEN/F-12 being this difference significant ($p<0.05$). With DMEN/F-12, greater viability (80%) and greater integrity of the mitotic spindle (71%) were achieved tan TCM-199, Therefore, we can conclude that, with the results shown, DMEN/F12 is the best medium for the culture and maturation of oocytes obtained after OPU.

Comparative study of oocyte recovery and embryo production using OPU- IVP techniques in six indigenous cattle breeds of India

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Keywords: In vitro Fertilization, Ova pick up, embryo

In Vitro Embryo Production (IVP) is still an emerging technology under Indian context. The present experiment was undertaken to determine oocyte recovery rate and embryo production using In-Vitro Fertilization technique (IVF) in six indigenous cattle breeds of India. BAIF Development Research Foundation, Central Research Station, Uruli Kanchan, Pune, Maharashtra, initiated work for IVP and studied the performance of six zebu cattle breeds namely Gir (17 donors), Sahiwal (10 donors), Deoni (02 donors), Dangi (03 donors), Gaolao (05 donors), and Red Kandhari (05 donors). All these experimental donors were maintained under the same managemental practices with the same feeding regime. Data was generated from 901 OPU sessions performed during a study period from October 2019 to January 2022 for IVP. Ovum Pick Up (OPU) procedure was carried out 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 and temperature range maintained of vacuum pump was in between 37 to 38 ^{0}C. OPU was performed without using pre-stimulation protocols for the non-lactating donors. All OPU donors have lactation range from 1 to 4 and their average age ranged from 4 to 10 years. Throughout the study period, these donors were not inseminated to make them pregnant. Media used for IVP were of IVF Bioscience, UK and Vitrogen, Brazil both. Use of media was random for IVP. No separate records were maintained to study the embryo production within the breed using the two different types of media. All the recovered oocytes were further processed in laboratory for IVP with protocol of 20 to 22 hrs of maturation period, 16 to 18 hrs for fertilization and 6 to 7 days post fertilization of culture period for embryo production. The parameters studied were oocyte recovery and embryo production per OPU session.
The best average oocyte recovery resulted in Dangi (9.32 ± 0.75) followed by Gir (8.94 ± 0.36) and Sahiwal (7.15 ± 0.38) breed. In the remaining three breeds, the average oocyte recoveries were 6.91 ± 0.98, 6.74 ± 0.92 and 3.86 ± 0.61 per OPU session in Deoni, Gaolao and Red Kandhari breeds, respectively. In terms of embryo production, Gir breed produced on average 2.66 ± 0.14 embryos per OPU session, followed by 1.96 ± 0.15 in Sahiwal, then 1.90 ± 0.30 in Dangi breed. Results of oocyte recovery and IVP are significantly influenced by the breed ($P< 0.01$). One-way ANOVA and Duncan's multiple range test were used to identify the critical differences among the breeds. All results are depicted as mean ± standard deviation. With the present experiment we can infer that three breeds namely Dangi, Gir and Sahiwal have better performance with regard to oocyte recovery and embryo production as compared to remaining three breeds. Amongst all the six breeds, Red Kandhari cattle breed was a poor performer in terms of oocyte recovery (average 3.86 ± 0.61) and embryo production per OPU session (average 0.92 ± 0.24). It requires more data to conclude the performance of Zebu cattle in respect to OPU and in vitro embryo production under Indian conditions.

The effect of IGFBP-4 on IGF-2 stability in bovine cumulus-oocyte cells during in vitro maturation

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Keywords: IGF-2, IGFBP-4, oocyte

The insulin-like growth factor 2 (IGF-2) is essential for oocyte maturation, cumulus cell steroidogenesis and oocyte viability. Nonetheless, free IGF-2 demonstrated a short lifespan. Six high-affinity binding proteins (IGFBP-1, -6) regulate the biological functions of IGF-2 by prolonging its lifespan and regulating its bioavailability on the target cells. From the IGFBPs, especially IGFBP-4 inhibited IGF-2, and it has been linked with the appearance of follicle atresia. To gain an inside on the regulation of IGFBP-4 on IGF-2 during cumulus-oocyte cells (COCs) maturation, the *in vitro* maturation medium (TCM 199 based, supplemented with 1 mg/ml of fatty acid free bovine serum albumin, /Sigma-Aldrich, Taufkirchen, Germany/; 10 I.U./ml equine chorionic gonadotropin and 5 I.U./ml human chorionic gonadotropin /Suigonan® 80/40 I.U./ml lyophilizate and injection solution, MSD Animal Health, Unterschleissheim, Germany/) was supplemented with recombinant human IGF-2 (rhIGF-2; 50 ng/ml, R&D systems, Bio-techne, Abingdon, United Kingdom) alone or in combination with recombinant bovine IGFBP-4 (rbIGFBP-4; 2,000 ng/ml, InVivo Biotech Services, Hennigsdorf, Germany) in the presence or absence of COCs. Bovine COCs were collected from abattoir-derived ovaries. Groups of 25 COCs were randomly assigned to each experimental group and set to *in vitro* maturation for 24 h at 38°C, 5% CO_2. Three biological repetitions were performed. The IGFBP-4 binding capacity was evaluated by taking samples directly after medium preparation (0 h) and after 3, 6, and 24 h of *in vitro* maturation. Free IGF-2 concentrations were measured using a competitive radioimmunoassay (Mediagnost®, Reutlingen, Germany). The rhIGF-2 was bound to rbIGFBP-4 by 49.8 ± 24.6% directly at medium preparation (0 h), 74.7 ± 21.3% after 3 h, 55.4 ± 33.9% after 6 h, and 44.3 ± 40.5% remained bound after 24 h of incubation. Similarly occurred in the absence of COCs, IGF-2 was bound to IGFBP-4, 49.8 ± 24.6, 72.2 ± 23.5, 48.5 ± 38.1 and 32.5 ± 58.2% at 0, 3, 6, and 24 h respectively. The concentrations of rhIGF-2 added to the medium and incubated in the presence of COCs declined 13.2 ± 8.6, 16.0 ± 10.8 and 77.2 ± 6.2% after 3, 6 h, and 24 h incubation respectively. Contrary, the concentrations of rhIGF-2 without COCs remained stable throughout 24 h. We conclude that rbIGFBP-4 was able to bind rhIGF-2 with a maximum binding capacity around 3 h after incubation and decreased at the final stage of IVM. The binding capacity was not affected by the presence of COCs. Moreover, COCs used the free available rhIGF-2 in the medium. Finally, rhIGF-2 demonstrated to be stable for 24 h under

Folliculogenesis, Oogenesis, and Superovulation

Superovulation efficiency and embryo production by using recombinant FSH (bscrFSH) vs. pituitary-derived FSH (FSH-p) in Brangus heifers

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Keywords: recombinant FSH, embryo production, heifers

During the last decades the use of the conventional pituitary-derived FSH in superovulation (SOV) protocols have been associated with low yield and quality of the obtained embryos. The objective of the present study was to determine whether the stimulation with a single dose of bovine long-acting recombinant FSH (bscrFSH) could achieve an efficient SOV response compared to the conventional pituitary-derived FSH (FSH-p) in Brangus cattle. Sixteen healthy Brangus heifers (BW: 450± 50 kg; BCS: 3.5± 0.5) were randomly distributed into two groups: conventional [FSH-p: FSH from Purified Pig Pituitary Extract (Pluset, Buenos Aires, Argentina); n= 8] vs. recombinant group [bscrFSH: recombinant FSH (Cebitropin B, Concepción, Chile); n= 8]. The conventional SOV protocol was applied as follows: Day 0: intravaginal P4 device (CIDR: 1.38 gr) + 2.5 mg (i.m.) 17-ß Estradiol + 50 mg P4 (i.m.); Day 4: 330 IU FSH-p/12 h intervals/4 d/8 decreasing doses, 60-60, 50-50, 35-35, 20-20, in total 8 injections; Day 6: 5th and 6th FSH-p dose + two PGF2α i.m. doses (500 µg D-cloprostenol each); Day 7: CIDR removal (p.m) at the 8th FSH-p dose application; Day 9: 0.0105 mg (i.m.) Buserelin Acetate (a.m) + AI (p.m.); Day 10: AI (a.m.). Regarding the recombinant group, the same protocol was applied with modifications (150 µg bscrFSH/24 h intervals/4 d/4 decreasing doses, 55-45-30-20, in total 4 injections). Ovarian structures [follicles (FL), corpora lutea (CL), and non-ovulated follicles (NOF)] were monitored by using ultrasonography on Day 8 (estrus; FL number) and Day 15 (embryo collection; CL and NOF number). Morphological embryo classification and quality were performed according to the IETS guidelines. The data were analysed by GLMM (SPSS® 25, USA). No statistical differences were observed between SOV protocols regarding FL, CL, or NOF ($p > 0.05$). Significant differences were observed in the number of total structures collected (15.8±2.9 vs. 6.0±1.3; bscrFSH-derived vs. FSH-p-derived SOV protocol, respectively; $p = 0.01$). Although no significant differences were observed in the number of non-transferable embryos (3.5±0.8 vs. 1.6±0.4; $p=0.07$), significant differences were observed in the number of non-fertilised oocytes (UFOs: 1.8±0.6 vs. 0.5±0.2; $p=0.04$) as well as in the number of degenerated embryos obtained (DE: 1.5±0.5 vs. 1.0±0.3; $p = 0.02$). However, no statistical differences were detected in the number of viable embryos obtained (9.2±2.6 vs. 4.38±1.2) when bscrFSH and FSH-p-derived protocols were compared ($p = 0.08$). In conclusion, the number of total structures, UFOs, and DE increased substantially per donor by using the bscrFSH-derived SOV protocol. Despite of the increase in the number of viable embryos obtained in Brangus heifers by using the bscrFSH-derived SOV protocol no differences were observed between groups. ANID 21201280.

Prolonged application of recombinant FSH (bscrFSH) in superovulation protocols: in vivo embryo production in Bos taurus cows in tropical environments

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Keywords: recombinant FSH, embryo production, cows

Bovine recombinant follicle-stimulating hormone (bscrFSH) has been used very occasionally in *Bos taurus* superovulation (SOV) protocols. The main aim was to study the effects of prolonged bscrFSH application in SOV protocols to test the differential effects on *in vivo* embryo production in *Bos taurus* cows under tropical environments. A total of 10 healthy Charolais cows (age: ~60 mo.; BW: ~750±50 kg; BCS: 3.75-4.0) located in Morona-Santiago province, Ecuador (Köppen-Geiger: Af; Precip.:~1,200 mm; R.H.:~92%; M.T.:~22 ºC latitude: 2°18'22.41"S / longitude: 78°6'55.34"W; altitude: ~1,020 m.a.s.l.) were divided randomly into 2 groups (G1: 4-day bscrFSH (Cebitropin B, Concepción, Chile), and G2: 5-day bscrFSH application; n=5 each). The G1 SOV protocol was applied as follows: Day 0: intravaginal P4 device (CIDR: 1.38 gr) + 2.5 mg intramuscular (i.m.) Estradiol Benzoate E2B + 100 mg P4 (i.m.); Day 4: 180 µg bscrFSH-r/24 h intervals/4 d/4 decreasing doses; Day 6: 3rd bscrFSH dose + two PGF2α i.m. doses (12 h interval/ 500 µg D-cloprostenol each, am/pm); Day 7: CIDR removal at the 4th bscrFSH dose application; Day 8: 0.02 mg GnRH + AI; Day 15: embryo collection. Regarding G2, the same protocol was applied with modifications: Day 0: same; Day 4: 180 µg bscrFSH-r/24 h intervals/5 d/5 decreasing doses; Day 7: 4th bscrFSH dose + two PGF2α i.m. doses (12 h interval/ 500 µg D-cloprostenol each, am/pm); Day 8: CIDR removal at the 5th bscrFSH dose application; Day 9: 0.02 mg GnRH + AI; Day 16: embryo collection. Ovarian-derived traits scored: number of corpora lutea (NCL) and non-ovulated follicles (NOF). Embryo-derived traits scored: total structures (TS), transferable embryos (TE), morulae (M), early blastocysts (EBL), blastocysts (BL), degenerated embryos (DE), unfertilized oocytes (UFOs), and non-transferable structures (NTS). The data were analysed by GLMM (SPSS® 25, USA). Significant differences were observed in EBL (7.75±2.65 vs. 0.75±0.75; $p=0.04$) and BL (2.50±1.55 vs. 0.20±0.20; $p=0.03$) in G2 and G1, respectively ($p<0.05$). Non-significant differences were detected between G1 and G2 SOV protocols when ovarian-derived traits and several embryo-derived parameters (TS, TE, and DE) were compared ($p> 0.05$). However, significant differences were observed in UFOs (5.75±2.90 vs. 2.00±0.90 for G1 and G2, respectively; $p =0.003$) and NTS (7.25±2.92 vs. 3.75±0.75 for G1 and G2, respectively; $p =0.015$) between protocols, being G2 lower in both parameters. In conclusion, no differences were observed regarding ovarian-derived traits between bscrFSH-derived protocols. The G2 protocol was the most efficient for EBL and BL production together with lower values of UFOs and NTS. These differences may be related to a prolonged ovarian stimulation during the application of the G2 SOV protocol in *Bos taurus* cows under tropical conditions. ANID 21201280.

Optimal blood anti-oxidant concentrations at the time of breeding may enhance preovulatory granulosa cell functions after negative energy balance in dairy cows. A transcriptomic insight.

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Keywords: antioxidants, granulosa cell transcriptome, metabolic stress

Early postpartum metabolic stress in high-yielding dairy cow s is strongly linked with reduced fertility. We have recently demonstrated that negative energy balance (NEB) and the increase in non-esterified fatty acids (NEFAs) at week 2 postpartum (pp) are associated with long-term changes in the granulosa cell (GC) transcriptome in the preovulatory follicles at the time of breeding (w8 pp) (Marei et al. 2022, *JDS*). Genes involved in cellular stress and inflammatory responses, and responses to lipids and ketones were upregulated in the GCs of NEB cows compared to those with basal blood NEFAs at w2. In the present study we aimed to evaluate the potential interaction between blood antioxidants (AO) and NEFAs on GC functions. We hypothesized that an optimal AO status may attenuate the long-term effects of NEFAs on the ovarian follicle.
To test this hypothesis, we used our RNAseq database of w8 preovulatory (pre-LH surge) follicle GCs collected from cows with known w2 and w8 blood AOs (ß-Carotene, ßC; Vitamin E, VitE) and NEFA concentrations (n=16). For this study, we selected the cows with above median w2 blood NEFAs (0.78±0.19 mM, n=10). These cows were then split into 2 subgroups (n= 4-6) based on median values of blood ßC and VitE, either at w2 (High AOs: 3.0±0.9 mg/dL ßC and 2.9±0.4 mg/dL VitE *vs.* Low AOs: 1.3±0.3 mg/dL ßC and 1.7±0.3 mg/dL VitE) or at w8 (High AOs: 5.5±1.9 mg/dL ßC and 6.5±1.4 mg/dL VitE *vs.* Low AOs: 2.3±0.6 mg/dL ßC. and 3.0±1.0 mg/dL VitE). The GC transcriptomic profiles of these subgroups were compared using a DESeq2 analysis at each timepoint to determine the differentially expressed genes (DEGs: *P*-adj<0.05, 5% FDR). DEGs were functionally annotated using Bioconductor packages in R.
Only 3 DEGs (3↑, 0↓) could be detected in the w2 comparison, whereas 194 DEGs (48↑, 146↓) were detected in the w8 comparison. The enriched upregulated pathways of the w8 comparison are related to activation of meiosis, MAPK signaling, IGF and EGF receptor signaling, as well as genes involved in fertilization. These are indicators of a better oocyte supportive capacity. High AOs also appear to increase active RNA biosynthetic processes, amino acid and carbohydrate metabolism, and mitotic activity (cell proliferation) as indicated by the upregulated pathways, suggesting better cell viability and follicle quality. The downregulated pathways indicate lower levels of inflammation and cellular stress because genes related to mitochondrial fragmentation, DNA breakdown, sphingomyelin biosynthesis and apoptosis were downregulated.
In conclusion, these results strongly suggest that GCs from follicles exposed to elevated NEFA during their early growth phases exhibit reduced cell stress levels and lower oxidative damage when the final follicle preovulatory development takes place under optimal antioxidant concentrations. In other words, a high blood antioxidant profile in dairy cows at the time of breeding may alleviate, at least in part, the impact of NEB on GC functions.

Effects of the endocrine disruptor ketoconazole on bovine oocyte maturation and blastocyst development

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Keywords: endocrine disruptor, oocyte, bovine

Endocrine disrupting chemicals (EDCs) can negatively affect the reproductive system, as evidenced by studies on animal models and human cell lines (Ratan S. et al., J. Endocrinol, 233(3), 2017). Exposure to such chemicals may cause detrimental effects on female reproductive health, increasing the need to improve regulatory reproductive toxicity assessment (van Duursen M. et al., Int. J. Mol. Sci, 21(3215), 2020). Broadening of the methods currently adopted, for example to include assessment of effects on oocyte maturation and developmental competence acquisition, is needed. Here, we present an assay identifying EDCs eliciting reproductive toxicity based on a bovine model of *in vitro* oocyte maturation and embryo production. To show the applicability of the assay, the known human-relevant endocrine disruptor ketoconazole (KTZ; a CYP450 inhibitor) was used. Endpoints explored include nuclear maturation, cumulus cell expansion, steroidogenesis and blastocyst rate.

Immature cumulus-oocyte complexes (COC) were isolated from ovaries excised from slaughterhouse cows post-mortem. The COCs were *in vitro* matured for 24h in defined culture media, as previously described (Brinkhof B. et al., BMC Gen., 16(1), 2015), and supplemented with KTZ (10^{-8} M, 10^{-7} M, 10^{-6} M; Sigma-Aldrich, Missouri, USA) diluted in DMSO (vehicle; Sigma-Aldrich, Missouri, USA). Oocytes were stained with DAPI to evaluate nuclear maturation based on the presence of an MII plate and a polar body. KTZ- and vehicle-treated oocytes had comparable nuclear maturation rates (ascending KTZ M; 81%±3 ,76%±3, 65%±15 vs 84±1 vehicle-treated, n=486). Similarly, KTZ did not affect cumulus cell expansion (n=725); measured as fold-increase of the projected COC surface and calculated from images acquired pre- and post-IVM. To further investigate cumulus cell function, steroid hormone secretion into the media was quantified by LC/MS. Progesterone, estrone, and 17β-estradiol were present in levels above the detection limit. Progesterone secretion by COCs exposed to 10^{-6} M KTZ was reduced by 88% (733±168 pg/ml vs 6326±1169 pg/ml, one-way ANOVA, $p < 0.001$, n=1050), not observed at lower KTZ doses. To determine effects on developmental competence, exposed COCs were used for IVF and zygotes were cultured *in vitro* (IVC) for 8 days without KTZ. Oocyte exposure to 10^{-8} M KTZ resulted in a reduction of the D8 blastocyst rate (21%±5 vs 31%±3, n=1246, one-way ANOVA, $p < 0.05$), not observed at higher KTZ doses. To assess impact on early embryo development, zygotes produced from non-exposed oocytes were exposed to KTZ in IVC. No effect on D8 blastocyst rate was observed at any KTZ dose (n=991). At least three biological replicates were performed for all endpoints.

In conclusion, exposure of COCs to KTZ impacted developmental competence and steroidogenesis. Oocytes were more sensitive to KTZ than cumulus cells, while embryos were not sensitive to KTZ. In the future, we will be expanding this assay to include more endpoints (e.g. cytoplasmic maturation, blastocyst quality) and verify the validity of the model for a range of EDCs with distinct action.

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Optimization of a superovulation protocol for the collection of in vivo matured oocytes from Holstein Friesian heifers

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Keywords: OPU, oocyte, cattle

In vivo matured oocytes have superior developmental competence to those matured *in vitro*. As such, the study of *in vivo* oocyte maturation supports the refinement of the *in vitro* maturation system. Therefore, this work aimed to optimize a protocol to collect *in vivo* matured oocytes from Holstein Friesian heifers. To do so, animals (n=5) were synchronized using an intravaginal device (CIDR, Zoetis Belgium). Eight days later, the device was removed and heifers received 0.5 mg of cloprostenol (2 ml of Cyclix (prostaglandin; PG) Virbac, France). Estrus (=day 0) was confirmed two days later by ultrasound. To induce ovulation, the heifers were treated with 10 µg of buserelin (2.5 ml of Receptal (GnRH), Intervet, Germany) on day 8 of the estrous cycle. From day 10, the heifers were superovulated with 180 mg of FSH (Folltropin, Vetoquinol, Canada) administered twice daily for four days in decreasing doses (1.5, 1.5, 1.25, 1.25, 1, 1, 0.75 and 0.75 ml respectively). On day 12, animals received 0.75 mg (3 ml) of PG and LH surge was induced with 10 µg (2.5 ml) GnRH administered 40 h after the PG injection. To determine the optimum timing of follicle aspiration, we tested four timing protocols (P1, P2, P3, P4) based on the time-post-GnRH treatment (P1=20h, P2=21.5h, P3=22.5h and P4=24h) at which OPU was performed. Only one animal was used in each protocol, except in P4 which included two heifers. Follicles ≥ 8mm in diameter were aspirated using a 18 gauge needle. A 130 mmHg vacuum pressure was used due to the presence of expanded and sticky cumulus cells characteristic of oocyte maturation. Before follicle aspiration, the OPU tubing system was rinsed with PVP medium (0.3% PVP (PVP-360; Sigma) in Ca- and Mg-free PBS + 10 IU/ml heparin (Sigma)). The follicular content was collected in a 50 ml conical tube and cumulus-oocyte complexes were recovered under a stereomicroscope and stored in EmXcell medium without BSA (imv-technologies, France) until the end of the collection procedure. To assess maturation status, all oocytes (n=45) were denuded in 0.1% hyaluronidase (Sigma) in PVP medium for approximately 3 min and by pipetting until all cumulus cells were removed from the oocyte. Subsequently, the presence of the first polar body (PBI) was evaluated and the denuded oocytes were stored for further molecular analysis. In P1, a total of 8 oocytes were collected and the PBI-extrusion rate was 0%. Similarly, in P2, we collected 3 oocytes and the PBI-extrusion rate was 0%. Interestingly, at 22.5h after GnRH injection (P3), the PBI extrusion rate was 20% (2/10). A greater PBI extrusion rate was observed in P4 where the rate was 37.5% for both heifers (3/8 and 6/16) used in this protocol. The present results suggest that the optimum timing of aspiration of *in vivo* matured oocytes is beyond 24h after GnRH injection. Moreover, the hormonal stimulation treatment we used could be considered an efficient method to collect in vivo matured bovine oocytes. This work also provides insights into using defined and serum-free media when performing non-standard experiments on livestock for further molecular studies on *in vivo* oocyte maturation.

Physiology of Male Reproduction and Semen Technology

Evaluation of Dithiothreitol and Glutathione antioxidants supplemented during cryopreservation of Large White boar semen and subsequent to fertilization of porcine oocytes

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Keywords: dithiothreitol, glutathione, semen

Cryopreservation is the most practical approach for the long-term storage of sperm in boars. However, the freezing and thawing processes result in compromised sperm function and *in vitro* fertilization (IVF) success. The study aimed to evaluate the fertilizing ability of frozen-thawed semen on matured porcine oocytes following IVF. Semen was collected from three Large White boars and then transported to the laboratory for evaluation. Semen was diluted with Beltsville Thawing Solution then equilibrated at 17ºC for 120 min, later centrifuged at 800 x g for 10 min at 15ºC. Semen pellets were resuspended with egg yolk citrate base extender and placed back for 90 min at 5ºC. Semen was supplemented with 4 different fraction B (control, 5 mM DTT, 5 mM GSH and a combination of 2.5 mM DTT + 2.5 mM GSH) extenders and loaded into 0.25 mL freezing straws, then placed in vapour for 20 min, later plunged into nitrogen tank (-196ºC). Semen was thawed at 37ºC then evaluated for sperm motility. Pig ovaries were collected from the local abattoir and transported to the laboratory within an hour in 0.9% saline water in a thermos flask at 38ºC. The slicing method was used to retrieve the oocytes from the ovaries. Oocytes were washed three times in modified Dulbecco phosphate buffered saline and modified Medium 199. Only good quality oocytes were *in vitro* matured for 44 hrs in North California State University-23 medium supplemented with 10 ng/mL of follicle-stimulating hormone, 10 ng/mL of luteinizing hormone and 10% porcine follicular fluid. The oocytes were washed five times in pre-warmed (37ºC) 100 µl of IVF medium drops and then distributed into 50 µl of the IVF drops. A drop of 50 µl of capacitated diluted (1×10^6) fresh and frozen-thawed sperm was used for IVF. Sperm and oocytes were co-incubated at 38.5°C in a moist atmosphere of 5% CO_2 in the air for 6 hrs. The fertilization rate was evaluated by the presence of a pronucleus with the aid of an inverted microscope using Hoechst 33342 staining. Data were analyzed using the GLM procedure. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at a 0.05 level of significance. Percentage data are presented as mean ± standard deviation values. The sperm total motility of frozen-thawed semen ranged from 22.4 to 32.0% for all treatments (P>0.05). The percentage of polyspermy differed significantly among the treatments (P<0.05). The total fertilization rate ranged from 31.9 to 48.7%. Raw semen (11.8±9.4) and combination of 2.5 mM DTT + 2.5 mM GSH (14.1±10.4) treatments recorded higher percentage of polyspermy as compared to the 5 mM GSH (0.8±1.9) and 5 mM DTT treatments (2.2±4.9), (P<0.05). The 5 mM DTT treatment (19.7±9.9) non-significantly had a high percentage of oocytes showing normal fertilization (2 PN) as compared to all the treatments. The 5 mM Glutathione treatment (31.9±8.6) non-significantly recorded the least percentage of total fertilization rate as compared to all the treatments (P>0.05). In conclusion, both Glutathione and Dithiothreitol did not have any effect on the fertilization rate by cryopreserved boar semen.

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The effect of cryoprotectants combination at different concentrations during cryopreservation of semen from windsnyer boars.

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Keywords: boars, cryoprotectants, cryopreservation

Boar sperm is associated with the post-thawed sperm quality reduction due to the low cholesterol and high polyunsaturated fatty acid contents in their sperm plasma membrane (Li J, 2017, Cryobiology, 80, 119-125). The objective of the study was to investigate the efficacy of the cryoprotectants combination at different concentrations on the Windsnyer boars sperm quality during cryopreservation. A total of 18 ejaculates (6 replications/boar) were collected from three Windsnyer boars of proven fertility with the use of the hand-gloved technique method, twice per week. Boars semen were pooled and was extended with Beltsville Thawing Solution [(BTS) IMV Technologies, France], held at 18°C for 3 hours and centrifuged. The sperm pellet was re-suspended with Fraction A (20% egg yolk + BTS) and cooled at 5°C for 1 hour. Following cooling, semen was divided and diluted into the combination of the cryoprotectants [Ethylene glycol {(EG) Sigma-Aldrich®, Munich, United State of America} + Glycerol {(GLY) Laboratory Consumables & Chemicals Supplies cc, Johannesburg, South Africa} + Propanediol {(PDO) Rochelle Chemicals & Lab Equipment, Johannesburg, South Africa}] at equal contribution to make the total concentrations of 4, 8, 12 and 16% and the 0% (control; without cryoprotectant) 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. However, the conventional boar semen cryopreservation protocol (4% GLY + 20% egg yolk + BTS) was tested, and the results were compared with the current experiment. 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 the Sperm Class Analyser® (Microptin, Spain) system. Eosin-Nigrosin staining was used to evaluate sperm viability and morphology at 100X magnification under a phase-contrast microscope (Olympus, BX 51FT, Tokyo, Japan). A total of 200 sperm per slide/treatment was counted for sperm viability and morphology characteristics. 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 and the significant differences were determined by P-value at a significant level of $P<0.05$. The highest post-thawed sperm total motility (21.4±7.2) percentage was recorded in the treatments supplemented with 16% combination of EG + GLY + PDO, significantly different from 4% GLY (18.0±8.2). The least post-thawed sperm progressive motility percentage was recorded in the treatments supplemented with 0% (1.1±1.3) and 8% (4.7±3.3) combination of EG + GLY + PDO, significantly different from 4% GLY (8.0±6.4). However, semen samples supplemented with a 4% (28.8±4.4) and 16% (30.4±6.2) combination of EG + GLY + PDO recorded the highest post-thawed live normal sperm percentage which was significant to 4% GLY (32.1±6.7). There was no post-thawed sperm abnormality percentage recorded in the semen supplemented with the 0% combination of EG + GLY + PDO ($P<0.05$). The 16% combination of EG + GLY + PDO maintained boars sperm survival during cryopreservation.

Impact of diets enriched with omega-3 fatty acids or antioxidants on Belgian blue bull semen

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Keywords: belgian blue bull, semen, antioxidants

Improving bull semen quality and quantity is an important issue for artificial insemination (AI) centers. Spermatogenesis is influenced by many factors, including genetics and nutrition. The objective of the study was to evaluate the impact on blood and semen parameters of enriching the diet of Belgian blue bulls with 1. Omega-3 fatty acids: concentrate enriched with extruded linseed (Linamix, Dumoulin) providing an omega-3 concentration of 14g/kg instead of 3.7 and extra vitamin E to prevent lipid peroxidation (160mg/kg instead of 50) = O3 diet; 2. Antioxidants :concentrate supplemented with 47mg/kg beta-carotene, 200mg/kg encapsulated grape extracts rich in polyphenols (Nor-Grape BP-0, Nor-Feed), 600 mg/kg melon extracts rich in superoxide dismutase (Melofeed, Lallemand) and 1mg/kg selenium instead of 0.8 = AX diet; 3. in comparison with the basic concentrate without enrichment = CT diet. After at least one month of receiving the CT diet, 24 Belgian blue bulls (1 to 9 years old) housed in the AI center of Inovéo (Ciney, Belgium) were randomly assigned to 3 groups. Each group received successively the three diets (basic diet + 1.25kg concentrate per 100 kg BW per day) for a period of 4 months in a different order. At the end of each 4-month period, specific analysis were conducted on semen (volume, concentration and computer assisted sperm analysis for motility and morphological parameters; fatty acid profiles analysis using gas chromatography) and blood samples (concentration in selenium, vitamin A and vitamin E). Statistical analysis was performed using a mixed model for repeated measurements with diet, period of the year and their interaction as fixed effects. A majority of the measured parameters was influenced by the period of sampling. As expected, blood concentrations in selenium and vitamin A were on average higher with the AX diet ($p=0.0011$ and 0.034, respectively), while the vitamin E concentrations were higher with the O3 diet by comparison with the two other diets ($p<0.0001$). A higher semen concentration was found with the AX diet by comparison with the CT diet (mean: 1.45 vs 1.28 billion spz/ml; $p=0.037$). The proportion of motile spz also tended to be higher for the AX by comparison to the CT regime (mean: 60.6 vs 56.1 %; $p=0.098$). No significant difference was observed for the semen parameters between the AX and the O3 diet or between the O3 and the CT diet. The diet did not significantly influence the other measured sperm characteristics, including the proportion of progressive spermatozoa. The proportion of saturated, monounsaturated or polyunsaturated fatty acids in sperm cells was similar between diets. However, the O3 diet significantly increased the proportion of docosahexaenoic acid (DHA; $p=0.0001$) and its precursor alpha-linolenic acid (ALA; $p=0.0009$) by comparison with the two other diets. A high DHA content in sperm membranes usually correlates with a better sperm quality, which was not demonstrated in the present study. In conclusion, the diet enriched in linseed increased the DHA content of the sperm, while supplementing the diet with a cocktail of natural antioxidants had a positive impact on semen concentration and motility.

Evaluation of a new sperm purification device for preparing bovine frozen-thawed semen for in vitro fertilization

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Keywords: cattle, sperm, purification

Density gradient centrifugation is a common approach for preparing frozen-thawed semen for *in vitro* fertilization. This method is time and labor intensive, requires experience and sperm cell recovery is limited. Therefore, we tested a novel sperm purification device, the VetCount™ Harvester (MotilityCount ApS, Copenhagen, Denmark). The core elements of the VetCount™ Harvester are two chambers which are separated by a microporous membrane with a pore diameter of 10 µM. Spermatozoa are selected when actively swimming out of the semen-filled chamber, i.e., through the membrane, into the other chamber which contains a sperm collection medium. The handling of the VetCount™ Harvester is simple. Semen and collection medium, in this experimental approach a TALP (Tyrode's Albumin Lactate Pyruvate) based medium, are injected into the chambers and, after 30 minutes incubation at 38°C, the medium is aspirated and the spermatozoa are ready for further use. In a preliminary evaluation, we assessed sperm recovery and semen quality of frozen-thawed but otherwise untreated bull semen and frozen-thawed semen treated with the VetCount™ Harvester or BoviPure™ gradient centrifugation (Nidacon International AB, Mölndal, Sweden), a standard technique in our laboratory. Frozen semen samples from six different bulls (n = 6), ten straws of one ejaculate per bull, were analyzed. Sperm concentration was determined using a hemocytometer chamber and the total sperm count was calculated. Motility parameters were assessed using IVOS II, a computer assisted sperm analysis (CASA) system, and flow cytometry was used to simultaneously evaluate viability, acrosome integrity, membrane fluidity and intracellular Ca^{+2} concentration. Results were tested for significant differences using Wilcoxon's signed rank test with Bonferroni correction. A *p*-value of <0.05 was set as significance level. BoviPure™ and VetCount™ Harvester treatment increased the progressive sperm motility compared to frozen-thawed semen samples (82.4±18.3%, 78.8±8.4%, and 41.2±18.4%, respectively; p<0.05). The proportion of viable, acrosome intact sperm cells with low intracellular Ca^{+2} concentration and low membrane fluidity was increased after VetCount™ Harvester or BoviPure™ treatment (78.6±6.0%, 76.5±4.4%, and 37.1±13.2%, respectively; p<0.05). Following VetCount™ Harvester filtration, viable, acrosome intact sperm cells had a lower normalized intracellular Ca^{2+} concentration (67±10% of the concentration in untreated semen; p<0.05) compared to spermatozoa following gradient centrifugation (84±14%; p<0.05) or untreated sperm cells (normalized to 100%). There was no significant difference in recovery rate of sperm cells between the VetCount™ Harvester and BoviPure™ treatment (12.4±3.6% and 14.4±5.1%; p>0.05). The data demonstrate that the VetCount™ Harvester treatment selects a high-quality fraction of sperm from frozen-thawed bull semen with even lower free intracellular Ca^{2+} concentrations than a BoviPure™ gradient centrifugation. We are currently investigating whether sperm treated with a BoviPure™ gradient or the VetCount™ Harvester differ in cleavage rate, blastocyst rate and quality when they are used in bovine *in vitro* fertilization.

Characterization of extracellular vesicles in seminal plasma of fertile and subfertile rabbit bucks

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Keywords: extracellular vesicles, semen quality

Extracellular vesicles (EVs) 40-120 nm in diameter are secreted particles present in all biological fluids, transporting molecular components that can affect gamete maturation, fertilization, early embryonic development, and embryo-maternal communication. The study of the composition of the EVs present in the seminal plasma (SP) and its correlation with the fertilizing capacity can help us modulate or enhance the productive results of the rabbit bucks. This study aimed to establish a standardized procedure for isolating and characterizing SP-EVs from rabbit bucks of different semen qualities. Fourteen sexual mature rabbit bucks Cal x NZW (10-12 months old) were used. During a month, 2 ejaculates/week were obtained with an artificial vagina to determine their semen quality (CASA, Microptic S.L., Barcelone, Spain), choosing 3 rabbits of high (HSQ) and 3 of low (LSQ) semen quality. A total of 25 rabbit females were artificially inseminated (seminal dose: $5x10^6$ spermatozoa/ml) with the diluted ejaculate of each male, confirming their high or low fertilizing capacity (fertility: 71.9 and 40.9%; prolificacy: 11.6± 0.3 and 8.1±0.4 liveborn, 0.1±0.1 and 0.7±0.1 stillborn in HSQ and LSQ, respectively; $p<0.001$). Then, 6 ejaculates of each animal obtained in 3 weeks (2 ejaculates/week) were centrifugated (800 × g 20 min, 2000 × g 20 min, and 16000 × g 60 min), and the resulting SP was pooled and frozen at −80 °C. The isolation of SP-EVs from ejaculates of each male was based on size exclusion chromatography analysis PURE-EV® (Hansa BioMed Life Sciences). SP-EVs were quantitative and qualitatively characterized by transmission electron microscopy (Jeol, Ltd Tokyo, Japan), nanoparticle tracking analysis (Nanosight NS500: Marven, INC) using software NTA 3.1, and western blot to confirm the expression of the classical exosome markers (HSP70, CD9 y ALIX). A correlation analysis between seminal parameters and the concentration and size of SP-EVs from the two groups of males was made (SAS, 2001). Different SP-EVs concentrations ($8.53x10^{11}$±$1.0x10^{11}$ and $1.84x10^{12}$±$1.75x10^{11}$ particles/ml of SP; p=0,008) with a similar average size (143.9 ± 11.9 and 115.5 ± 2.4 nm; p=0.7422) in HSQ and LSQ males, respectively were observed. The concentration of SP-EVs was positively correlated with the percentage of abnormal forms (r=0.94; $p<0.05$) and with the percentage of immotile spermatozoa (r=0.88; $p<0.05$). Particle size was not correlated with any kinetic parameter. We can conclude that the methodology used for the extraction and characterization of the SP-EVs is valid by confirming their existence in the SP of rabbits and the SP-EVs concentration depends on semen quality and its fertilizing ability. *Supported by projects RTI 2018-094404-B-C-21 and PID2019-111641RB-I00.*

Bovine oviductal fluid, the physiological additive for bovine sperm selection.

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Keywords: Oviductal fluid, bovine, spermatozoa

Today, during a cycle for IVP of bovine embryos, the current blastocyst yield per oocyte is similar to the figures reported a decade ago, with only 30-40% of the collected oocytes reaching the blastocyst stage (Lonergan et al., Theriogenology, 81(1), 49-55, 2014). Since bovine oviductal fluid (BOF) is the physiological environment in which the last stages of capacitation and fertilization take place, we hypothesized that the inclusion of BOF into the composition of the sperm preparation medium might select a sperm population of better quality in terms of motility, viability and capacitation status, which will contribute to increasing the efficiency of the IVP process. Sperm quality was evaluated in terms of motility, viability, apoptosis, plasma membrane fluidity, and state of the acrosome. Asturian Valley bull sperm straws were thawed at 38 °C for 30 s. Each replicate (N=5) consisted of 4 straws from the same bull and a different bull was used in each replicate. BOF from the late follicular phase was acquired from EmbryoCloud (Murcia, Spain). Spermatozoa were selected by the swim-up method (Ruiz et al., Reproduction in Domestic Animals, 48(6) e81-e84, 2013). Two different media were used: Swim-up BSA, containing 6 mg/ml BSA (Parrish et al., Biology of Reproduction, 40(5), 1020-1025, 1988); and Swim-up BOF, where BSA was replaced by 1% BOF (v/v). To analyse sperm motility, sperm samples were evaluated at 38 ºC and 200X magnifications under a negative phase-contrast microscope coupled to a CASA system (ISASv1, ProiserR+D, Valencia, Spain). To evaluate viability, acrosomal integrity, and membrane fluidity of the samples by flow cytometry, the sperm concentration was set at $2x10^5$ spermatozoa per mL and the sperm suspension was incubated for 15 min at 37 °C with the following combinations of fluorochromes: i) 2.5 µg/mL propidium iodide and 1 µg/mL *Pisum sativum* lectin I conjugated with fluorescein isothiocyanate to assess sperm viability and acrosome integrity; and ii) 2.7 mM merocyanine-540 and 25 nM Yo-Pro1 to assess sperm viability and plasma membrane fluidity. Subsequently, samples were subjected to analysis by a Guava Easycyte 6-2L flow cytometer (Merck Millipore, Hayward, USA). Data, presented as mean ± SEM, were analyzed by the Student's t-test using the IBM SPSS Statistics package (IBM, Armonk, USA). Differences were considered significant when $P < 0.05$. BOF enabled a higher ($P < 0.01$) total and progressive motility, and higher kinematic parameters after swim-up, except for ALH and BCF. The percentages of spermatozoa viable, non-apoptotic and with non-reacted acrosome were similar in both groups. The proportion of spermatozoa with high membrane fluidity was higher after swim-up in BOF (37.5 ± 4.2%) than in BSA (21.9 ± 1.6%; $P < 0.05$). To sum up, the inclusion of BOF into the composition of the sperm preparation medium selects a population of functional spermatozoa with improved motility, which might contribute to increasing the fertilizing ability of impaired semen samples and so, enhance the efficiency of the IVP of bovine embryos.
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The fate of porcine sperm CRISP2 from the perinuclear theca before and after in vitro fertilization.

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Keywords: CRISP2; porcine; sperm; perinuclear theca; decondensation; fertilization; oocyte.

In a previous study (Zhang et al., Biol Reprod 105:1160-1170; 2021) we reported that porcine sperm cysteine rich secretory protein 2 (CRISP2) is localized in the post-acrosomal sheath (PAS)-perinuclear theca (PT) as reduction-sensitive oligomers. In the current study, the decondensation and removal of CRISP2 was investigated during *in vitro* sperm capacitation, both after induction of the acrosome reaction and after *in vitro* fertilization. Confocal immunofluorescent imaging revealed that additional CRISP2 fluorescence appeared on the apical ridge and on the equatorial segment (EqS) of the sperm head following capacitation, likely a result of the local de-oligomerization of CRISP2. After an ionophore A23187 induced acrosome reaction, CRISP2 immunofluorescence disappeared from the apical ridge and the EqS area partly due to the removal of the acrosomal shroud vesicles but also partly due to its presence in a subdomain of EqS (EqSS). The fate of sperm head CRISP2 was further examined post-fertilization. *In vitro* matured porcine oocytes were co-incubated with boar sperm cells for 6-8 h and the zygotes were processed for CRISP2 immunofluorescent staining. Notably, decondensation of CRISP2, and thus of the sperm PT, occurred while the sperm nucleus was still fully condensed. CRISP2 was no longer detectable in fertilized oocytes in which sperm nuclear decondensation and paternal pronucleus formation was apparent. These data indicate that PT decondensation and degradation may be executed in advance of sperm DNA decondensation post-fertilization. This rapid dispersal of CRISP2 in the PT is likely regulated by redox reactions for which its cysteine rich domain is sensitive. Reduction of disulfide bridges within CRISP2 oligomers may be instrumental for PT dispersal and PT elimination. These results raise important questions such as whether the dispersed PT proteins in the oocyte cytoplasm may be involved in oocyte activation, as well as in male nuclear chromatin decondensation in order to form the male pronucleus.

Genomic analysis of bovine beta-defensin genes implicated in sperm function fertility

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Keywords: CNV, Beta-defensin, Fertility, Cattle

Beta-defensins are short secreted peptides that have traditionally been characterised as antimicrobial. However, it is known that some beta-defensins have important functions in fertility. For example, beta-defensin 126 is an important component of the sperm glycocalyx and is involved in capacitation of sperm prior to fertilisation in macaques, and promotes sperm motility in cattle. Genes encoding beta-defensins are known to show extensive deletion and duplication (known as copy number variation (CNV)) in cattle and other mammals, including humans. However this has not been characterised in detail in cattle. In this study we mapped publicly available sequencing data (1000 Bulls Consortium) from 100 bulls of different breeds to the bovine genome (ARS-UCD1.2-bosTau9) assembly based on long read PacBio sequencing, which is likely to have a more accurate assembly of complex repeated regions of the genome, such as the beta-defensin regions. We show that the chromosome 13 beta-defensins, including beta-defensin 126, are commonly duplicated in Holstein bulls. Other beta-defensins on chromosome 27, in particular DEFB103, show extensive copy number variation across breeds, including complete loss of the gene. In addition we selected positive control bulls target that high and low copy numbers associated with beta-defensin by the mapping. We are currently testing an extended fertility panel of Holstein bulls and the positive control bulls for beta-defensin genes showing CNV using digital droplet PCR, with the aim of investigating the relationship between CNV at bovine beta-defensin genes and fertility.
Our results may improve our understanding of CNV, which is known as an important genomic structural variation in cattle. We also believe that beta-defensin will provide important information about potential CNV effects on reproductive performance, which forms the basis for its inclusion in a future dairy cattle breeding programme.

Embryology, Developmental Biology, and Physiology of Reproduction

Collection of embryos and fluid from the bovine oviduct

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Keywords: embryo, oviduct, cattle

In contrast to in vitro culture systems which are most commonly performed on a static medium culture basis, embryo development in the oviduct is directed by a dynamic supply system. In order to get more insight into stage specific needs of a developing embryo the aim of the present study was to determine, whether embryos and the corresponding tubal fluid can be simultaneously obtained from the bovine oviduct. Fifteen Austrian Fleckvieh heifers were synchronized by two intra muscular injections of PGF2a (Estrumate, Cloprostenol, MSD Animal Health, Austria) 11 days apart and GnRH (Veterelin, Buserelin, Calier, Barcelona, Spain) 48 hrs after each PGF2a injection. Fixed time artificial insemination has been performed using fresh semen. At Day 3 after expected ovulation, oviduct flushing and embryo collection has been done using an endoscopy set for transvaginal access (STORZ, Tuttlingen, Germany). Following epidural anesthesia and genital disinfection a trocar set was placed in the tip of the vagina and introduced via dorsomedial perforation through the vagina wall into the peritoneal cavity. Prior to flushing the ovulation side has been determined and the morphology of the growing corpus luteum has been assessed. Flushing was performed in two steps: first the ampulla was repeatedly flushed using 0.5 ml PBS medium. Finally, 0.2 to 0.4 ml medium/fluid has been recovered, adjusted to 0.5 ml and transferred into an Eppendorf tube and centrifuged for estimation of total protein using colorimetric Protein Quantitation Assay (Pierce BCA Protein Assay Kit, ThermoFisher, Austria). In a second step 50 ml medium (PBS, 1 % FCS) was flushed through the oviduct, collected in the tip of the uterine horn via an embryo flushing catheter which has been connected to an Emcon embryo filter. Additionally, 300 ml medium were used to extra flush the uterine horn via the flushing catheter. The medium collected in the filter was checked for the presence of an embryo using a stereo microscope. Overall, 12 heifers have been synchronized 3 times and 3 heifers 2 times (in total: 42 treatments). In 35 cases ovulation has been confirmed and oviduct fluid has been collected successfully. Additionally, 21 embryos (recovery rate 60%) have been recovered at the 4 to 8-cell stage. Protein quantitation analyses of the collected tubal fluid ranged between 0.28 and 1.4 mg/ml total protein. Taken these preliminary results together it is concluded, that is possible to repeatedly collect tubal stage embryos and the corresponding fluid as a prerequisite to analyze the stage-specific environment of the embryo. It was also assumed that the obtained total protein amount was sufficient for further examining tubal components. It is expected that consecutive studies including the collection of tubal fluid and corresponding embryos throughout the migratory phase in the oviduct will provide much more detailed information about the needs of an embryo especially during in vitro culture.

Effect of serum in maturation medium on the birth weight of Holstein calves

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Keywords: IVP, birthweight, serum

It has been known for years that IVP calves generally have a higher birth weight as compared to AI calves. Serum in the IVP media is often blamed for this. Indeed removing serum from the IVC medium reduced the amount of heavy calves (Wagtendonk et al. 2000), but the level is still higher than that in AI calves.
The aim of this study was to investigate whether the removal of serum from the maturation medium could further decrease the birth weight and the percentage of heavy calves.
Oocytes were collected from female HF donors between 9 and 24 months of age. The oocytes were incubated for 24 hours at 38.5°C in IVM medium (M199 supplemented with 0.1 mM Cysteamine, 0.01 U/ml FSH (SigmaAldrich) and LH 0.02Ug/ml (SigmaAldrich)) either with 10% Fetal Calf Serum or without (no further additions to compensate for the serum removal). After 24 hours, oocytes were fertilised with different bulls and subsequently cultured for another 7 days. Day 7 embryos were transferred fresh or frozen at different commercial farms throughout The Netherlands. Birth weight of the calves were recorded by the farmers and are a mix of estimated weights and real measured weights.
In total 1198 and 689 different OPU sessions were performed for control and serum free medium respectively. On average 10 oocytes were collected per session.The percentage of transferable day 7 embryos was significantly (Chi-square test, $P<0.05$) smaller in the IVM medium without serum as compared to the control with serum (21% transferable embryos for the control compared to 19% for the serum free IVM medium). Subsequently, 860 control embryos and 620 embryos from the serum free group were transferred. Pregnancy rates (on average 45%) of the embryos from both groups were comparable. The mean birth weight of the 97 animals from the serum free IVM medium was not different from that of the 148 animals in the control group (42.5 and 43.0 kg respectively). Also the percentage of heavy calves (i.e. calves of 50 kg or heavier) was not significantly (Chi-square test) different between the two groups (9% and 7% for the serum free IVM and control respectively). As expected male calves were about 3 kg heavier, which was the same in both groups.
These results indicate that the removal of serum from the IVM medium does not have a significant effect on lowering the birth weight of IVP calves. Therefore further research is needed to reduce the birthweight of the IVP calves. Potentially further modifications in the media are required.

Comparative proteomic analysis of bovine embryos developed in vivo or in vitro up to the blastocyst stage

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Keywords: Embryo, proteomics, cattle.

Despite many improvements in *in vitro* systems and embryo culture media, *in vitro* derived embryos still display morphologic and metabolic differences making them less viable and cryoresistant compared to their *in vivo* counterparts. To bring knowledge to this issue, we used a quantitative proteomic approach to compare early bovine embryos developed *in vivo* or *in vitro*.
Eleven Holstein females were synchronized for estrus, treated for ovarian superovulation and inseminated twice with frozen-thawed semen. Between days 1.7 and 7.5 after the first artificial insemination, *in vivo* embryos were recovered after slaughter by flushing of the oviducts and uterus. In addition, embryos were produced *in vitro* using slaughterhouse bovine ovaries, the same male semen and a culture medium with no serum or protein supplementation. All embryos were washed three times and stored at −80°C before analysis. Proteins from pools of grade-1 embryos at the 4-6 cells, 8-12 cells, morula, compact morula and blastocyst stages (4 embryos/pool; 3-4 pools/stage, total of 38 pools) were analyzed by nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Proteins were identified using the UniProt *Bos taurus* database and quantified by label-free spectral counting. Proteins quantified with minimum 2 normalized weighted spectra (NWS) in at least one condition were analyzed using principal component analysis (PCA) and ANOVA. The hierarchical clustering of differentially abundant proteins (DAPs; ANOVA p-value < 0.05) were done using Spearman correlations and the gplots package of RStudio. Functional analysis of DAPs was carried out using the Metascape on-line tool.
A total of 3,028 proteins were identified in embryos, of which 227 were specific to *in vivo* embryos and 49 to *in vitro* embryos. The PCA of the 2,186 proteins quantified with more than 2 NWS showed a clear separation of embryo pools according to their stage of development and origin (*in vivo* vs. *in vitro*). Oviductin, also known as oviduct-specific glycoprotein 1 (OVGP1), and clusterin were among the most overabundant proteins in *in vivo* compared to *in vitro* embryos at all stages. Three clusters of 999 DAPs (ANOVA's p-value <0.05) according to the origin were evidenced: 463 DAPs with higher abundance *in vivo* than *in vitro* across development (cluster 1); 314 DAPs with less abundance *in vivo* than *in vitro* before the morula stage (cluster 2); and 222 DAPs with less abundance *in vivo* than *in vitro* after the morula stage (cluster 3). Proteins in cluster 1 were mainly involved in carbohydrate metabolic pathways, cellular detoxification and cadherin binding. Proteins in cluster 2 were mainly involved in protein synthesis. Proteins in cluster 3 were mainly involved in mitochondria-dependent activity and cytoskeleton organization.
These data provide a first exhaustive proteomic comparison between *in vivo* and *in vitro* embryos in cattle and bring new insights into the molecular contribution of the maternal environment (ovarian follicle, oviduct and uterus) to the preimplantation embryo. Moreover, the DAPs identified constitute valuable markers of embryo quality for the assessment of new *in vitro* systems, closer to *in vivo* conditions.

Sub-optimal paternal diet accelerates pre-implantation embryo development in mice

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Keywords: embryo, diet, paternal

The embryo is critically sensitive to altered environmental conditions *in utero*, which can subsequently direct fetal growth and influence offspring health in later life. Emerging evidence suggests the father's pre-conception diet/lifestyle also has a role in regulating fetal development and ultimately alters disease risk in the adult offspring. Defining the early embryonic dynamics that underlie this impact on offspring health is key to improving our understanding of the developmental origins of health and disease. Therefore, the aim of this study was to examine how impaired paternal nutritional status influences early embryo development in a mouse model.
Male C57/BL6J mice were fed either a low-protein (LPD: 9% casein, 24% sugar, 10% fat; n=6), Western (WD: 19% casein, 34% sugar, 21% fat; n=7) or control (CD: 18% casein, 21% sugar, 10% fat; n=6) diet for a minimum of 8 weeks, to ensure all periods of spermatogenesis were exposed to dietary factors. Males were mated to virgin 8-12 week old females (n=6-7 per group), with pregnancy confirmed by the presence of a copulation plug and termed embryonic day (E)0.5. Pregnant dams were euthanized on E1.5 and the oviducts flushed to retrieve 2-cell embryos. Embryos were cultured individually in EmbryoMax® KSOM media (37°C; 5% CO_2) in an EmbryoScope time-lapse incubator, with images acquired every 10 minutes. Embryo cleavage rate and blastomere/blastocyst area were determined using EmbryoViewer software. Fully expanded blastocysts were fixed in 4% paraformaldehyde and stained for quantification of inner cell mass (Oct4) and trophectoderm (Cdx2) cell numbers. Statistical analysis was performed using IBM SPSS Statistics v25. Data were assessed using a linear mixed model or a one-way ANOVA with Tukey's post-test where appropriate.
The sub-optimal paternal diets did not alter number of embryos per dam (CD: 8.2±1.0, LPD: 8.2±0.2, WD: 8.1±0.3). Embryos generated by WD fed-males demonstrated an increased average blastomere size at the 2-cell stage (2282±31μm^2 vs 2108±47μm^2, $p<0.05$) compared to CD. Embryos from both LPD and WD fed males developed through all pre-compaction cell cleavage stages faster than those from CD males (time to 4-cell stage; LPD: 6.0±0.6h, WD: 5.3±0.4h vs CD: 9.8±0.8h; $p<0.01$, $p<0.001$ respectively, and to 8-cell stage; LPD: 19.1±1.0h, WD: 19.0±0.5h vs CD: 22.9±0.8h; $p<0.01$). Post-compaction, no differences were seen between diet groups. Blastocysts from LPD fed males demonstrated a faster time to expansion compared to CD (54.1±1.0h vs 59.5±1.3h, $p<0.01$ (h= hours from start of culture)), yet were found to have a smaller area than both CD and WD blastocysts (6745±127μm^2 vs 7647±278μm^2 and 7886±245μm^2, $p<0.05$, $P<0.01$ respectively). We observed no differences in inner cell mass or trophectoderm cell number.
We found patterns of early embryonic development were accelerated in embryos derived from LPD and WD fed males. Whilst this accelerated rate of developed declined post-compaction, differences in blastocyst formation were still observed. Altered rates of early embryonic development have been linked with impaired implantation, and even altered fetal development later in gestation. Our data suggests that a poor paternal pre-conception diet plays a key role in regulating the rate of early embryonic development, which could have implications for the future health of the offspring.

Lipids characteristics in bovine preimplantation embryos originated from in vitro fertilization or parthenogenetic activation

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Keywords: embryo, cattle, lipids

Lipids are essential elements of the cells since they build biological membranes and are involved in many processes, including energy metabolism. They are stored in the cytoplasm in lipid droplets (LD). Lipid content alters during the preimplantation development of embryos, mainly due to the changes in energy metabolism requirements. Except for classic in vitro fertilization (IVF), embryos have the ability to activate their development without the involvement of male gametes (parthenogenesis, PA). The question arises of whether it affects embryonic lipid content.
The aim of this study was lipid characteristics in embryos: 1. at crucial stages of development, 2. originating from IVF and PA systems.
Bovine oocyte-cumulus complexes were matured in vitro and either in vitro fertilization (bIVF group) or parthenogenetic activation (5uM ionomycin/2mM 6DAMP; bPA group) was performed. Embryos were cultured in SOF medium and collected in the following developmental stages: zygote, 2-cell, 4-cell, 8-16-cell, morula, early and expanded blastocyst (approx. 20 per group). They were stained with BODIPY 493/503 and DAPI for LD and chromatin visualization, respectively, then captured and z-stacked with Zeiss LSM 880 confocal microscope. The following parameters were analyzed in ImageJ Fiji software: total lipid content, LD number, LD size, and % area of LD. Statistics included appropriate tests in the R statistical package.
Our results show that in bIVF group, total lipid content reaches the highest level at the zygote stage, and it drops to the lowest values at the 8-16-stage ($P<0.01$), following a significant increase at the expanded blastocyst stage ($P<0.05$). A similar decrease at the 8-16-cell stage is observed for the LD number (113+/-41 vs 57+/-21 μm^2), LD size (5.15+/-2.59 vs 2.16+/-0.77), and % area occupied by LD (3.3+/-1.14 vs 1.06+/-0.83) ($P<0.01$). When bIVF and bPA at 2-cell, 4-cell, and expanded blastocyst stages are compared, a significantly lower value of total lipid content ($P<0.05$) is observed at the 2-cell stage in bIVF embryos. Yet, there areno differences between expanded blastocysts.
LD parameters decrease at the 8-16-cell stage is observed at the same time-point as the embryo genome activation in cattle. It suggests that early embryos may strongly utilize lipids as a source of energy without the possibility of replenishment due to the lack of transcription. Moreover, others suggest a lower contribution of lipids in the late preimplantation embryo development since embryos switch their energy metabolism into glucose. However, it is inconsistent with our data due to the observed rise of lipid parameters at the blastocyst stage. Our results also indicate that lipid content is altered depending on the embryo origin (IVF or PA) only in the early stages of development (2-cell embryos). It suggests that during further development, embryos are able to compensate for the lipid deficiencies, however further studies in every step of development are necessary.
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Effect of astaxanthin on post-thaw viability of bovine vitrified oocytes: preliminary results

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Keywords: astaxanthin, oocytes, vitrification

Although various techniques for cryopreservation of bovine oocytes are known, their developmental potency after thawing is still unsatisfactory. Astaxanthin (AX) is a powerfull antioxidant, which improved post-thaw viability of pig oocytes but its effects on bovine vitrified oocytes are to be examined. Our goal was to examine whether AX, added to the post-thaw medium, can improve the viability of bovine vitrified oocytes. Oocytes, recovered from cow´s ovarian follicles at slaughtering, were *in vitro* matured (IVM) and then vitrified in minimum volume on the nickel electron microscopy grids by ultra-rapid cooling technique. Following several months the oocytes were warmed and incubated 3 hours for post-thaw recovery in the maturation medium (TCM199, 10% FCS, 0.25mmol.l^{-1} sodium pyruvate, 50µg/ml gentamicin, 1/1 I.U FSH/LH (Pluset)) either without AX (Sigma Aldrich, Missouri, USA; 0µM; vitrified group; n=186) or with 2.5µM (the dose was chosen according to the previous reports) of AX (vitrified+AX group; n=179). Fresh IVM oocytes (n=157) served as a control. Afterwards, oocytes of all groups were fertilized *in vitro* using frozen bull semen and cultured in B2 medium (prepared according to Menezo) with 10% FCS, 10mg/ml BSA, 31.25mM sodium bicarbonate and 50µg/ml gentamicin 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 four replicates. Total blastocyst rate (D6-D8) was significantly less in vitrified (12.90%) and vitrified+AX (13.41%) groups compared to control group (25.48%), whilst cleavage rate was different only in vitrified group (53.26%), but not in vitrified+AX (55.87%) compared to control (64.33%). However, AX significantly ($p<0.05$) increased (Chi-square test) the proportion of embryos that reached the blastocyst stage earlier (Day 6; 20.83%), compared to the vitrified group without addition of AX (8.33%), thus approaching this value to those in the control group (25.00%). Vitrification led to slight decrease ($p>0.05$; Student´s t-test) in the blastocyst cell number from 103.03±4.42 (control group) to 92.24±6.20 (vitrified), whilst AX reversed this suppressive effect (102.87±6.00). Apoptotic occurrence (TUNEL-index) did not differ significantly among control (10.33±1.84%), vitrified (13.02±3.24%) and vitrified+AX (13.93±3.35%) groups (Student´s t-test). AX indicated a trend to improve quality of actin cytoskeleton by increasing the proportion of embryos with excellent actin quality (grade 1) in vitrified+AX oocytes (82.61%) in comparison to the fresh (64.87%) or vitrified (61.90%) groups, although differences were not significant (Mann-Whitney U-test). In conclusion, astaxanthin, added to vitrified/warmed oocytes during post-thaw recovery period, accelerated development to the blastocyst stage, what was reflected in increased rate of faster developing blastocysts with no effect on the total blastocyst yield.
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Effects of palmitic acid-induced lipotoxicity on epigenetic programming in zygotes and morulas during bovine in vitro embryo production

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Keywords: bovine in vitro embryo, epigenetics, lipotoxicity

Maternal metabolic disorders are associated with subfertility. Upregulated lipolysis causes a rise in non-esterified fatty acids in the blood, which is reflected in the follicular and oviductal micro-environment. This has a lipotoxic effect on oocyte and embryo development, mainly due to elevated palmitic acid (PA) concentrations. Surviving embryos may exhibit persistent defects in later life due to epigenetic alterations as oocyte maturation and early embryo development involve dynamic changes in epigenetic reprogramming and may therefore be vulnerable to changes in their micro-environment. We hypothesized that short-term exposure to PA during oocyte maturation and early embryo development can alter epigenetic patterns in the resulting embryos. To test this hypothesis, a validated bovine IVP model was used, where oocytes were exposed to standard (CONT) or solvent (SCONT) media, or a pathophysiological concentration of PA (150µM, BSA 7.5 mg/ml) during IVM (24h). Oocytes were then *in vitro* fertilized (for 20h) and presumptive zygotes were cultured in the corresponding CONT, SCONT or PA (230µM, BSA 20 mg/ml) media, respectively (3 groups in total). 12 replicates were performed (858-1630 COC's/treatment). Cleavage rates were recorded at 48h post insemination (p.i.) (12 replicates) and blastocyst rates at 8d p.i. (4 replicates). Zygotes (60/treatment, 6 replicates) were collected at 20h p.i. and morulas (70/treatment, 8 replicates (not used to record blastocyst rates)) were collected at 4.7d p.i. and were fixed for 5mC and H3K9ac/H3K9me2 immunostaining and confocal microscopy to assess global DNA methylation and histone acetylation/methylation, respectively. Developmental competence data were analysed using logistic regression, and numerical data with one-way ANOVA (5mC/H3K9ac) or Kruskal-Wallis test (H3K9me2) with post-hoc Bonferroni correction depending on normality of distribution. For developmental competence, 5mC, and H3K9ac, no effects of the solvent could be detected compared to CONT. H3K9me2 mean gray intensity was significantly lower in SCONT compared to CONT in zygotes (22.2% reduction, P=0.011) and morulas (13.5% reduction, P=0.001). Exposure to PA during IVM and IVC resulted in significant reduction of cleavage (63.9 ± 4.7 % vs. 79.5 ± 2.6 %, P<0.001) and blastocyst rates (25.2 ± 4.9 % vs. 39.2 ± 2.6 %, P=0.005) compared to SCONT. 5mC mean grey intensity was not altered in PA-exposed zygotes (P=0.432) but was increased in morulas (27.4% increase, P<0.001). H3K9ac was significantly increased in zygotes exposed to PA (32.5% increase, P<0.001), but not in morulas (P=0.913). H3K9me2 was significantly increased in PA-exposed zygotes (46.3% increase, P<0.001) and morulas (15.5% increase, P=0.039). We conclude that a lipotoxic micro-environment during bovine IVM results in increased histone acetylation and methylation already at the zygote stage. Continued exposure during IVC was associated with increased histone and DNA methylation in the morulas. These upregulated epigenetic marks may cause altered gene expression and imprinting, resulting in aberrant embryonic cell differentiation. We are currently investigating potential mechanisms through which these changes occur.

Glyphosate affects mRNA expression pattern of bovine oocytes and belonging cumulus cells

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Keywords: glyhosate, IVM, bovine

Glyphosate (Roundup®) is a non-selective systemic herbicide widely used. There is some evidence that Roundup® (R-Gly) can act as an endocrine disruptor. Cleavage and developmental rates were significantly decreased for embryos stemming from cumulus-oocyte complexes generated with 300 µg/mL R-Gly during IVM (36.2% ± 16.6; 4.9% ± 4.5) compared to those of two other groups (0 and 30 µg/mL R-Gly; 73.9% ± 11.1, 80.3% ± 7.1; 31.7% ± 11.2; 29.5% ± 11.5). A significant P4 and E2 increase was detected in the maturation medium from the 300 µg/mL group. These data indicate that a supraphysiological R-Gly concentration during IVM affects steroid synthesis of cumulus cells and subsequent embryo development (Blad-Stahl et al. 2020; Reprod Dom Anim, 55, 6-7). The aim of this study was to evaluate the effect of different Roundup® concentrations (0, 30, 300 µg/mL R-Gly) supplemented during in vitro maturation (IVM) on mRNA expression patterns in oocytes and belonging cumulus cells before and after in vitro maturation which means for oocytes at the GV and MII stage, respectively. Gene transcripts were analyzed via a RT-qPCR assay as described previously (Blaschka et al. 2019; Theriogenology 131, 182-192). MessengerRNA (mRNA) from single oocytes and total RNA from the belonging cumulus cells was directly used for the reverse transcription (RT). The relative expression ratio of a target gene is calculated based on efficiency and the crossing point deviation (Ct-values) of an unknown sample versus a reference gene. At least 6 IVM runs and 3 replicates from different IVM for the RNA analyses were undertaken. Selected transcripts are involved in steroid metabolizing processes [cytochrome P450 monooxygenase (CYP19), hydroxysteroid dehydrogenase 3 beta (HSD3ß), luteinizing hormone/choriogonadotropin receptor (LHCGR), follicle-stimulating hormone receptor (FSHR), estrogen receptor alpha (ERα), progesterone receptor (PGR), progesterone receptor membrane component 1 (PGRMC1), progesterone receptor membrane component 2 (PGRMC2) in cumulus cells and PGR, PGRMC1, PGRMC2, and steroidogenic acute regulatory protein (STAR) in oocytes]; and in oocyte growth and development [zygote arrest protein 1 (ZAR1), growth differentiation factor 9 (GDF 9), bone morphogenetic protein 15 (BMP15), glucose-6-phosphate dehydrogenase (G6PD9]. In cumulus cells, all transcripts, except PGRMC2, showed a significant increase in the relative abundances after IVM. For PGRMC2, a decrease was detectable. Supplementation of the high R-Gly concentration affected the mRNA expression of HSD3ß, LHCGR, FSHR, ERα, PGR and PGRMC1. During IVM of oocytes, a significant decrease of GDF9, ZAR, and STAR transcripts was determined, whereas it was an increase for PGR mRNA. A significant increase was also measurable in oocytes being matured in medium supplemented with the high R-Gly concentration for G6PD, whereas the relative mRNA abundance was similar compared to immature oocytes for GDF9, ZAR, and STAR transcripts.
These data indicate that during IVM a supraphysiological R-Gly concentration affects the mRNA expression of gene transcripts related to steroid metabolism in cumulus cells and that in oocytes especially related to oocyte growth.

Effects of a polylactic acid, 3-D printed scaffold, on bovine embryo development in vitro

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Keywords: Polylactic acid (PLA), IVF, embryo development, bovine

The development of 3D printers and the advances in materials science and tissue engineering in the last years have allowed the implementation of this technology in several research areas. So, it has been used as a tool to create a 3D-oviduct-on-a-chip with poly(dimethylsiloxane) that mimics de physiological environment, which has proved to be useful in reducing the differences between in vitro and in vivo produced embryos (Ferraz et al, Nat commun, 9(1), 1-14, 2018). As an alternative, we suggested polylactic acid (PLA) as a great candidate to generate engineered 3D scaffolds due to its high biocompatibility and mechanical properties (Chi et al, BMC chem, 14(1), 1-12, 2020). Our goal was to evaluate for the first time, the feasibility of PLA scaffolds printed by the fused filament fabrication method to support IVF, by means of resulting blastocyst rates and total cell number. IVF was performed under 3 different conditions (N=4 replicates): i) Conventional IVF (Parrish et al., Biol Reprod, 38(5), 1171-1180, 1988) (control group, N=360) ii) IVF in a medium conditioned by the scaffold for 24h (rinse group, N=215) and iii) IVF inside the scaffold used for the rinse group (scaffold group, N=190). Before IVF, the scaffolds were sterilized in 70% ethanol for 1h, washed 4 times for 5 min in PBS and air-dried at room temperature. For IVF, in vitro matured oocytes were washed and cultured in Fert-TALP medium (Parrish, Theriogenology, 81(1), 67-73,2014) for 22h with frozen-thawed bull sperm ($1x10^6$spz/ml) selected by Bovipure gradient (Nidacon, Sweden). Putative zygotes were cultured in microdrops of SOF medium (Holm et al, Theriogenology, 52(4), 683-700, 1999) supplemented with 0.3% BSA (w/v) covered with paraffin oil (Nidoil, Nidacon). Cleavage (48h) and blastocyst rates (day 8) were evaluated. At day 8, blastocysts were fixed in glutaraldehyde and stained with Hoechst 33342 to assess their cell number by fluorescence microscopy. The parameters were analyzed by Kruskal-Wallis one-way ANOVA test when the distribution was not normal and by one-way ANOVA when it was. Differences were considered significant when $p<0.05$. Regarding cleavage rate, it was significantly higher in the control group (79.4±2.1%[a]) than in the rinse and scaffold groups (30.7±3.2%[b] and 52.6±3.6%[c], respectively). Furthermore, the control group showed a higher blastocyst rate (25.6±2.3%[a]), than the scaffold and rinse groups (14.1±2.5%[b] and 2.3±1.0%[c], respectively). As for the total cell number, no significant differences were found (78.2±3.6 for control, 89.6±22.3 for rinse, and 79.4±7.0 for scaffold). It's known that the incubation of PLA in PBS causes a rapid drop in pH on the first day, probably due to the release of lactic acid, a known PLA degradation byproduct (Diomede et al, Stem Cell Res Ther, 9(1), 1-21, 2018). This fact could explain the differences between groups, since the rinse group contained the medium that had been in contact with PLA for 24 hours. Further experiments controlling the post-culture pH could corroborate such hypothesis. In conclusion, the current data demonstrates that PLA does not seem to be applicable to use for 3D scaffolds for IVF. Supported by Fundación Séneca reference 21651/PDC/21.

Can a maternal obesogenic diet influence offspring oocyte lipid droplets and mitochondria?

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Keywords: intergenerational effects, oocyte quality, obesogenic diet

Consumption of an obesogenic (OB) diet is linked with infertility. Hyperlipidemia alters the ovarian follicle microenvironment and induces lipotoxicity in the oocytes, mainly characterized by mitochondrial (MT) dysfunction and lipid accumulation. Increased reactive oxygen species (ROS) production by the defective MT leads to oxidative stress and reduced oocyte quality. Since MT are exclusively maternally inherited, transmission of aberrant MT from the oocyte to the embryo may alter MT functions in the offspring germline. In addition, the obesogenic maternal uterine and lactation environment can also impact the developing offspring oogonia, which may lead to defective oocyte MT in newborns. Therefore, we hypothesized that oocyte lipid content and MT are not only affected by an OB diet but also by the mother's obesogenic background.
To test this hypothesis, female Swiss mice were fed a control (C, 10% fat, 7% sugar) or OB diet (60%fat, 20% sugar) for 7 weeks (w), then mated with the same males. Female offspring from each litter were equally weaned on a C or OB diet in a 2x2 factorial design, resulting in 4 treatment groups: C»C, C»OB, OB»C and OB»OB. Per treatment group, at least 5 oocytes per offspring (at least 7 females) from 7-8 C mothers or 6-8 OB mothers were collected at 10w of age after hormonal stimulation (10IU PMSG and 10IU hCG i.p.). Oocytes were stained and imaged (LeicaSP8 Confocal microscope) to quantify lipid droplet (LD) content (BODIPY, x10^3 μm^3), MT inner membrane potential (MMP) (JC1) and ROS (CellRox Deep Red) (x10^3 pixel intensity). Data were analyzed in SPSS using two-way ANOVA, and shown as mean±SEM. In addition, active MT distribution patterns were categorized as peri-cortical, diffuse or aggregated, analyzed using generalized linear models and presented as mean%±SEM.
No interactions between maternal and offspring diets were observed. However, LD content was affected by offspring diet (P=0.000), irrespective of the maternal diet, as it was significantly increased both in C»OB compared to C»C (7.2±0.3 vs 5.7±0.3) and in OB»OB vs OB»C (6.8±0.3 vs 5.5±0.3). Similarly, MMP was significantly increased only by offspring diet (P=0.025), both in C»OB vs C»C (55.7±2.9 vs 46.9±2.3) and in OB»OB vs OB»C (56.3±2.7 vs 52.9±3.0). Comparably, ROS accumulation was only affected by offspring diet (P=0.007), and was also higher in C»OB vs C»C (31.9±2.6 vs 24.2±2.0) and in OB»OB vs OB»C (30.6±3 vs 25.3±2.4). MT distribution was not affected by offspring diet. In contrast, maternal diet significantly increased the proportion of MT aggregation (P=0,016), irrespective of the offspring diet, as it was increased in OB»C vs C»C (6.3%±4.7 vs 0%±0.0) and in OB»OB vs C»OB (7.8%±4.1 vs 0%±0.0). This category exhibited high ROS accumulation and very low MT MMP.
In conclusion, while we could confirm the increase in LD content, MMP and ROS in oocytes upon direct exposure to an OB diet, it appears that oocyte MT in offspring born to obese mothers have more MT aggregation with an increased ROS accumulation and a low MMP. This study stresses the importance of a heathy dietary intake for both mother and offspring to guarantee oocyte quality.

Role of secreted proteins and heparan sulfate on sperm binding to oviduct epithelial cells in cattle

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Keywords: spermatozoa, oviduct epithelial cells, oviduct fluid

After insemination in mammals, the elite of spermatozoa reaches the first part of the oviduct (isthmus) where they can bind to oviduct epithelial cells (OEC) to form a sperm reservoir (Lefebvre et al., Biol Reprod, 1995). In cattle, sperm receptors on OEC surface include glycoproteins which are also secreted in the oviduct fluid (OF) and interact with sperm as soluble proteins (Lamy et al., Reproduction, 2018). Sulfated glycosaminoglycans (sGAG) are other macromolecules secreted in the OF and known to interact with sperm surface (Plante et al., Cell Tissue Res, 2015). Moreover, heparin, a sGAG which is not present in the bovine OF, was shown to release bovine sperm from OEC (Talevi and Gualteri, Biol Reprod, 2001). How the proteins and sGAG present in the OF before ovulation modulate sperm adhesion to the oviduct reservoir is currently not known. The objective of this study was to assess the roles of soluble proteins and heparan sulfate, one of the most abundant sGAG in the bovine OF, on the binding of sperm to OEC.

Bovine isthmic OEC were collected from pre-ovulatory oviducts at a local slaughterhouse and cultured for 4 days (TCM199, 10% FCS, 38.8°C) to form spheroids with the apical side of OEC oriented outside. Groups of 20 spheroids ~100 µm in diameter were incubated with frozen-thawed Percoll-washed sperm from 3 bulls at 1.10^6/mL for 1 h at 38.8°C in a non-capacitating medium as control (TALP-HEPES + pyruvate/lactate/PVA; pH=7.4) ; OF at 1, 2 and 4 mg/mL of proteins; fractions of OF at 1 mg/mL after ultrafiltration (cut-off at 3 kDa); OF after heating (complement inactivation, 56°C, 30 min); OF after boiling (protein denaturation, 95°C, 5 min) or protein digestion (proteinase K); OF after heparan sulfate lysis (heparinase I/II/III treatment). After incubation, sperm-spheroid complexes were washed, fixed and stained with Hoechst for determination of bound sperm density using confocal microscopy. Sperm viability and motility were analyzed during incubation by flow cytometry and CASA, respectively. ANOVA followed by Tuckey post-tests were used to analyze the data.

Co-incubation of sperm with OF at 1, 2 and 4 mg/mL of proteins had no effect on sperm motility and viability but decreased significantly the sperm density on spheroids compared to controls with a dose effect ($p<0.001$). The OF fraction > 3kDa (containing most proteins and sGAG) but not < 3kDa reproduced the effect of OF on sperm density ($p<0.001$). Complement inactivation and protein denaturation did not reproduce the effect of OF on sperm binding. OF after protein or heparan sulfate digestion tended to reduce sperm binding compared to control ($p=0.08$ but to a lesser extent than native OF ($p<0.01$).

In conclusion, macromolecules in the pre-ovulatory OF decreased the ability of a sperm subpopulation to bind to OEC. Heparan sulfate together with other sGAG/proteoglycans and glycoproteins seem to be responsible of this effect.

MALDI-TOF lipidomic imaging of the oviduct after short and long-term exposure to an obesogenic diet in outbred mice.

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Keywords: obesogenic diet, oviduct, MALDI imaging

Metabolic disorders associated with the consumption of an obesogenic (high fat/high sugar (HFHS)) diet are strongly linked with reduced fertility in women. Direct detrimental effects of such metabolic alterations on oocyte quality have been documented, however the impact on the oviductal microenvironment where fertilization and early embryo development take place, is less characterised. Furthermore, when such changes appear after the start of a HFHS diet remains unclear. The aim of this study was to test whether the introduction of a HFHS diet in mice can lead to changes in lipid composition in the oviductal epithelial cells (OECs) and when these changes start to appear. Seven week old female outbred Swiss mice were fed with either control (CTRL; 10% fat) or HFHS (60% fat in diet, 20% fructose in drinking water) diet. Mice (n=3 per treatment per time point) were sacrificed and oviducts were collected at 3 days (3d), 1 week (1w), 4w, 8w, 12w and 16w after the start of dietary treatment. MALDI mass spectrometry imaging was performed to image lipids on sections of the oviduct (ampulla) using a Rapiflex MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with norharmane as matrix. Spectra at mass range of m/z 400-2000 were obtained in positive and negative reflectron modes (10µm resolution). Data sets were analyzed with SCiLS Lab 3D, version 2016b. Spatial segmentation of ion spectra showed differences in spatial distribution of lipid species between the oviductal epithelium and stroma. For further analysis we focused on the OEC layer, which was identified by co-registering the MS images with the optical scans of the H&E staining of the same section. Spectra from the OEC cluster were subjected to Receiver Operating Characteristic (ROC) analysis to calculate discriminative m/z values and determine differentially regulated lipids (DRLs) in the HFHS versus CTRL mice at each time point. Assignments were done from MS/MS spectra. ROC analysis revealed a different lipid profile in HFHS oviducts compared to the CTRL, which was shown by the detection of 303 and 247 discriminative masses (DMs) between HFHS and CTRL mice in negative and positive mode respectively, when including all time points. The total number of detected DMs in both reflectron modes increased over different time points, in negative mode this is from 10 DMs at 3d, 40 at 1w, 44 at 4 and 8w, to 55 at 12w and finally 110 DMs between CTRL and HFHS mice at 16w. The DRLs (across all time points) were focused in specific mass ranges, namely around 700-900 m/z in both modes which indicates differential abundance in phospholipids (phosphatidyl(P)-choline, P-serine, P-ethanolamine, P-inositol) and sphingomyelin. A few DRLs were detected around 1500 m/z suggesting a differential abundance of the mitochondrial lipid cardiolipin. In conclusion, exposure to an obesogenic diet results in changes in lipid profile in the oviductal epithelium even after a short exposure time of only 3 days. These changes progressively increase after longer exposure. Further analysis is ongoing to functionally annotate the detected DRLs and study their potential pathophysiological impact on the oviductal microenvironment and ultimately on the growing embryo.

Monounsaturated oleic acid addition during early embryonic development increases bovine blastocyst rates.

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Keywords: embryo; free fatty acid; stearoyl-CoA desaturase.

Metabolic stress, characterized by elevated levels of free fatty acid (FFA), have been linked to reduced female fertility. Saturated FFA (stearic acid (SA)) appears to have a dose-dependent negative effect on oocyte developmental competence while monounsaturated oleic acid (OA) is shown to be harmless. Oocytes are protected against FFA by cumulus cells via stearoyl-CoA desaturase 1 (SCD1) activity, which converts saturated SA into mono-unsaturated OA. To study FFA effects on early embryonic development, embryos were cultured in the presence of SA and OA and SCD activity was analyzed.
Cumulus-oocyte-complexes (COCs), collected from 2-8 mm sized follicles of bovine slaughterhouse ovaries, were *in vitro* matured (n=400/run) and fertilized (Aardema et al., Biol Reprod; 85: 62-69, 2011). During the first five days of embryo culture (day 1-5; i.e. oviductal period), embryos were cultured in SOF without (control) or with FFA (FFA conc.= 25 and 50 µM OA; 25 and 50 µM SA; 25 or 50 µM OA + 25 or 50 µM SA). Fatty acids were complexed to fatty acid free BSA (10 mM) (FFA:BSA ratio of 5:1). FFA was conjugated to albumin, likewise the transport of FFA *in vivo*, to solubilize FFA in an aqueous solution. At day 8 the number of blastocysts was counted. With RT-qPCR the mRNA expression of *SCD1* was measured in all the conditions. The general linear model was used for statistical analysis with SPSS 27.0. The day 8 embryos, COCs (positive control) and oocytes (negative control) were fixated in 4% PFA and incubated with a primary antibody, SCD1, diluted 1:100 in PBST overnight at 4°C for immunostaining. Thereafter, embryos were washed 3 times in PBST for 15 min and incubated with the second antibody, goat anti-rabbit Alexa™ fluor 647, diluted 1:100 in PBST for 1h in the dark at RT. Confocal microscopy was performed using an inverted Nikon A1R confocal microscope to determine the presence of SCD1 protein.
Exposure to 25 and 50 µM SA from day 1-5 resulted in a significantly lower blastocyst rate of respectively 18.9 ± 1.6% and 2.6 ± 4.6%, compared to the control condition 25.9 ± 3.1% (n=3; $p<0.05$). Interestingly, exposure to 25 and 50 µM OA resulted in a significantly higher blastocyst rate, 36.4 ± 6.3% and 34.6 ± 7.3% respectively (n=3; $p<0.05$). Exposure to 25 µM OA + 25 µM SA resulted in a blastocyst rate of 26.0 ± 3.7% comparable to the control condition and was not significantly different from exposure to 50 µM OA + 50 µM SA (25.6 ± 5.5%) (n=3; $p>0.05$). Interestingly, exposure to 50 µM OA + 25 µM SA resulted in a blastocyst rate of 33.7 ± 9.3% comparable to the 50 µM OA condition (n=3; $p<0.05$). SCD1 was faintly expressed at RNA and protein levels in day 8 embryos. Nevertheless, SCD1 was stronger detected in embryos compared to oocytes.
Previously, our group demonstrated no detectable SCD1 protein levels in oocytes, but solely in cumulus cells. We here showed that day 8 embryos express SCD1, which may protect embryos against saturated FFA. The current culture data show that OA counteracted the negative effect of SA on embryos. Future studies should investigate the role of OA and SCD1 in embryos.

The oviduct expresses the protein stearoyl-CoA desaturase 1 that converts saturated into mono-unsaturated fatty acids

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Keywords: oviduct, fatty acids, stearoyl-CoA desaturase

Reduced reproductive performance is an important problem in dairy cattle and has been related with the negative energy balance (NEB) during the first weeks postpartum. A major characteristic of the NEB are elevated concentrations of non-esterified free fatty acids (FFA), after mobilization from body fat reserves, in the blood and follicular fluid. Previous studies demonstrated that saturated FFA, like stearic acid (SA), have a negative effect on the developmental competence of the oocyte. In contrast, unsaturated FFA like oleic acid (OA) can reverse the negative effect of saturated FFA on oocytes (Aardema et al., Biol Reprod, 85, 62-69, 2011). Cumulus cells that surround the oocyte are proven to protect the oocyte for SA stress via the enzyme stearoyl-CoA desaturase 1 (SCD1), which converts saturated into unsaturated fatty acids (Aardema et al., Biol Reprod, 96, 982-992, 2017). However it is not clear whether the embryo is protected after release of the cumulus cells. In this study the focus lays on the oviduct, where the bovine embryo resides during the first 5 days after fertilization. The possibility that SCD1 is present in oviductal cells, and may act as a protector, was tested. Both fresh and cultured bovine oviductal epithelial cells (BOECs) originated from slaughterhouse material, were lysed before Western blot examination for the presence of SCD1. The antibody against SCD1, was kindly provided by Dr. Corl (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA). Fresh oviductal tissue was isolated of bovine genital tracts, treated with liquid nitrogen and the piece of fresh oviductal tissue was smashed with a hammer. Then it was lysed with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, US). Udder material, which was the positive control, was treated the same way. To collect BOECs, epithelial cells were squeezed out of the oviduct and lysed with RIPA buffer. Alternatively, the BOECs were cultured on a porous membrane for two weeks in order to obtain a confluent BOEC monolayer which was confirmed by TEER and Tracer-Flux measurements (Leemans et al., Biol Reprod, 106, 710-729, 2022). After one week BOEC differentiation was initiated by an air-liquid culture period of one week. The BOEC monolayer was lysed with RIPA buffer. The positive control showed the SCD1 specific 37 kDa band, which was also present in the isthmus region of the oviduct. However the band was not detectable in the ampulla region, squeezed epithelial cells and BOECs. However, SCD1 expression was visualized in the BOECs by the Nikon A1R confocal microscope, using the primary antibody of SCD1 by Dr. Corl, and its binding was visualised using goat anti-rabbit Alexa Fluor 647 (Thermo Fisher). Beyond the predicted 37 kDa band, an additional band was observed around 50 kDa in lysates of the isthmus and ampulla region, and the positive control. This 50 kDa band has been described for an commercial anti-SCD1 antibody (ab19862, Abcam, Cambridge, UK). The extra band could indicate an isoform of SCD1 or PTMs. The presence of SCD1 indicates that the oviduct is capable to convert saturated into unsaturated fatty acids, which may protect the bovine embryo during metabolic stress during early development.

Effects of endocrine disruptors ketoconazole and diethylstilbestrol on BOEC air-liquid interface monolayer culture

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Keywords: oviduct epithelium, endocrine disruptor, cytoskeleton abnormalities

Reproductive disorders, a worldwide public health concern, have been associated with exposure to endocrine disrupting chemicals (EDCs). Two well-known EDCs to influence female reproductive health are diethylstilbestrol (DES; a synthetic estrogen agonist) and ketoconazole (KTZ; a CYP450 steroidogenesis enzyme inhibitor). Here, the effects of DES and KTZ on a bovine oviduct epithelial cell (BOEC) culture, an *in vitro* animal model of the first embryo-maternal contact site (i.e. the oviductal epithelium), are explored. To establish a BOEC monolayer an air-liquid interface (ALI) culture approach was adopted, which supports cell differentiation (Chen et al., Sc. Reports, 7, 2017). Reported KTZ and DES effects include ALI-BOEC monolayer permeability, confluency, and actin organisation.
BOECs were mechanically isolated from the lumen of oviducts, obtained from slaughterhouse cows post-mortem. BOECs ($5x10^5$ cells) were seeded and cultured for 8 days in Transwell® cell culture inserts (Corning, USA, NY, CLS3413, 6.5mm). To introduce an ALI apical media was removed, while basolateral media was maintained. At day 14 of ALI, BOEC monolayers were exposed for 4 days to DES (10^{-9} M, 10^{-7} M, 10^{-5} M) or KTZ (10^{-8} M, 10^{-7} M, 10^{-6} M), or 0.01% v/v DMSO (vehicle). Effects of DES and KTZ on the permeability of the BOEC monolayer were assessed by transepithelial electrical resistance (TEER) measurement and paracellular tracer flux assay. TEER measurements confirmed confluency in all BOEC monolayers, with no significant difference between DES and KTZ treated vs DMSO treated monolayer (one-way ANOVA). The cell-impermeable tracer fluorescein disodium salt (12 µg/mL, 0.4kDa) was used for the tracer flux assay. Apical media was supplemented with the tracer and, after a 2h incubation, the basolateral media was collected to measure fluorescence. There was low percentage (<1.2%) of total tracer transferred from the apical to the basolateral side, and not significantly different between DES- or KTZ-exposed vs vehicle treated monolayers (one-way ANOVA). Consistent with TEER and tracer flux data, confocal microscopy of stained (phalloidin, acetylated α tubulin, Hoechst 33342) BOEC monolayers further supported confluency after DES and KTZ exposure. Clear basolateral cell-cell and cell-membrane adhesion was observed for all monolayers. In contrast to the membrane-adhering side of DES- or KTZ-treated BOECs, lateral cell-cell connections were scarce towards the apical side of the cells. This effect was similar in all doses of DES or KTZ and evidenced by gaps between phalloidin staining of individual cells, and it was not observed in DMSO treated BOECs.
In conclusion, DES and KTZ induced abnormalities in BOEC cytoskeletal organisation. We hypothesize that cell-cell junctional contacts in BOECs are disturbed by these EDCs. This distortion has an effect on cell polarity, and may also change epithelial cell binding and secretion properties. The possibility that this may indirectly cause aberrant early embryo-development, which could differ from direct exposure of these EDCs to the embryo cultures, is under current investigation.
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Uptake evaluation of bta-miR-181d present in extracellular vesicles from bovine oviductal and uterine fluids by in vitro produced embryos

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Keywords: mimic miRNAs, early embryo development, bovine

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through post-transcriptional mechanisms. We have found that bta-miR-181d was more abundant in extracellular vesicles (EVs) isolated from uterine fluid during mid-luteal phase (Days 5-10 of the estrous cycle) than in EVs from the oviductal fluid recovered during the early luteal phase (Days 1-4 of the estrous cycle) in cattle. In addition, bioinformatic analysis indicated that this miRNA is related to Hippo and WNT biological pathways, both critical for lineage segregation and blastocyst formation. Therefore, we aimed to determine whether bta-miR-181d is uptaken in bovine in vitro produced embryos by passive transfection (gymnosis). Presumptive zygotes (PZ) produced by in vitro maturation and fertilization were cultured in SOF (Control) or supplemented with 1 µM miR-181d mimics (miRCURY LNA miRNA Mimics; Qiagen, Maryland, USA); or 1 µM control mimics fluorescently labeled (miRCURY LNA miRNA Mimic 5′FAM, N° 339173, Qiagen). Embryos were collected at ≥16-cell (≥16C) and D7 blastocyst (BD7) stages and snap-frozen in LN_2 (3 pools n=10/ group) to examine the expression pattern of miR-181d by qPCR using miRCURY LNA miRNA PCR Assay. To confirm the uptake of control mimics fluorescently labeled, BD7 (n=10/group) were fixed, stained with Hoechst 33342, and observed under a widefield fluorescence microscope. Data were transformed by arcsine square root and tested for normality prior to One Way ANOVA. Embryo development was not affected by the presence of miR-181d or control mimics in the media (cleavage rate/PZ: 88±6.0%, 86±1.6%, 87±1.5% and blastocyst yield/PZ on Day 7: 25±0.8%, 25±2.2%, 24±0.1% for miR-181d mimics, control mimics and control respectively, P>0.05). Fluorescent staining showed that the control mimics can be taken up in blastocysts by gymnosis; however, expression of miR-181d mimics did not differ between groups, suggesting that embryos failed to incorporate this miRNA via this mechanism. Consequently, a second experiment was conducted to test Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, USA) for the delivery of miR-181d mimics. Hence, presumptive zygotes were cultured in SOF (Control) or supplemented with 50 nM miR-181d mimics and 1.5 µL Lipofectamine; or 50 nM control mimics and 1.5 µL Lipofectamine. Embryos (≥16C and BD7) were snap-frozen in LN_2 for future miR-181d expression analyses. Supplementation of Lipofectamine to the culture media did not have any deleterious effect on embryo development (cleavage rate/PZ: 82±4.0%, 81±2.1%, 82±4.1% and blastocyst yield/PZ on Day 7: 25±8.5%, 18±6.4%, 25±3.1% for miR-181d mimics, control mimics and control respectively, P>0.05). In conclusion, despite the fact that the control mimics is uptaken, the miR-181d mimics was not able to internalize in bovine embryos by gymnosis. Lipofectamine does not impair embryo development, hence, it could potentially be used as a carrier for miR-181d. Ongoing work will confirm whether miR-181d is internalized in embryos via this system.

Abnormalities in centrosome behavior are frequent in the first mitotic division of non-rodent mammalian zygotes

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Keywords: zygote, centrosomes, aneuploidy

Post-zygotic or "mosaic" aneuploidy, i.e. the presence of a subset of cells with an aberrant number of chromosomes, is a frequent feature of human preimplantation embryos. A high incidence of aneuploidy within an embryo is recognized as the major cause of developmental arrest and miscarriage. Post-zygotic aneuploidy often arises during the first cell divisions in the embryo and it is not only exclusive of human embryos, but has a similar occurrence in nonhuman primate, bovine, and equine embryos. Despite the wide incidence and often severe developmental consequences of postzygotic aneuploidy, it is still unclear why the early cleavages are so prone to errors.
In somatic cells the centrosomes, formed by two centrioles surrounded by the pericentriolar material, are the two major microtubule-organizing centres (MTOCs) and play an essential role in spindle assembly and chromosomes segregation. Mammalian oocytes lack of centrosomes and, although two centrioles are re-introduced by the spermatozoon at fertilization, we recently showed that centrosomes make only a minor contribution to zygotic spindle assembly. Although not essential for spindle assembly, the role of centrosomes in ensuring fidelity during the zygotic division is still unclear. Here, we evaluated the incidence and consequences of centrosomes abnormalities in the zygotic division of bovine embryos, a species which, similarly to human embryos, inherit centrioles paternally at fertilization. To this end we imaged the first mitotic division in real-time live bovine zygotes (n=55) injected with mRNA encoding for H2B-mCherry and MAP4-eGFP to allow visualization of chromatin an microtubules respectively.
Abnormalities in centrosome behaviour were observed in 40% of the zygotes imaged. The most observed abnormality was failure (15%) or delay (10%; range: 10-30min after nuclear envelope break down) of one of the centrosome to engage to the metaphase spindle. Premature fragmentation of one of both of the centrosomes (10%; range 6-21 min before anaphase) and abnormal positioning of one of the centrosomes (5%) within the mitotic spindle were also observed. Centrosome failure to engage to the mitotic spindle resulted in 60% of the cases in the inability of one or more chromosomes to be captured by spindle microtubules. In contrast, chromosomes lagging after anaphase onset (50%) was observed with the same frequency also in zygotes displaying normal centrosomes behaviour. Non injected zygotes (n=350) fixed at different stages of the first mitotic division showed similar type and frequency of centrosome abnormalities as the ones observed in mRNA injected zygotes. Taken together our observations suggest that centrosomes partially contribute to chromosomes segregation fidelity during early embryonic development, however other players are probably responsible for the high incidence of lagging chromosomes. Further studies are needed to elucidate the reason why in mammalian zygotes centrosomes are less active as MTOCs than in somatic cells.

Towards serum-free culture conditions for bovine endometrial explants: some preliminary results

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Keywords: Endometrium, explant culture, serum-free médium

Bovine endometrial explant culture has emerged as a model for the study of *in vitro* uterine function. Culture medium for endometrial explants is routinely supplemented with fetal bovine serum (FBS) as it contains hormones, vitamins, transport proteins, and growth factors that optimize *in vitro* endometrial function. However, there is an increasing interest in using serum-free conditions as a more defined medium and due to sanitary and animal-welfare concerns (FBS is an animal-derived product). Yet, the optimal serum-free culture medium composition and incubation time window for endometrial explants remain to be elucidated. This study aimed to evaluate the effect of serum and serum-free culture medium and incubation time on endometrial explant viability markers. Five uteri from healthy Belgian Blue cows at the diestrus stage (stage I corpus luteum) with no evident signs of gross inflammation were collected from the slaughterhouse and transported to the laboratory within 1 h. Intact endometrium tissue samples (8 mm in diameter; 1-2 mm in thickness) were obtained using a sterile 8-mm punch biopsy. The explants were cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 50 µg/mL gentamicin and supplemented with, (1) control (no supplementation), (2) 10% FBS, (3) 10% serum replacement (SR) (Knockout™ SR, Gibco), and (4) 1% bovine serum albumin (BSA). Spent culture medium samples were collected at 6, 12, 18, 24, 30, 36, 42, and 48 h of incubation and assayed for interleukins (IL-1ß and IL-6) using bovine-specific ELISA kits. Indirect assessment of tissue viability was measured through lactate dehydrogenase (LDH) activity (colorimetric test) in the spent culture media. The effect of culture medium composition, incubation time, and their interaction on IL-1ß, IL-6, and LDH concentrations were fitted in linear regression models in RStudio. No differences ($P > 0.05$) within the first 24 h of culture were found among experimental groups for IL-1ß. Reduced IL-1ß concentrations ($P < 0.05$) were found in FBS compared with SR at 30 and 42 h of incubation. Control and FBS culture medium had lower IL-6 concentrations at 12, 18 and, 24 h compared with the other groups ($P < 0.05$). The LDH activity was higher ($P < 0.05$) for FBS than SR (6 and 18 h) and BSA (12, 18, 24, and 30 h). The present study shows that a serum-free medium is a valid alternative for short-term bovine endometrial explant culture. However, high LDH activity may suggest that endometrial explants viability significantly declines after 24 h of incubation irrespective of culture conditions.

Overview on the metabolism of buffalo oocyte during in vitro maturation

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Keywords: Buffalo, Oocyte, Metabolomic

The interest in IVEP in buffalo (Bubalus bubalis) has increased worldwide in the last decade, due to the recognized role of this livestock in different environments. It is known that an appropriate oocyte maturation is critical for the acquisition of oocyte competence. The aim of this study was to analyze the variation of metabolite content during IVM of buffalo oocytes, in order to acquire information on oocyte metabolism during maturation. Abattoir-derived buffalo oocytes were divided into two groups (N=50/group, over 5 replicates): immature oocytes (IO) and oocytes in vitro matured (MO) according to standard procedures (Gasparrini et al.,Theriogenology,54,1537-1542, 2000). Immediately after collection and 22h post IVM oocytes were denuded, pooled (N=10) and stored at -80°. To extract the polar fraction, samples were re-suspended in methanol, sonicated for 30 sec for cell lysis, centrifuged (4000 rpm, 10 min) in the presence of chloroform and analyzed by Proton Nuclear Magnetic Resonance (1H-NMR). Pathway analysis on polar metabolites was performed using the Metabo Analyst tool (Xia et al., Nucleic acids research,37,652-660,2009). The Orthogonal Projections to Latent Structures Discriminant Analysis plot evidenced that the two groups clustered separately, suggesting the presence of significant differences in metabolites in relation to maturation stage. The Variable Importance in Projection plot showed the proton signals corresponding to metabolites that showed the greatest variation. Interestingly, among the huge number of detected metabolites, some amino acids such as ornithine, arginine, trimethylamine, asparagine, leucine, proline and alanine were found in lower concentration in MO compared to IO, with the exception of tryptophan that showed an opposite trend. The reduction of the amino acid content may be due to the use of these substrates during maturation for both energetic and biosynthetic processes. After IVM also glucose was reduced while the ATP content was higher, suggesting an increased energy production during maturation. Finally, in MO lower levels of glutathione were found compared to IO, suggesting that oocytes use this thiol to counteract oxidative stress. The pathways enrichment analysis revealed that the metabolites showing variations during IVM are mainly involved in glutathione metabolism, as well as glycine and serine metabolism. These preliminary results can be considered as a starting point to better understand the metabolism of buffalo oocytes during the maturation process. The significantly changes in the metabolite content and the higher ATP levels found in matured oocytes are probably related to the need of producing energy for the subsequent phases of development. However, further studies are needed to deeply investigate buffalo oocyte metabolism, providing information on metabolic needs, useful to improve the IVM system in this species.

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

Seasonal variation of morphometrical characteristics of fresh and cryopreserved Saanen goat sperm

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Keywords: Goat sperm, cryopreservation, morphometry, seasonality

Cryopreservation of sperm allows use of assisted reproductive technologies (ART) in animal husbandry more efficiently. Various studies have shown that the survival rate of sperm after cryopreservation depends on their initial morphofunctional characteristics. Cryopreservation of sperm with the best morphofunctional characteristics may allow them to be used for ART in goats throughout the year to effectively increase livestock. Therefore, the aim of the study was to evaluate the effect of seasonality on the morphometric characteristics of goat sperm before and after cryopreservation.

Ejaculates of 3 mature Saanen bucks were obtained by artificial vagina twice a month during the breeding season (September-December, n = 24) and non-breeding seasons (February-July, n = 36). Cryopreservation of sperm was performed in a HEPES buffer medium with 10% glycerol and 20% egg yolk. Sperm suspension with cryoprotectant was transferred into straws with a 0.25 ml volume, equilibrated for 30 min at room temperature (+20 °C), 2.5 h at +5 °C, 15 min in nitrogen vapors 4 cm from the liquid nitrogen and plunged into liquid nitrogen. The samples were thawed in a water bath (+37 °C) for 30 sec. Smears of fresh and cryopreserved sperm were fixed and stained using the Spermac Stain kit (FeriPro, Belgium) according to the manufacturer's protocol and visualized under a light microscope at a magnification of x1000. For morphometric measurements, sperm micrographs were taken and analyzed using ImageJ software (version 1.51j8, NIH, USA). Mann-Whitney U-test was used to compare the two samples, the difference was considered significant at $p \leq 0.05$.

Morphometric analysis of sperm showed that the size of the head and tail decreased after cryopreservation, compared with these measurements before cryopreservation. After cryopreservation, the length and width of the head decreased significantly ($p \leq 0.05$) compared to the corresponding sperm size before cryopreservation. Moreover, these changes were observed in both breeding and non-breeding seasons. When analyzing the morphological characteristics of the sperm tail, a reduction after cryopreservation was observed, probably due to the twisting, the formation of loops, and the separation of its part.

To conclude, the morphometric characteristics of Saanen goat sperm change significantly after cryopreservation, regardless of the season. After cryopreservation, the size of the head and tail of sperm is significantly reduced.

Placental vascularization in in vitro-derived pigs: a preliminary study.

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Keywords: placenta, vascularization, pig

The placenta plays a critical role in maintaining and protecting the developing fetus. Placental vascularization abnormalities, including a decrease in arterial number, lumen size, and branching, have been extensively described in humans born from in vitro-produced (IVP) embryos (Riesche and Bartolomei, Seminars Reprod Med, 36:240-247, 2018) but studies on IVP pigs are very limited (Ao et al., Placenta 57:94-101, 2017). The objective of this study was to compare the placental vascularization in pigs born from in vitro- and in vivo-produced embryos (the latter born by artificial insemination; AI group). IVP embryos were produced after in-vitro fertilization (IVF) of in vitro matured oocytes and further culture (IVC) up to blastocyts stage in media supplemented with or without 1% porcine oviductal fluid and 1% uterine fluid (more details in Paris-Oller et al., J AnimSci and Biotech 12:32-44, 2021). Blastocysts produced with (RF-IVP group) and without (C-IVP group) reproductive fluids were surgically transferred at day 7 post-IVF. Both AI and IVP embryos were produced with spermatozoa from the same boar. After birth, placenta samples were collected at 3-5 cm from the insertion of umbilical cord, and fetal parameters were recorded. The placenta of 9 animals (3 per group) from different litters was selected following these criteria among animals: similar uterus position, birth weight, and crown-rump length; and a close male/female ratio among groups. Samples were fixed (10% formaldehyde solution) and paraffin embedded. Two complete placental sections (5 µm thickness) were stained (hematoxylin–eosin), photographed at 5x (ZEN 3.2, ZEN lite, Zeiss) and images processed (ImageJ) for a detailed study to record vessel number, area occupied by each vessel (µm^2), and total vascular area (%). Based on their size and histological characteristics, vessels were categorized by an expert operator as capillary (1-500 µm^2), arteriole/venule (501-1000 µm^2), small artery/vein (1001-3000 µm^2), medium-sized artery/vein (3001-30000 µm^2), and large artery/vein (>30000 µm^2). Data (mean±SEM) were analyzed by one-way ANOVA (Systat v13.1), and differences (P<0.05) were compared by Tukey's test. The total placental area observed, and total number of vessels analyzed was higher in AI (86.1±7.5 mm^2, 726 vessels) than C-IVP (45.9±6.8 mm^2, 544 vessels), and RF-IVP (52.8±5.1 mm^2, 637 vessels) (P<0.05). However, no differences were found in the total vascular area being 14.9±3.3% (AI), 19.9±2.7% (C-IVP), and 17.8±2.2 (RF-IVP) with similar pattern distribution in all groups: over 85% microvessels, 10-15% medium-size vessels and 5% macrovessels. However, the vascular area occupied by medium-sized vessels (arteries and veins) was significantly higher in the AI group (7.2±0.5%) than in IVP groups (2.1±0.3% and 1.8±0.2%) regardless of the addition of reproductive fluids (P<0.05). No differences in vascular areas of micro and macrovessels were observed. Preliminary results show that impaired placental vascularization in ART-derived pigs might occur due to a reduction of medium size vessels.

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Transcriptional differences between cattle and buffalo growing follicles: possible effect over oocyte competence.

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Keywords: Buffaloes, gene expression, oocyte competence, Cattle, Apoptosis

Buffaloes are exceptional specie used to produce meat and milk under challenging conditions, especially in tropical areas. Since this is a relatively new production system, many practices used in cows have been applied to buffaloes, especially in the field of reproduction. Our team and others have been reported that the use of follicle-stimulating hormone (FSH) before aspiration increases the number of follicles and the number of oocytes recovered, and although FSH has its effect on the follicle, oocytes produced are no more competent than controls to form embryos producing similar blastocyst rate. To explore the different outcomes with a similar stimulation protocol, we studied the transcriptome of early dominant and subordinate follicles using granulosa cells as an indicator of follicular differentiation.
The somatic RNA was obtained from granulosa cells from growing follicles > 7mm obtained from ovaries of 10 buffaloes and 10 cattle recovered at the local slaughterhouse, sequenced with NovaSeq, and the differential expression was analyzed using EdgeR in Bioconductor. Files from differential expressed genes were cleaned, removing 9669 genes (low count) to be analyzed using Ingenuity Pathway Analysis.
Of 5393 genes that were retained for the analysis and with significant p-value, the most interesting canonical differences are related to eukaryotic initiation factor 2 (EiF2, growth), mammalian target of rapamycin (mTOR, energy), signal transducer and activator of transcription 3 (STAT3, differentiation), and protein kinase A (PKA, stimulation) on the upstream side. Progesterone and progesterone receptor (PGR) differences may indicate a difference in changes preparing for ovulation but transforming growth factor-beta (TGFB), p53, tumor necrosis factor (TNF), and phosphatase and tensin homolog (PTEN). These genes are all related to differentiation and apoptosis and may indicate a different timing in follicular dynamics between the two species. From a practical perspective, this result may indicate that coasting -FSH withdrawal prior to ovum picK-up- creates atresia faster in buffalos than in cows.

Evaluation of two IVP bovine embryo sexing techniques according to their ability to preserve embryo viability after vitrification/warming

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Keywords: Cell-free DNA, Blastocoele collapse, trophectoderm biopsy

Trophectoderm (TE) biopsies are frequently used for embryo genotyping, although they are invasive and harmful to further embryonic development. Cell-free DNA (cfDNA) found in blastocoele fluid (BF) can be considered as a non-invasive sexing method. The purpose of this study was to compare the accuracy of a non-invasive method using cfDNA present in BF, to the biopsy procedure in terms of determining the embryo's sex and its effect on embryo survival following vitrification/warming. Expanded Day 7 IVP embryos were randomly assigned to two groups: VIT-Collapsed (n=37), blastocysts artificially collapsed by aspiration of BF with an ICSI pipette; VIT-Biopsied (n=56): blastocysts biopsied by cutting off a small portion of the TE using a microblade. After sample collection, all embryos were vitrified/warmed by the Cryotop method and individually cultured *in vitro*. Intact embryos individually cultured (VIT-Single) (n=58) or cultured in group (VIT-Control) (n=56) after vitrification/warming were used as vitrification controls, whereas intact non-vitrified embryos were used as fresh controls (n=45). The survival of vitrified blastocysts was assessed as re-expansion and hatching rates at 24 h post-warming. Sex identification was performed in BF or biopsies as well as in the corresponding surviving embryos of VIT-Collapsed and VIT-Biopsied groups. BF samples underwent a whole genome amplification using REPLI-g single cell kit (Qiagen, Germantown, MD, USA), whereas biopsies and blastocysts were lysed by incubation with 100 µg/mL proteinase K at 55°C for 2h. Embryo sex was analyzed by PCR using two sets of primers: Y-chromosome specific primer (*BRY4a*) and bovine specific satellite sequence primer (*SAT1*). Products were visualized on a SafeView stained 2% agarose gel. Samples with *BRY4a/SAT* bands were considered male, while samples with only the *SAT1* band were assigned as female. Data were analyzed with a one-way ANOVA ($P \leq 0.05$). VIT-Collapsed blastocysts showed similar post-warming survival rates (87.55±16.1%) to those of fresh non-vitrified blastocysts (100%) and significantly higher than blastocysts from the VIT-Single and VIT-Control groups (79.0±9.1% and 72.0±14.3%, respectively). Blastocysts vitrified after biopsy showed the lowest ($P \leq 0.05$) survival rate (53.5±12.6%). No differences ($P>0.05$) between the two sources of DNA were observed either in their amplification efficiency (72.0% (18/25) in BF samples; 79.3% (23/29) biopsies) or in their accuracy in sex diagnosis (83.3% (15/18) in BF samples; 82.6% (19/23) in biopsies). In conclusion, the results of this study indicate that cell-free DNA analysis is an efficient and minimally invasive approach to sex IVP cattle embryos. Moreover, artificial collapse of blastocoel had a positive effect on embryo viability after vitrification/warming. Further studies to improve the efficiency of cell-free DNA collection and amplification are guaranteed.
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PRACTITIONERS´and CLINICAL REPORTS

Influence of body condition score and lactation status on oestrus response and pregnancy rate in dairy and beef cows inseminated with sex-sorted or non-sex-sorted semen

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Keywords: synchronization, pregnancy, cattle

Oestrous synchronization and artificial insemination (AI) are two advanced reproductive technologies that dairy and beef cattle productions are increasingly using to enhance their herds' reproductive performance. However, oestrus synchronization can be influenced by many intrinsic and extrinsic factors. Therefore, the objectives of this study were to compare the effects of body condition score (BCS) and lactation status on oestrus response rate and pregnancy rates of dairy and beef cows submitted to timed artificial insemination (TAI) with sex-sorted or non-sex-sorted semen. For this study 231 cows (dairy; n = 134 and beef; n = 97) with BCS of ≤2.5, 3 and ≥3.5 (scale 1: emaciated to 5: obese), lactating or dry and at 90 days postpartum were used. On any given day throughout the oestrous cycle (Day 0) the cows received a controlled intravaginal drug release (CIDR®, Pfizer Laboratories) device, with 2 mL intramuscular (i.m.) of estradiol benzoate® (EB; VTech). On Day 5, 2.5 mL i.m. of pregnant mare serum gonadotrophin (Chronogest®, Intervet International B.V.). On Day 8, 2 mL i.m. of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (Estrumate®, Intervet, South Africa), with adhesive tail-head heat mount detectors (HMD) (Kamar®, USA) and CIDR® was removal. On Day 9, 1 mL i.m. of EB. TAI was performed by the same inseminator 55 hours following CIDR® removal using frozen-thawed X-sorted or non-sex sorted semen from eight sires (4 Holstein Friesian and 4 Angus). At AI, oestrus behaviour was assessed by activation of the HMD colour either as are red (oestrus/activated patch) or white (no oestrus/ not activated patch). Pregnancy diagnosis was performed 95 days following TAI using transrectal ultrasound scanner (5.0-MHz linear transducer; Ibex pro™, USA). Chi-square test was used to compare the proportion of oestrus response and pregnancy. The model included sires as a fixed effect. The proportion of oestrus response by BCS of ≤2.5 (79.0%), 3 (89.0%) and ≥3.5 (92.6%) were higher in dairy cows as compared to ≤2.5 (68.4%), 3 (61.1%) and ≥3.5 (70.8%) beef cows ($P < 0.05$). Lactating (86.2%) and dry (81.5%) dairy cows had higher oestrus responses, compared to beef lactating (67.7%) and dry (59.4%) cows ($P < 0.05$). The proportion of pregnancy was higher in BCS ≥3.5 in dairy (64.3%) cows compared to beef (40.0%) cows inseminated with non-sex-sorted semen ($P < 0.05$). However, BCS of 3 on beef cows had higher (41.9%) pregnancy rate compared to dairy (31.6%) cows inseminated with sex-sorted semen. Lactating dairy cows inseminated with sex-sorted (42.5%) or non-sex-sorted (50.0%) semen, had higher pregnancy rate compared to beef (sex-sorted; 31.2% and non-sex-sorted; 34.4%) ($P < 0.05$). However, pregnancy rate in dry cows was higher in beef (sex-sorted; 47.4% and non-sex-sorted 46.1%) cows compared to dairy (sex-sorted; 36.4% and non-sex-sorted; 36.4%) ($P < 0.05$). In conclusion, BCS and lactation status of dairy and beef cows do affect negatively on oestrus response and pregnancy rate.

Differences in reproductive parameters between two close related bovines, buffalo and cattle raised in the same environment conditions

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Keywords: Reproductive parameters, buffaloes, cattle, differences

Buffalo and cattle are bovines that belong to the subfamily Bovinae, used worldwide to produce milk and meat. Reproductive parameters are different *in vivo* and *in vitro,* mainly when applying reproductive technologies. It has been reported that buffalo has a smaller number of primordial follicles than cattle do (10 000–19 000 vs. 150 000, respectively), smaller antral follicles, and a higher incidence of atresia (82–92%) (Kumar A. Anim Reprod Sci. 1997;47:189) as a consequence buffalo ovaries are smaller than cattle., few reports compare the consequences over reproduction of these differences, especially those produced natural mating system and their reproductive parameters. This report performed in 2021, aims to compare reproductive parameters in two bovine species located in the same geographical area, with the same reproductive management, cattle (brahman breed N=912).and buffaloes (crossbred N=262). The farms are located in Middle Magdalena Region in Colombia (6°18′48″N 73°57′00″O). Data from reproductive parameters (calving, inter calving period (IEP), days open (DO) were collected and pregnancies were diagnosid by rectal palpation, and compared. Cattle and buffaloes were culled at 10 and 15 years.Pregancies were diagnosed by rectal palpation.Data were analyzed using descriptive statistics and comparison using the student T-test. $P<0.05$ was considered statistically significant. Calving numbers were 3.04 and 4.36 for cattle and buffaloes, respectively ($P< 0.05$). Calving, IEP, and DO were 67.8% vs. 96.5%, 528 days vs. 420, and 275 days vs. 133 days, respectively, for cattle and buffaloes and statistically significant.For other parameters such as first calving age and abortion rate based on pregnancy detection was 42.76 months vs. 35.09 months, and 10.80% vs. 6.04% were are also statistically significant. If the results in reproductive parameters are associated with differences in calving number, they remain to be clarified, but there are undoubtedly paradoxical because the specie with lower quantity of primordial follicles has better reproductive performance. To date, there are no explanations for this phenomenon. Many reports that show the differences in many other reproductive parameters *in vivo* and *in vitro*. Indeed, the reproductive parameters of buffaloes and cows are different; and from this resutls are better than cattle. This performance makes the production of buffaloes more attractive to breeders specially in lands where cattle dont produce well (wet lands and humid foresr) and for reproductive biology researchers to study the causes of this observation,

Difference in performance between OPU and slaughterhouse derived oocytes

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Keywords: IVP, OPU, slaughterhouse

CRV is continuously working on further improvement the IVP embryo production process. In that process new commercial media or modification of in the standard CRV (in house made) media are tested. These media are always first tested in several experiments using slaughterhouse derived oocytes. If the embryo production rate of those experiments look good, i.e. are at least similar or better than the control (=CRV standard medium), a trial using OPU derived oocytes is started.
The aim of this study is to check whether the results of these media trials obtained with slaughterhouse oocytes are representative for those obtained with OPU derived oocytes.
Oocytes for the slaughterhouse trials are derived from ovaries of animals (mainly HF) that are slaughtered because of low production, fertility problems, etc.. These ovaries are transported (transport time ~6Hrs) at 30 °C to the IVP lab. Upon arrival oocytes are collected and quality 1 and 2 oocytes (50:50 ratio) are used for the experiments.
Oocytes for the OPU trials, are collected from animals (mostly heifers) at our own CRV station. In this case also mainly quality 1 (28%) and 2 (66%) is used in the experiments (remaining part are some quality II and IV).
Subsequently, both groups of oocytes (slaughterhouse and OPU derived) are used for in vitro production of embryos using the standard CRV media (=control) or using a new serum free medium (=test medium). The standard CRV protocol starts with a 24 hr maturation in M199 supplemented with 10% FCS, LH&FSH plus cysteamine, followed by a fertilisation for 24 hrs and a culture of 7 days in SOF-BSA medium with 0.2% serum. In the test group we used serum free media (both IVM and IVC), but supplemented the IVM and IVC media with EGF. For the slaughterhouse we did 5 different sessions for the test and control medium. In the OPU experiments we did 125 different sessions for the test medium and 178 for the control.
The results of the slaughterhouse clearly indicated that the test medium is better than the control (% total embryos 29% and 24%, respectively, $P<0.05$ Chi square test). However, the OPU results were completely different. In that case the test group was significantly worse than the control group (36% compared to 43% total embryos, $P<0.05$ Chi square test). If you look at only the quality 1+2 embryos (i.e., the transferable ones) the same pattern was observed.
From these results it is clear that the test medium, when used in combination with slaughterhouse derived oocytes, is significantly better than the control medium. However, when OPU derived oocytes are used, the test medium is significantly worse than the control medium. This is a phenomena we also see in other trials with different media.
It is difficult to explain these results. It might be because of the higher percentage of class 2 oocytes in the OPU derived group. Potentially class 2 oocytes are more sensitive to different (potentially less optimal) media.
We therefore conclude that you have to be very careful in making a decision to switch to new IVP medium based on slaughterhouse experiments only.

Efficiency of repeated in-vivo embryo collections in Holstein heifers

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Keywords: in-vivo collection, bovine, breeding program

Worldwide, the in-vitro production (IVP) of embryos within Holstein breeding programs has increased significantly in recent years. Overall, the collection of embryos after superovulation and flushing is declining (IETS,2020). Compared to IVP embryos, embryos obtained in vivo are characterized by higher pregnancy rates, lower losses until calving and higher cryotolerance (Hansen,2020).

The aim of this study is to show that larger numbers of embryos transferable for transfer can be obtained within a short time by repeated flushing. For this purpose, the data of four ET teams in Schleswig-Holstein, Lower Saxony and Hessen from the period January 2019 to March 2022 were evaluated. The calculation was carried out with Excel ®. The means were presented as arithmetic mean (AM) and median (MED). The first ($Q_{0.25}$) and third quartiles ($Q_{0.75}$) were also calculated.

1.353 embryo collections (day 7) of 712 different heifers of the Holstein breed were taken into account. The embryos were classified according the IETS standard. The frequencies of the flushings were distributed as follows: 347 heifers 1x; 223 heifers 2x; 76 heifers 3x; 35 heifers 4x; 15 heifers 5x; 8 heifers 6x; 4 heifers 7x and 1 heifer each 8x, 9x, 10x or 14x.The superovulation was done with Folltropin® (Vetoquinol) in 676 flushes and with Pluset® (Calier) in 677 flushes. 446 recoveries took place on a donor station and 907 on the farm of the breeder. On the donor station the animals were usually given an intravaginal progesterone releasing device (CIDR®; Zoetis) after each collection.

In the 1.353 flushings 12.783 embryos/oocytes (AM=9,4;MED=9; $Q_{0.25}$=5; $Q_{0.75}$=13) were recovered. 9.129 embryos (AM=6,4; MED=6; $Q_{0.25}$=3; $Q_{0.75}$=10) were suitable for transfer. At the first collection the heifers had an average age of 13.2 (MED = 13 $Q_{0.25}$=12; $Q_{0.75}$=14). Animals flushed more than four times were on average 11 months old on the day of first embryo collection (MED = 11; $Q_{0.25}$=10.0; $Q_{0.75}$=12.0). The interval between the collections averaged 51 (MED =46; $Q_{0.25}$=35; $Q_{0.75}$=59) days. 52.7% of the heifers were superovulated again at intervals of 31 to 50 days. In this group, 5,5 embryos suitable for transfer could be obtained per flush (MED=5;$Q_{0.25}$=2; $Q_{0.75}$=8). In heifers on a donor station, the average intervall was only 43 days (MED=36 $Q_{0.25}$=35; $Q_{0.75}$=48).

When the heifers were flushed four times, 25 transferable embryos could be obtained within 152 days.

It could be shown that a sufficient number of embryos can be obtained as part of a breeding program using conventional embryo transfer. The better pregnancy results of the embryos obtained in vivo (fresh and frozen) help the farms to use their limited number of recipients economically and effectively.

An increase of the effectiveness of embryo recovery seems possible. In many cases it seems to make sense to start using the donor animals even earlier. In many cases, the interval between flushings can also be shortened further (see flushings on station). Here, the farms need to be advised on rearing intensity and measures to shorten the intervals between embryo collections.

IETS (2020), Data Retrieval Report 2020; https://www.iets.org Hansen (2020), Journal of Animal Science, 2020, Vol. 98, No. 11, 1–20

Fertility of heifers after superovulation and embryo collection – breaking the myth

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Keywords: superovulation, reproductive performance, embryo donor

There exists a myth among farmers in Finland, that superovulation and embryo flushing can jeopardize further reproductive performance of embryo donors. Data of 866 heifers of Holstein and Ayrshire breeds, which had undergone superovulation and embryo collection on Finnish dairy farms (donor group) were analyzed. The control group (n=824) consisted of untreated herd mates, which had their first service at the same time period as the donors. The average age at first service was 16.1 and 15.5 months for donors and controls, respectively. The following reproductive parameters were analyzed: first service conception rate, services per conception, length of the breeding period, and culling due to infertility. Independent samples t-test was used to analyze the differences between the groups.

Conception rate at the first service was similar in both groups, 52.2 and 52.1% for donors and controls, respectively. When donors were separated into two groups according to their embryo production, conception rate was 43.5 % for those which didn't produce any viable embryos (n=92) and 53.5 % for those which produced embryos (n=720). Double inseminations (>1 AI per estrus) were performed for 8 % of the donors and 4 % of the controls. Because of the higher frequency of double inseminations in the donor group, consecutively a more services per conception were required for donors compared to the controls (1.95±1.35 vs. 1.73±1.05, $P<0.001$). However, there was no difference in the duration of the breeding period for donors and controls (26.2±43.8 and 25.3±48.1, $P=0.693$). Within the donor group, 6.2 % of heifers never calved and were culled. In the control group, 10.3 % of heifers were culled without a preceding calving.

These findings indicate that farmers need not to be concerned of embryo production being a risk for reproductive performance of donor dairy heifers. More services per conception were needed after superovulation, but this was because of more double AIs. The reason(s) for increased frequency double AIs in the donor group were not investigated in this study. It is possible that the farmers' fertility management strategies were different for donors, as they were slightly older at the time of first service compared to their herd mates and represent the top animals of the herd, resulting to multiple AIs in order to ensure the pregnancy possibility. Also, the estrous behavior after a superovulatory treatment could be more vague and thus timing of insemination more difficult to predict. Further research is needed to investigate if there is a relationship between viable embryo production and conception rate, as the poor-responding donors showed a lower conception rate at first service. However, the most important parameter from the practical and economical point of view, the time from first service to conception, was similar for the donors and untreated controls. Also, superovulation didn't increase the risk of the heifer being culled before first calving.

Workshop I: Sperm in the female tract - what can we learn in vivo and in vitro?

Insights into the multifunctional roles of bovine host defence peptides in reproduction and immunitye host defence peptides in reproduction and immunity

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Keywords: Defensins, Cathelicidins, immune, fertility

Previous work by our group uncovered a panel of novel host defence peptides, consisting of both ß-defensin and cathelicidicin families which show evidence of expansion in the bovine genome compared to humans and mice. We hypothesised that the peptides these genes encode for would play important antimicrobial and immunomodulatory roles. Expression patterns suggested a role for the ß-defensin genes in the male reproductive tract with higher levels of expression in the caudal epididymis. Analysis of evolutionary orthologues showed that one of these proteins, ß-defensin 126, creates an 'invisibility cloak' on human sperm to prevent their recognition by the female immune system as they pass through the cervix and uterus. More recent analyses have also suggested that restoration of ß-defensin levels on human sperm can improve antimicrobial function. Taking a lead from these human studies we assessed the antimicrobial and reproductive roles of the bovine peptides using genetic association studies, antibacterial assays and a range of *in vitro* functional assays. In an analysis of bulls used in artificial insemination with divergent field fertility, we uncovered a ß-defensin haplotype representing a single nucleotide polymorphism (SNP) panel significantly associated with reduced fertility. Within the haplotype was the bovine ß-defensin 126 gene and functional assays confirmed a role for this protein in sperm agglutination, motility and binding to oviductal epithelium. Three-dimensional analysis of the protein structure using the recently released artificial intelligence program *AlphaFold*, shows a very distinctive extended C-terminal tail on some ß-defensins which may yield important insights into their precise biological roles. In contrast, a highly coiled α-helical structure is apparent in the cathelicidin peptides. The reproductive tract of the male represents a very dynamic environment with sequential expression of specific peptides to equip sperm for transit and survival after ejaculation. Our research shows that these peptides play important reproductive and immunological roles, critical to protection of gametes within a highly regulated immune environment. Although more detailed functional investigation is required, these multifunctional peptides exhibit relevant species-specific adaptations and thereby offer exciting potential for targeting pathogenic bacteria and improving fertility via modulation of the semen microbiome.

Regulation of sperm transit across the ovine cervix

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Keywords: cervical mucus, sperm interaction, sialic acid

In species where semen is deposited in the vagina, the cervix and its secretions are the major barriers for sperm transport. The ewe is an excellent model for investigating how sperm are selected in the cervix as semen is deposited vaginally and cervical artificial insemination (AI) is limited due to poor pregnancy rates when frozen–thawed semen is used. Worldwide, pregnancy rates rarely exceed 30% when frozen–thawed semen is used in conjunction with cervical AI. However, Norway is the exception to this, since they routinely achieve pregnancy rates of 60–70% with vaginal (shot-in-the-dark) AI to a natural oestrus. This has been reported to be due to the breed of the ewe used in Norway and specifically the inability of frozen–thawed sperm to traverse the cervix of some ewe breeds. During oestrus the cervix is filled with mucus, which is a gel made up predominantly of mucins, which are heavily *O*-glycolysated proteins that can be modified by the addition of terminal sugars such as either fucose or sialic acid. In order to identify the components in the cervical mucus that could explain why frozen-thawed ram sperm can traverse the cervix of some ewe breeds but not others we used a novel sheep model composed of six ewe breeds with known differences in cervical sperm transport following cervical AI with frozen–thawed semen. These were Suffolk and Belclare (low and medium fertility, respectively) in Ireland, Ile de France and Romanov (both with medium fertility) in France and Fur and Norwegian White Sheep (NWS), both with high fertility in Norway. Cervical mucus was collected at the follicular phase of both a synchronised and a natural oestrous cycle. By combining ultra-performance liquid chromatography (UPLC), exoglycosidase digestions and mass spectrometry, a total of 124 *O*-glycans were identified across a range of mucin-type cores, from which core 2 and core 4 glycans had higher abundance in the low fertility Suffolk breed compared with high fertility ewe breeds (Fur and NWS). Differences in sialylated glycans were also identified between high and low fertility ewe breeds. For example, the sialylated glycan (2,3)-sialyl-T-antigen had lower abundance in the low-fertility, Suffolk, compared with Fur (high fertility). Using other biochemical techniques such as reverse phase UPLC and weak anion exchange UPLC, Suffolk had higher levels of sialic acid compared to high fertility ewe breeds (NWS and Fur). From over 50 different sialic acid structures in nature, we characterised seven. The two most prevalent were *N*-acetyl-neuraminic acid (Neu5Ac) and *N*-glycolyl-neuraminic acid (Neu5Gc), acetylated and glycosylated, respectively. There was no effect of oestrous synchronisation on sialic acid species although there was an effect of ewe breed, which was represented by Suffolk having higher levels of Neu5,9Ac2 compared to NWS. The results of this study suggest that cervical sperm transport is regulated by sialylated cervical mucins, which could be also involved in the regulation of the cervical immune response against sperm.

Regulation of the uterine environment by paternal factors: Insights from the bovine model

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Keywords: Seminal plasma, sperm, endometrium, bovine

During transit through the male reproductive tract, mammalian sperm are sequentially bathed in testicular, epididymal and accessory gland (AG) secretions which collectively constitute seminal plasma (SP). It has been suggested that SP components can modulate the maternal immune response, creating a tolerogenic environment for the semi-allogeneic embryo and leading to improved fertility. Indeed, exposure of sows and mice to SP has a positive effect on embryo survival and implantation. Due to the characteristics of mating in these species, the ejaculate comes into direct contact with the endometrium. However, bulls ejaculate in the vagina, and it is questionable whether any SP reaches the uterus. This makes cattle an interesting model for the study of the regulation of the uterine environment by paternal factors in species that ejaculate intravaginally. As a first approach to study this interaction, we generated endometrial explants from heifers in oestrus, and incubated them with sperm or SP. Interestingly, SP had a detrimental effect on endometrial RNA integrity which was blocked by addition of an RNase inhibitor, demonstrating a role for a SP-RNase. Both cervical and vaginal explants were more resilient to the SP-induced RNA degradation. These data gave weight to our hypothesis that, in species that ejaculate intravaginally, paternal modulation of the uterine environment is not due to direct contact with SP. To determine whether SP can have an indirect effect on the uterus and/or whether the presence of sperm can affect the endometrial response, we designed an in vivo study where heifers were mated to an intact bull (that ejaculate sperm and SP), a vasectomized bull (that only ejaculate SP), or were left unmated. Surprisingly, no differentially expressed genes (DEG) were observed in the endometrium of unmated heifers and those mated to vasectomized bulls, 24 h after mating. In contrast, the endometrium of heifers mated to intact bulls exhibited 22 and 24 DEGs in comparison with unmated heifers and heifers mated to vasectomized bulls, respectively. These data suggested that sperm might play a more critical role than SP in the regulation of the uterine environment in species that ejaculate in the vagina. To determine whether the effect observed was driven by intrinsic sperm factors, or rather, by AG factors that bind to sperm at ejaculation, we incubated endometrial explants alone or with epididymal (which have had no exposure to AG secretions) or ejaculated sperm. Both epididymal and ejaculated sperm induced changes in the endometrial transcriptome. However, the response elicited by the former was more dramatic than the later (1912 vs. 115 DEGs, respectively). In both cases, the top pathways associated with these genes included T cell regulation and NF-KB and IL17 signaling. Altogether, the data derived from our studies demonstrate the different ability of AG factors, and those acquired during spermatogenesis and epididymal maturation, in the regulation of the bovine endometrial transcriptome by sperm. At the same time, it highlights the species-specific nature of the modulation of the female environment by paternal factors in mammals.

Workshop II: Rigor in the IVF laboratory

Optimal conditions for the culture system: a review of QC/QA recommended practices

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Keywords: embryo culture, IVF practices, IVF program, culture conditions

The goal of an IVF program is to achieve high rates of embryo development in vitro and healthy offspring. This success is dependent on many factors, one of the most important, is the embryo culture system, which must support and provide the best conditions for the correct development of healthy embryos. Improper culture conditions have been shown to lead to environmental stress, compromise cell function and embryo development. Therefore, a careful selection and screening of culture system components is mandatory for achieving optimal results and consistency over time. The implementation of a robust QC/QA program in the IVF lab can guarantee an optimal and stable culture environment and allow to identify sub-optimal products, before they are put in contact with gametes or embryos. In this talk, we will review some practices that can be incorporated as part of the routine of QC/QA programs, which may help to guarantee the best results.

Potential applications of timelapse technology in animal agriculture

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Timelapse technology is not a new concept, though recent advances in digital capture technology, computing power, and incubation systems have made this technology more accessible to clinics and laboratories worldwide. While these systems have been embraced by human fertility clinics, their integration into animal reproduction laboratories has been hindered by cost and a lack of research into the potential benefits of this technology. However, these limitations are easing as academic laboratories and research institutions strive to understand the potential benefits and uses of modern timelapse technology. One benefit is the ability to observe embryos across all stages of development without having to remove them from the culture environment, rather than evaluating snapshots of development under a microscope on the bench. Timing of embryo development events and visualization of aberrations in development may be used to predict which embryos will reach the blastocyst stage or be appropriate for transfer. For example, a study of equine ICSI-derived embryos demonstrated that evaluating 2 and 8-cell stage embryos while being cultured in a timelapse incubator allowed embryologists to reasonably predict if an embryo would reach the blastocyst stage of development (Meyers et al., 2019). Abnormal developmental kinetics observed with timelapse have also been associated with chromosomal abnormalities in cattle embryos and may be an indication of embryo viability (Magata et al., 2019). This talk will provide an overview of current research on timelapse technology in animals as well as the potential applications of timelapse technology in research and commercial animal IVF labs.

Notes

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