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Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

33ème COLLOQUE SCIENTIFIQUE

33rd SCIENTIFIC MEETING

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Dr. Cesare Galli

Special Celebration

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Bath, UK, 8th and 9th September 2017

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Dr. Cesare Galli

A.E.T.E. Medalist 2017

Dr. Cesare Galli
A.E.T.E. Medalist 2017

Professor Cesare Galli:
AETE Pioneer Award 2017

The first time I met Cesare Galli was in 1991, on the occasion of the Annual Conference of the Italian Society of Veterinary Science. These were the heydays of embryo micromanipulation and nuclear transfer of embryonic cells, a field I had endorsed with passion a couple of years earlier. Cesare's contribution was entitled: *Nuclear transplantation (by electrofusion) of cultured embryonic cells with Met II cytoplasts in the sheep. Galli, C., Laurie, S., Lazzari, G. & Moor, RM. Annual Conference, Italian Society of Veterinary Science, (1991) vol XLV 299-303*. The work reported the first pregnancies (till day 45) of ewes carrying embryos produced by nuclear transfer of somatic cells (fibroblasts). I was struck by the contrast between the energy springing from the terrific experimental data, and the relaxed attitude sported by Cesare. I have introduced myself to Cesare on that occasion, and since then we have been constantly in contact, working on different projects.

I have arranged the *laudatio* in two section, a formal one, that provides a necessarily short summary of Cesare' contribution to Embryo Transfer field and Reproductive Biology in general, and a second, more informal, where I would like to describe my personal view of Cesare as a man and as a scientist.

1) Cesare Galli was born the 15-8-1961 in Erba (Como), Italy. His family had a dairy farm where he spent many years working with animals, all sorts of farm animals and became passionate about animal breeding. From 1981 he studied at the Faculty of Veterinary Medicine, University of Milan, becoming Doctor in Veterinary Medicine in 1986. During the university he met Giovanna Lazzari, who later became his wife and main co-worker, and they have 3 children, Francesca, Paolo, e Marco.

Cesare started to be passionate about science already as under-graduated students under the guidance of Prof. Antonio Lauria, from Milan University. Cesare spent three years working on the development of superovulation regimes in cattle, data that later provided the test trials for the registration of the FSH-LH superovulation brand for Serono, marketed still today as "Pluset". Thus, it really was a first sight love for embryology and embryo transfer. Always with Prof. Lauria,

Cesare developed a collagen embedded system for follicular growth in cattle. I have personally seen Prof. Lauria proudly showing this pioneer work no later than 6 years ago in Teramo. After his degree, Cesare moved in UK, where he has been working as research scientist at Animal Biotechnology Cambridge Ltd, 307 Huntingdon, Cambridge, a start up company set up by Dr. Chris Polge (the scientist who discovered how to freeze spermatozoa) from 1986-1988. Subsequently, he was appointed Higher Scientific Officer, Department of Molecular Embryology, Institute of Animal Physiology and Genetics Research, Babraham Hall, Cambridge, from 1989-1991. In Cambridge Cesare continued his embryology studies working with a Bob Moor (a former recipient of the AETE Pioneer Award), a prominent scientists in the field of Developmental Biology, working on the biology of the oocyte and the culture requirements of mammalian embryos and on nuclear transfer. Next again at Babraham, working with the Nobel Laureate, Professor Martin Evans, Cesare and Giovanna focused on the production of ES cells from domestic animals many years before anyone else in the world. Despite the difficulties associated with this topic, these workers have persisted, have produced calves from ES cells aggregated with tetraploid embryos and now lead the world in stem cell research in ungulates.
While at Cambridge Cesare and Giovanna collaborated with Twink Allen in the early steps of in vitro embryo production in horses as early as 1989 that is now well established clinical practice.

At the end of his three years research fellowship he returned to Italy to start his own Institute in Cremona. This he did from scratch, and since his return in 1992, has built up one of the leading European Institutes on Assisted Reproduction in domestic species: The Laboratory of Reproductive Technologies, that become Avantea in 2009. Avantea is one of the strongest Embryo Biotechnology Companies in the world. Under Cesare direction, Avantea gained over the years an impressive portfolio of Embryo Technologies carried essentially on all farmed animals: Cattle, Pig, Horse and Sheep. Besides routine *in vitro* embryo production and freezing, Avantea has developed and offers as a service: Microfertilization through ICSI, Monoparental (androgenetic/Parthenogenetic) embryo production, Cloning and Transgenic Animals.

In 2008 Cesare was awarded the Simmet Prize by ICAR. Cesare was selected by the Scientific Committee from a group of highly qualified scientist, with the following motivation "for significant progress and exemplary work within and outside Science. A number of papers have been published by Dr. Galli, among other, constitutive work for the establishment of a standard protocol for in *in vitro* production of bovine embryos. The first successful cloning of a horse is to his credit also. An outstanding contribution for research and application of assisted reproduction was the foundation of LTR (Laboratorio di Tecnologie della Riproduzione) in 1992. His long-time membership in the AETE, whose president he was for 4 years, also points out his efforts for technical progress and knowledge transfer from research to practice." The 50000 euros award were used by Cesare to fund "Fondazione Avantea Onlus", whose mission is to disseminate Science between scientists and general public.

Despite his full time involvement in Avantea, where besides the duties as a leader, Cesare works also as practitioner, doing ovum pick up on 8-9 mares three times a week. Thus, one might

wonder where and how he finds the time to deal with the other responsibilities outside Avantea, briefly summarized below:

-1996- 2000, President, European Embryo Transfer Association.
-2004 – 2017, Associated Professor University of Bologna.
-2006 – 2010, member of the International Advisory Board, Institute of Genetics and Animal Breeding, Polish Academy of Sciences.
-2010- present, Co-founder and President Fondazione Avantea Onlus
-2014 - Co-founder Xenothera SAS, Nantes, France
-2017 – Co-founder Equigea LLC, Ocala, Florida

Other activities
-2000-2001 contract professor at Univ. of Bologna and lecturer at several Italian universities; - member of the editorial board of Cellular Reprogramming (formerly Cloning & Stem Cells) and Journal of Reproduction and Fertility
- 2004 member of the editorial board of Biology of Reproduction
-ad hoc reviewer for Reproduction, Theriogenology, Biology of Reproduction, Transgenic Research, and others;
-invited speaker at several international conferences.
-member of the Ministry of Health working group for the preparation of the guidelines for the use of mesenchymal stem cells in veterinary medicine.

Cesare international recognition as a leading scientist and his trustworthiness, makes of him the partner every consortium wishes to have; these recent collaborative projects, some still on going, are self-explanatory:

Superpig. Technological platform for the use of pigs in biomedical (organs and tissues transplantation) and biotechnological (animal model) research. Grant n 14388 (01-06-2011 / 30-11-13), coordinator

Xenoislet. " Macroencapsulated Porcine Pancreatic Islets to cure Diabetes Mellitus type 1/2 " Grant agreement no: 601827 (01-09-13 / 31-08 16)

Translink. Defining the role of xeno-directed and autoimmune events in patients receiving animal-derived bioprosthetic heart valves " Grant agreement no: 603049. (01-09-13 / 31-08 17)

MitCare. Mitochondrial Medicine: developing treatments of OXPHOS-defects in recombinant mammalian models. ERC Advanced Grant, ERC-2012-ADG_20120314, Grant agreement n 322424. (01-07-13 / 30-06-18) (Partner)

Amyotrophic lateral sclerosis (ALS) swine models: production and characterization. GR-2010-231252. Italian Ministry of Health (01-12-12 /30-11-2015)
EU-rhythmy - Molecular strategies to treat inherited arrhythmias. ERC Advanced Grant, ERC-ADG-2014. Grant agreement n 322424. (01-11-15 / 31-10-20) (Partner)

Cesare current research covers all aspects of biotechnology of reproduction in farm animals, basic and applied research in molecular embryology with particular emphasis on *in vitro* oocyte maturation, micro-fertilisation, embryo culture, micromanipulation, embryonic and somatic nuclear transfer, genetic modification of animals to generate pig models of human diseases, embryonic stem cells, mesenchymal stem cells. Genetic engineering of pig for the purpose of obtaining cells, tissues and organs for xenotranplantation research, pre-clinical and clinical trials.

Cesare has published 250 publications (110 peer reviewed full papers), and has also contributed as an inventor/co-inventor to 4 international PCTs.

2) Below is my informal view of Cesare as a scientist, that I have decided to frame under three **C**s, (CCC).

Courage. *In vitro* embryo production and culture in farm animals was almost in its infancy, thus rather "fluid", when Cesare decided to start its company. Therefore, true courage was required to translate unsettled embryo technologies into a marketable product, indeed one of the first and best examples of "translational research" in Developmental Biology ever. Cesare took the risk, and the results stand in front of all of us. Cesare demonstrated courage and determination also on two other separate occasions.
Following the publication of Dolly the sheep paper by Wilmut and collaborators, the Italian minister of health banned all nuclear transfer experiments - animal included - for 5 years, with our disappointment. Cesare has been among the very first "cloners" worldwide, and he was stranded in the most exiting phase of cloning research. Few weeks before the main agricultural show in Cremona, Cesare phoned me informing he was determined to present in the show "Galileo", a calf cloned from a nucleated blood cells taken from "Zoldo", a semen donor bull. As anticipated, the duo Galileo/Zoldo was exposed in the show. Italian Authority's response was unusually rapid, and resulted in the jailing of the clone and its cloner. A very sad story that underlines, should it be necessary, the dull attitude toward Science in our Country. Another scary event I remind, is Cesare standing against animal liberation front activists that launched a very aggressive attach against experiments of stem cell therapy undertaken by Cesare and his Co-Workers on horses while working in Bologna University. Even on that occasion, Cesare courage and cold blood were unparalleled.

Cloning. As mentioned in the introduction, Cesare has published his data on nuclear transfer with differentiated cells (fibroblast) in sheep in 1991. The reconstructed embryos were able to implant, and developed till day 45 of gestation. No lambs were derived on that occasion, simply

because the number of recipient ewes was too small to ensure success. This was an incredible achievement, anticipating all other groups dealing with cloning. Cesare did not manage to convince his seniors to continue the experiments: too early, too risky, perhaps, for the time. Later, within the frame of the Bovine Embryo Multiplication Agreement project (BEMA) between Babraham and the Roslin Institute, Cesare has showed his data to Ian Wilmut, who, more forward tinker than the other, decided instead to invest heavily on nuclear transfer using differentiated/somatic cells. These efforts culminated with the production of Dolly the sheep. In other words, Dolly could have had a different name, perhaps Sofia …..(Loren).
Even though he lost the opportunity to be the first on that historical moment, Cesare's contribution to cloning has been influential and continuous. Prometea, the first horse clone ever, was produced by Cesare and Co-Workers in Avantea.

Consistency - Cesare's activity did not show any backward, but rather a steady growth over the years. The number of employees in Avantea has continued to increase, in parallel with the numerous services that the company now offers. Once the horse breeders started to consider reproductive technologies as a breeding tool, Cesare was the first on the market, making cloning and micro-fertilization through ICSI widespread between elite horse breeders, Arabian Emirates included. At the end of the nineties, the funding for reproductive research started to decline irreversibly. Cesare counteracted it by steering Avantea toward the new opportunities offered by joint research with biomedical scientists. He understood that the only way to thrive was to "convert" reproductive technologies as a tool to produce animal models for human medicine. Again, the initiative was a big success, with Cesare leading the field of transgenic research using pigs in biomedical (organs; xeno-transplantion; tissue transplantation: porcine pancreatic islets to cure diabetes mellitus type 1/2) and biotechnological (animal models: Amyotrophic Lateral Sclerosis (ALS) swine models) research. On the top of all of it, since 2015 Cesare is part of an international, multidisciplinary team whose goal is to rescue extremely endangered species, namely northern white rhinoceros, through "Conservation by Cellular Technologies". Even on that occasion I have had the opportunity to see the "magic" touch of Cesare with oocytes/embryos, findings that will rise again surprise and admiration, once made public.

Besides the scientific community, Cesare disseminates the outcomes of his activities also to the general public, informing them on the benefits arising from the application of the technologies he's been working at. Cesare lectures also at service clubs, and releases interviews to international, national and local TV and radio programmes. This is the reason why Cesare counts many prices/honours from public/private associations, just to mention the most recent ones. 2003 Cremonese of the Year; 2004 Giovanni Marcora Award; 2005 Gino; Bogoni Award; 2008 Silver Violin from Lions Club Cremona Stradivari.

To conclude, I feel privileged to have been selected to write Cesare Laudation for the 2017 AETE Meeting. While completing the draft I had a flash in my mind suggesting I should add two **G**s to the three **C**s, to complete the description of Cesare as a man. The first **G** could stand for

Giovanna, sure, the synergy springing from their collaboration is simply incredible (after all, the duo **CG** matters even at genomic level), but Cesare will have more to say about. The second **G** would stand for Generosity, no doubts. Cesare willingness to support colleagues, and to share his expertise for societal benefits is just unique.

On Behalf of the AETE, (of which Cesare is a Past president), but I include without fear all the scientists working on reproduction worldwide, I would like to express my deep gratitude for the incredible contribution given by you, Cesare, to our discipline: thank you!

Lino Loi
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Achievements and unmet promises of assisted reproduction technologies in large animals: a personal perspective

Cesare Galli

Avantea and Fondazione, Avantea, Cremona, Italy.

Abstract

This paper gives an overview of assisted reproductive technologies (ART) in livestock species coming from the author's direct experience and contribution to the development of several of them. The assessment is conducted on the basis of the progress achieved since the early eighties and the impact on the clinical/practical use of such procedures. Artificial insemination (AI) is still the leading technology used on a large scale in livestock with most favourable cost benefit ratio. All the other ARTs have niche applications compared to AI. Significant progress has been achieved in embryo culture, somatic cell nuclear transfer and on the identification of the many unknown variables affecting the success rate, while in areas such as superovulation, oocyte maturation, IVF, embryonic stem cells and cryopreservation progress has been limited or absent. It is the opinion of the author that ARTs have reached a plateau whereby only minimal improvement of efficiency can be achieved. Significant advances can only come from major breakthrough in the understanding of the underlying biological mechanisms.

Keywords: ARTs, large animals, oocytes, embryos, stem cells, SCNT.

Introduction

Assisted reproduction technologies (ARTs) as we know them today have been the result of a long process that started conventionally with the first artificial insemination performed by Spallanzani (Spallanzani 1780) in a bitch and the first embryo transfer in rabbit performed by Heape (Heape 1890). The former laid down the principle and basic knowledge for the development of the artificial insemination (AI) industry on the male side while the latter marked the beginning of embryo technologies on the female side.

The theoretical and scientific basis for these developments came in fact much earlier (see (Cobb 2012)for review) when William Harvey published his book De Generatione Animalium (Harvey 1651) and when Leeuwenhoek discovered the spermatozoa (Leeuwenhoek 1678).

It has been only in the second half of the last century that ARTs reached the high level of efficiency necessary to find a practical application in livestock breeding for diseases eradication and genetic selection and to serve as a model for clinical application in human clinics. Artificial insemination rapidly developed as a cost effective and reliable technique in the cattle industry (Vishwanath 2003) where it is now the standard because of the limited amount of semen needed per

individual female. However, in other species, like the pig or the horse, the requirement for higher amounts of semen per insemination dose did not allow the same widespread use of AI in breeding programmes. Moreover, significant research investments would be required still today to overcome sensitivity to cryopreservation and achieve further improvements.

Much less modest has been the impact of ARTs on the female germ line because the current state of the art allows to exploit only a very minute fraction of the oocyte pool present in the ovary to generate offspring.

In this paper I will review what were the expectations or the promises thirty years ago at the beginning of my scientific career, what has become a reality and what has remained undeveloped or has become an illusion based on a personal opinion.

The sperm

Quantity and quality of semen per se has rarely been a limiting factor for artificial insemination in cattle both as frozen or refrigerated. In other livestock species the use of frozen semen is still highly variable and in general is associated with lower fertility, due to cryo-injuries of various kind (Bailey et al. 2000), and often requires laparoscope or deep intrauterine insemination. Advances in insemination with lower sperm numbers has come from the developments of sperm sexing by flow cytometry (Cran 2007). It is now possible with the refinements introduced in the sexing procedure to achieve pregnancy rates in cattle comparable to non sexed semen, with a fraction of the number of spermatozoa used for conventional artificial insemination (Gonzalez-Marin et al. 2016). Methods of sperm sorting other than those based on DNA content have failed to become established in practice (Seidel 2012) so far. Male germ cell transplantation (Brinster and Zimmermann 1994) was also proposed as a way to increase sperm production, rescue particular genotypes or to alter the germ line (Dobrinski 2005) but it has not progressed to a level of practical use for livestock breeding.

The superovulation

The MOET (Multiple Ovulation and Embryo Transfer) is the most cost effective way to exploit the female genetics in cattle and small ruminants. The products that are used to induce superovulation have changed over the years as well as the average number of embryo produced (Bo and Mapletoft 2014). What has changed over time however are the protocols that have become more user and animal friendly (Mapletoft and Bo 2011). In the horse, the use of equine derived FSH induces a good superovulatory response that however does not correspond to the expected embryo recovery rate (Logan et al. 2007) probably due to the constrains typical of the anatomy of the horse ovary. Therefore horse superovulation is not being used in practice.

The oocyte

Despite being a finite number at birth, contrary to the sperm, primary oocytes are present in the order of tens of thousands (Lazzari et al. 1992) on livestock ovaries. In theory all of them could be harvested and recruited for maturation and fertilisation rather than being lost due to atresia. However the specific requirements, including sequential media and prolonged culture time, to grow a primordial oocyte to a stage of full competence for meiotic resumption and embryo development has been optimised only in mice (Eppig and O'Brien 1996). In livestock species attempts have been made to grow ovarian follicles at various stages of development both in vitro and in xenografts but with very modest success (Silva et al. 2016). Only two calves have been produced from growing early antral follicles (Miyano and Manabe 2007) and an improvement of blastocyst production could be obtained again by growing oocytes collected from early antral follicle cultured in vitro for 2 weeks (Makita et al. 2016), thus the supply of competent oocytes is still limited to the advanced antral follicles where the oocyte has completed the growth phase and acquired the full developmental competence.

The recovery of oocytes from pre-pubertal animals has been always proposed as a way to shorten the generation interval and increase the number of offspring from any given female. The advent of genomic selection, that allows the identification of superior genotypes soon after birth, has been an incentive to select oocyte donors at a very young age. For the same reason genetic screening has been optimised starting from embryo biopsies anticipating at the pre-implantation stage the identification of the required genotypes. In ruminants the competence of pre-pubertal oocytes is limited in the ability both to develop to blastocyst (Galli et al. 2001) and to establish pregnancy rates compared to adult donors (Ptak et al. 2006)Galli C. 2017; Galli & Lazzari unpublished observations).

The protocols to mature in vitro competent oocytes capable of giving rise to offspring after fertilization, culture and embryo transfer has long been developed (Staigmiller and Moor 1984, Gandolfi and Moor 1987). It is well established that oocyte competence is correlated to follicular diameter (Galli and Moor 1991), nevertheless even after the most accurate selection according to the state of the art and the best in vitro conditions, the average blastocyst production has remained fairly stable despite many scientific papers reporting every time few percentage points increase of blastocyst rates for any given treatment. After 30 years of developments of the *in vitro* technology, we still average at best 30% blastocyst rates in cattle under experimental conditions and half that in a clinical context (Galli et al. 2014b). The situation is even more inefficient in the buffalo and the horse where only 10% of the oocytes eventually develop into a transferable embryo (Galli et al. 2012, Galli et al. 2007). Nevertheless for these species this success rate is higher that with in vivo production of embryos by superovulation and embryo flushing.

If the oocytes are matured in vivo and harvested from pre-ovulatory follicles their developmental competence is higher (Rizos et al. 2002, Scott et al. 2001) indicating that the majority of oocytes that we are using for in vitro maturation are either coming from regressing follicles (advanced atresia) or from growing follicles that are not yet ready for maturation and require more than the canonical in vitro maturation time necessary to reach metaphase II. The introduction of the concept of pre-maturation *in vitro* to allow time for the oocyte to complete cytoplasmic maturation before the resumption of meiosis has found no application because of the

modest, if any, improvements (Lodde et al. 2013, Dieci et al. 2013) despite the fact that such inhibited oocytes at least maintain their developmental competence (Ponderato et al. 2001) and allow better scheduling of the work. This approach has found application in the equine. It is remarkable that by simply holding equine oocytes at room temperature for 24 h maturation can be arrested without loss of viability (Choi et al. 2006, Galli et al. 2014a) and the same blastocyst rate can be obtained compared to freshly matured oocytes following in vitro production. This procedure allows shipping of oocytes from equine clinics where they are recovered, to a centralized in vitro production laboratory for ICSI and embryo culture, and returning to the same clinics the frozen blastocyst for transfer (Galli et al. 2016).

In vitro fertilization (IVF)

This event critical for the successful trip of the oocyte towards becoming an embryo has been almost neglected in recent years and has not been the subject of much research. In humans IVF has become a reality after the first success obtained with the birth of Louise Brown (Steptoe and Edwards 1978), celebrated also with the 2012 Nobel Prize to R. Edwards. This event was the turning point where human IVF began to be the model for livestock species. It was not until 1982 that the first success was reported in cattle (Brackett et al. 1982) and later became routine procedure with the use of heparin to capacitate bull spermatozoa (Parrish et al. 1986) and other ruminants but no significant progress has been made since then. Most of the bulls will fertilize in vitro under standard conditions but the variation can be high ranging from no fertilisation to polyspermy, both compromising embryo development. This problem has its origins both in the variable quality of the frozen semen batches, in the intrinsic genetic variability of the semen donors but also in the quality of the oocyte itself and in its competence and ability to block polyspermy. In species other than ruminants IVF has remained an unreliable procedure. In pig the unresolved problem of polyspermy still dominates the field (Romar et al. 2016) but no solutions are on the horizon. Still porcine embryos can develop in vitro to reasonable rates (Grupen 2014) and probably adjust to the diploid state during early embryo development. In the horse the situation has been the opposite: IVF does not work, in fact no reproducible advances have been made since the only successful IVF report (Palmer et al. 1991). Again the human model helped to overcome this limitation in the horse with the introduction of ICSI (Palermo et al. 1992) that is now largely used on a routine basis in human IVF. In the horse ICSI is currently the only option to obtain fertilization in vitro and luckily the horse is the livestock species where it gives consistent results (Lazzari et al. 2002a) like in humans. Given the paucity of oocytes that can be harvested from a mare, ICSI will remain the technique of choice even if IVF ever would become available in the future. With the introduction of ICSI *in vitro* embryo production in horses has become possible and it has been developed to a level of being used in the clinical practice (Galli et al. 2014b). ICSI would be of help also in cattle to use limited amount of sexed semen or semen of poor quality recovered from young bulls; however, so far results have been disappointing (Galli et al. 2003b), and the rates of blastocyst remains below those obtained by conventional IVF. The advent of piezo-ICSI could offer still some options that could be developed also for cattle or pigs.

The preimplantation embryo

Embryo development in vitro is the area where significant progress has been made in recent time. Forty years ago in vitro derived livestock embryos could be hardly kept alive in culture and the only successful report was from Tervit (Tervit et al. 1972). Then the use of embryo co-culture with oviductal cells (Gandolfi and Moor 1987) was the beginning of a new era. To overcome the limits in early technology and lack of scientific knowledge, in vitro produced zygotes have been cultured for a long time in the surrogate sheep oviduct that ensured cryotolerance and viability after transfer to recipients comparable to that of in vivo generated embryos (Galli and Lazzari 1996, Lazzari et al. 2010). For many years and still now in some laboratories, co-culture with primary cells like oviductal or cumulus cells (Galli and Moor 1989) or with established cell lines like BRL and Vero became the routine also in the human field (Menezo et al. 2012). The presence of a monolayer of somatic cells however required the use of complex media and the presence of serum to ensure the viability of the somatic cells. The cells function was to create a microenvironment with low oxygen, besides promoting detoxification and/or providing secretion of factors suitable for embryos to develop. It turned out however that serum (and probably some secreted factors) was detrimental for post implantation embryo development in ruminants and identified as primarily responsible for the Large Offspring Syndrome (LOS;(Young et al. 1998). The presence of serum was also found detrimental for cryopreservation because it was responsible for the accumulation of lipids (Lonergan and Fair 2008, Galli and Lazzari 1996). Therefore media formulations shifted towards a serum free and cell free solutions where the still undefined component remains only Bovine Serum Albumin. The medium used today by many laboratories is based on the SOF formulation of Tervit (Tervit et al. 1972) with the addition of amino acids and various energy substrates (Gardner et al. 1994) as well as the reduction of the level of oxygen to 5% by feeding nitrogen to the gas mixture to lower oxidative damage.

With the use of modified SOF medium the incidence of LOS was decreased (van Wagtendonk-de Leeuw et al. 2000) but still the presence of high levels of BSA is accountable for such problems (Lazzari et al. 2002b) and occasionally LOS is still reported. LOS is typical of ruminants. In the horse it has never been reported: the foals born out of *in vitro* embryo production are not oversize (Galli et al. 2007). This suggests that the problem is associated with the type of placentation and or placental abnormalities (Farin et al. 2006, Ptak et al. 2013).

The embryos that are selected for freezing or for transfer are chosen by experienced embryologists based essentially on morphological criteria. Attempt to develop more objective non-invasive measurements that could be use to select embryos for transfer have not found the way to the clinical use. Both the measurement of oxygen consumption (Lopes et al. 2007) or the amino acid turnover (Brison et al. 2004) were investigated for this purpose. Another aspect that is not taken into consideration is the embryo genotype. Usually beef breeds perform better in embryo production *in vitro* than Holstein, notoriously a breed with higher inbreeding. We have shown that crossbred embryos develop better that inbreed ones (Lazzari et al. 2011).

An important part of ARTs is the cryopreservation of embryos both for practical and commercial reasons. Despite the development of vitrification (Rall and Fahy 1985), that found its application in research laboratory or in human clinics for oocyte cryopreservation (Vajta 2013), the industry standard in livestock for both ruminants and horses embryos is still the slow cooling method (Willadsen et al. 1978). Embryos frozen in glycerol require very simple thawing procedures for cryoprotectant removal that any practitioner can perform on farm conditions. The development of direct transfer has further simplified the transfer of bovine embryos (Voelkel and Hu 1992) allowing many more practitioners, without the ability to handle an embryo in a dish, to perform embryo transfer.

The stem cells and somatic cell nuclear transfer

At the beginning of my scientific career Steen Willadsen (AETE Pioneer Award recipient) had already cloned sheep and cattle using blastomeres of early stage pre-implantation embryos (Willadsen 1986) and companies were being set up by the breeding industry to exploit this technology. But soon it became evident that embryonic cloning had limitations (number of nuclei available, unpredictable genotype, etc.). It was from this limitation that the scientific community got interested in developing embryonic stem cells to have an unlimited source of nuclei for nuclear transfer. In collaboration with Martin Evans (later 2007, Nobel Prize for Medicine) we started a long and painful path in the attempt to establish embryonic stem cells in sheep and pig (Notarianni et al. 1991) and later on in cattle (Galli et al. 1994, Lazzari et al. 2006) but never succeeded to obtain naïve ESC lines and to date no one has reported success in this endeavour. At most we and others obtained ES-like cells that probably were not much different from somatic cells. Because of the difficulties in generating stable ESC lines the interest for nuclear transfer shifted towards this type of ES like cells (Galli et al. 1991, Campbell et al. 1996) and eventually to somatic cells (Wilmut et al. 1997, Galli et al. 1999). It turned out that somatic cells can be used for cloning animals and indeed surprisingly perform better that the supposedly less differentiated cells (Sung et al. 2006).

In the horse the development of in vitro maturation and embryo culture has also benefited the cloning of this species (Galli et al. 2003a) that fortunately, despite the low efficiency of development to term, as in other species, it turned out to be free of LOS and late pregnancy losses. In the pig the situation is intermediate and no dramatic phenotypes are reported (Kurome et al. 2013). The low efficiency is compensated by the transfer of many cloned embryos per recipients to ensure a high pregnancy rate and a reasonable number of newborn piglets.

The recipient

The success of any ART procedure is measured at the end of the day on the birth of a viable offspring. Therefore the recipient that is selected for the transfer plays an important part in the successful outcome. Synchronization procedures have been greatly improved and simplified with the introduction of the Ov-Synch protocols (Thatcher et al. 2004, Baruselli et al. 2010) for cattle

together with a better management of the health and nutrition of the recipient animals and the collection and registration of precise information in electronic formats. Maiden recipients are generally preferred and give the highest pregnancy rates across livestock species. However in cattle there is always a struggle to maintain the pregnancy rate close to 50% especially with cryopreserved embryos or embryos produced in vitro. This relatively low efficiency seems to be associated with the gradual decrease in fertility observed in cattle herds (Diskin et al. 2016) that hopefully will be reversed by genomic selection for fertility traits. On the contrary in vitro produced frozen-thawed equine embryos can achieve a consistent and remarkable pregnancy rates up to 60% with a foaling rate of 50% (Galli et al. 2007, Galli et al. 2016). Also in pig the use of gilts ensures after embryo transfer a pregnancy rate in excess of 50%.

The unknowns of the laboratory

The outcome of ART procedures depends heavily, besides the gametes and the surrogate mothers mentioned above, also on two other components: the human factor and the laboratory set up. After 30 years in the profession I have meet and seen all sorts of people: the good, the bad, the ugly. Gametes and embryos are like little babies and they need a lot of care to be kept alive and thrive. Over the years this human factor has been and still is very important despite some technological advances and better equipment. Therefore it is crucial to find the right people to work in the laboratories moreover a long period of training, trial and testing is needed. Unfortunately these people are rarely found. The other critical aspects are the equipment, reagents and the disposables used in the various procedures. A great help has come from the enormous development of the human ART industry, in terms of varieties of supplies and technical solutions. However livestock gametes and embryos and in particular cattle are, in our experience, far more sensitive that mouse or human ones. This is why the most common quality control test used by the suppliers of disposable, chemicals or media, i.e. the MEA (mouse embryo assay; (Punt-van der Zalm et al. 2009) does not detect toxicants or conditions that can affect cattle embryo development. For this reasons we have introduced our own testing for quality control using bovine embryos, both for disposables plastic, BSA and culture media once a new batch is prepared. A typical example of chemical that requires testing is mineral oil (Otsuki et al. 2007). After several years there is still an issue in discussion forums about its toxicity and the use of paraffin oil instead of mineral oil. We are not using oil at all for long term culture. Only during cloning or ICSI procedures we cover the micromanipulation drops with mineral oil but exposure is limited to short time. The reason is that even batches that we tested and found suitable for embryo culture, over time became toxic.

The unknowns of the biology

What has emerged with the implementation of ART in livestock, in particular in ruminants, is the incidence of some abnormalities that might result in low pregnancy rates, high pregnancy losses and abnormal offspring with a higher birth weight that can cause dystocia (Lazzari et al. 2002b). Several factors can contribute to this phenomenon and the causes of these perturbations are

likely to be a response to stressors (Thompson et al. 2002) . Sub-optimal in vitro environment, inadequate culture medium and untested disposable material can contribute to alteration of cellular parameters such as pH and redox state ultimately affecting embryo development. Factors such as diet and metabolic conditions can be involved in epigenetic effects while specific procedure such as somatic cell nuclear transfer, can directly affect the methylation status of the cloned embryos. These findings in the animal models have fuelled concerns for human ART (Thompson et al. 2002) where these alterations might have far greater impact on the health of the resulting babies at birth and later on during adult life on the incidence of diseases such as diabetes type 2, cardiovascular diseases and obesity (Chen and Heilbronn 2017). This issue will be addressed more fully in the near future with the increasing amount of data collected and analysed from ART adult offspring. In livestock species the long term effects impact essentially on the efficiency of the technology while animal welfare questions emerge mainly in ruminants and in relation to pregnancies and offspring derived from somatic cell nuclear transfer procedures. On a positive note these epigenetic alterations do not appear to be transmitted to the progeny (Tamashiro et al. 2002, Shimozawa et al. 2002).

Conclusions

ARTs have come a long way in the last 30 years both in animals and humans and are well established in the clinical practice. Progress has been slow but steady especially in the area of embryo culture (Table 1). It seems that we have now reached a plateau with only small margins for improvement because of the intrinsic biological and/or technical limitation /variation of the source of gametes and of in vitro conditions. We have to live with that unless a major breakthrough occurs in our understanding of the underlying biological mechanisms.

Table 1. Current status of assisted reproductive technologies in livestock, a personal opinion.

	progress	practical use	research activity
	last 30 years	present	present
AI	+	++++	+
MOET	+	+++	-
Oocyte	++	+++	+
IVF	+	+++	+
embryo culture	++++	+++	+++
cryopreservation	+	+++	+
embryonic stem cells	-	-	+
SCNT	+++	+	++
recipient animals	++	+++	+
unknowns	+++	+++	++
	++++ intense	+++ moderate	++ low
	+ minimal	- absent	

Acknowledgments

The opinions that the author has expressed in this paper have accumulated over the course of 30 years in the profession, as well as through collaborations and discussions with many colleagues, who are too many to be mentioned, in the occasion of scientific conventions such as the AETE or the IETS. All my work and contributions to the field would have not been possible without good mentors and I am particularly indebted to Dr. Robert Moor who, during my years in Cambridge, was an example, setting the stage and illuminating the road ahead. At the same time I acknowledge the support of my family: my wife Giovanna Lazzari, as a partner but most importantly as a colleague, with her sharp comments, wise judgement and uncompromising dedication, and my children (Francesca, Paolo and Marco) to whom we endeavoured to set an example but also subtracted many hours and weekends because of the on-going "magical" experiments in the lab.

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Commercial

Embryo Transfer Activity

in Europe 2016

Marja Mikkola. Finland

National data collectors

Country	Collector
Austria	Friedrich Führer
Belgium	Peter Vercauteren, Isabelle Donnay
Bosnia Herzegovina	Teodor Markovic
Croatia	Mario Matkovic
Czech Republic	Pavel Bucek
Denmark	Henrik Callesen
Estonia	Jevgeni Kurykin
Finland	Seija Vahtiala
France	Serge Lacaze
Germany	Hubert Cramer
Greece	Foteini Samartzi
Hungary	Ferenc Flink
Ireland	Patrick Lonergan
Italy	Giovanna Lazzari
Latvia	Vita Antane
Lithuania	Giedrius Palubinskas, Vytuolis Žilaitis, Jonas Kutra
Luxembourg	Marianne Vaessen
Macedonia	Toni Dovenski
The Netherlands	Helga Flapper
Norway	Eiliv Kummen
Poland	Jędrzej M. Jaśkowski
Portugal	João Nestor Chagas e Silva
Russian Federation	Denis Knurow, Viktor Madison
Serbia	Aleksandar Milovanovic
Slovakia	Jozef Bires, Dalibor Polak
Slovenia	Janko Mrkun
Spain	Daniel Martinez Bello
Sweden	Renée Båge
Switzerland	Rainer Saner
Turkey	Ebru Emsen
Ukraine	Viktor Madison
United Kingdom	Roger Sturmey

Bovine *In vivo* embryo production

Country	Collections	Viable embryos	Viable embryos / collection	Dairy (%)	Beef (%)	Sexed semen (%)
Austria	259	2038	7,9	75 %	25 %	10 %
Belgium	1135	5753	5,1	14 %	86 %	1 %
Bosnia and Herzegovina	0	0				
Croatia	0	0				
Denmark	679	4593	6,8	94 %	6 %	0 %
Estonia	0	0				
Finland	329	2090	6,4	100 %	0 %	5 %
France	6260	34896	5,6	79 %	21 %	9 %
Germany	3201	21311	6,7	91 %	9 %	0 %
Greece	0	0				
Hungary	111	917	8,3	17 %	83 %	2 %
Ireland	857	6013	7,0	20 %	80 %	0 %
Italy	1975	15487	7,8	96 %	4 %	0 %
Latvia	0	0				
Luxembourg	180	1242	6,9	89 %	11 %	0 %
Macedonia	0	0				
Netherlands	1836	11370	6,2	100 %	0 %	0 %
Norway	60	361	6,0	58 %	42 %	0 %
Poland	192	1215	6,3	100 %	0 %	21 %
Portugal	131	720	5,5	89 %	11 %	30 %
Russian Federation	1298	8022	6,2	45 %	55 %	35 %
Serbia	11	35	3,2	100 %	0 %	18 %
Slovakia	0	0				
Slovenia	9	14	1,6	100 %	0 %	22 %
Spain	610	3624	5,9	65 %	35 %	27 %
Sweden	204	994	4,9	97 %	3 %	2 %
Switzerland	485	3390	7,0	95 %	5 %	28 %
Turkey	0	0				
Ukraine	2	6	3,0	100 %	0 %	0 %
United Kingdom	959	4786	5,0	78 %	22 %	0 %
Total	**20783**	**128877**	**6,2**	**77 %**	**23 %**	**7 %**

Bovine *In vitro* embryo production – OPU

Country	Total sessions			Non-stimulated sessions				Stimulated sessions				Breed distribution		
	Total sessions	Oocytes	Embryos	Sessions	Oocytes	Embryos	Embryos/ session	Sessions	Oocytes	Embryos	Embryos/ session	Dairy (%)	Beef (%)	Sexed semen
Austria	1	7	1	0	0	0		1	7	1	1,0	100 %	0 %	0 %
Finland	214	1477	462	103	687	142	1,4	111	790	320	2,9	100 %	0 %	0 %
France	611	5331	1628	0	0	0		611	5331	1628	2,7	95 %	5 %	9 %
Germany	1352	12505	1919	1352	12505	1919	1,4	0	0	0		97 %	3 %	0 %
Italy	581	7197	1510	581	7197	1510	2,6	0	0	0		62 %	38 %	12 %
Netherlands	4726	45662	8385	0	0	0		4726	45662	8385	1,8	100 %	0 %	0 %
Poland	13	64	34	9	47	24	2,7	4	17	10	2,5	100 %	0 %	0 %
Russian Federation	1519	6076	911	1519	6076	911	0,6	0	0	0		100 %	0 %	100 %
Serbia	14	80	35	6	24	11	1,8	8	56	24	3,0	100 %	0 %	0 %
Spain	734	9892	2579	573	7621	1853	3,2	161	2271	726	4,5	79 %	21 %	60 %
United Kingdom	886	6116	1415	346	2995	710	2,1	540	3121	705	1,3	44 %	56 %	11 %
Total	10651	94407	18879	4489	37152	7080	1,6	6162	57255	11799	1,9	91 %	9 %	21 %

Bovine *In vitro* embryo production – abattoir

Country	Donors	Oocytes	Embryos	Dairy (% donors)	Beef (% donors)
France	1	17	10	100 %	0 %
Italy	8	302	70	50 %	50 %
Netherlands	61	8998	141	100 %	0 %
Portugal	1	740	43	100 %	0 %
Spain	65	1060	471	69 %	31 %
United Kingdom	120	7200	360	0 %	100 %
Total	**256**	**18317**	**1095**	**44 %**	**56 %**

Bovine embryo technologies

Country	Sexed embryos		Genotyped embryos	
	In vivo	In vitro	In vivo	In vitro
France	2688	0	1595	0
Germany	966	0	416	0
Netherlands	0	0	381	0
Total	**3654**	**0**	**2392**	**0**

Bovine *In vivo* embryo transfer

Country	Dairy breeds			Beef breeds			Non-separated breeds			Total transfers
	Fresh	Frozen domestic	Frozen foreign	Fresh	Frozen domestic	Frozen foreign	Fresh	Frozen domestic	Frozen foreign	
Austria	396	773	40	242	269	12	0	0	0	1732
Belgium	189	440	1426	1006	3030	34	0	0	0	6125
Bosnia and Herzegovina	0	0	0	0	0	0	0	0	0	0
Croatia	0	0	0	0	0	0	0	0	0	0
Denmark	2498	966	0	54	124	0	0	0	0	3642
Estonia	0	0	0	0	0	6	0	0	6	12
Finland	892	1415	252	0	0	0	0	0	0	2559
France	15590	14321	1134	1729	6588	190	48	36	0	39636
Germany	7781	11215	0	0	0	0	350	972	0	20318
Greece	0	0	0	0	0	0	0	0	0	0
Hungary	28	79	0	124	232	0	0	0	38	501
Ireland	573	629	0	2294	2517	0	0	0	0	6013
Italy	6000	0	0	300	0	0	0	0	0	6300
Latvia	0	0	0	0	0	0	0	0	0	0
Luxembourg	0	0	0	0	0	0	600	0	0	600
Macedonia	0	0	0	0	0	0	0	0	0	0
Netherlands	2522	9519	0	0	0	0	0	0	0	12041
Norway	0	0	70	0	0	0	0	0	0	70
Poland	611	524	0	0	0	3	0	0	0	1138
Portugal	120	434	4	10	9	63	0	0	0	640
Russian Federation	717	1290	82	22	4466	143	0	0	0	6720
Serbia	12	23	0	0	0	0	0	0	0	35
Slovakia	0	0	0	0	0	0	0	0	0	0
Slovenia	10	6	4	0	0	6	0	0	0	26
Spain	988	752	50	736	408	58	0	0	0	2992
Sweden	0	0	0	0	0	0	78	744	181	1003
Switzerland	859	1657	374	21	29	16	0	0	0	2956
Turkey	0	0	0	0	0	0	0	0	0	0
Ukraine	0	0	35	0	0	0	0	0	0	35
United Kingdom	70	173	1	326	585	154	0	0	0	1309
Total	**39856**	**44216**	**3472**	**6864**	**18257**	**685**	**1076**	**1752**	**225**	**116403**

Bovine *In vitro* embryo transfer

Country	OPU			Abattoir		
	Fresh	Frozen domestic	Frozen foreign	Fresh	Frozen domestic	Frozen foreign
Finland	61	226	0	0	0	0
France	526	543	2	8	2	0
Germany	1556	265	0	0	0	0
Italy	479	372	0	0	20	0
Luxembourg	0	0	50	0	0	0
Netherlands	6448	1242	0	23	111	0
Portugal	0	0	0	9	0	0
Russian Federation	81	47	0	0	0	0
Serbia	5	30	0	0	0	0
Spain	1196	413	149	0	0	0
Switzerland	0	0	91	0	0	0
United Kingdom	55	195	0	0	0	0
Total	**10424**	**3343**	**292**	**40**	**133**	**0**

Embryo production and transfer in other species

Country	Sheep, *In vivo*				
	Embryo production		Embryo transfer		
	Collections	Embryos	Fresh embryo	Frozen domestic	Frozen foreign
France	20	64	0	0	0
Hungary	6	24	20	0	96
Norway	0	0	0	0	250
Sweden	0	0	0	0	465
Turkey	29	151	117	34	0
United Kingdom	2500	12000	0	300	0
Total	**2555**	**12239**	137	334	811

Country	Goat, *In vivo*			
	Embryo production		Embryo transfer	
	Collections	Embryos	Fresh embryo	Frozen domestic
France	17	73	0	0
Spain	20	42	0	42
Turkey	5	43	43	0
United Kingdom	25	200	0	0
Total	**67**	**358**	**43**	**42**

Country	Horse, *In vivo*			
	Embryo production		Embryo transfer	
	Collections	Embryos	Fresh	Frozen
France	983	529	0	0
Poland	17	17	11	0
Spain	594	300	203	10
Sweden	22	14	14	0
Total	**1616**	**860**	**228**	**10**

Country	Horse, *In vitro*				
	Embryo production			Embryo transfer	
	OPU sessions	Oocytes	Embryos	Fresh	Frozen
Italy	965	11056	800	61	229

Embryo exports

Country	Embryos exported			
	Bovine, dairy	Bovine, beef	Sheep	Goat
Austria	35	21	0	0
Belgium	20	58	0	0
Denmark	97	0	0	0
Finland	67	0	0	0
France	1883	419	0	0
Spain	37	20	0	0
Switzerland	48	0	0	0
United Kingdom	0	222	1200	200
Total	**2187**	**740**	**1200**	**200**

INVITED LECTURES

Follicular environment and oocyte maturation: roles of local peptides and steroids

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Running title: Intra-follicular peptides and steroids control differentiation of the cumulus-oocyte complex.

Abstract

A large amount of data on the mechanisms regulating cumulus-oocyte maturation in mammals has been generated in the last 20 years. It has been made clear that oocyte-secreted factors play a central role in the control of cumulus differentiation and oocyte developmental competence. However, more recent data indicate that cumulus-derived factors are also involved. In this mini-review, we have compiled and discussed data produced in our laboratory about the involvement of oocyte and cumulus-derived peptides, including fibroblast growth factors, bone morphogenetic protein 15, Kit ligand and natriuretic peptide C, in the regulation of cumulus metabolism and oocyte nuclear maturation. In addition, we discuss the interaction of follicular steroids with natriuretic peptide C in the control of meiosis progression.

Introduction

There is great interest to improve efficiency of *in vitro* maturation of oocytes (IVM) in animal species and humans as IVM has been considered the main technological bottleneck to improve embryo *in vitro* production following *in vitro* fertilization (IVF). It has been clearly demonstrated that current IVM systems do not adequately reproduce the follicular environment where the cumulus-oocyte complex (COC) physiologically differentiates, which compromises cumulus cells function and oocyte developmental competence (Rizos *et al.* 2002, Brown *et al.* 2017). Therefore, understanding the mechanisms that regulate COC differentiation is critical for the improvement of IVM systems.

The bidirectional interaction between the oocyte and cumulus cells is essential for oocyte developmental competence and constitutes a valuable parameter for improving IVM/IVF outcomes (Gilchrist 2011). A lot of attention has been given to secreted paracrine factors as mediators of the oocyte-cumulus communication, mainly to oocyte secreted factors (OSF). There is robust evidence that OSF, particularly bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), both members of the transforming growth factor-ß (TGF-ß) superfamily, and fibroblast growth factors (FGF) regulate various aspects of cumulus cells differentiation such as expansion, metabolism, steroidogenesis and apoptosis (Eppig 2001, Sugiura *et al.* 2007, Gilchrist *et al.* 2008, Caixeta *et al.* 2013b). However, data obtained in cattle and pigs suggest that the importance of OSF in the control of cumulus expansion and metabolism may vary between species (Buccione *et al.* 1990, Vanderhyden 1993, Ralph *et al.* 1995, Sutton *et al.* 2003, Caixeta *et al.* 2013b). Although the other direction of the

oocyte-cumulus communication has been less explored, there is recent evidence that cumulus derived peptides regulate nuclear maturation and gene expression in the oocyte (de Lima *et al.* 2016).

Apart from secreted factors, oocyte-cumulus communication is also mediated by transzonal cytoplasmic projections (TZP), which are extensions of cumulus cells that cross the zona pellucida transporting ions, metabolites and regulatory molecules (Albertini *et al.* 2001). The delivery of glucose metabolites and small regulatory molecules through gap junctions connecting the end of the TZPs with the ooplasm appears crucial for the control of meiosis, chromatin configuration, transcriptional activity and metabolism of the oocyte (Conti *et al.* 2012, Luciano *et al.* 2014, Gilchrist *et al.* 2016, Brown *et al.* 2017). In addition to the transport through gap junctions, recent studies indicate that TZPs can also deliver larger molecules such as RNA transcripts via micro-vesicles in a transport mechanism designated as the gametic synapse (Macaulay *et al.* 2014). In fact, cumulus-derived RNA has been identified in oocyte polyribosomes suggesting that the gametic synapse can influence the translational activity of the oocyte (Macaulay *et al.* 2016).

This paper aims to review and discuss some of the recent data on paracrine mediators of the oocyte-cumulus interaction, as well as mechanisms regulating periovulatory differentiation of cumulus cells and oocyte nuclear maturation with potential practical implications for IVM.

Oocyte *vs.* cumulus secreted factors: who runs the show in the cow?

A large body of data produced in mice points to a leading role for the oocyte in the regulation of cumulus cells differentiation and metabolism (Matzuk *et al.* 2002, Gilchrist *et al.* 2008). However, studies using microsurgical removal of the oocyte from the COC and co-culture of oocytectomized COCs with secreting denuded oocytes indicate that OSF are needed for cumulus expansion in mice, but not in cattle, pigs or rats (Buccione *et al.* 1990, Ralph *et al.* 1995, Vanderhyden *et al.* 2003). More recently, the same approach demonstrated that OSF also play a central role in the regulation of glycolytic activity of cumulus cells in mice (Sugiura *et al.* 2005), whereas in cattle, utilization of oxygen, glucose, pyruvate and lactate by cumulus cells was not affected by removal of the oocyte (Sutton *et al.* 2003). Taken together, these data point to differences between species with regard to the participation of the oocyte in cumulus differentiation, raising speculation that autocrine and paracrine signaling within the cumulus may be more influential in species other than the mouse. In mono-ovulatory mammals, the functional relevance of intra-cumulus TGF-ß signaling is controversial. Studies assessing the expression patterns of BMP15 and GDF9 in the COC are conflicting. While expression of BMP15 and GDF9 has been consistently detected in the oocyte, in cumulus cells it was observed in one but not all studies in cattle (Hosoe *et al.* 2011, Crawford & McNatty 2012).

Alternatively, FGF2 and Kit Ligand (KL) are two potential and less controversial cumulus-derived regulators of COC maturation. A microarray study pointed *FGF2* as an important up-regulated gene in the predicted pathways activated by the LH surge to induce final differentiation of bovine cumulus cells (Assidi *et al.* 2010). In addition, we have shown that transcription of *FGFR2C* and *FGFR3C*, two receptors efficiently activated by FGF2, is drastically and rapidly increased in cumulus cells from bovine COCs subjected to FSH-stimulated IVM, suggesting that sensitivity to FGF2 is enhanced with activation of the ovulatory cascade (Zhang *et al.* 2006, Caixeta *et al.* 2013a). Taken together, these studies indicate that FGF2 signaling is enhanced in preparation for ovulation and final

COC maturation. In fact, recent data from our laboratory suggest the involvement of FGF2 in the regulation of meiosis progression, cumulus expansion and apoptosis (data not published). Moreover, the involvement of FGF2 in the control of COC maturation is also consistent with our previous finding that FGF2 increases phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) in granulosa cells, two pathways known to regulate meiotic resumption and cumulus expansion (Jiang *et al.* 2011, Prochazka *et al.* 2012).

On the other hand, we have recently reported that mRNA levels of KL increase around 10 times in bovine cumulus cells during FSH-stimulated IVM and presented evidence of a role for KL in the regulation of meiosis progression under the influence of OSF in cattle (Lima *et al.* 2016). These data are compiled and discussed in further details below. Therefore, whilst further studies are needed to address the importance of cumulus derived peptides for COC maturation, the data collected so far suggest that OSF are less influential in the cow than in the mouse and that an interaction of oocyte and cumulus derived factors likely run the show in the cow.

Oocyte secreted factors in the control of cumulus expansion and metabolism

Although OSF are not absolutely required for cumulus expansion in cattle, they appear to influence this process (Ralph *et al.* 1995, Zhang *et al.* 2010a, Caixeta *et al.* 2013b). BMP15 and FGF10 are expressed by the oocyte and when added to the IVM medium they can enhance cumulus expansion and embryo production in cattle (Hussein *et al.* 2006, Buratini *et al.* 2007, Zhang *et al.* 2010a, Crawford & McNatty 2012). We have provided evidence that BMP15 and FGF10 control the expression of key genes in the ovulatory cascade. Supplementation of the IVM medium with BMP15 increased transcription of disintegrin and metalloprotease 10 (*ADAM 10*), *ADAM 17*, amphiregulin (*AREG*) and epiregulin (*EREG*) in cumulus cells from bovine COCs. Alternatively, treatment with FGF10 promptly increased mRNA levels of prostaglandin (PG)-endoperoxide synthase (*PTGS2*), and subsequently of pentraxin 3 (*PTX3*) and tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*) in bovine cumulus cells (Caixeta *et al.* 2013b). Therefore, BMP15 appears to enhance the ovulatory cascade right at its beginning by increasing production, cleavage and release of EGF-like factors, the last two events being a consequence of ADAM10 and ADAM17 activity (reviewed by Ben-Ami *et al.* 2006). These effects of BMP15 are consistent with its positive impact on developmental competence in cattle (Hussein *et al.* 2006) and with evidences of suboptimal EGF signaling in bovine COCs matured *in vitro* (Brown *et al.* 2017). On the other hand, FGF10 would act downstream of EGF-like factors by increasing the expression of cross-linking proteins that stabilize the extracellular matrix (PTX3 and TNFAIP6). This appears to be at least in part mediated by the preceding prompt increase in *PTGS2* expression, since PGE2 is required for *TNFAIP6* expression (Ochsner *et al.* 2003 reviewed by Russell & Robker 2007). These findings are in agreement with the positive effect of FGF10 on embryo production following IVM/IVF and with higher expression of *TNFAIP6* in cumulus cells from *in vivo* matured compared with *in vitro* matured bovine COCs (Tesfaye *et al.* 2009, Zhang *et al.* 2010a).

Despite the evidences that BMP15 and FGF10 act at different steps of the ovulatory cascade, they appear to act similarly with regard to their influence on glucose metabolism. They both increased glucose uptake without altering lactate production during IVM of bovine COCs, which was

accompanied by increases in mRNA levels of glucose transporters (*GLUT1* and *GLUT4*). Interestingly, BMP15 and FGF10 also increased mRNA abundance of glucosamine:fructose-6-PO_4 transaminases (*GFPT1* and *GFPT2*) and hyaluronan synthase 2 (*HAS2*), which are rate-limiting enzymes in the hexosamine pathway of glucose metabolism that leads to the production of hyaluronic acid, the major component of the extracellular matrix (Sutton-McDowall *et al.* 2010, Caixeta *et al.* 2013b). Therefore, collectively, these data suggest that after the activation of the ovulatory cascade BMP15 and FGF10 direct the metabolism of glucose towards the synthesis of hyaluronic acid to support the formation of extracellular matrix for cumulus expansion.

Nevertheless, at earlier stages of COC maturation, before the activation of the ovulatory cascade, the influence of BMP15 and FGFs on glucose metabolism may be different. BMP15 and FGF8 were shown to cooperate to increase the expression and activity of glycolytic enzymes phosphofructokinase (*PFKP*) and lactate dehydrogenase (*LDHA*) in cumulus cells from COCs arrested at the germinal vesicle (GV) stage in mice (Sugiura *et al.* 2007). On the other hand, in bovine COCs undergoing IVM, the combination of BMP15 with FGF17, a member of the FGF8 superfamily also expressed by the bovine oocyte and capable of activating the same receptors that FGF8, did not alter *PFKP* mRNA levels in cumulus cells (Zhang *et al.* 2006, Machado *et al.* 2009, Machado *et al.* 2015). Therefore, additional studies dissecting the influence of species, COC developmental stage and culture conditions are needed for a clearer understanding of the roles of OSF in the regulation of glucose metabolism in the COC.

Like FGF10, FGF17 was shown to enhance expansion of bovine COCs during IVM (Machado *et al.* 2015). However, FGF17 did not alter the expression of *PTGS2*, or any of the genes in the ovulatory cascade investigated [*ADAM10, ADAM17, AREG, EREG, PTX3, TNFAIP6, VERS* (versican) and *HAS2*]. Therefore, different FGFs appear to impact on cumulus expansion and differentiation through different mechanisms. Although no additional effect on cumulus expansion or meiosis progression was observed when FGF17 was combined with BMP15 during IVM, this combination increased mRNA levels of the nuclear progesterone receptor (*nPR*) in cumulus cells after IVM, as well as the number of cells in the inner cell mass (ICM) of blastocysts produced by IVF/IVC (Machado *et al.* 2015). These data therefore suggest that FGFs and BMP15 interact during COC maturation to improve developmental competence, which may be at least in part a consequence of increased progesterone sensitivity. Previous studies using inhibitors of progesterone synthesis and nPR antagonists have elegantly demonstrated that progesterone signaling is crucial for cumulus expansion and oocyte developmental competence (Aparicio *et al.* 2011).

Oocyte and cumulus-derived factors in the regulation of nuclear maturation and cumulus-oocyte communication

A major and well recognized limitation of IVM is the asynchrony between oocyte nuclear and cytoplasmic maturation. Chromatin condensation is precipitated and transcriptional activity diminishes abruptly when the COC is removed from the follicle (Hyttel *et al.* 1987, Lodde *et al.* 2007). Therefore, pre-IVM cultures containing agents capable of delaying nuclear maturation such as natriuretic peptide precursor C (NPPC) and phosphodiesterase inhibitors have been proposed to improve the outcomes of IVM/IVF in cattle (Albuz *et al.* 2010, Franciosi *et al.* 2014), although these strategies have not yet

provided consistent results in different breeds and laboratories (Gilchrist *et al*. 2015).

Robust studies using *Nppc* and *Npr2* (natriuretic peptide receptor B) mutant mice first demonstrated the importance of NPPC signaling for meiotic arrest. A model has been proposed and widely accepted in which NPPC produced predominantly by mural granulosa cells activates natriuretic peptide receptor B (NPR2) on cumulus cells to induce production of cGMP, which is then transferred to the oocyte through gap junctions, deviating the activity of phosphodiesterase 3 from cAMP. This would maintain cAMP at levels required to prevent the synthesis of maturation promoting factor (MPF), thus holding the oocyte in meiotic arrest (Zhang *et al.* 2010b, Conti *et al.* 2012). Later studies demonstrated that *NPPC* is expressed by bovine cumulus cells, and that, like in the mouse, NPPC also inhibits germinal vesicle breakdown (GVBD) in cattle (Franciosi *et al.* 2014, De Cesaro *et al.* 2015). For meiosis resumption to occur, LH inhibits NPPC production by granulosa cells and reduces the flow of cGMP from the outer layers of the cumulus to the oocyte (Kawamura *et al.* 2011, Shuhaibar *et al.* 2015). Reduced gap junction functionality after the LH surge is believed to be a consequence of the production/secretion of EGF-like peptides that bind to the EGFR to induce mitogen activated protein kinase (MAPK) dependent phosphorylation of connexins, the main components of gap junctions (Conti *et al.* 2012).

Therefore the influence of NPPC on nuclear maturation depends on the functionality of gap junctions between cumulus cells and the oocyte. The importance of gap junction mediated communication for meiotic arrest and developmental competence was unequivocally demonstrated by studies where chemically induced gap junction uncoupling led to chromatin condensation and decreased transcriptional activity in the bovine oocyte (Luciano *et al.* 2011). And since these effects were neutralized by co-treatment with cilostamide, an oocyte specific phosphodiesterase inhibitor, it was concluded that the impact of gap junction functionality is mediated by intra-oocyte cAMP. This is in agreement with a later study from the same group reporting positive effects of NPPC and cilostamide on gap junction functionality in cattle (Franciosi *et al.* 2014).

Interestingly, the influence of NPPC appears to be regulated by intrafollicular steroids. In mice, estradiol is required to maintain the ability of NPPC to stimulate cGMP production and to prevent GVBD in culture, and both estradiol and testosterone can increase mRNA levels of *Npr2* in cumulus cells (Zhang *et al.* 2011). The enhancement of NPPC action by steroids also occurs in cattle. We have recently demonstrated that intrafollicular steroids cooperate with NPPC to slow nuclear maturation and to increase gap junction mediated cumulus-oocyte communication in the bovine COC. More specifically, co-treatment with estradiol, progesterone and adrostenedione at physiological concentrations enhanced the ability of NPPC to inhibit GVBD and to increase the transfer of a dye from the oocyte to cumulus cells, which was accompanied by an increase in *NPR2* mRNA levels. Therefore, the enhanced effects on nuclear maturation and gap junction functionality were interpreted as a consequence of greater NPPC signaling and cGMP production in the presence of steroids. Moreover, the combination of NPPC with follicular steroids in a pre-IVM culture promoted improved embryo quality (assessed by total cell number), suggesting that this strategy may be useful to improve IVM/IVF outcomes (Soares *et al.* 2017).

The NPPC system is also regulated by cumulus and oocyte-derived factors. We have recently reported evidence of a link between NPPC and KL under the influence of OSF in cattle. In mammals,

Kit ligand is expressed by granulosa cells since very early stages of folliculogenesis and activates the receptor KIT on the oocyte and theca cells (Hutt *et al.* 2006, Thomas & Vanderhyden 2006). The roles of KL signaling in periovulatory COC differentiation have not been deeply investigated and are controversial; KL delayed 1st polar body extrusion in rats (Ismail *et al.* 1997), but did the opposite in mice (Ye *et al.* 2009). In cattle, first we demonstrated that mRNA levels of both isoforms of *KL*, *KL1* and *KL2*, increase during the first 12 hours of FSH-stimulated IVM in cumulus cells, suggesting that *KL* transcription is enhanced in preparation for ovulation. Secondly, we observed that KL supplementation during IVM of bovine COCs does not affect cumulus expansion, but enhances oocyte maturation as assessed by the percentage of oocytes reaching metaphase II. To investigate the mechanisms by which KL impacts on nuclear maturation, we assessed its effects on the expression of genes regulating meiosis in the bovine COC. Kit ligand did not alter mRNA levels of *NPR2*, but decreased mRNA abundance of *NPPC* in bovine cumulus cells. In addition, KL increased expression of Y-box binding protein 2 (YBX2) in the oocyte, a protein that regulates RNA stability and protein synthesis and is required for normal spindle formation (Medvedev *et al.* 2011). Finally, we assessed whether the oocyte regulates *KL* expression in cumulus cells using the oocytectomy model, and observed mRNA levels around 5 times more abundant in oocytectomized compared with intact COCs at the end of IVM. The increase in *KL* expression was completely abrogated by co-culture with denuded oocytes, indicating that the influence of the oocyte on *KL* expression is mediated by OSF. Conversely and in agreement with the inhibitory influence of KL on *NPPC* expression described above, oocytectomy markedly decreased mRNA levels of *NPPC* in cumulus cells. The specific OSF that mediate the effects of KL on cumulus *NPPC* expression appear to vary between species and remain to be completely identified. In our studies, treatment with FGF10 during IVM decreased *KL2* mRNA expression, suggesting that FGF10 may be one of these OSF in cattle (Lima *et al.* 2016). Taken together, these data suggest that the oocyte and cumulus derived factors interact to control meiosis. It is tempting to speculate that an increase in cumulus *KL* expression overcoming the inhibitory effect of the oocyte through NPPC signaling may be part of the mechanisms leading to meiosis resumption in the periovulatory period in cattle.

Concluding remarks

In this mini-review we compile data indicating that oocyte and cumulus derived factors interact to regulate cumulus differentiation, nuclear maturation and oocyte developmental competence in cattle. In addition, we present published evidence that steroids modulate the influence of cumulus-derived factors on meiosis progression and cumulus-oocyte communication. The data compiled herein widens our view of the mechanisms that regulate meiosis and cumulus function in cattle, and represent useful parameters for the improvement of IVM/IVF outcomes.

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Effects of nutrition on sexual development of bulls

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Abstract

In the last decades a series of attempts have been made to improve reproductive performance of bulls via optimizing nutrition. Although an increase in energy uptake during the post-weaning period of calves led to a faster growing rate, it had no positive effects on sexual development. In contrast, a high-nutrition diet during the prepubertal period reduced the age at puberty of the bulls and increased the size/weight of the testis and the epididymal sperm reserves. This faster sexual development was associated with an increased transient LH peak, which seemed to be mediated by an increase in serum IGF-I concentrations. However, the exact mechanisms responsible for the interaction between nutrition and the subsequent development of calves are still not clear. Sexual development of bull calves depends not only on nutrition of the calves after birth but also on the feed intake of their mothers during pregnancy. A high-nutrition diet fed to the mother during the first trimester has negative effects on the reproductive performance of their offspring. In summary, growth, health and reproductive performance can be improved by nutrition, but further studies are necessary to obtain a better understanding about the mechanisms responsible for this phenomenon.

Keywords: puberty, nutrition, reproductive performance

Introduction

Because of the introduction of genomic selection in cattle breeding a few years ago, the relevance of the reproductive performance of bulls has increased tremendously. Using this new method, the breeders are able to obtain information about the genetic value of the bulls at either the embryonic period or immediately after birth. With this information, they may be able to obtain semen from bulls with high genetic value earlier. One factor that limits this goal of the breeders is the high variability in the onset of puberty and sexual maturation in bull calves (Brito et al., 2012).
It is well known that there is a relationship between body weight and sexual development (Brito et al., 2012). Therefore, a series of attempts have been made to improve the growth of bull calves. However, contradictory results have been obtained via the supplementation of feed. There are even reports that a high-energy feed intake during the pubertal period has negative effects on the health and reproductive performance of bulls (Coulter and Kozub, 1984; Coulter et al., 1987). In addition, there is now evidence that the nutritional differences in feed during the prepubertal period (Brito et al., 2007 a; b; d) or even the feed intake of the mothers (Sullivan et al., 2010; (Jaquiery et al., 2012) can affect the development of the calves during later stages of life. The aim of this paper is to give a review of the literature dealing with the effects of nutrition on sexual development of bull calves.

Physiology of sexual maturation in bulls

For a better understanding of the effects of nutrition on sexual development it is important to be familiar with the physiological alterations occurring during this time period in bulls. An excellent overview of this topic has been given from Rawlings et al. (Rawlings et al., 2008). Therefore, only some aspects of the alterations occurring during sexual development in bulls will be described in this paper.
Testicular growth follows a sigmoidal pattern in bull calves, with small changes occurring up to 25 wk of age, followed by a distinct increase in changes until puberty, and a slowing down of growth as the bull reaches sexual maturation (Abdel-Rauf, 2008; Amann, 1983a; Macmillan and Hafs, 1968). In bulls, puberty is commonly defined as the time when the scrotal circumference (SC) is at least 28 cm and the ejaculate has a concentration of at least 50 million sperm/mL with ≥10% progressively motile sperm, and sexual maturation is defined as the first time when the ejaculate consists of ≥70% morphologically normal sperm (Wolf et al., 1965).
Based on the gonadotropin and testosterone concentrations in blood plasma, the reproductive development of bulls can be divided into three periods: the infantile, prepubertal and pubertal periods. During the infantile period, which lasts from birth to up to 8 wk of age, there are low concentrations of both gonadotropins and testosterone (Rawlings et al., 2008; Amann et al., 1986). In the following prepubertal period, ranging from 8 to 20 wk of age, a transient increase in gonadotropin concentration and a concurrent small increase in testosterone secretion occur (Rawlings et al., 2008; Amann et al., 1983b; Barth et al., 2008). The concentration of LH starts to increase at 4 to 5 wk and is at a maximum concentration from 12 to 16 wk of age. It then falls, reaching a baseline at 25 wk of age (Amann et al., 1983b; Barth et al., 2008). The early postnatal increase in LH secretion is clearly triggered by an increase in the frequency of pulses of GnRH secretion (Rodriguez and Wise, 1989). High LH concentrations during the prepubertal period have a

positive effect on sexual development (Secchiari et al., 1976). Calves with a higher LH secretion at this time period reach puberty earlier than calves with lower LH concentrations during the prepubertal period (Amann et al., 1983b; Evans et al., 1995). Blood FSH concentrations are generally also elevated during the prepubertal period, but changes in FSH are less pronounced than the corresponding changes in LH. Calves that achieve a greater FSH concentration during calfhood are expected to develop larger testes and possibly reach puberty at an earlier age. The age at which SC first reaches 28 cm has been shown to occur earlier in FSH-treated calves than in saline-treated (control) calves. The concentration of FSH decreases to baseline levels by approximately 25 wk of age (Bagu et al., 2006; Evans et. al., 1996; Miyamoto et al., 1989). Serum concentrations of testosterone increase slowly from birth to approximately 20 wk of age; subsequently, testosterone concentrations increase rapidly until 35 wk of age (Evans et. al., 1996; Secchiari et al., 1976; Miyamoto et al., 1989; Lacroix et al., 1977; Sundby et al., 1980; Rawlings and Evans, 1995). The subsequent distinct increase in testosterone concentrations after 20 wk of age occurs during the period of rapid growth of the testes, but interestingly, it also occurs during a time period with low gonadotropin secretion (Bagu et al., 2006). The period of the most active spermatogenesis in bulls is at the end of the early postnatal increase in LH secretion and is at the time when FSH declines from its maximum concentration during the prepubertal period (Rawlings et al., 2008).

It has been hypothesized by Brito et al. (2007c) that endogenous metabolic hormones such as leptin, insulin, GH and IGF-I, which have distinctly altered concentrations during the pubertal period, could have distinct effects on the sexual development of bulls. The authors have shown characteristic alterations of the serum concentrations of leptin, insulin, GH, IGF-I and testosterone and moderate correlations of these hormones with body weight, backfat, SC and paired testes volume. Leptin, insulin, GH and IGF-I concentrations together accounted for 63% of the variation in SC and 59% of the variation in paired testes volume. Therefore, Brito et al. (2007c) assumed that these hormones might also be involved in testicular development during the pubertal phase.

Effects of modifications in nutrition staring during the pubertal period

Most studies dealing with the effect of nutrition on sexual development have been performed after the weaning of beef bulls (Brito et al., 2012; Coulter et al., 1987; Mwansa et al., 1991; Pruitt et al., 1986; Ohl et al., 1996). As bulls are usually weaned at an age of approximately 7 to 8 mo (Brito et al., 2012), this means that the modification of nutrition was started during the pubertal period after the transient increase in gonadotropin concentration. Most often a positive effect of feeding a high-nutrition diet during the pubertal period on SC and testis weight at 12 to 15 mo of age but no effect or even negative effects on sperm production and semen quality was reported (Wolf et al., 1965; Amann et al., 1986; Secchiari et al., 1976). These deleterious effects of an excessive average daily gain (ADG) during the post-weaning period are likely due to fat deposition around the testicular vascular cones and the testes, thus, causing heat stress during spermatogenesis (Coulter et. al., 1997). Furthermore, there is some evidence that excessive energy intake in young bulls may cause laminitis (Greenough et al., 1990), as well as abnormal bone and cartilage growth, resulting in stiffness and lameness. In a recent study, where beef bulls were fed diets with a low-, medium- or high-nutritional content from 6 to 16 mo of age (Brito et al., 2012),

no associations between ADG and the sexual development of the bulls could be detected. These observations are consistent with the hypothesis that the effects of a high-nutrition diet after the early increase in gonadotropin are negligible (Brito et al., 2007b; Rawlings et al., 2008).

Effects of modifications in nutrition starting during the prepubertal period

Also Brito et al. (2007 a; b; d) were among the first authors to investigate the effects of modifications to their nutrition on bull calves before weaning on their later reproductive performance. In several trials (Brito et al., 2007 a; b; d) they clearly demonstrated that a high nutrition during the prepubertal period resulted in a more sustained increase in LH pulse frequency and bigger testicles at maturity. Therefore, LH secretion during the prepubertal period may prime testicular development and determine maximum adult testicular size (Barth et al., 2008). Circulating IGF-I concentrations increased constantly during the prepubertal and pubertal periods, indicating that IGF-I may be involved in regulating sexual development. The temporal association between GnRH/LH secretion and IGF-I concentration is a strong indicator for a regulatory role of IGF-I on GnRH secretion, but more studies should be conducted to determine whether IGF-I can indeed promote GnRH secretion in bulls (Barth et al., 2008). Nutrition also affected testosterone concentrations, which suggests effects on Leydig cell number, their function, or both. A consistent observation was that leptin, insulin, and GH concentrations did not differ among the groups with different nutrition levels during the period of the early gonadotropin increase and therefore were not involved in the differences in LH secretion. Therefore, the role of these hormones, if any, in regulating GnRH secretion is permissive. However, leptin and insulin had moderate to good correlations with SC and paired testes volume in some of the experiments, indicating that these hormones may promote testicular development (Barth et al., 2008).

Effects of modifications in nutrition starting during the infantile period

There is very little information regarding the effects of a modification of nutrition starting during the infantile period. In one study (Bratton et al., 1956) carried out more than 60 years ago, restriction of feed intake in Holstein bull calves from 1 to 80 wk of age had a tremendous negative effect on pubertal development. In recently published studies, Dance et al. (2015, 2016) examined the effects of early life nutrition on reproductive development in Holstein bulls. Twenty-six Holstein bull calves were randomly allocated into 3 groups at approximately 1 wk of age to receive either a low-, medium-, or high-nutrition diet from 2 to 31 wk of age. Afterwards, all animals were fed the medium-nutrition diet. While there were no effects of nutrition on basal FSH concentrations, the increase in LH during the prepubertal period was advanced by 8 wk (11 *vs.* 19 wk) and mean LH concentrations were higher in bulls fed the high-nutrition diet than in those of the other 2 groups. Furthermore, bulls fed the high-nutrition diet had greater testosterone concentrations than those fed the low-nutrition diet from 11 to 27 wk. Bulls fed the high-nutrition diet were younger at puberty and when they reached a SC of 28 cm than bulls fed the low-nutrition diet. In a similar study performed recently on Holstein-Friesian and Jersey bull calves between 3 to 49 wk of age these results could be confirmed (Byrne et al., 2017). In addition, Dance et al. (2015) noticed at 72 wk, that bulls fed the high-nutrition diet had greater paired testes weights. The estimated number of

sperm produced by day in the bulls of the high-nutrition group was about 9% higher compared to the bulls of medium-nutrition group and about 30% higher than the number estimated in bulls of the low-nutrition group, while no differences in sperm quality were noticed noticed depending on nutrition of the bulls (Dance et al., 2016). Overall, the results obtained by Dance et al. (2015, 2016) and Byrne et al. (2017) are consistent with the studies from Brito et al. (2007 a; b; d), who modified the nutrition of beef bulls starting at the prepubertal period. All these studies provide clear evidence that nutritional modulation of bull calves before puberty has profound effects on reproductive development. Because Dance et al. (2015) and Byrne et al. (2017) modulated the nutrition during the infantile and prepubertal periods, it is unclear, however, whether a nutritional modulation is required for both periods to obtain these effects on reproductive development or whether a shorter time interval would be as effective.

Effects of modifications in nutrition limited to the infantile period

We conducted two own studies to characterize the effects of an *ad libitum* feeding of milk within the first three wk of life compared to the effects of an established restrictive feeding protocol on performance, health status, metabolism and the onset of reproductive activity in bull calves.

In the first experiment (Maccari et al., 2015; Prokop et al., 2015), 48 Holstein bull calves were randomly assigned to a group fed milk *ad libitum* for three wk or to a group of restrictively fed calves. Calves were transferred from calf hutches into a group pen at either the second or third wk of life. After the third wk, the housing, feeding and management of calves from both groups were identical. Restricted amounts of milk replacer were offered, as well as a total mixed ration, concentrates and hay, and water was available *ad libitum*. The volume of milk replacer offered was reduced constantly from wk 5 to 10 of life. Subsequent fattening was based on an established concentrate-based ration until slaughter at an age of eight mo. Average daily gains differed markedly: restrictively fed calves achieved an average gain of 380 g per d within the first three wk of life, and the ADG of the *ad libitum* calves was threefold higher (1,280 g). As a result of the different feeding protocols within the first three wk of life, some *ad libitum*-fed calves achieved a weight well above 80 kg by the fourth wk, whereas most restrictively fed calves did not reach a body weight of 60 kg during this period. On average, *ad libitum*-fed calves were 20 kg heavier than the restrictively fed calves at an age of 22 d.

Calves fed restrictively in their first three wk of life had lower testosterone plasma concentrations at an age of 10 wk than *ad libitum*-fed calves, i.e. sexual development was accelerated by an intensified feeding within the first three wk of life.

The effect of nutritional programming was obvious only at the time of slaughter in those calves that did not suffer from severe bronchopneumonia at some point. Calves with a history of pneumonia grew significantly less than healthy calves, and the effects of a higher level of nutrition were totally abolished.

We recently performed another study (unpublished results) for which the results are not yet complete but that would be useful to include here for the discussion of this issue. The objective of this study was to investigate whether a short-term postnatal nutritional trigger of 4 wk affects daily weight gain, health status and the onset of puberty in Brown Swiss calves. Twenty-four bull calves

were fed milk either restrictively (RES) or *ad libitum* (AdL) for four wk. Housing was identical throughout this initial feeding period and also thereafter, when calves were transferred to group pens. Feeding was similar for all calves from wk 5 of life and beyond when a conventional ration for the fattening of bulls was offered. The reproductive performance of the calves was assessed by frequent measurements of SC. As soon as it reached 26 cm, electroejaculations were performed biweekly to determine the quantity and quality of the semen. There were clear differences in the results with respect to milk ingestion. The high inter-individual variance in the group of *ad libitum*-fed calves indicated that roughly one third of those calves did not consume more milk than calves fed according to the established restrictive feeding protocols while other *ad libitum*-fed calves ingested 12 L per d or more. Thus, we subdivided the group of *ad libitum*-fed calves into a group of calves with a substandard milk intake (designated as AdL-low) and calves with a greater-than-normal level of milk consumption (designated as AdL-high). In fact, AdL-high calves consumed roughly twice as much milk as the RES calves and calves in the AdL-low group, both in the first two wk of life and in the subsequent two wk under the different feeding protocols. In agreement with milk intake, daily weight gains were not markedly different between RES calves and AdL-low calves (Fig. 1). However, AdL-high calves achieved a ADG of 600 g by the first two wk of life and gained almost 1,200 g/d in the subsequent two wk. When we followed these bull calves until the end of the study, AdL-high calves remained 30-40 kg heavier during the entire period than RES calves and AdL-low calves (Fig. 1). These results demonstrate the impact of energy intake in early calfhood on subsequent development.

Fig. 1

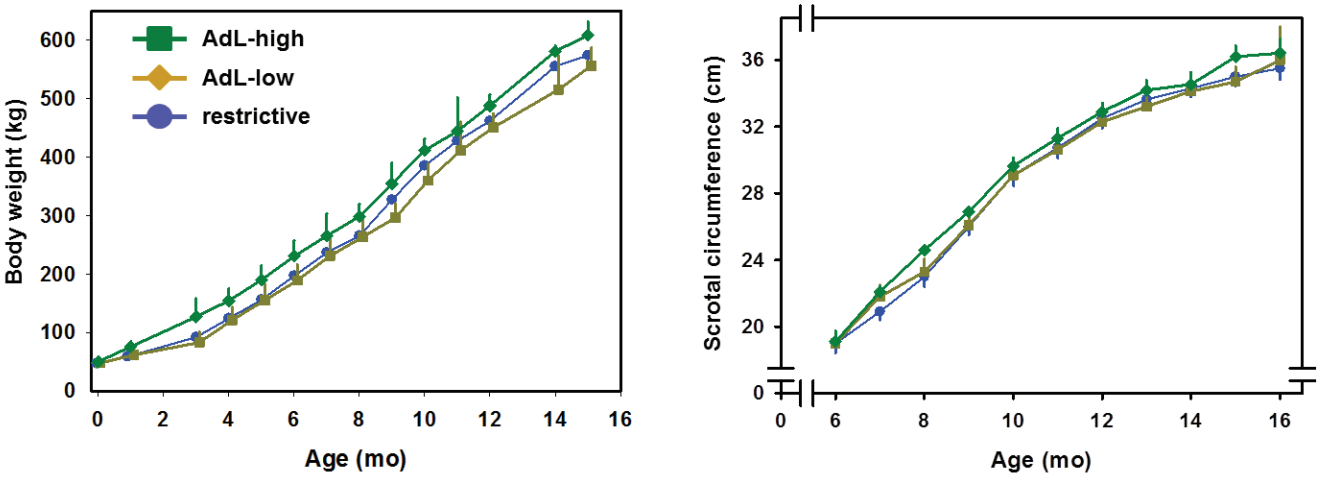

Figure 1:
Differences in milk feeding intensity during the first four wk of life induces long-lasting differences in weight gain and scrotal development in Brown Swiss calves (Bollwein et al., 2016)

With respect to reproductive performance, the onset of puberty based on the analysis of ejaculates did not differ between the groups (278 vs. 274 vs. 275 d) despite the considerable variance within each group. An SC of 28 cm was reached somewhat earlier (Fig. 1) in AdL-high calves (252 d) than in the AdL-low (271 d) and the RES calves (268 d). Testosterone plasma concentrations assessed between 6 and 13 mo of age were significantly higher in AdL-high calves than in calves fed restrictively for the first four wk of life. A significant difference also occurred in the proportion of morphologically normal sperm, which was higher in AdL-fed calves than in RES-fed calves between 10 and 16 mo of age. In conclusion, there was a trend in the data suggesting that a high level of nutrition limited to the infantile period may also affect long-term reproductive performance but significant differences were rare. This might be due to the enormous inter-individual differences within each group. These differences also demonstrate that the onset of puberty is determined by a variety of factors and the intensity of pre-weaning milk feeding is only one of these factors. In addition, the number of animals analyzed in this study could simply be too small to demonstrate an effect of pre-weaning feeding intensity on the subsequent sexual performance of bull calves. Alternatively, the period of intensive milk feeding might have been too short to provoke more profound and long-lasting consequences. In that context, it should be taken into consideration that ADG was reduced markedly in all calves after transfer from individual hutches to a group pen. This setback may be harmful because it still falls in the critical period when the regulatory systems of the calves are responsive to epigenetic factors, and therefore, positive effects of events occurring during the first wk of life may be abolished. Whether this speculation is valid should be investigated in forthcoming studies.

Effects of modifications in nutrition during the fetal period

The hypothesis that in male calves the development of the testis and the hypothalamic–pituitary axis along with the associated synthesis of gonadotropins would be affected by maternal dietary intake and genotype and would correlate with IGF-I and leptin levels was proven by Sullivan et al. (2010). For this purpose, pregnant heifers were divided into two treatment groups stratified by body weight and genotype. For the first trimester of gestation, the groups were fed either high (H) or low (L) protein and energy diets. During the second trimester, half of the animals in each treatment group were switched to the alternate treatment group. This resulted in four treatment groups: high/high (HH), high/low (HL), low/high (LowH), low/low (LL). During the third trimester all heifers were fed a standard diet. Paired testicular weight was positively associated with the LowH nutritional group. There was a tendency for bull calves in the HL group to have smaller paired testicle volumes than those in the LowH group. Bull calves in the LL group had higher FSH than those in the HL group. There was a tendency for HL bull calves to have higher LH concentrations than LowH bull calves. Serum testosterone concentrations were not associated with nutritional group. Reduced circulating concentrations of FSH and lower paired testicular weights in prepubertal bull calves whose dams received higher dietary levels of protein and energy during early gestation suggest that the reproductive axis of beef bulls may be susceptible to nutritional perturbations *in utero* (Sullivan et al., 2010). According to their results, the authors of this study concluded that a compromised development of the hypothalamic–pituitary–gonadal axis in the early

fetal stage may result in a reduced prepubertal gonadotrophin surge in male calves. Overall, the findings of this study suggest a deleterious effect of elevated dam dietary protein and energy in the first trimester of gestation on the reproductive development of their bull calves.

Effects of modifications of nutrition during the periconceptional period

To the best of our knowledge up to know there have been no studies on the effect of nutrition around the time of conception in cattle on the reproductive performance of their offspring. However, such investigations have been performed in sheep. Growth and body composition from birth to adulthood was measured in male and female singleton offspring of ewes that were undernourished before, before and after, or only after conception, and they measured carcass and organ weights after slaughter (Jaquiery et al., 2012). Five-yr-old Romney ewes were randomly allocated to a control group or to one of three groups that were undernourished around the time of conception. Normally nourished controls (N) were fed a maintenance ration. The diets of the ewes in the three undernourished groups were adjusted to achieve and maintain a weight loss of 10–15% body weight from 61 d before mating to the time of mating (d 0) (UN-61-0), from 61 d before to 30 d after mating (UN-61-30), or from 2 d before to 30 d after mating (UN-2-30). Thereafter, all animals were fed in the same way as the control group. Total gonadal weight was affected by periconceptional undernutrition in animals of both sexes but in opposite ways. UN males had heavier testes and UN females had lighter ovaries than N animals. As all time periods of undernutrition were associated with a similar effect on male body composition, it was assumed that the results were via a direct effect on the blastocyst or early embryo, and not on the ovum prior to conception, or via an indirect effect mediated by the altered adaptations to pregnancy observed in UN-61-0 and UN-61-30 groups. The authors of this study also mentioned that future investigations are necessary to obtain a better understanding of the signals in embryonic life that determine sex-related growth differences. However, it would be interesting if these effects were also observed in cattle.

Conclusions

Sexual development of bull calves can be influenced by a modulation of nutrition during their infantile and prepubertal periods. However, the frequent practice of feed supplementation during the pubertal period after weaning does not seem to have positive effects on sexual development (Fig. 2). Interestingly, the development of bull calves is affected by their nutrition not only after but also before birth. In contrast to the positive effects of high feed intake by the bull calves during the first wk after birth, a high-nutrition diet fed to the mother during the first trimester seems to have negative effects on the development and the reproductive performance of their offspring later in life. It has not yet been determined whether there is also an effect of periconceptional feed intake by the cows on the development of their male offspring, as has been reported in sheep. Several studies have demonstrated that modifications in feed intake have an influence on the hypothalamic–pituitary–gonadal axis, which might be mediated by serum IGF-I concentrations, but the exact mechanisms responsible for the interaction between nutrition and the subsequent development of offspring are not yet clear. Therefore, further studies are necessary to

obtain a better understanding of the phenomenon of effects of nutrition on sexual development in bulls to be able to optimize the performance of young bulls.

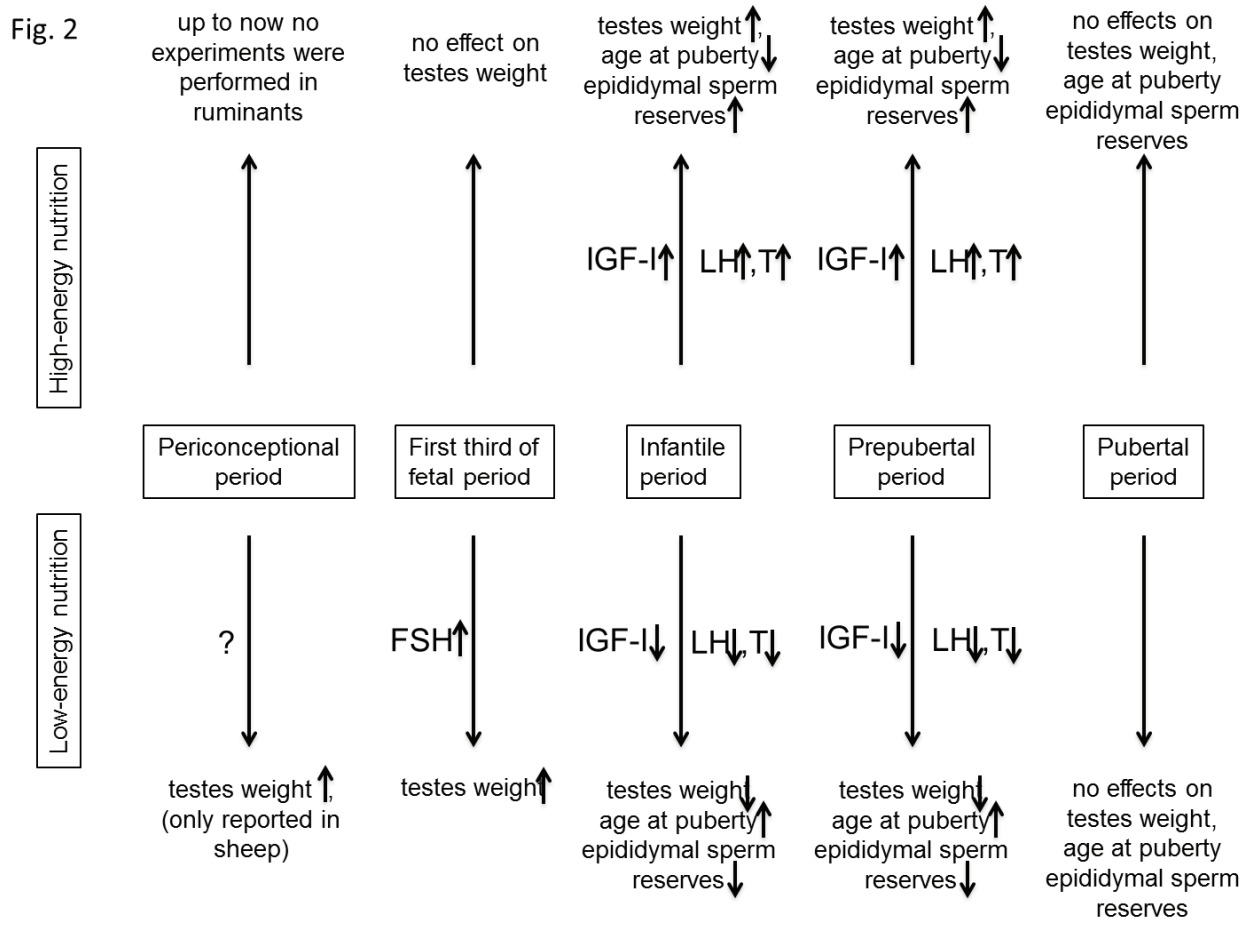

Figure 2:
Scheme summarizing the nutritional effects through the different developmental stages (T = testosterone) (Bollwein et al. 2016)

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Postpartum uterine infection and endometritis in dairy cattle

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Abstract

After parturition, uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus is required before cows are likely to conceive again. However, pathogenic bacteria often cause uterine disease in modern dairy cattle, leading to decreased productivity and reduced fertility. This review aims to provide an overview of postpartum uterine infection and disease in dairy cattle. Metritis and endometritis are the main postpartum clinical conditions; although, subclinical endometritis is an emerging issue. Postpartum uterine disease is associated with the isolation of *Escherichia coli, Trueperella pyogenes,* and anaerobic pathogenic bacteria. Sensing of bacteria or their pathogen-associated molecules, such as lipopolysaccharide, by the innate immune system generates inflammatory responses. Endometrial inflammation includes increased expression of complement, calgranulins, interleukins and acute phase proteins, as well as the chemotaxis of neutrophils and macrophages to the site of infection. Uterine disease is also characterised by tissue damage, including endometrial cytolysis caused by the cholesterol-dependent cytolysin, pyolysin. The responses to pathogens are energetically expensive, and depletion of the key cellular nutrients, glucose or glutamine, impairs inflammatory responses by endometrial tissues. For sustainable intensification of the dairy industry over the next 50 years, it is vital to understand why high-milk-yield cows are so susceptible to uterine pathology and develop new ways to prevent uterine disease.

Keywords: Cow, metritis, infertility, immunity, uterus, ovary.

Introduction

Bacterial infections of the endometrium that cause uterine disease are common in modern dairy cattle after parturition, and lead to decreased productivity and subfertility (Sheldon *et al.*, 2009). The rising incidence of postpartum metritis and endometritis over the last 50 years has generated interest in better understanding the characteristics of the diseases and the impact of the disease on animal health. There has also been a parallel increase in understanding of the mechanisms underlying uterine disease in dairy cattle. Here we provide an overview of postpartum uterine infection and disease in dairy cattle.

Definitions of uterine diseases

The definitions of the various uterine diseases in the literature varied considerably until 2006, when a series of definitions were formulated, with consensus amongst about 20 international experts and referees prior to publication (Sheldon *et al.*, 2006). The initial definitions are now widely used, as set out in 2006 or with minor modifications (Sheldon *et al.*, 2009; de Boer *et al.*, 2014).

The two main postpartum clinical conditions are metritis and endometritis. Metritis is most common within 10 days of parturition, and is characterized by an enlarged uterus containing a watery red-brown fluid to viscous off-white purulent uterine discharge, which often has a fetid odour. The severity of metritis is categorized by the signs of the animal's health, from mild disease to toxaemia. The incidence of metritis varies between breed, country and herd, but in a study of the records from 97,318 cows in the USA, the lactation incidence of metritis, including retained placenta, was 21% (Zwald *et al.*, 2004). Clinical endometritis is defined as the presence of a purulent discharge detectable in the vagina 21 days or more post partum, or mucopurulent discharge detectable in the vagina after 26 days post partum. The incidence of clinical endometritis is around 10 to 20%, with variation between breed, country and herd; a typical study reported that 16.9% of 1,865 cows were affected in Canada (LeBlanc *et al.*, 2002). One of the determinants of the likelihood of uterine disease is the incidence of risk factors. These risk factors can be divided into factors that are associated with damage to the uterus, metabolic stress, or deficits in hygiene. Interestingly, the latter is the least important in the majority of epidemiological models that quantify the risk factors for uterine disease (Dubuc *et al.*, 2010; Potter *et al.*, 2010; Sheldon, 2014). The risk factors most frequently associated with uterine infection are those that likely lead to some trauma to the endometrium, including stillbirth, twins, male and beef-sire calves, dystocia, caesarean section operation, and retained placenta (Hussain *et al.*, 1990; Peeler *et al.*, 1994; Dubuc *et al.*, 2010; Potter *et al.*, 2010).

The diagnosis of metritis and clinical endometritis should include an inspection of the contents of the female genital tract by speculum or insertion of a clean-gloved-hand into the vagina (Sheldon, 2004; Sheldon *et al.*, 2006; de Boer *et al.*, 2014). Whilst somewhat invasive, examination of the vagina carries little risk of further microbial contamination of the uterus in postpartum dairy cattle (Sheldon *et al.*, 2002a). Manual examination of the vagina also facilitates collection of fluid from the vagina to evaluate the presence and odour of pus, which can be used to score the severity of disease and predict the likely success of treatment (Sheldon *et al.*, 2006). Vaginal examination also allows the operator to detect damage to the wall of the vagina and cervix, indicative of obstetric injuries, vaginitis, and cervicitis. However, as with any clinical examination, the evaluation of uterine disease is subjective and there is inter- and intra-operator variation (Sannmann and Heuwieser, 2015).

The absence of pus in the postpartum genital tract does not mean that the tract is normal. The importance of subclinical endometritis has emerged over the last 15 years, with the realisation that

cytological evidence of inflammation of the endometrium is associated with reduced fertility (Kasimanickam *et al.*, 2004; Gilbert *et al.*, 2005). The cause of subclinical endometritis is not yet clear, and may include resolving bacterial infections, immune-pathology without pathogenic bacteria, or even aberrations of postpartum tissue regeneration and repair. Subclinical endometritis is characterized by inflammation of the endometrium that results in a significant reduction in reproductive performance in the absence of signs of clinical endometritis. Subclinical disease is defined by the proportion of polymorphonuclear neutrophils (PMNs) exceeding operator-defined thresholds, usually about 5% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of clinical endometritis, about 35 to 40 days post partum (Sheldon *et al.*, 2006; de Boer *et al.*, 2014).

Pyometra is characterized by the accumulation of purulent or mucopurulent material within the uterine lumen and distension of the uterus, in the presence of a closed cervix and an active corpus luteum. Postpartum pyometra is uncommon, with an incidence rate of less than 2%, and is thought to be caused by the growth of pathogenic bacteria within the uterine lumen after the formation of the first corpus luteum (Noakes *et al.*, 1990). Although there is functional closure of the cervix, the lumen is not always completely occluded and pus may occasionally discharge through the cervix into the vaginal lumen. Pyometra is sonographically characterised by mixed echodensity fluid in the uterine lumen with distension of the uterus, and a corpus luteum in an ovary (Sheldon *et al.*, 2006).

The postpartum period

Uterine disease reflects a disturbance of the normal postpartum period, which usually lasts about 40 days, and is defined as the time between parturition and completion of uterine involution (Sheldon, 2004). After parturition, four concomitant events need to be completed before cows are likely to be able to conceive again: uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus is required before cows are likely to conceive again. (Sheldon, 2004; Sheldon *et al.*, 2006). Failure to resist the growth of pathogenic microbes in the endometrium commonly results in uterine disease.

Uterine involution

Involution is the term used to describe the physical reduction in size of the uterus and cervix after parturition. Involution is thought to be driven by uterine muscular contractions, turnover of the extracellular matrix, necrosis and sloughing of the uterine caruncles, and regeneration of the endometrium (Gier and Marion, 1968). It is often difficult to insert a hand through the cervix 24 h after parturition, and it only admits two fingers by 96 h postpartum. By about 2 weeks post partum, the entire genital tract is palpable per rectum in normal animals; although, the previously gravid horn can still be identified because it is wider and longer than the previously non-gravid horn, and this difference is evident up to 4 weeks postpartum (Okano and Tomizuka, 1987; Tian and Noakes, 1991b; Risco *et al.*, 1994). In parallel with the changes in dimensions, the weight of the uterus decreases from about 9 kg at parturition to 1 kg by 30 days postpartum (Gier and Marion, 1968).

Uterine involution can be monitored by repeated estimation of the size of the uterus, using transrectal palpation or transrectal ultrasonography (Okano and Tomizuka, 1987; Sheldon *et al.*, 2000; Sheldon *et al.*, 2003). It should be noted that dimensions estimated by transrectal palpation are often about 1 to 2 cm greater than ultrasound measurements; presumably because operators include the thickness of the rectal wall when using transrectal palpation. The changes in uterine horn diameter are almost imperceptible by 4 weeks postpartum, and are probably complete by 6 weeks. In the literature, the time to completion of uterine involution is often reported, but this endpoint is difficult to estimate in clinical practice. On the other hand, factors that delay uterine involution are important because completion of involution is associated with fertility (Fonseca *et al.*, 1983). The factors that delay involution include dystocia, hypocalcaemia, retained placenta, metritis, and endometritis.

Regeneration of the endometrium

The epithelium of the endometrium is often damaged during parturition, the caruncular tissue sloughs as part of the physiological process of the puerperium, and there is considerable tissue remodelling during the postpartum period (Gier and Marion, 1968; Wagner and Hansel, 1969; Tian and Noakes, 1991a). It is thought that the endometrium takes 3 to 4 weeks to fully recover the normal tissue architecture, and it is assumed that a normal endometrium is important for fertility.

Return of ovarian cyclic activity

Within a few days of parturition, circulating steroid hormone concentrations decrease to basal values, and there is an increase in plasma FSH concentration, with subsequent recurrent increases in FSH concentrations every 7 to 10 days (Crowe *et al.*, 1998; Duffy *et al.*, 2000). The first postpartum dominant follicle, with a diameter > 8 mm, is usually selected about 10 days after parturition. This dominant follicle may ovulate to form the first postpartum corpus luteum, the dominant follicle may undergo atresia with subsequent emergence of a second dominant follicle, or it may abnormally persist as an ovarian cyst (Savio *et al.*, 1990; Stagg *et al.*, 1995; Beam and Butler, 1997). The fate of the first postpartum dominant follicle depends on LH pulse frequency, and failure to ovulate is usually a consequence of inadequate LH pulse frequency and reduced ovarian follicle estradiol (Beam and Butler, 1999; Duffy *et al.*, 2000; Cheong *et al.*, 2016). In dairy cattle, metabolic stress - most often negative energy balance - is the main cause of reduced LH pulse frequency, although a range of other factors can impact ovarian cyclic activity (Cheong *et al.*, 2016).

Microbes that cause uterine disease

Postpartum uterine disease is associated with the isolation of pathogenic bacteria, particularly *Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum*, *Prevotella* and *Bacteroides* (Elliott *et al.*, 1968; Griffin *et al.*, 1974; Huszenicza *et al.*, 1991; Noakes *et al.*, 1991). Indeed, *T. pyogenes, F. necrophorum* and *Prevotella* act synergistically to increase the likelihood and the severity of endometritis (Ruder *et al.*, 1981; Olson *et al.*, 1984). More recent studies using

aerobic and anaerobic culture confirm the importance of *E. coli*, *T. pyogenes* and anaerobic bacteria (Dohmen *et al.*, 2000; Sheldon *et al.*, 2002b; Williams and Sheldon, 2003; Westermann *et al.*, 2010). Novel endometrial pathogenic *E. coli* have been isolated from animals with uterine disease (Sheldon *et al.*, 2010); and, *T. pyogenes* is associated with the severity of endometrial pathology and clinical disease (Bonnett *et al.*, 1991; Westermann *et al.*, 2010). The link between *T. pyogenes* and disease may be explained by the cholesterol-dependent cytolysin pyolysin (PLO) secreted by *T. pyogenes*, which causes cytolysis particularly of endometrial stromal cells (Amos *et al.*, 2014; Preta *et al.*, 2015).

The role of *E. coli* and *T. pyogenes* is highlighted by infusing *E. coli* and *T. pyogenes* into the uterus of naïve cows to create animal models of endometritis (Ayliffe and Noakes, 1982; Amos *et al.*, 2014). In addition, vaccines containing components of *E. coli*, *F. necrophorum* and/or *T. pyogenes* protect animals against postpartum uterine disease (Nolte *et al.*, 2001; Machado *et al.*, 2014). However, metagenomics techniques have found associations between uterine disease and bacteria that are not readily cultured by standard techniques (Machado *et al.*, 2012; Santos and Bicalho, 2012; Peng *et al.*, 2013; Knudsen *et al.*, 2015; Wagener *et al.*, 2015). Whilst some of the studies find *E. coli*, *T. pyogenes* and the expected anaerobic bacteria, others report finding *Bacteroidetes* and *Firmicutes*. There remains a gap in understanding how "unculturable" bacteria contribute to the pathogenesis of uterine disease. A consistent finding among most microbiology studies is that anaerobic bacteria are more abundant in the diseased endometrium than in healthy uteri. Perhaps this is not surprising as the endometrium is a microaerophilic environment, with tissue damage likely reducing the oxygen tension further. Taken together the evidence is that *E. coli*, *T. pyogenes* and anaerobic bacteria are probably the main pathogens causing the clinical signs of postpartum uterine disease (Fig. 1).

One note of caution about our understanding of microbes in the endometrium is that recent evidence counters the traditional view that the uterus is sterile outside the postpartum period. There is evidence from studies using fluorescent probes for bacteria and from 16S ribosomal RNA gene sequencing, that there is a sparse microbiome in the uterus, even during pregnancy (Karstrup *et al.*, 2017; Moore *et al.*, 2017). The bacteria include *Trueperella, Fusobacteria and Prevotella* species, but the abundance of these bacteria is a small fraction of those present in animals with postpartum uterine disease. Whilst postpartum uterine bacteria may also derive from the vagina, skin and the environment, it is possible that the pathogenic bacteria present in the uterus before parturition grow and cause pathology after parturition.

Host defence against infections of the uterus

The host has a range of defences against microbial contamination of the uterus and infection of the endometrium. Whilst the animals' environment is heavily contaminated with bacteria, the vulva, vagina and cervix provide anatomical barriers to ascending infections, except during parturition (Fig. 1). Whether the resident flora of the vagina or the pH of the vagina might also compete with pathogens to limit disease is a contentious matter. However, there is a range of

antimicrobial peptides, glycoproteins and mucins in the vagina, cervix and uterus, that counter bacterial contamination and restrain bacterial growth (Davies *et al.*, 2008; Chapwanya *et al.*, 2013; Kasimanickam *et al.*, 2014).

Of course, microbial invasion of the female genital tract is not unnoticed. Adaptive immune responses are evident, with increase abundance of antibodies (Dhaliwal *et al.*, 2001); which, concur with the ability to vaccinate against uterine pathogens (Nolte *et al.*, 2001; Machado *et al.*, 2014). A recent advance in knowledge has been about the role of innate immunity in the female genital tract (Fig. 1). Innate immunity depends on the binding of pathogen-associated molecular patterns from microbes to pattern recognition receptors in host cells. There is a range of pattern recognition receptors found in the plasma membrane or cytoplasm of mammalian hematopoietic cells. The two most widely investigated pattern recognition receptor families are the Toll-like receptors and components of the inflammasome (Moresco *et al.*, 2011; Lamkanfi and Dixit, 2014). The Toll-like receptors bind components of bacteria, such as lipopolysaccharide, lipopeptides and nucleotides, which leads to production of inflammatory mediators; typically interleukin (IL)-6 and IL-8. Similarly, pathogen-associated molecules that reach intracellular compartments activate the inflammasome. However, the inflammasome can also be activated by a range of generalized cell perturbations, including the ion fluxes that are associated with pore-forming toxins secreted by bacteria. Activation of the inflammasome typically leads to cleavage of pro-IL-1β and secretion of the mature form of IL-1β (Lamkanfi and Dixit, 2014). The Toll-like receptor system is present and active in the cells of the endometrium, both epithelium and stroma, as well as in bovine hematopoietic cells (Herath *et al.*, 2006; Cronin *et al.*, 2012; Turner *et al.*, 2014; Cronin *et al.*, 2016). However, endometrial cells secrete little IL-1β protein, and so inflammasome activity may be more important in hematopoietic cells.

The innate immune system provides a non-specific and rapid response to pathogens and damage. However, excessive inflammation leads to immunopathology or septic shock, and so innate immunity is carefully calibrated. A series of checks and balances are in place to scale inflammation to meet the level of microbial threat, and to limit inflammation when infections are cleared (Blander and Sander, 2012). One example in the bovine endometrium, is the role of STAT3 to regulate the secretion of IL-6 and IL-8 in stromal cells (Cronin *et al.*, 2016). Another example is the apical secretion of IL-6 and IL-8 from bovine endometrial epithelial cells, toward the invading pathogens in the uterine lumen and away from the underlying stromal cells (Healy *et al.*, 2015).

Figure 1:

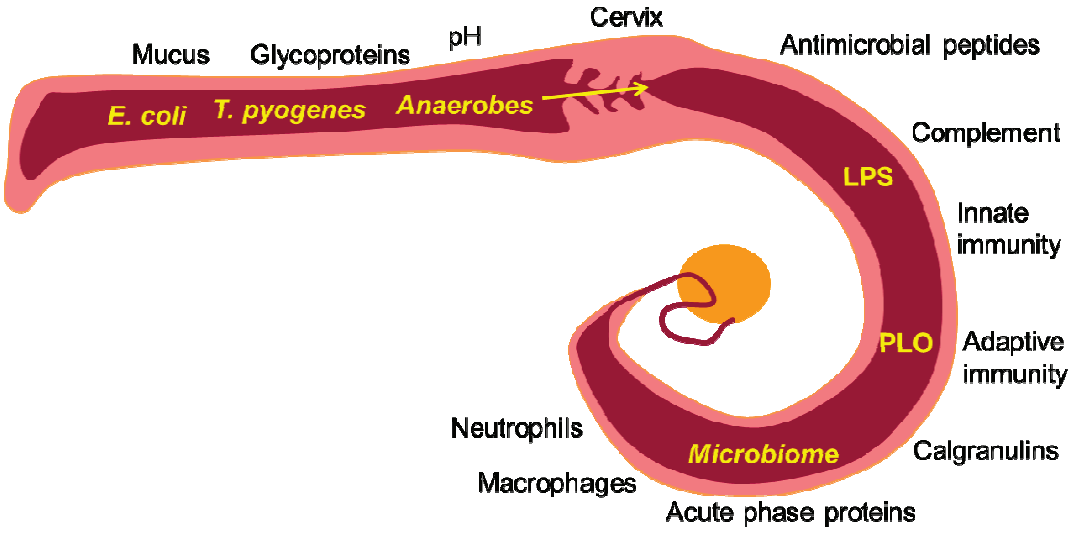

Figure legend

Figure 1. Schematic outline of factors contributing to postpartum uterine health. After parturition the anatomical barriers of the vulva, vagina and cervix are breached, introducing bacteria into the uterus, including pathogens, along with bacteria that constitute the uterine microbiome. However, tissue factors such as mucus, glycoproteins, the pH of the genital tract, and antimicrobial peptides help counter bacterial invasion. If bacteria or their pathogen-associated molecules, such as lipopolysaccharide (LPS), are sensed by the innate or adaptive immune systems then an inflammatory response ensues, including increased expression of complement, calgranulins and acute phase proteins, and chemotaxis of neutrophils and macrophages to the site of infection. As well as inflammation, uterine disease is characterised by tissue damage, including cytolysis caused by the cholesterol-dependent cytolysin, pyolysin (PLO).

Beyond recognition of microbes, one of the features of infection is tissue damage, which in the endometrium is often caused by secretion of pyolysin by *T. pyogenes* (Amos *et al.*, 2014; Preta *et al.*, 2015). Damaged cells release damage-associated molecular patterns, such as nuclear and cytoplasmic molecules that are not normally encountered in the extra-cellular compartment (Kono and Rock, 2008). Some pattern recognition receptors, primarily in hematopoietic cells, sense damage-associated molecular patterns, leading to inflammatory responses. Damaged endometrial tissue cells, primed with LPS, produce the damage-associated molecular patterns, IL-1α, which is normally retained in the cytoplasm of healthy cells (Healy *et al.*, 2014). Furthermore, endometrial stromal cells express the receptor for IL-1 and generate inflammatory responses to IL-1α, including secretion of more IL-6 (Healy *et al.*, 2014).

Innate immunity is an evolutionary ancient system and so it is not surprising that it is integrated with other cellular homeostatic and metabolic pathways (Kotas and Medzhitov, 2015). Dairy cattle are under metabolic stress after parturition, with reduced concentrations of nutrients and changes in metabolic hormones, including reduced abundance of glucose, glutamine and insulin-like growth factor 1 (Chagas *et al.*, 2007; Kerestes *et al.*, 2009). Negative energy balance may impair the inflammatory response and clearance of bacteria from the endometrium, leading to chronic endometritis (Esposito *et al.*, 2014). Certainly, the response to pathogen molecules is energetically expensive *in vivo* and *in vitro* (Turner *et al.*, 2016; Kvidera *et al.*, 2017). A striking example is that animals use > 1 kg of glucose in the first 6 h after challenge with LPS (Kvidera *et al.*, 2017). Furthermore, the depletion of the key cellular nutrients, glucose or glutamine, reduces inflammatory responses by endometrial tissues *in vitro* (Turner *et al.*, 2016; Noleto *et al.*, 2017). If metabolic stress compromises the ability of animals to respond sufficiently to pathogens, this may result in persistence of infections and chronic inflammation.

Impact of uterine disease on animal health and fertility

Clinical uterine disease has a marked impact on reproductive health in cattle, causing subfertility and infertility. In a meta-analysis of records from more than 10,000 animals, there was evidence that postpartum metritis caused subfertility by increasing the time to first insemination by 7.2 days, reducing conception rate to first insemination by 20%, and increasing the calving to conception interval by 18.6 days (Fourichon *et al.*, 2000). Similarly, clinical endometritis increased the interval to first insemination by 11 days, and delayed conception by 32 days, compared with animals that did not have endometritis (Borsberry and Dobson, 1989). Although less common than subfertility, uterine disease also cause infertility. Cows with clinical endometritis between 20 and 33 days post partum were 1.7 times more likely to be culled for reproductive failure than cows without endometritis (LeBlanc *et al.*, 2002).

Pathology in the endometrium is likely to be detrimental to fertilization and conception. In addition, extension of infection or inflammation to the oviduct likely disrupts the delicate balance of the immune systems that are required for fertilization (Marey *et al.*, 2016). However, an important observation for mechanisms that perturb fertility, is that postpartum uterine infection also impacts

fertility after resolution of the clinical disease (Borsberry and Dobson, 1989). Several mechanisms may underlie the wider effects of uterine infection on fertility, beyond the tubular genital tract. First, there is evidence that bacterial infections disrupt the endocrine signalling in the hypothalamic-pituitary-gonadal axis, and the secretion of gonadotrophins (Karsch *et al.*, 2002). Secondly, uterine infections disrupt ovarian follicle growth and function, with smaller and less steroidogenic ovarian follicles (Sheldon *et al.*, 2002b). Finally, uterine infections may reduce oocyte quality, with increased rates of meiotic arrest and germinal vesicle breakdown failure (Bromfield and Sheldon, 2011). Oocyte development lasts about120 days, between the primordial follicle stage to ovulation of a cumulus-oocyte complex. Thus, in cows inseminated 60 to 120 days post partum, the oocytes that are ovulated may have been exposed to pathogen molecules and inflammatory mediators throughout the postpartum period, if the animal had uterine disease. Therefore, limiting uterine disease is not only important for the affected animals, but also for their offspring. Further discussion of the mechanisms linking uterine disease and reproductive biology are published elsewhere (Sheldon *et al.*, 2014; Bromfield *et al.*, 2015).

Outstanding questions

Whilst there is a clear understanding of the clinical aspects and implication of postpartum uterine disease, and some of the mechanisms of pathology, there are important outstanding questions. The most obvious question is why are modern high-milk-yield cows so susceptible to metritis and endometritis? Allied to this, is what can be done to prevent uterine disease? Answering these questions is vital for sustainable intensification of the dairy industry over the next 50 years.

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COST-Action GEMINI and EPICONCEPT: What we learned after 8 years?

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Abstract

Scientific societies have a major role in facilitating and disseminating scientific discoveries. Here, we are all members of societies related to reproductive biology, such as AETE (European Association of Embryo Transfer), SRF (Society for Reproduction and Fertility) or ESHRE (European Society of Human Reproduction and Embryology). However, many of you may be unfamiliar with COST Actions. These are atypical, EU-funded temporary societies, that can have a huge impact upon the lives and careers of their members. The objective of the present paper was to capture the influence that one specific COST Action, EPICONCEPT, and to a lesser extent also the earlier COST Action GEMINI, has had on European scientists involved in animal reproduction and embryo transfer. We discuss the intrinsic value of belonging to EPICONCEPT, we focus on how EPICONCEPT advanced the careers of the scientists involved and the lessons learned. We conclude that such specific short-lived societies as granted by COST can be the basis of permanent collaborative ties and networking within Europe. Moreover, EPICONCEPT has been a very useful tool to raise awareness about epigenetics among animal scientists and breeders.

Keywords : COST – Epigenetics – periconception environment - Researcher

Introduction

Young scientists are not always aware of the importance of attending different conferences and the value of engaging in networking activities. My[1] former boss told me: "If you attend a conference it will benefit your network". But do we actually need a network to be successful in science and to advance in our career? I can now wholeheartedly say: "Yes" to this question.

The network that was of major importance to me, and also to many other European researchers, was EPICONCEPT, short for "Epigenetics and Periconception Environment".

EPICONCEPT really started about ten years ago. I received an e-mail from a scientist, Prof Alireza Fazeli, that was at that time only affiliated to the University of Sheffield in the UK. It read: "Dear Dr. Van Soom, we may have never met before, but I know from your published work that you are interested in sperm-oviduct interaction. I plan to apply for a COST Action that is focusing on this broader topic. Are you willing to take part in this Action and if so, can you give me the names of other people who may also intend to join? If you are interested, we can also talk on the phone, so I can explain the background."

I had never heard of a COST Action before, so I agreed to talk on the phone to learn more. I asked Alireza if this was a way to raise European money for research. "No", he said, "you can get money for organizing conferences and workshops, for visiting each other's laboratories and for exchanging PhD students."

At first, I did not really think that the COST Action was going to be a useful approach at all, but I agreed to contribute nonetheless. I produced a list of emails from people involved in research regarding oviducts and spermatozoa, and thought that would be the last thing that I would ever hear from it. Little did I know! Alireza Fazeli, together with many others, turned from an unknown person into a dear lifelong friend. I will review in this personal testimony, how EPICONCEPT affected our lives and careers, and we will point out what we have learned from EPICONCEPT.

History of COST Action

The COST-Action website (http://www.cost.eu/), states **"**COST is a unique means for European researchers, engineers and scholars to jointly develop their own ideas and new initiatives across all fields of science and technology through trans-European networking of nationally funded research activities**."**

The first COST Action I was involved in was GEMINI (Maternal Interaction with Gamete and Embryo 2008-2012). Until then, I had been mainly involved with bovine embryos and how they interact with their environment (the Petri dish), but during this action, we learned about maternal interaction in insects, fish, reptiles (Holt and Lloyd, 2010) and even apprehended information on *in silico* models (Burkitt et al. 2011). I started writing (opinion) papers with other European scientists, we exchanged students with other labs, we organized workshops and meetings and we were inspired by all these contacts and communications: it broadened our view, it encouraged us to apply

[1] *Just for the reader's information, wherever in this manuscript the words "I/me/my" are used, it refers to my own (Ann Van Soom) experiences. However, whenever "we" is used it will refer to my coauthor and/or the wider COST community.*

for more nationally funded projects and we were able to start common research with other EU-groups, with our own funding of course. The annual meetings and workshops increased the bonds we had and they created the sense of belonging to a large scientific family.

COST Actions can be a very useful tool, and we used it very well: GEMINI turned out to be important for my career and also for many young investigators and their supervisors. Scientific societies, including COST Actions, have a major role in facilitating scientific discoveries and disseminating them (Bahr, 2008). To use a metaphor: The network that is created by a COST Action is like a spider web, connecting different people with sticky threads, and when a new fly is caught, it is signaling by its movement its presence to the central spider, who can easily catch it for a presentation at one of the upcoming conferences. The outstanding feature of COST is that it provides a platform to young investigators, to researchers that are underrepresented (many of whom are female), to researchers from countries within and outside Europe that is very different from the "Old Boy Network" (see Merriam Webster : an informal system in which wealthy men with the same social and educational background help each other), which is not always in favor of young researchers belonging to a minority to present his or her research.

In this digital age, one could wonder if the network that is provided by COST and other societies could not be replaced by a Facebook page, or by twitter, or by a comparable social media connection. The answer is probably "No". A questionnaire which was filled in by trainee members of the Society for Study of Reproduction provided indeed more evidence that people need to interact in a personal way, to connect and to stay current (Table 1). Meeting in an informal manner removes many of the prejudices people may have when they receive an unsolicited e-mail from somebody they have not met before. Contrary to the common expectation, a discussion at a poster session or even having a drink at the bar with another scientist can be the start of a lifelong scientific collaboration!

So we can conclude that COST Actions are indeed useful, contrary to what I expected after my first contact with Alireza Fazeli. During this period, my career moved on to the fast track. I applied for many more grants than before and as a result, I was also successful in achieving more funding. At the end of GEMINI, I even decided to apply for a second COST action, EPICONCEPT, as the Chair this time. The topic was on epigenetics, and although I was not a molecular biologist, I had always been intrigued by genetics, by evolution, by Lamarck and Darwin, and the link it had with embryology, as in the discredited theory of Ernest Haeckel, where he stated that ontogeny is a recapitulation of phylogeny. EPICONCEPT would give me the opportunity to delve deeper into this topic. I considered the fact that I was not a geneticist to be an advantage, since I had to make the topic understandable for non-geneticists. This is often the key to success: convey your message in a simple, understandable way, both to other scientists and to the general public. That too, I learned during the COST Actions.

Why is epigenetics interesting when you are working with embryos?

So why did I think EPICONCEPT was an interesting line of research? Many things had evolved in the field of assisted reproduction since the birth of the first test-tube baby, Louise Brown, in 1978. Cattle were the first species, after the human, in which transfers of *in vitro*

Table 1. Survey filled in by trainees that were member of Society for the study of Reproduction (SSR) (adapted from Bahr 2008)

Question	Most popular answer	Second important answer
What is the primary reason for joining a scientific society?	To attend annual meeting	To share knowledge with other researchers
What is the value of belonging to a scientific society?	To interact with people who share common interest and to meet experts in the field	To stay informed about the latest advances in the field
What can scientific societies do to advance trainees' scientific careers?	To facilitate networking and collaboration	To hold annual meetings

produced embryos were performed on a large scale during the 1990s. Coinciding with the first reports of the birth of the first cloned calves and sheep, troubling anecdotal reports emerged of congenital abnormalities associated with cloned animals and later in a broader perspective, also of abnormal offspring born after *in vitro* culture of ruminant embryos (Willadsen et al., 1991; Van Soom et al., 1994; Walker et al.,1996). The most obvious characteristic of the abnormal offspring was an overgrowth phenotype, and thus the syndrome was termed "Large Offspring Syndrome" or "Abnormal Offspring Syndrome" (for review see Farin et al., 2010).

As early as 1998 it was hypothesized that the mechanism was probably related to changes in DNA-methylation of imprinted genes, which were imposed upon the embryo by its exposure during a critical period to a perturbing environment (Young et al.,1998).

Also in humans there were similar reports on the influence of the intrauterine or perinatal environment on fetal development. Barker postulated that a baby with a low birth weight has a higher risk to suffer from cardiovascular disease as an adult (Barker et al., 1989). This hypothesis was later called the "Developmental Origins of Health And Disease" or DOHAD hypothesis, and by the mid-1990s the concept that late-onset diseases are related with earlier prenatal events, was

well established (Grace et al., 2009; Barker, 1995). Barker studied mainly fetal undergrowth, but also fetal overgrowth has been reported in humans. Assisted reproduction, which is currently accounting for 5-6 % of the live birth rates in Belgium, has indeed been associated with increased risk of imprinting diseases such as Beckwith–Wiedemann syndrome, which is a fetal overgrowth syndrome (Owen and Segars, 2009). Both "Large Offspring Syndrome" in cattle and the "Developmental Origins Of Adult Health And Disease" hypothesis in humans are reflections of the fact that small changes in the environment to which the embryo is exposed can either lead to obvious phenotypical changes in the neonate (oversized calf, Beckwith-Wiedemann baby) or to more subtle, long-term programming effects, which can lead to impaired health during adulthood (Sinclair and Singh, 2007) (Fig. 1).

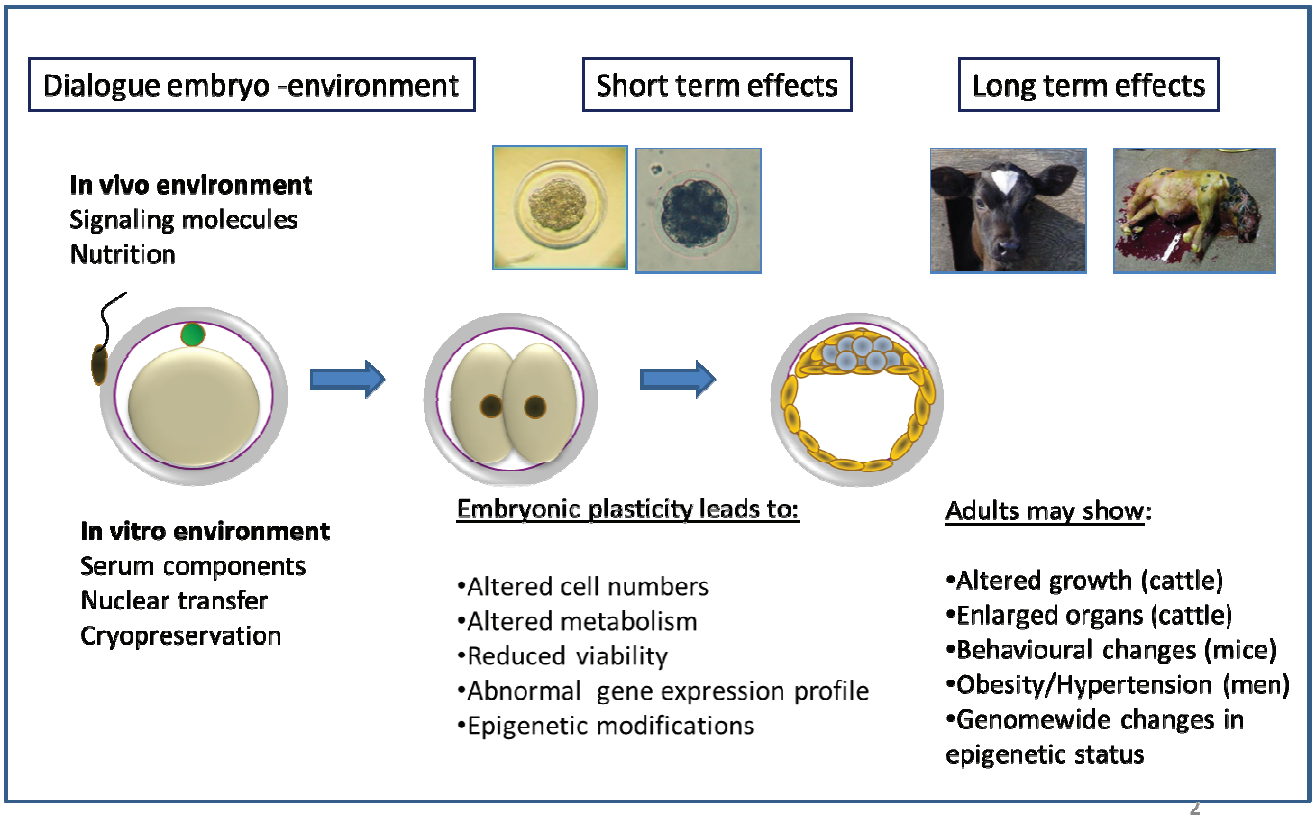

Fig. 1. An unusual environment to which the embryo is exposed will lead to short and long term effects, both of which are caused by epigenetic modifications and which in some cases can be transgenerational. At present, these effects have been shown to be induced by *in vitro* embryo culture in mice, man and cattle.

Such an important concept (DOHAD) called for more in depth research. The field of environmental epigenetics, which was closely related to the concept of Developmental Origins Of Health And Disease, was studied extensively by using various animal models. These models provided a means to understand how environmental factors, which are present at periconception, may induce heritable changes in gene expression and as such, can cause diseases that cannot be explained by

conventional genetic mechanisms (Rosenfeld, 2010). These changes were called epigenetic changes: in Epiconcept , we aimed to apply our animal models to the search for an answer on how environment affects offspring health and performance in the adulthood (Van Soom et al., 2010, 2013, 2014). Understanding the epigenetic mechanisms involved in embryonic development will help to address such issues as (a) the risks associated with stress, illness or dietary restrictions and metabolic imbalances during the peri-conceptional period, which is including prenatal and early postnatal life (Mossa et al., 2013; Fleming et al., 2015; Velazquez, 2015); (b) the effects of maternal and paternal nutritional status/stress on epigenetic programming through the germline; and (c) transgenerational effects where, in future, greater emphasis in livestock species should be placed on traits of agricultural importance (Gonzalez-Recio et al., 2012; Opsomer et al., 2017).

Epigenetic changes may be less harmful than genetic mutations since they are reversible. Understanding the healthy settings of the periconception environment that avoid deleterious epigenetic changes will allow to potentially improve this environment to attain the ideal conditions to which breeding animals and embryos should be exposed in order to prevent epigenetic mutations to occur. The periconception environment encompasses ontogenesis and the organs and tissues in which gametogenesis, embryogenesis, implantation and placentation take place. Although sexual reproduction is globally robust, it is also a vulnerable process. Gametes and embryos are especially vulnerable to epigenetic changes. Most epigenetic marks are systematically erased in the preimplantation embryo and in the primordial germ cells in order to down-regulate the inheritance of epigenetic (acquired) information between generations, and appear again later on. Likewise, epigenetic processes are responsible for laying down the gender-specific imprinting that allows for gender-specific gene expression, which is of paramount importance for embryonic development and placentation.

What have we learnt during EPICONCEPT

Parental stress before, during and after conception (i.e. the periconception period), induces epigenetic changes in gametes and embryos. Such epigenetic changes may adversely affect the future health, development, productivity and fertility of those offspring. While there is increasing evidence for this in agricultural species, most of this knowledge is derived from epidemiological studies in humans and controlled studies in laboratory animals. In EPICONCEPT, time frames and mechanisms during which the gametes and early embryo are susceptible to epigenetic modifications were investigated in livestock in order to optimize their health and productivity. The objectives were to:

1. Develop an epigenomic toolbox for large scale screening of epigenetic changes in gametes and embryos.
2. Define the factors that can influence the epigenetic profile during the periconceptional period of gametes and embryos.
3. Define the time-window during which most epigenetic changes take place
4. Define the range of the optimal periconception environments to ensure healthy offspring.

5. Compare the susceptibility of different species (livestock, poultry, fish) and different model systems (*in vivo* vs *in vitro*) to epigenetic disturbances.

We achieved these objectives by discussing these topics at our conferences and workshops. Here, we need to acknowledge the generosity of fellow scientists who were not members of Epiconcept, but who travelled from around the world to participate in our meetings, to share their knowledge with us and whose presence meant that we were exposed to cutting edge science and methodologies. As material output we published several review papers on the topic (O'Doherty and McGettigan, 2014; Anckaert and Fair, 2015; Salvaing et al., 2014; Gutierrez-Adan et al. 2014; Brevini et al., 2014), in a Research Front entitled Epigenetics and Periconception environment in Reproduction, Fertility and Development (Editors: Ann Van Soom and Alireza Fazeli), and a book entitled Periconception in Physiology and Medicine (Editors: Alireza Fazeli and William V. Holt), which is in press by Springer.

Public engagement activities were carried out during the COST Action to inform the general public on the importance of the epigenome via the periconception environment in future food production, health and welfare. We communicated via organized Weeks and Nights of Science at our universities, we raised awareness and had an impact on young students even at the level of the secondary school pupils. We used our website (cost-epiconcept.eu) to inform scientists and the public and we produced a facebook page (www.facebook.com/Epiconcept-COST-Action-1381626895453232/?fref=ts) to interact with scientists, stakeholders, clinicians and practitioners to improve gamete and embryo handling and animal husbandry and breeding. We informed different companies and invited them to our workshops and courses, to learn about the possible impact of gamete and embryo handling on later life. A major success story from our action was to convince stakeholders, such as companies involved in semen freezing and cattle breeding by artificial insemination, to become involved in new research EU-projects on this topic.

Final conclusions

With this short review I wanted mainly to point out how important interaction is between scientists. We have not only got to know each other better during Epiconcept, we have also become like friends and family. Some of us are still collaborating, either in an EU Project, or in a project based on national funding or as a member of a new Cost Action, Cell-fit (https://www.facebook.com/COST-Action-16119-CellFit-1660173010901682/). I do not have a final message for you, suggesting to do this or that to prevent epigenetic changes to occur, in order to prevent diseased offspring resulting from your research. But I do think that we have raised awareness, that scientists now know that the addition of certain ingredients, such as serum, to the culture medium can have far reaching consequences, and that the introduction of novel techniques should be carefully investigated for subsequent epigenetic effects. But since epigenetics is part of life, and since we are all influenced by our environment; it is also important to realize that we cannot prevent this interaction with the environment. We should live healthy lives and we need to expose our gametes and embryos to a healthy environment, but we should not be terrified or reluctant towards change.

Acknowledgements

COST – office is acknowledged for creating this excellent tool to interact between scientists and laboratories. We also wish to thank Dr. Laszlo Tecsi for his invaluable services to the members of the Epiconcept Cost Action.

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SHORT COMMUNICATIONS

TAI/FTET/AI

What could be the impact of cervical mucus removal after oestrus synchronization on artificial insemination outcome in INRA180 prolific sheep?

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Keywords: Oestrus synchronization, mucus, artificial insemination

Artificial insemination (AI) is an important tool that improves the chance of using superior rams (Arranz et al., Renc. Rech. Rum, 15, 359-362, 2008) and helps to control genitally diseases. During the AI process, the administration of exogenous progestagen to synchronize oestrus in ewes is one of the most important steps. However, the administration of such hormones has resulted in contradictory reports of both increased and decreased mucus production, which could affect the outcome of AI. The present work aimed to study the effect of mucus removal after oestrus sychronisation of INRA180 prolific adult ewes. A total of 84 ewes (2.5 to 3 years old) that have been managed under natural reproduction system were used to make 3 groups: group 1 (control) reproduce naturally, group 2 inseminated without removing the mucus and group 3 inseminated after mucus removal. For each group, two different doses of eCG (250 IU vs 300IU) have been used. The ewes were treated with intravaginal progesterone sponges (20 mg Flurogestone acetate, Pharmavet) for 14 days, and then injected with equine chorionic gonadotropin (eCG) at 250 IU or 300IU (Folligon®, Pharmavet) during Jun 2016. They were naturally mated (n=20 with 250 IU, and n=20 with 300 IU) or inseminated after removing the mucus (n=12 with 250 IU, and n=12 with 300 IU) or not (n=12 with 250 IU, and n=12 with 300 IU). The mucus was removed from animals in standing position using vaginal speculum. A split-plot design was adopted and all analyses were performed using JMP SAS v11. Fertility and prolificacy data were assessed by $\chi 2$ analysis of contingency tables. The results showed that the natural mating revealed the highest fertility rates (80 to 90%). In both 250 IU and 300 IU groups, the treatment (removing the mucus or not) was highly significant ($P<0.05$). The conception rates were 32% and 40% respectively for the ewes receiving 250 IU and 300 IU of eCG. The mucus removal has significantly improves the fertility as this parameter increased to 59% and 67% respectively in 250IU and 300 IU. Recently, it has been reported that the cervicovaginal mucus proteome of the ewe undergoes natural variation across the oestrous cycle, and is significantly altered by progesterone synchronisation (Maddison et al., Journal of Proteomics, 155, 1-10, 2016,). This could explain a part of the result obtained in this work. The prolificacy was not improved ($P>0.05$) and it varies from 1.44 to 1.89. In conclusion, the present study revealed that it is possible to improve the fertility rate after artificial insemination by means of mucus removal after oestrus induction. Further protocols are planned on a large group of animals and will focus mainly on the eCG doses (300 IU) showing the greatest fertility rate.

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Comparative study of ewe's cervixanatomy of two Moroccan breed: A necessary step before the artificial insemination

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Keywords: Boujaâd ewes, D'man ewes, cervix anatomy

The anatomy of the cervix represents a major constraint for developing the trans-cervical artificial insemination (TCAI) in sheep. Thus, the aim of this study was to compare this parameter in two Moroccan indigenous breeds. A total of 250 uteri Boujaâd (non-prolific,n = 187) and D'man (prolific, n = 64), with different ages (2,4,6 and 8 teeth) (Hemming, J.Wildl. Manag. 33, 552–558,1969) were collected from slaughterhouses at Bejaad and Errachidia respectively. The uteruses were transported at 5°C to the laboratory, and were cleaned in order to perform various measurements. For each sample, the weight of the uterus, length of the cervix, depth of penetration (using the artificial insemination gun), cervical grade (Kershaw et*al*.,Theriogenology 64, 1225-1235, 2005), and the number of cervical rings were recorded.All data were analyzed using the Statistical Analysis System software JMP (SAS version 11), by means of a factorial design ANOVA. The statistical model included the breed and age as fixed effects. When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SD.The cervical grade data were assessed by $X2$ analysis of contingence tables. Independently on the ewes age, this study showed significant differences in the cervical grades between the two breeds. According Kershaw et *al*., (Theriogenology 64, 1225-1235, 2005) the grades were as follow: grade 1 (11.90%) vs (12.82%), grade 2 (52.38%) vs. (69.23%) and grade 3 (35.71%) vs. (17.95%) respectively for Boujaâd and D'man. Furthermore, the average length of the cervix was 54.47 ± 13.60 mm for Boujaâd ewes instead of 41.53 ± 9.54 mm for D'man ($P <0.05$). In addition, there were significant differences between breeds in the number of cervical rings (4.56 ± 1.32 for Boujaâd *vs.*3.91± 1.09 for D'man), weight of the uterus (46.04± 18.21 g for Boujaâd *vs.*37.39±13.15 g for D'man) and the depth of penetration percentage (34.18% for Boujaâd *vs.*41.70% for D'man). While the penetration depth of the insemination gun was not significantly different between the two breeds(18.06± 7.95 mm). Age had a significant effect on all studied parameters within each breed. Generally, in ageing ewes, the cervix tended to become longer with loose folds. As a conclusion, there was an apparent difference in the complexity of the cervix between Boujaâd and D'man breeds and between age within each breed, with a marked complexity in the Boujaâd ewe,which may make trans-cervical artificial insemination more difficult in this breed.

Analysis of sperm-induced neutrophil extracellular traps (NETs) formation in the bovine system

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Keywords: sperm, PMN, neutrophil extracellular traps (NETs)

The natural site of semen deposition is the vagina in cattle and sperm migrate into the uterus leaving the bulk of the seminalplasma (SP) behind. However, current artificial insemination introduces variable amounts of SP into the uterus, which naturally remains in the vagina. While neutrophils combat microbial contamination and eliminate excess/dead sperm, their presence at the time of semen deposition reduces fertility. Beside phagozytosis and secretion of immune modulators, polymorphonuclear neutrophils (PMNs) are able to form "Neutrophil Extracellular Traps" (NETs) extruding their DNA into the extracellular environment. These are web-like structures, mainly composed of chromatin. Bovine PMN were isolated via Ficoll gradient centrifugation from peripheral blood. Frozen/thawed sperm cell suspensions (SCS) of bulls with proven fertility were used. The visualization and identification of NETs was achieved by scanning electron microscopy (SEM) or via fluorescence microscopy analysis, respectively. For NET induction, PMN and SCS were co-cultured for different time points (0, 15, 30, 45, 60, 120, 180 min). NET induction of sperm and supernatant alone was also measured. Zymosan was used to induce the formation of NETs in bovine PMN as positive control. Quantification of NETs formation was performed by spectrofluorometric analyses using an automated plate monochrome reader (Varioscan Flash; Thermo Scientific). Scanning electron microscopy as well as fluorescence microscopy analyses revealed that the exposure of bovine PMN to frozen/thawed bovine SCS trigger the formation of NETs. After quantification, fluorescence intensities (FI in arbitrary units, AU) indicate that sperm alone led to significantly reduced fluorescence intensities suggesting that the extender and the remaining seminal plasma are affecting NET formation to a higher extent. A significant increase in FI was seen until 60 min of incubation indicating that NET formation might be finished at that time point. Furthermore, no differences in FI were assessed with motile or immotile sperm indicating that the release of NETs is independent on sperm motility. These data show that bovine sperm are able to induce NETs formation.

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Automated activity monitoring of estrus in recipient heifers: a retrospective study in an embryo transfer center

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Keywords: automated activity monitor, recipient, estrus

For several years, EVOLUTION has set-up a new strategy to optimize its selective breeding programs by genotyping embryos after biopsy, keeping them frozen until their transfer once selected based on their estimated breeding value. Since only the best embryos are transferred, the management of recipients become essential and to succeed in this task, a recipients center has been opened to transfer embryos from our dairy breeding programs. The aim of this retrospective study is to highlight some zootechnical results obtained with an automated activity monitoring of estrus in recipient heifers.

209 Holstein heifers weighting more than 400 kg were used to receive frozen biopsied embryos. Most of them wear activity monitors (HEATIME®) and their heats were synchronized by groups of 5 to 15 using the following protocol: D0, Norgestomet implant insertion, Norgetsomet and Buserelin injection (CRESTAR Pack®) – D10 Cloprostenol (ESTRUMATE®) injection – D11 implant removal – D12 to D14 heat observation. The objective of this protocol is to transfer embryo between 6 and 7 days after heat detection, at D19. 383 heats were detected and followed by a transfer for 295 of them while the full activity profile was recorded for 219 of them. Each heifer was allowed up to 3 transfers before leaving the breeding program. The HEATIME® profile allows the record of the beginning of the peak activity, the increase of activity at the peak and the duration of the peak activity. The pregnancies were checked by ultrasonography at day 30 and confirmed between day 55 and day 65.

419 synchronization protocols were performed : 87 heifers were synchronized once, 62 twice, 40 three times and 20 more than three. Regarding the beginning of activity peak, 2% occurred in less than 12 hours after implant removal, 12% between 12 and 24 h, 41 % between 24 and 36 h, 17% between 36 and 48 h, 17% between 48 and 72 h, 9% between 72 and 96 h and 2% after more than 96 h. The mean activity increase at the peak during heat is 85 % ±16 and the mean of peak activity duration is 14.5 h ±6.1. For the 295 transfers, the pregnancy rate is 56%. This rate differs according to intensity of peak activity ($p<0.05$) with 42.5% when the increase was strictly less than 90 % and 57.5% for increase of 90% or more. No effect of protocol or transfer rank can be observed. No significant effect either of the recipient's stage could be shown as the pregnancy rates are 54 % (7/13), 61 % (27/44), 56% (128/227) and 45 % (5/11) for transfers done between [4-5[, [5-6[, [6-7[and [7-8] days after the heat, respectively.

This kind of automated activity monitoring allows us to have detailed description of recipient's heats which is not the case when recipients are in partners' farms. Recorded information will allow us to discriminate more closely the recipients based on estrus characteristics or stage.

Progesterone concentrations on the day of re-insemination on farms using artificial insemination services and on herd-owner insemination farms

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Keywords: cow, oestrus, pregnancy

The objective was to examine whether training background of artificial inseminator (herd-owner inseminators=OWNER, AI technicians=AI-T and fertility consultants=FC) has an effect on pregnancy rate and if additional training helps AI professionals to detect cows not in oestrus. A total of 1584 re-AI occasions on 754 farms were included. Whole milk samples were collected for progesterone (P4) analysis (RIA) from all cows submitted to re-AI and data including farm type, previous breeding attempts, oestrus signs and evaluation of uterine tone, slipperiness of the cervix and co-operation of the cow was collected. Further breeding attempts and next calving or culling date were sought from registers. AI occasions were divided into three categories based on P4 concentrations; <6 nmol/l: no luteal activity and cow could be in oestrus, 6 to 10 nmol/l: some luteal activity and >10 nmol/l: high luteal activity and cow was not in oestrus. A proportion of 7.7% of cows offered for re-AI had P4 concentration >10 nmol/l. There was no statistical difference between OWNERs and farms using AI service. OWNER farms chose for AI more cows with intermediate P4 values than farms using AI service (OWNERs: 82.7% <6 nmol/l, 9.8% from 6 to 10 nmol/l and 7.5% >10 nmol/l and farms using AI-service: 86.2%, 5.9% and 7.8%, respectively). AI-Ts recommended no AI significantly less than FCs: 1.6% versus 4.9%. Both groups were equally right: 71% and 68% of cows recommended no AI had high P4 concentration. Finally, in the three P4 categories, AI-Ts and FCs inseminated 86.3%, 6.5%, 7.2% and 89.2%, 5.5%, 5.3% of cows, respectively. As of more courageous rejection of cows with high P4, FCs inseminated statistically significantly more cows at <6 nmol/l and less cows at 6 to 10 nmo/l than OWNERs. 36.7% of cows finally inseminated got pregnant and there was no significant difference between OWNERs and farms using AI service (37.1% versus 36.4%). FCs had significantly higher pregnancy rates than AI-Ts (39.6% versus 32.6%).

The proportion of cows inseminated during the luteal phase has increased in Finland from 4.4% (Laitinen 1983, Oestrus confirmation, pregnancy diagnosis and postpartum ovarian follow-up of the Finnish dairy cows by milk progesterone assay: Effects of breed, season, feed and sampling on milk progesterone levels, PhD thesis) to 7.7% in the past 30 years. This should be taken into account in the education of AI professionals and OWNERs. The ability of the inseminators to detect the cows not in oestrus and to reject them can be strengthened through training. More accurate rejections yield a higher pregnancy rate. The best indicative and predictive oestrous signs detected by the inseminator at the time of AI are uterine tone and consistency of the vaginal mucus. Behaviour of the cow at AI did not predict the P4 concentration.

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OPU – IVF and ET

Addition of seminal plasma reduces binding of stallion spermatozoa to bovine oocytes

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Keywords: seminal plasma, stallion, binding assay

Equine *in vitro* fertilization (IVF) is currently not a repeatable process; a heterologous zona binding assay (HZBA) using *in vitro* matured (IVM) bovine oocytes could be an alternative to test the fertilizing capacity of stallion spermatozoa. Survival of stallion spermatozoa during cryopreservation varies considerably between individuals. Seminal plasma (SP) has been shown to repair cryodamage to sperm membranes (Bernardini et al., Theriogenology 76:436-447; 2011) and therefore may influence binding to the zona pellucida. Objective: To investigate the effect of adding SP from "good" (GF) or "bad" (BF) freezer stallions on sperm binding capacity. Ejaculates (one from each of six stallions) were processed by Single Layer Centrifugation (SLC) to remove SP and were frozen using the standard protocol at a commercial stud (Schober et al., Theriogenology 68:745-754; 2007). Straws were thawed at 37°C for 30s; the contents were gently layered on a low density colloid and were centrifuged to separate spermatozoa from cryoprotectant. The pellet was harvested and resuspended in modified Whitten's capacitation medium (MW) containing sodium bicarbonate and BSA. Salt-stored IVM bovine oocytes with intact zona pellucida, were obtained from several batches of ovaries, pooled and stored until needed. They were washed several times in prewarmed PBS/PVA, equilibrated for one hour in 37°C and transferred in groups of 25-27 to four-well plates containing: i) control (C) 500 µL MW (n=152); ii) 500 µL MW supplemented with 5% pooled BF-SP (BF) (n=161); or iii) 500 µL MW containing 5% pooled GF-SP (GF); (n=164). Sperm samples (final concentration 5×10^6 spermatozoa/mL) were added to the drops; the plates were incubated for 14-18 h in 38°C in 5% CO_2 incubator, 95% humidity atmosphere. The sperm-oocyte complexes were pipetted several times then rinsed gently three times to remove loosely attached spermatozoa. They were fixed in 2% (V/V) paraformaldehyde in PBS/PVA overnight at 4°C, washed, stained with Hoechst 33342 (5 µg/mL) and mounted under anti-fade medium (Vectashield) on glass slides. The coverslip was sealed with nail polish and allowed to dry. The number of spermatozoa bound to the zona pellucida (ZP) was assessed using confocal microscopy at 200x. Data were analyzed by General Linear Model using the SAS® software (version 9.3); significance was set to $P \leq 0.05$. All values are LSMEAN ± S.E. The number of spermatozoa bound to ZP was higher in C than in BF or GF (C 21.89±0.67; BF 2.86±0.65; GF 2.50±0.64; C vs. BF $P\leq0.0001$; C vs GF $P\leq0.0001$). No differences were found between BF and GF. In conclusion, addition of SP impaired stallion sperm binding to the zona pellucida of bovine oocytes, independently of whether the SP came from a good freezer or a bad freezer. This effect may be due to the presence of sperm decapacitation factors in the SP.

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Biobanking the first collection of oviductal and uterine fluid from hysterectomised patients

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Keywords: biobank, human reproductive fluids, surgery patients

The safety of procedures in assisted reproductive technologies (ART) and the effect of culture conditions on embryo and fetal development, it is raising a great deal of concerns, mainly due to the lack of information about the formulations of commercially available culture media used in human IVF/ICSI treatments. As it was already described, the different IVF culture media influence the rates of successful implantation, pregnancy and birth weights (Kleijkers, Human Reproduction, Vol.31, No.10 pp. 2219–2230, 2016). It has been recently shown that culture media supplemented with natural female reproductive fluids have improved IVF efficiency, morphological embryo quality and epigenetic reprogramming profiles in pig blastocysts, compared with culture media without these supplements (Canovas, Elife, 6: e23670, 2017). This has encouraged the development of strategies that allow a noninvasive collection of reproductive fluids in humans, in order to validate them as supplements in the future. The first objective of this study was the development of a method to collect human oviductal and uterine fluids. A second objective was the initial characterization of reproductive fluids by measuring volume, protein concentration (Bradford Reagent, Sigma, Madrid, Spain), osmolality (Wescor Vapro 5520 Vapor Pressure Osmometer) and pH (pH OxyMini FOR PRESENS, Germany). The fluids were collected from 33 premenopausal women undergoing a total abdominal hysterectomy in the scheduled gynecological surgery of 'Virgen de la Arrixaca' University Clinical Hospital, whose indication was a benign uterine pathology. The oviductal fluid was collected according to the method previously described in Carrasco et al. (Reproduction, 136: 833–842, 2008). The collection of uterine fluid was carried out with a device normally used for mucus sampling. Once collected, the fluids were centrifuged at 7000 g for 10 min at 4°C to remove cellular debris and stored at −80 °C in BIOBANC-MUR IMIB. It was possible to collect a mean volume of 23.9 ± 14.6 µl (n=22) of oviductal fluid and 62.8 ± 33.0 µl (n=26) of uterine fluid. The mean total protein concentration was 30.9 ± 14 µg/µl (n=22) for oviductal fluid and 48.9 ± 17.9 µg/µl (n=26) for uterine fluid. Mean value of osmolality was 316.6 ± 35.9 mmol/kg (n=22) for the oviductal fluid and 283.8 ± 69.5 mmol/kg (n=22) for the uterine fluid. Finally, mean pH values for oviductal and uterine fluids were 7.4 ±0.7 (n=22) and 7.8 ±0.3 (n=22), respectively. Although the selected methods allowed the reproductive fluids collection, they should be improved in order to obtain higher volumes without endometrial damage, to perform clinical trials that could validate their use as a supplement in culture media for ART. Besides the volume limitations, we can conclude that it is possible to establish a biobank of reproductive fluids, which meets sanitary conditions and legal requirements for research and future medical applications.

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Seminal plasma proteins increase *in vitro* fertility rate of frozen-thawed ram semen

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Keywords: Apoptosis, capacitation, fertility

Ram seminal plasma proteins (SPP) have an antiapoptotic effect (Mendoza et al., FEBS J 279: 62-63, 2012) and can protect spermatozoa from the cryopreservation damage (Barrios et al., Biol. Reprod. 63: 1531-1537, 2000). Therefore, their use in frozen-thawed seminal doses might increase fertility results.

In order to test this hypothesis, ram semen was frozen after adding SPP. SPP were obtained by semen centrifugation at 12000 x g for 5 min at 4 °C, the supernatant was loaded in >3 kDa filters (Filtron Tech, Northborough, MA, USA) and centrifuged for 6 h at 3000 x g at 4 °C. Protease and phosphate inhibitors (Sigma Chemical Co, St. Louis, MO, USA) were added and SPP were stored at -20 ºC until use.

Ram semen collected from nine Rasa Aragonesa rams using an artificial vagina was frozen in plastic straws with (P) or without (NP) 40 mg/ml SPP, in a Tris-glucose-citric acid-egg yolk based medium (Evans, Aust. J. Biol. Sci. 41: 103-116, 1988), following the Fiser's et al. method (Theriogenology 28: 599-607, 1987). After thawing at 37ºC for 30 sec in a water bath, seminal parameters of frozen-thawed samples (P and NP) and a fresh semen sample (control, C) were analyzed (n=4). Viability (by the double staining with carboxyfluorescein diacetate/propidium iodide (Sigma Aldrich; Harrison and Vickers, J Reprod Fertil 88: 343-352, 1990)) and apoptotic markers (phosphatidylserine translocation by FITC-Annexin V (Thermo Fisher Scientific, Waltham, MA, USA) combined propidium iodide, and DNA damage by TUNEL assay (Sigma Aldrich, San Luis, MO, USA)) were measured by flow cytometry. The capacitation state was assessed by the chlortetracycline staining (Grasa et al., Reproduction 132: 721-732, 2006), and fertility by IVF of ewes' oocytes (n=103, 99 and 98 for P, NP and C, respectively in 4 replicates) and subsequent embryo development (Forcada et al., Span J Agric Res 11: 366-370, 2013). Obtained results were analyzed by chi-square test (SPSS Statistics, IBM analytics, Armonk, NY, USA).

The frozen-thawed processes lowered ($P<0.05$ when P and NP are compared to C) both sperm viability (21.6±7.6% in P, 22.8±7.4% in NP and 61.6±4.3% in C) and the rate of viable spermatozoa without phosphatidylserine translocation (15.1±8.4%, 15.3±9.5% and 27.0±8.1% for P, NP and C, respectively). No differences were found in DNA damage (9.2±2.6%, 12.2±5.8% and 7.2±0.9% for groups P, NP and C, respectively). The addition of SPP resulted in significant differences in the rate of non-capacitated spermatozoa (11.5±0.5% in P, 5.0±1.1% in NP, and 58.0±7.3% in C; $P<0.05$ for all groups), which was reflected in a higher *in vitro* fertility rate (88.5%, 72.2% and 98.7% for P, NP and C, respectively; $P<0.05$) and embryo cleavage (67.0%, 51.5% and 76.5% for P, NP and C, respectively; $P<0.05$ when NP is compared with P and C). Furthermore, blastocyst rate was also higher in groups P and C when compared with group NP (53.6%, 33.3% and 51.3% for P, NP and C, respectively; $P<0.05$).

In conclusion, ram SPP can increase fertility results after frozen-thawed procedures.

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Effect of reproductive tract O2 levels during *in vitro* fertilization and porcine embryo culture

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Keywords: oxygen; in vitro fertilization; embryo culture

Oxygen concentration is a key factor for many physiological reactions in cells. However, most of the *in vitro* processes during pig embryo production are performed under environmental O_2 conditions (20%). These levels are far away from those recently measured in oviduct and uterus of sows and gilts (7 and 10%, respectively) (López Albors *et al*., Society for Reproduction and Fertility, vol. 2, P045, 2015). Indeed, 20% O_2 have a negative impact on embryo development in several species (Mantikou *et al*., Hum Reprod Update, vol. 19 (3), p. 209, 2013). Therefore, the effect of atmospheric vs. reproductive tract O_2 concentration during IVF and embryo culture (EC) in pig was compared. Porcine oocytes collected from gilts at slaughterhouse were *in vitro* matured and, 44 hours later, *in vitro* fertilized with sperm selected by swim up (Cánovas *et al*., eLife, vol.6, p. e23670, 2017). Gametes were co-cultured (2000 spz/oocyte) in TALP medium with 1% oviductal fluid from the late follicular phase (NaturARTs® PIG OF-LF, Embryocloud, Murcia, Spain), from 0 to 8 hours post insemination (hpi). Putative zygotes were cultured until blastocyst stage in NCSU23 medium with 1% oviductal fluid from the early luteal phase (NaturARTs® PIG OF-EL) from 8 to 48 hpi and 1% uterine fluid (NaturARTs® PIG UF-EL) from 48 to 180 hpi. Two groups were distinguished depending on whether 20% O_2 or 7% O_2 was used during IVF and subsequent EC. After 18-20 hpi, putative zygotes (182/653 from the 20% O_2 and 174/641 from the 7% groups) were fixed and Hoechst stained to evaluate IVF by fluorescence microscopy. After 48 hpi, cleavage rate was assessed. After 180 hpi, kinetic of development was evaluated classifying blastocysts as early, late, hatching or hatched. Later, they were fixed and Hoechst stained to quantify the number of nuclei in each blastocyst by fluorescence microscopy. Data were analysed by one-way ANOVA. A P-value <0.05 was considered to denote statistical significance. Oocytes fertilized under 7% O_2 showed the same penetration and monospermy rates, mean number of spermatozoa inside oocytes, and attached to the zona pellucida than oocytes fertilized under atmospheric O_2 levels. However, embryos cultured under 7% O_2 showed a significant increase in cleavage rate (60.0 ± 2.3%) compared with those cultured under 20% O_2 (32.0 ± 2.2%). Embryos cultured under 7% O_2 showed also a higher mean number of cells per blastocyst (88.9 ± 5.9) compared with those cultured under 20% O_2 (59.0 ± 5.0). Although no significant differences were observed for different embryo developmental stages between the groups due to the limited number of blastocysts (25 from the 20% O_2 and 50 from the 7% groups), absolute values for hatching and hatched blastocysts were larger in embryos cultured under 7% O_2 than under 20%. Overall, O_2 is an important factor to take in consideration during ART. The use of O_2 levels closer to those found in the reproductive tract not only enhances embryonic development but also improves the quality of the blastocysts produced.

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The effect of the presence or absence of a cavity in the corpus luteum on progesterone concentrations and pregnancy rate in heifers following embryo transfer

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Keywords: Corpus luteum, recipients, pregnancy rate

Objectives

The aim of the study was to compare pregnancy rate and concentration of progesterone (P4) in embryo recipients in which the presence of compact corpus luteum (CL_{com}) or corpus luteum with a cavity (CL_{cav}) was observed at the day of embryo transfer.

Materials and methods

279 heifers recipients were used in the study. Oestrus was synchronized with two i.m. injections of 25 mg of dinoprost tromethamine (5 ml of Dinolytic, Zoetis, Warsaw, Poland) administered at 14 day intervals. On Day 7 after oestrus, the ovaries were examined with the use of ultrasound (linear probe, 7,5 Mhz, iScan, Draminski). Corpora lutea were divided based on the presence or absence of a cavity into CL_{com} (n=187) and CL_{cav} (n=92). With the use of ultrasonography, the diameter, area and volume of CL and cavities (where present) were measured. Simultaneously, blood samples were taken from 41 heifers (25 recipients with CL_{com} and 16 recipients with CL_{cav}). Serum concentrations of P4 in the samples were evaluated by RIA. Fresh embryos (one embryo per recipient) were placed into the ipsilateral horn of the uterus. Pregnancy was diagnosed by ultrasonography 2 months after embryo transfer. Data were analysed by ANOVA and logistic regression using the STATISTICA 9,0 software PL.

Results

The mean diameter, area and volume of CL_{com} and CL_{cav} were 21.7 ± 2.57 mm vs. 23,0±2,56 ($p<0,001$), 384.6 ± 94.5 mm^2 vs. 458.8 ± 98.4 mm^2 ($p<0,0001$) and 7301.7 ± 2416 mm^3 vs. 8849.5 ± 2579 mm^3 ($p<0,0001$), respectively. The mean cavity diameter, area and volume were 9.4 ± 2.91 mm, 140.6 ± 50.4 mm^2 and 1177.5 ± 296 mm^3, respectively. The area and volume of luteal tissue were greater in CL_{cav} compared to CL_{com}. Mean concentrations of P4 12.1 ± 3.58 and 8.1 ± 3.96 ng/ml in CL_{cav} and CL_{com}, respectively ($p<0.0001$). Pregnancy rate two months following embryo transfer were 51.1% and 34.7% for CL_{cav} and $CL_{com,}$ respectively ($p<0.02$). In recipients with CL_{cav}, transfer to the right uterine horn resulted in a pregnancy rate of 41.8% compared to 59,5% for the left uterine horn ($p>0,05$). For recipients with CL_{com} pregnancy rate following transfer to the right horn was 30.4% compared to 37.3% for transfers to the left horn ($p>0,05$). Moreover, when P4 concentration was higher than 10.88 ng/ml in 87.5% of CL there was a cavity, whereas when P4 concentration was lower or equal to 10.88 ng/ml in 88% CL was compact ($p<0.01$).

Conclusions

The presence of cavities in the CL 7 days after ovulation appears to have a beneficial effect on the results of fresh embryo tranfer in recipients. Regardless of the type of CL, the placement of the embryo in the left horn of the uterus provided a higher percentage of pregnancies. However, in the case of CL_{cav} placing the embryo in the left horn resulted in higher pregnancy rate than CL_{com}. It seems possible to predict the occurrence of cavity inside the CL basing on the P4 concentration in the blood.

Bovine in vitro maturation medium with different protein supplementation influences the maturation and fertilization rates

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Keywords: bovine follicular fluid, IVM, IVF

While some studies show beneficial outcomes on the use of bovine follicular fluid (bFF) in in vitro maturation (IVM), others display neutral or even detrimental effects. The main problem is related to the inhibitory effect on the meiosis resumption when high concentrations are used (Kim, Theriogenology, 45, 798, 1996). We hypothesized that inactivation of bFF might avoid this feature, thus we conducted 2 experiments (Exp) to evaluate the effect of bFF (either heat-inactivated or not) on the oocyte competence assessed by different parameters related to the nuclear and cytoplasmic maturation (Exp1) and the IVF efficiency (Exp2).

Cumulus-oocyte complex's (COC) were obtained from slaughterhouse ovaries and IVM was performed using TCM-199 with 10% of either Fetal Bovine Serum (Control), bFF or bFF heat-inactivated (bFFin – 30' 56ºC). COC's were incubated for 22-24h and either denuded for Exp1, or submitted to IVF for Exp2. Frozen semen was used for IVF in TALP medium and incubated for 20-22h with oocytes. After fixation and Hoechst staining, oocytes and zygotes were evaluated under a fluorescence microscopy to assess nuclear status or fertilization parameters. In addition, cumulus cell expansion was measured in fresh oocytes before and after IVM. Total number of oocytes and replicates were as follows: 387 within 4 replicates for nuclear status; 432 within 3 replicates for cumulus expansion; 691 within 5 replicates for IVF. Data were analysed by one-way analysis of variance (ANOVA) and Tukey test with a level of significance $p<0.05$. The software used was IBM SPSS Statistics (v22.0). Values are percentages ± S.E.M.

In Exp1, the nº of oocytes reaching metaphase II and showing a clear polar body were not significantly different among groups (69.29±3.91 for control, 68.29±4.21 for bFF and 68.55±4.19 for bFFin). Cumulus cell expansion showed no statistical difference between groups. In Exp2, the sperm penetration rate wasn't significantly different between control and bFF (91.29±1.9 and 83.37±2.5 respectively) but it was between control and bFFin (78.41±2.7). Monospermy, mean nº of penetrated sperm per oocyte (S/O) and male pronucleus formation (MPN) showed no significant differences among groups. Mean nº of sperm bound to the zona pellucida (S/ZP) was different between groups, with bFF showing the lowest value (1.98 S/ZP) and control the highest (4.5 S/ZP). The efficiency of the IVF was also different with the highest value for control 73.45±3.0 and the lowest for bFFin 59.49±3.2. However, there were no significant differences between bFF (63.48±3.2) and the other groups.

In conclusion, adding bFF/bFFin to the IVM medium did not improve nor decreased maturation rates. However, IVF efficiency was lower when using bFFin but not when untreated bFF was used. Most likely, as others studies have shown (Collins, Theriogenology, 43, 1, 189, 1995), heating might inactivate some crucial heat-labile proteins that will further influence the ability to form a viable embryo.

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In vitro viability and developmental competence of porcine morulae stored in liquid state for up to three days

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Keywords: embryo storage, porcine, morulae

The use of vitrified porcine embryos for non-surgical embryo transfer (Ns-ET) programs has disadvantages, as complying with strict air regulations for handling and transport of dewars, the risk of devitrification during transport, the need of LN_2 in the recipient farms or the number of embryos needed per Ns-ET. These drawbacks call for alternative procedures for short-term embryo storage in liquid state. This study aimed to evaluate storage of in vivo derived pig morulae in liquid state for up to 72 h on their further in vitro development. In Experiment 1, morulae (N=228) were stored at 25ºC or 37ºC in TL-HEPES-PVA defined medium (DM) or NCSU23-HEPES-BSA semi-defined medium (S-DM) for 48 h. After storage, embryos were assessed for viability (embryos with appropiate morphology according to the International Embryo Transfer Society criteria) and development, and then conventionally cultured (NCSU23-BSA-fetal calf serum, 38.5ºC, 5% of CO_2 and 95% humidity) for 48 h to assess their hatching competence. Non-stored morulae (N=44) cultured under conventional conditions were used as controls. Differences among groups were analyzed using Fisher's exact test. At 48 h of storage, DM at 25ºC was detrimental ($P<0.05$) for embryo viability (73.9%) compared to the control (93.2%) and the rest of the experimental groups (90.9% to 98.3%). Following conventional culture, S-DM at 37ºC was the only group able to maintain embryo viability in a percentage similar to the control group (96.7%). Embryo development at 48 h of storage was delayed ($P <0.001$) in all experimental groups compared with the controls, being the delay more severe at 25ºC. Most embryos stored at 37ºC reached blastocyst stage but, unlike controls, none of them hatched at the end of storage. After conventional culture, the hatching rate of embryos stored in S-DM at 37ºC was similar to that of controls (85.0%) but higher ($P<0.01$) than for the other groups (9.1% to 23.8%). In Experiment 2, morulae (N=59) were stored at 37ºC in S-DM for 72 h, assessed for viability and development, and conventionally cultured for 24 h. Non-stored morulae (N=50) cultured under conventional conditions were used as controls. There were no differences in embryo viability between S-DM and controls at the end of storage (98.3% vs 90.0%, respectively). Moreover, all viable embryos from S-DM group remained viable after 24 h of conventional culture. Although there was a development delay in the stored embryos compared with the controls, some stored embryos (6.9%) hatched at the end of storage. The hatching ability after conventional culture was similar for stored and control embryos (65.5% and 70.4%, respectively). In conclusion, morulae stored in S-DM at 37°C for up to 72 h maintain in vitro viability and developmental competence. In addition, most blastocysts derived from stored morulae conserved intact the zona pellucida at the end of storage. These findings open new possibilities for porcine embryo transport in liquid state.

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Comparison of two culture conditions during maturation on in vitro development of sheep embryos

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Keywords: *Ovis aries*, static culture, dynamic culture

Over the last two decades, the most studied variables to improve embryo development in vitro include the chemical composition of culture media. In fact, these approaches have proven to be beneficial and have contributed to improve success rates after assisted reproduction. However, not only the chemical requirements should be considered, but potential physical requirements may also be important factors in the continuous search for improving in vitro conditions. The objective of this study was to evaluate the effect of two culture systems (static and dynamic) during oocyte maturation in early ovine embryonic development. A total of 338 oocytes were obtained by aspiration of ovaries collected from a slaughterhouse. The oocyte control group (T1, n = 165) was placed for 24 h in a static culture, while another group (T2, n = 173) underwent dynamic culture receiving orbital movement with the aid of an electric stirrer agitator (AGO-1016, PRENDO, Mexico), for 5 seconds every 60 minutes for 24 hours. In both treatments the same maturation medium was used (TCM-199; In vitro S.A., Mexico), which was supplemented with 10% fetal bovine serum (Microlab, Mexico), 5 µg mL-1FSH (Folltropin, Vetoquinol, USA), 5 IU mL-1 hCG (Chorulon, Intervet, Colombia), 1 µg mL-1 17-β estradiol (Estrol, Pharmavet Argentina) and 50 IU heparin / mL (PISA, Mexico). The oocytes were fertilized with fresh semen using 55x106 mL-1 spermatozoa in medium TALP-Hepes (In vitro S.A., Mexico) and 18 hours later both groups were placed in Cleavage medium (COOK Medical, Australia), 60 hours later they were placed in Blastocyst medium (COOK Medical, Australia), performing the same management in both treatments. Embryo development was carried out in a CO2 incubator at 38.5 °C, 5% CO2 and 95% humidity. The size and development of the embryos was measured with an inverted microscope and a camera (AmScope) 144 hours after fertilization. The criterion for evaluating maturation in the cumulus-oocyte complexes (COCs) was by identifying the polar corpuscle and the level of expansion of the granulosa cells Fertilization was evaluated by the first cell division at 30 hours after performing Change to Cleavage medium (i.e.: 48 hours post insemination). The percentage of maturation, fertilization and blastocysts yield was calculated based on the initial number of COCs of each treatment. The means were compared by Student's t-test and chi-square according to the type of variable, using SAS. The percentage of maturation rate was higher ($P < 0.05$) in oocytes that underwent dynamic culture compared to static culture (78.3 ± 2.6 vs. 71.3 ± 2.7%). However, fertilization rate (72.8 ± 8.3 vs. 67.3 ± 13.0%), blastocyst yield (39.3 ± 6.8 vs. 36.36 ± 11.5%), and blastocyst diameter (166.5 ± 3.4 vs. 163.8 ± 2 µm) were similar ($P > 0.05$) in T1 and T2 groups. In conclusion, under the conditions of this study the use of dynamic culture for maturing sheep oocytes only improved maturation rate without any effect on embryo development.

The addition of ascorbic acid to the vitrification-warming media enhances the cryotolerance of in vitro produced porcine blastocysts

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Keywords: Ascorbic acid, blastocysts, vitrification

It is known that vitrification and warming procedures disturb the oxidation-reduction status increasing intracellular reactive oxygen species levels in porcine blastocyst. This study aimed to assess the effects of adding ascorbic acid (AsA) as antioxidant to vitrification-warming media on the post-warming survival and quality of IVP porcine blastocysts. Immature oocytes (N=3600) collected from prepubertal gilts were cultured in maturation medium supplemented with 10 IU/mL eCG and 10 IU/mL hCG for 22 h and then for an additional 22 h in maturation medium without hormonal supplements. Mature oocytes were inseminated with thawed sperm (1000 spermatozoa per oocyte) in fertilization medium for 5 h. Presumed zygotes were cultured in glucose-free embryo culture medium (supplemented with pyruvate and lactate) for 2 days and in embryo culture medium containing glucose for an additional 4 days. Blastocysts were vitrified and warmed with the superfine open pulled straw method using TL-HEPES as basic medium and ethylene-glycol and dimethyl sulfoxide as cryoprotectants (Sanchez-Osorio et al. Theriogenology, 2010, 73:300-308). We added 50 µg/mL of AsA both vitrification and warming media (VW+ group). Control group media were not supplemented with AsA. After warming, VW+ (N=281) and control (N=307) blastocysts were cultured for 24 h to assess embryo survival (ratio of blastocysts that reformed their blastocoelic cavities at the end of culture to the total number of embryos cultured) and hatching rates. To evaluate the quality of vitrified-warmed blastocysts, the number of inner cell mass (ICM) and trophectoderm (TE) cells was determined in each embryo using a differential staining based on an indirect immunofluorescence reaction. For that, a primary antibody (anti-CDX2), which specifically binds TE cells, and a secondary antibody (anti-Mouse IgG) conjugated with alexa Fluor® 568 that emits red fluorescence were used. Afterwards, all blastocysts cells were counterstained with the DNA-binding fluorochrome Hoechst-33342 to identify the ICM cells that displayed only blue fluorescence. Stained blastocysts were examined under fluorescence microscopy. Results are expressed as means ± SD of six replicates, and differences between groups were analyzed by an unpaired Student's t-test corrected for inequality of variances (Levene's test). The VW+ group showed a higher ($P < 0.02$) survival rate (51.1 ± 20.9%) than the control group (34.8 ± 21.4%). However, there were no differences between groups in hatching rates (10.7±12.0% vs. 6.0±8.1%). There were also no differences between VW+ and control blastocysts in terms of ICM (14.5 ± 6.5% vs. 16.4 ± 7.5%) or TE (44.2 ± 18.2 vs. 46.3 ±12.1%) cells. In conclusion, the addition of 50 µg/mL of AsA to vitrification-warming media considerably enhances the cryotolerance of IVP porcine blastocysts but does not affect the quality of embryos in terms of number of cells in the ICM or TE.

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Haematological and blood biochemical parameters in piglets derived from embryo transfer

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Keywords: Porcine, *in vivo*, embryos

Porcine embryo transfer (ET) has an important role in pig industry because it allows the transport of genetic material, avoiding the risk of diseases dissemination. However, there are not many studies that assess the characteristics of piglets born by ET and information on the haematological and biochemical parameters in the newborns is limited. Since those parameters can be indicators of metabolic disorders and other pathologies, we aimed to compare haematological and blood biochemical parameters from piglets obtained by ET (ETp, n=22, 2 litters) vs. piglets obtained by artificial insemination (AI) (AIp, n=27, 2 litters). For this study, sows were used with the same genetics, feeding and housing conditions and they were inseminated with the same Large White boar semen doses. *In vivo* produced embryos (7 days after AI) were transferred to recipient sows by non-surgical methodology (DeepBlue® Porcine ET catheter, Minitübe, Tiefenbanch, Germany). Piglets were weighed and blood samples were collected on days 3 and 15 after birth. Blood samples were analyzed by haematology analyzer (Siemens ADVIA® 120, Tarrytown NY, USA) and clinical chemistry analyzer (Olympus AU400, Tokyo, Japan). Statistical analysis was performed using Systat Software (v. 13, San Jose CA, USA) by ANOVA considering day of birth and group (ETp and IAp) as factors, and litter as covariable. Differences were considered to be statistically significant when $P \leq 0.05$.

Sex of the piglets and weight were not different between ETp and AIp after birth. An increase in the number of white blood (WBCB), red blood cells (RBC), RBC distribution width (RDW), mean platelet volume (MPV), platelet component distribution width (PCDW) and platelet mass distribution width (PMDW) was detected in ETp in comparison to AIp. On the other hand, a reduction in platelets counts (PLT), plateletcrit (PCT), mean PLT component (MPC) and corpuscular haemoglobin concentration mean (CHCM) were observed in ETp. Furthermore, higher alkaline phosphatase (ALP) values were observed on day 3 in ETp, while gamma-glutamyl transferase (GGT) values increased on day 3 and 15. No significant differences were observed on the other parameters measured.

In conclusion, these preliminary results (derived from 4 litters) suggest that changes in the haematological and biochemical parameters are associated to the ET, although there are not differences from the reference values in piglets (Ventrella, BMC Veterinary Research, 13: 23; 2017). The alterations in platelets related parameters could be explained by an immunological platelet injury, probably associated to presence of maternal antibodies incompatible with platelet antigens from the piglets (Forster, Can Vet J. 48:855-7; 2007). This hypothesis must be confirmed with further studies. Currently, we are evaluating the clinical significance of the data, as well as the gene expression and DNA methylation changes in blood cells and placental tissue from these animals.

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Supplementation of α-tocopherol in two sheep breeds: effect on *in vivo* embryo production

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Keywords: ewes, α-tocopherol, superovulation

The efficiency of a multiple ovulation and embryo transfer (MOET) program depends on the number of good quality embryos obtained, some studies suggest that supplementation with antioxidants can help to improve embryo quality. The objective of the study was to evaluate the effect of α-tocopherol supplementation on embryo quality of a MOET program in two sheep breeds. In total 43 females were superovulated, from which 12 Charollais and 12 Dorper were treated with 500 IU of α-tocopherol given 60 h before sponge removal, while 11 Charollais and 8 Dorper were not treated (0 IU). The ewes were synchronized with intravaginal sponges containing 20 mg FGA for 12 days and on day 10th were superovulated with a purified source of follicle stimulating hormone. Estrus was detected with teaser rams and ewes in estrus were inseminated by laparoscopy 18 h after estrus onset with 4 doses of fresh semen containing $100x10^6$ spermatozoa each. Embryo recovery was attempted 7 d after estrus by laparotomy. Ovulation rate, recovery rate, fertilization rate, and embryo quality were measured. The results were analyzed using ANOVA and t-test for means comparison or Chi-square tests as it was required. There was no effect ($p>0.05$) of α-tocopherol application, breed or their interaction on ovulation and recovery rates. Fertilization rate was similar ($p>0.05$) among ewes treated or not with α-tocopherol, but was higher ($p<0.05$) in Dorper than Charollais ewes (45.10 vs. 36.42%). Embryo quality was similar ($p>0.05$) among breeds, but lower ($p<0.05$) in treated (53.91%) than non-treated (70.33%) α-tocopherol ewes. The same trend occurred in the two breeds. In conclusión, the application of α-tocopherol did not improve fertilization rate and embryo quality of superovulated ewes under the conditions of the study.

The STEINER OPU System: A New Autoclavable Device for flushing follicles in Equine Oocyte Collection

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Keywords: eqine opu, flushing follicles, new autoclavable device

In equine IVF, oocyte collection is particularly challenging. Because equine IVF veterinarians must perform in vitro maturation (IVM), collecting oocytes from unstimulated follicles, it is essential to optimize the follicular flushing technique. The STEINER-TAN Needle (available in 17-, 19- and 21-gauge sizes) System was developed as a result of many years of research (Rose and Laky 2013) and practical work conducted in the field of human in vitro fertilization (IVF) (Schenk et al. 2017). It is manufactured by IVFETFLEX.com Handelsgmbh & Co KG (Graz, Austria). This needle combines the advantages of both single lumen and double lumen needles in that the option of flushing follicles is retained. Double lumen needles (12-gauge) are currently used for equine IVF in combination with epidural anesthesia and sedation. The STEINER (ovum pick-up) OPU System could also potentially be useful to veterinarians due to the fact that it is autoclavable and has the same functional properties as the low-cost STEINER-TAN Needle System currently used in human IVF. In this study, an autoclavable device (Steiner OPU System) was adapted to meet the needs of the veterinarian and demands of the IVF market. A 15-, 16-, or 17-gauge disposable needle or EchoTip® autoclavable needle, approximately 10-15 cm in length, is attached via a male luer lock to distal end of a piece of metal tubing. The aspiration tubing is inserted into this tubing with open end a few millimeters (proximal) from the male luer lock, enabling the free flow of fluid between outer (flushing) and inner (aspirating) tubing and facilitating follicle flushing. Female luer locks are attached to the aspiration and flushing tubing at the proximal end. The complete length of the tubing is 45 cm. This system can be used in combination with an autoclavable needle guide and elongated with a vaginal probe (available from IVFETFLEX.COM and manufactured to fit any US probe on the market). This tubing may be flushed manually. For optimal temperature control, a STEINER flush/valve, which is a flushing pump with a syringe warmer fitted for 50-cc syringes, may be used (also available from IVFETFLEX.COM), two models (mechanical or electrical) of which are available. Based on our research findings in humans, we hypothesize that the newly-designed OPU system could potentially be used by veterinarians to facilitate IVF in horses and openly welcome opportunities for scientific collaboration. Our results using this needle for oocyte retrieval in humans (Rose and Laky 2013) have led us to the conclusion that this new system could have significant advantages for IVF in horses over existing systems because it reduces pain (use of a much smaller OPU needle), obliterates the need for epidural anesthesia, allows for better temperature control (use of the syringe warmer), and helps keep the costs of the OPU procedure low.

References:

Rose BI and Laky DJ. 2013. J Assist Reprod Genet. 30(6): 855-860; Schenk M, Huppertz B, Obermayer-Pietsch B et al. 2016. J Assist Reprod Genet. 34(2): 283–290. YouTube videos may be viewed by entering the keywords: steiner, flushing, computer animation

Effect of resveratrol-cyclodextrin complex supplementation during oocyte maturation or embryo culture *in vitro* in bovine

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Keywords: resveratrol, embryo, bovine

The damaging effects of reactive oxygen species on *in vitro* embryo production have been widely studied on the past decade. Thus, many antioxidants such as resveratrol have been added to the *in vitro* production media mimicking endogenous antioxidants in an attempt to decrease their negative impact. Resveratrol has been reported to have a positive effect when added to *in vitro* maturation (0.1- 10 µM) or to culture media (0.25- 1.0 µM) in bovine and porcine *in vitro* embryo production. Higher concentrations of resveratrol in the embryo culture medium had been proved to have toxic effects on the developing embryos. Methyl ß-cyclodextrin, a group of cyclic oligosaccharides, has been used to improve the solubility of drugs. The present study evaluates the effect of the complex resveratrol-cyclodextrin during *in vitro* oocyte maturation (IVM) or *in vitro* embryo culture (IVC) on developmental competence and quantitative changes in gene expression of developmental important genes. In experiment 1, a concentration of 1 or 10 µM resveratrol (R1 or R10 respectively) diluted in 0.001% cyclodextrin was added to IVM media (TCM-199+10% FCS) and after 24 h a representative number of oocytes (n=330) were fixed to examine maturation level or snap frozen for gene expression analysis by RT-qPCR (n=120). The remaining were *in vitro* fertilized and cultured in SOF+3 mg/ml BSA to the blastocyst stage (n=1293). In experiment 2, 744 *in vitro* produced zygotes were cultured in SOF+3 mg/ml BSA supplemented with 0.5 or 1 µM resveratrol (R0.5 and R1 respectively) diluted in 0.0001% cyclodextrin. In both experiments, cleavage rate and blastocyst yield were recorded and blastocysts on Day 7 and 8 of the experiment 2 were snap frozen for gene expression analysis. A group without complex resveratrol-cyclodextrin (control$^-$) and a group with cyclodextrin (control$^+$) were included during IVM and IVC. A higher percentage of oocytes remained arrested in germinal vesicle when 10 µM resveratrol was added to the IVM medium (16.6 ± 2,6 %) compared to R1 (9.03 ± 0.6) and control groups (4.22 ± 0.6 and 10.3 ± 0.7 for control$^-$ and control$^+$ respectively ANOVA, $P<0.05$). No differences were found in cleavage rate or blastocysts yield between groups in both experiments. Regarding gene expression in oocytes, 10 µM of resveratrol during IVM decreased the expression of genes involved in competence of oocytes and subsequent embryo development (*NLRP2* and *BMP15* and *POU5F1,* ANOVA, $P<0.05$). Moreover, the expression of *BAX* was lower in oocytes treated with resveratrol compared to control$^-$group (ANOVA, $P<0.05$). Blastocysts produced with 0.5 µM f resveratrol showed a positive effect on the expression of genes related to lipid metabolism (*LIPE, CYP51, PNPLA2 and MTORC1)* compared to control groups (ANOVA, $P<0.05$) indicating that resveratrol could decrease lipid accumulation leading to a higher survival rate after vitrification. Further studies are needed to study the long-term effects of resveratrol supplementation on *in vitro* embryo production.

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Folliculogenesis, Oogenesis, and Superovulation

Effect of slow-release FSH on embryo recovery in dairy cows

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Keywords: bovine, superovulation, embryo flushing

The objective was to study if a slow-release FSH superovulatory treatment (SLOW/2FSH) differs from the traditional 4-day FSH superovulatory treatment (TRAD/8FSH) in the numbers and grades of viable embryos and the numbers of degenerated embryos and unfertilized ova (UFO). Reduction of FSH treatments from eight to two diminishes restraining, discomfort and pain to the cows and labor and human errors in administration of treatments.Eight dairy cows (parity 1 to 4) were randomly designated into the TRAD/8FSH and SLOW/2FSH protocols in a cross-over study. First, the oestrous cycles of the cows were synchronized using 25 mg of prostaglandin (PG) (Dinolytic vet. 5 mg/ml, Zoetis Finland Oy, Finland) i.m., and 9 to 12 days after the synchronized oestrus either TRAD/8FSH or SLOW/2FSH treatment was initiated. TRAD/8FSH treatment consisted of eight declining i.m. doses (total 1000 IU) of FSH (Pluset vet, Laboratorios Calier, S.A., Spain) for four days at 6:00 h and 18:00 h. SLOW/2FSH treatment consisted of Pluset combined with hyaluronic acid (Hyonate®vet 10 mg/ml, Bayer Animal Health GmBH, Germany), 666 IU i.m. as 1st treatment at 6:00 h on the first treatment day and 334 IU i.m. as 2nd treatment 48 hours later. In the evenings of the 3rd and 4th day of each treatment, the cows were treated with 25 mg of PG and were inseminated after induced oestrus three times 12 hours apart, beginning 12 h after standing oestrus (= Day 0). Embryos were flushed non-surgically on Day 7. After flushing, CIDR devices (CIDR depot 1.38g, Zoetis Finland Oy, Finland) were inserted in the vaginae, removed after 12 days and a day before removal, 25 mg of PG was administered i.m. After this induced oestrus, the second cross-over run of the experiment was initiated 9 to 12 days later.

The numbers of viable embryos, degenerated embryos and UFO were counted. All viable embryos were graded following IETS recommendations and cool-transported in straws to be analyzed in another study for their survival after 1, 3, 5 or 7 days in +4°C storage. Results are depicted as percentages of totals and averages ± SDs. Paired t-test was used to define difference between treatments.

The number of viable embryos did not significantly vary between treatments (p=0.47). The TRAD/8FSH treatment yielded an average of 12.50±7.11 and SLOW/2FSH treatment an average of 10.13±4.67 viable embryos. In the TRAD/8FSH treatment, 82.0% (100/122) of the recovered structures were viable embryos, 6.5% (8/122) were degenerated embryos and 11.5% (14/122) were UFO. In the SLOW/2FSH treatment, 86.2% (81/94) were viable embryos, 8.5% (8/94) were degenerated embryos and 5.3% (5/94) were UFO. In the TRAD/8FSH and SLOW/2FSH treatments, 72.0% and 79.0% were grade I, 16.0% and 12.3% were grade II and 12.0% and 8.6% were grade III viable embryos, respectively.

The results indicated no difference in the average number of viable embryos between treatments. However, slow-release FSH treatment yielded a higher percentage of viable embryos and less UFO than the traditional FSH treatment and therefore warrants further investigation. Olvi Foundation is acknowledged for funding the research.

Effects of docosahexaenoic acid on bovine granulosa cells in vitro: involvement of FFAR4 receptor.

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Keywords: n-3 PUFA, lipid, signalling pathways

Previous studies suggest a beneficial effect of dietary fish oil supplementation (enriched in n-3 polyunsaturated fatty acid (PUFA)) on reproductive variables in dairy cows. These PUFA influence female reproduction by acting at the uterine and ovarian levels (Leroy JL, et al. Reprod Domest Anim. 49:353-61; 2014). Recently, we showed that docosahexaenoic acid (DHA, the most active n-3 PUFA) was able to affect oocyte quality by increasing blastocyste rate after *in vitro* maturation and fecundation (Oseikria M, et al. Theriogenology. 85:1625-1634.e2; 2016), but no data is available on its potential effects on ovarian somatic follicular cells. Our objectives were to assess the effect of DHA on proliferation, steroidogenesis and signalling pathways in bovine granulosa cells (GC). The potential involvement of the receptor FFAR4 in the effects of DHA was investigated in bovine GC through FFAR4 expression and FFAR4 agonist (TUG-891) assessment in functional studies. Primary GC cultures were performed after dissection of ovarian small follicles (3-6 mm) collected from slaughterhouses. Recovered GC were cultured in serum-free McCoy's 5A medium with insulin (10 µg/L) in absence or presence of DHA (1, 10, 20 or 50 µM) or TUG-891 (1, 10 or 50 µM) for the appropriate times. Fatty acid composition of total lipids in GC after 24h DHA treatment was assessed by gaz chromatography. Cell proliferation after 24h and steroidogenesis after 48h were measured by tritiated thymidine incorporation in cells and by ELISA of secreted progesterone and estradiol in culture medium, respectively. Phosphorylation of MAPK14, AMPK, MAP1/3 and AKT signalling pathways were assessed by western Blotting in GC treated with DHA for 5 to 60 min. These parameters were statistically analysed using either Kruskal-Wallis test or non parametric permutational ANOVA. We showed that FFAR4 mRNA and protein were expressed in bovine GC. GC proliferation was stimulated after 10 and 50 µM DHA treatment and a similar increase was observed with TUG-891 at 1 and 50 µM. Progesterone secretion was enhanced after 20 and 50 µM DHA supplementation, whereas a slight decrease was observed with TUG-891 at 1µM. Estradiol secretion was increased after DHA 1, 10 and 20 µM treatment, whereas no effect of TUG-891 was reported. The DHA content in total lipids was increased in GC supplemented with 10 and 50 µM DHA for 24h compared to control GC. DHA had no effect on MAPK1/3, AKT and AMPK phosphorylation, whereas it stimulated transiently MAPK14 phosphorylation after 30 min DHA treatment at 10 µM and 50 µM similarly to TUG-891. In conclusion, this work showed that DHA is able to highly incorporate the GC total lipids after 24h supplementation. Moreover, both DHA and TUG-891 stimulated similarly GC proliferation and MAPK14 phosphorylation, whereas only DHA increased steroid secretion from GC, suggesting that DHA could influence female fertility by acting on GC partly through FFAR4 and MAPK14 pathway for GC proliferation and through other mechanisms on steroidogenesis.

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Polymers used to reduce a number of FSH-injections during superstimulation treatment for superovulation induction in cows

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Keywords: Bovine, FSH, prolongators

Over 50% of more than 1 million bovine embryos presently produced in the world are in vivo derived. Induction of superovulation has been one of the major methods for embryo production for the past 40 years. However, there were and still are many attempts to develop protocols allowing fewer injections and to find substances prolonging FSH effects during superovulation induction in cows. In our work we examined the ability of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) to affect the release of FSH after injection and to act as prolongators during hormonal treatment.

To achieve this goal, two experimental groups of cows were treated with FSH-Super (LLC Agrobiomed, Russia) plus either PVA or PEG. Animals were assigned semi-randomly to two experimental groups. Each of the two working compositions was prepared directly before injection. To inject a single animal the necessary amount of polymer (either 0.9 g of PVA or 2.5 g PEG) was mixed with 1000 IU of FSH and dissolved in 7.5 ml of 0.9% NaCl solution. Animals of the first group (group I; n=98) were injected once with FSH plus PVA. Animals of the second group (group II; n=96) were injected once with FSH plus PEG. Subcutaneous injection of FSH plus either PEG or PVA was conducted once at the 10th day of the cycle, if there was a well-defined corpus luteum on one of the ovaries. The injection was located in the shoulder blade area. Injection of the PGF2a (0.5 mg) was conducted intramuscularly 48 hours post FSH injection. Artificial insemination was conducted 48 hours after PGF2a injection and was repeated two more times at 12 h intervals. On day 7 after the first insemination, corpora lutea were determined by trans-rectal palpation and embryos were collected by a standard non-surgical flushing procedure. Embryo quality was assessed according to IETS Manual.

Portion of cows with a reaction to treatment was significantly higher ($P<0.05$) in the second group (86.4%) than in the first group (74.5%). Total number of ovulations and collected embryos were 1080 and 811 (14.2±8.2 and 10.7±8.1 per donor, respectively) in group I and 1181 and 922 (14.2±7.4 and 11.1±6.6 per donor, respectively) in group II. Relative portion of grade 1-2 embryos in each group differed significantly (64.2% in group I and 79.0% in group II; $P<0.001$). Total number of the collected embryos of grade 3 and lower was 114 in group I and 89 in group II. Their relative number was also significantly larger in group I compared to group II ($P<0.05$). There was also a significantly larger ($P<0.001$) portion of oocytes collected in group I (21.7%) than in group II (11.3%). The results of our study indicate that treatment of cows with a single injection of FSH plus PEG results in higher flushing outcomes after superstimulation compared to single treatment with FSH plus PVA. Thus PEG may be a more effective agent for optimization of the superovulation induction procedure using FSH in cows.

Physiology of Reproduction in Male and Semen Technology

Glycosaminoglycans isolated from follicular fluid reduce PKA activity during capacitation in porcine spermatozoa.

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Keywords: Glycosaminoglycans, hyaluronic acid, porcine spermatozoa

Introduction: Glycosaminoglycans are linear polysaccharides comprised of repeating hexosamine-containing disaccharides that are found in the female genital tract and follicular fluid. Different studies showed that they may be involved in capacitation and acrosome reaction *in vivo* and their presence in sperm reservoir could maintain membrane stability and viability due to their interaction with sperm membrane (Tienthai, Journal of Reproduction and Development, 61, 245-250, 2015).

The aim of this study was to investigate the effect of hyaluronic acid (HA) and glycosaminoglycans (GAGs) isolated from folicullar fluid (G-FF) and cumulus oophorus secretions obtained after oocyte porcine *in vitro* maturation (G-COS) on PKA activity and acrosome status after *in vitro* capacitation.

Material and Methods: Glycosaminoglycans were isolated from COS and FF (from ovaries in the periovulatory phase) by protease digestion, lipid extraction and by different precipitation conditions according to Bellin and Ax (J Dairy Sci 70:1913–1919. 1987). Spermatozoa from five fertile boars (N=5) were incubated for 3 h in TALP (at 38.5ºC and 5% CO_2) or PBS (38.5ºC in air) supplemented or not with G-FF, G-COS or HA (100 ug/mL or 500 ug/mL). PKA activity was assessed by Western Blot using anti-PKA antibody (9624, Cell Signaling Technology, Massachusetts, USA). The relative optical density (R.O.D.) was quantified with ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK). Viability was evaluated by propidium iodide 500 mg/mL (Sigma P 4170, Madrid, Spain) and carboxifluorescein 0.46 mg/mL (Sigma D6883, Madrid, Spain), while the acrosome status was analyzed by fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FICT). In each case, 200 spermatozoa were evaluated. Data were analyzed by one-way ANOVA followed by Dunnett´s multiple comparisons test ($p< 0.05$).

Results: PKA activity was significantly reduced after the incubation in TALP supplemented with G-FF compared to control (1.74 ± 0.39 and 3.77 ± 0.33, respectively). No effect was observed after the incubation with HA (100 ug/mL or 500 ug/mL) and G-COS. Spermatozoa incubated in TALP showed the lowest percentage (50.83%±3.02) of viability and the highest percentage of acrosomal damage (3.83%±0.54) than spermatozoa incubated in PBS groups (75.66%±1.71 and 1.66±0.40, respectively) ($p<0.05$). However, the addition of HA or GAGs had not effect on this parameter.

Conclusions: These results provide evidence that the supplementation of GAGs from FF might prevent sperm capacitation by reducing PKA activity. Nevertheless, GAGs were not able to maintain viability and to protect acrosomal damage of sperm.

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Effect of Saffron extract on Boujaâd ram semen liquid storage

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Keywords: Boujaâd ram, semen, aqueous saffron extract

The objective of this study was to evaluate the effect of aqueous saffron extract addition (ASE) (6%) to skim milk and tris on the Boujaâd rams (3-4 years) sperm liquid storage. To achieve this goal, this work was divided into two steps; step 1: Boujaâd ram semen was extended in skim milk based extender supplemented or not with 6% of ASE and stored at 15°C during 24h. While for the step 2: Boujaâd ram semen was extended in Tris egg yolk based extender supplemented or not with 6% of ASE and stored at 5°C during 24h. Ejaculates were collected once a week during 4 weeks for each step using an artificial vagina. Samples were extended to reach a final concentration of $0.8x10^9$ spermatozoa/ml. Then evaluated at different storage times (0 and 24h). A computer-assisted sperm motility analysis (ISAS, version 1.0.17) was used to determine total (TM), progressive motility (PM) and linearity (LIN). Nigrosine-eosin staining, hypo-osmotic swelling test (HOST), and Malondialdehyde (MDA) concentrations were used to determine viability, membrane integrity and lipid peroxidation. The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. A factorial design ANOVA analyzed the data of extended semen quality parameters. The statistical model included the addition of saffron extract, and storage periods (0 and 24h). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SD.

In the first step, it was observed that at 0h no significant difference was recorded between the two treatments (Control vs ASE addition) regarding all studied quality parameters: (PM, TM, LIN, viability, HOST and MDA: 79.2±1.66%, 94.25±1.51%, 63.1±3.45%, 95.97±2.89%, 89.41±1.84%, 1.6±0.08 TBARS, nmol/0.810^9 sperm) respectively. While, at 24h it was found that ASE significantly improved the PM (71.23±1.27 vs 74.64±1.25%), TM (91.93±1.2 vs 94.14±1.38%), LIN (61.65±1.83 vs 65±0.83%), viability (92.4±1.47 vs 94.6±0.97%) and HOST (77.9±1.88 vs 81.2±1.08%). As it decreased the MDA production (3.1±0.06 vs 2.6±0.05 TBARS, nmol/0.8 x 10^9 sperm), compared to the control. In the second step; at 0h, the addition of 6% of ASE to the tris eggs yolk extender increased significantly the PM (68.6±3.45 vs 78.6±2.14%). While TM, LIN, viability and HOST, were not affected by this supplementation. At 24h, ASE significantly improved the PM (40.77±3.79 vs 64.67±3.97 %), LIN (28.5±2.93 vs 41.2±1.47 %), viability (89.7±4.55 vs 91.8±2.31%), HOST (65.9±2.93 vs 74.5±1.46%), and decreased MDA production (3.27±0.8 vs 1.81±0.6 TBARS nmol/0.8 x 10^9 sperm) compared to control. While TM was not influenced by the treatments. In conclusion, the addition of the ASE (6%) improved the quality of Boujaâd ram sperm conserved, either at 5°C in Tris egg yolk or at 15°C in skimmed milk based extenders. A biochemical characterization of the aqueous extract and a confirmatory study using artificial insemination are necessary to complete this work.

Measurements of ram sperm quality under anaerobic and aerobic liquid storage conditions

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Keywords: ram semen, liquid storage, aerobic

The aim of the present study was to assess sperm quality of INRA180 ram, stored in skim milk extender (SME) at 5°C under aerobic and anaerobic conditions. Ejaculates were collected once a week during 9 weeks from four INRA180 rams, using an artificial vagina. The ejaculates containing spermatozoa with more than70 % total motility and concentrations higher than 3 x 10^9 spermatozoa/ml were pooled. The pool was divided into two parts and each part was extended in SM under aerobic or anaerobic (in sterile syringes) conditions, to reach a final concentration of 0.8 x 10^9 spermatozoa/ml. Samples were evaluated at different storage times; 0, 24, and 48 h. A CASA system was used to determine total motility (TM%) and progressive motility (PM%). Other tests such as nigrosine–eosin staining, Diff-Quick staining, hypo-osmotic swelling test (HOST), and Malondialdehyde (MDA) concentrations were used to determine viability(%), morphology (%), membrane integrity (%) and sperm lipid peroxidation. The statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. A factorial design ANOVA analyzed the data of extended semen quality parameters. The statistical model included the fixed effect of storage conduction (aerobic vs anaerobic), and storage periods (0, 24 and 48 h). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors, considering the significance level of $P < 0.05$. Data are expressed as the mean ± SE.The results of PM (71.72±1.34%), TM (90.78 ± 1.03%), viability (97.5±0.52%), abnormality (2.44±0.21%) and membrane integrity (94.5±0.84%), showed that, at 0h, there was no difference between the two storage conditions (anaerobic vs aerobic). However, lipid peroxidation was significantly higher in aerobic condition(0.59±0.03 TBARS, nmol/10^8 sperm) compared to the anaerobic one (0.47±0.03 TBARS, nmol/10^8 sperm). At 24 h, semen stored in the anaerobic condition shows the highest PM (64.22±1.17 vs 53.88±2.31%), membrane integrity (88.44± 0.68 vs 84.22±0.95%) and the lowest lipid peroxidation (1.32±0.01 vs 2.05±0.08 TBARS, nmol/10^8 sperm) compared to the aerobic storage ($p<0.05$). While the TM (80.72±1.22%), viability (91.14±0.91%) and abnormality (6.25±0.31%) were not affected by the storage condition ($P>0.05$). At 48 h, the best semen quality results were obtained in anaerobic condition. And that concerned; PM (50.66±1.75 vs 33.66±1.12%), viability (81±0.46 vs 76.22±1.31%), abnormality (10.5±0.32 vs 13.05±0.52%), membrane integrity (63±1.49 vs50.44±1.12%) and lipid peroxidation(1.35±0.03 vs 3.56±0.09 TBARS, nmol/10^8 sperm) compared to aerobic one. Whereas, for the TM (74.28 ±2.21%) is was not affected by the two treatments. In conclusion, INRA180 ram semen stored at 5 °C in a skim milk-based extender exhibited highest quality parameters under anaerobic exposure compared to aerobic exposure.

Cyclin/Cdk complexes are involved in control of actin dynamics during boar sperm capacitation

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Keywords: Sperm capacitation, Cyclin/Cdk complexes, Aminopurvalanol A.

Mammalian spermatozoa are virtually infertile immediately after ejaculation and will only reach their full fertilizing ability after they reside within the female genital tract for hours to days, depending on the species. This process of capacitation implies marked changes in the whole biochemical machinery expressed by spermatozoa. Thanks to the adoption of high-throughput technologies (Chronowska, *Biomed Res. Int.* vol 2014 (2014)) it was demonstrated that male gametes express proteins involved in cell cycle control, that are thought to be not present or active in sperm cells (Hydbring et al., *Nat. Rev. Mol. Cell Biol.* 17, 280–292, (2016)).

Objectives

To identify the cell cycle proteins potentially involved in sperm capacitation by a computational modelling approach and to assess their actual role in vitro capacitation and IVF by inhibiting these with a potent and specific inhibitor of the identified proteins, Aminopurvalanol A (AA).

Materials and methods

Cell cycle network was created and analysed using Cytoscape 3.3.0, by previously using the pathway database Reactome as data source.

All the chemicals were purchased by Sigma Aldrich and were of the purest analytical grade.

Semen samples were processed using a validated protocol (Barboni et al., *PLoS One* **6**, e23038 (2011)) and spermatozoa were incubated under capacitating conditions with or without AA at different concentrations (20, 10 and 2 μM) during 4 hours. Then, acrosome integrity (PSA staining), actin polymerization (Phalloidin staining), tubulin relocation (immunocytochemistry assays), membrane lipid remodelling (FRAP), and fertilizing ability (IVF) were evaluated in vitro.

Results

The network representing the molecules involved in cell cycle control was created by using an in silico approach (Bernabò et al., *OMICS* 19, 712–21, (2015); Bernabò et al., *BMC Syst. Biol.* **5**, 47, (2011a)). Among the whole proteins involved in cell cycle, it was possible to highlight the central role of Cyclins/Cdk in signal transduction during capacitation. With this information, in vitro experiments were performed to confirm the finding. By adding the Cyclins/Cdk inhibitor AA at different concentrations (20,10 and 2 μM) during capacitation it was possible to evince a dose-dependent inhibition of actin polymerization (phalloiding staining), with the consequent loss of acrosomes (PSA staining) and a decrease of in vitro fertilizing ability of spermatozoa (IVF), far-reached with the highest concentrations of AA ($p<0.05$). Otherwise, AA showed not to interfer with membrane lipid remodelling (FRAP analysis, DILC12 staining) or cytoskeleton tubulin dynamics (immnunocytochemistry assays).

Conclusions

Cyclin/cdk complexes could be a new element in control system of actin polymerization during boar sperm capacitation.

This data could revamp the knowledge on biochemistry of capacitation and could suggest new perspectives in studying male infertility.

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Variation of melatonin, testosterone and antioxidant enzymes in seminal plasma of three ram breeds under tropical conditions

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Keywords: Ram seasonality, rainy season, drought season

In temperate regions, pineal melatonin and photoperiod regulates sheep seasonality (Chemineau *et al. Reprod Dom Anim* 43 (Suppl. 2), 40–47. 2008). In tropical conditions, with equal duration of day light, small ruminant reproduction is regulated by the annual cycle of rainfall and food availability more than photoperiodic changes (Morales *et al*. *Small Rum Res* 137, 9-19. 2016). Therefore, melatonin must be playing other functions, like the regulation of the antioxidant defense system (Mayo *et al. Cell Mol Life Sci.* **59**, 1706–1713. 2002). Although melatonin, testosterone and antioxidant enzymes (AE) are present in the ram seminal plasma of seasonal breeds (Casao, *et al. Reprod Biol Endocrinol* **8**, 59. 2010), there is no information on hormonal concentration and AE activity in the ram seminal plasma from tropical regions such as Colombia, located at equatorial level, with a bimodal regime of rain in the Andean region. Thus, the aim of this study was to evaluate the variation of melatonin, testosterone and AE in the seminal plasma form three sheep breeds (Colombian Creole, Romney Marsh and Hampshire) under tropical conditions.

Semen from twelve rams (four rams from each breed) was collected weekly for one year by artificial vagina. Sires were housed at the National University of Colombia, located in Mosquera (4°40'57'' N. 74°12'50'' W) at 2510 m above the sea level. Seminal plasma was extracted by double centrifugation at 9000xg for 10 min at 4ºC, filtered through a 0,22 µm Millipore membrane (Merck, Darmstadt, Germany) and kept at -20 ºC until use. Melatonin and testosterone concentration were measured by a commercial competitive immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Switzerland and Testosterone-ELISA, DiaSource InmunoAssays S.A., Belgium), following the manufacturer's instructions. The activity of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRD) were analyzed as previously described (Casao *et al. Anim Reprod Sci* **138**, 168–174. 2013). Results were grouped into four environmental seasons; two rainy (March-May, September-November) and two dry (December-February, June-August) and analyzed by two-way ANOVA followed by Bonferroni post-test (GraphPad Software, La Jolla, CA, USA).

Melatonin concentration was lower ($P<0.05$) in the March-May rainy season (25.8±0.7 pg/mL) than in the other seasons (32.5±1.5 pg/mL for Sep-Nov, 35.2±1.7 pg/mL for Dec-Feb and 36.3±1.8 pg/mL for Jun-Aug). Testosterone showed higher concentrations ($P<0.05$) in Romney Marsh and Hampshire breeds than in Creole (3.4±0.1 and 3.5±0.2 *vs*. 2.3±0.3 ng/mL) during the June-August dry season. GPx activity were higher ($P<0.05$) between Sep-Nov (10.4±0.9 nmol/min.mL) and Jun-Aug (10.6.±1.3 nmol/min.mL) compared with Dec-Feb (5.2±0.9 nmol/min.mL) and Mar-May (5.4±1.1 nmol/min.mL).

In conclusion, melatonin, testosterone and antioxidant enzymes are present in seminal plasma of rams under tropical conditions, and show seasonal or breed differences.

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Effects of short exposure of bull semen to Roundup® on sperm kinetics and on *in vitro* embryo production

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Keywords: Roundup, semen, embryo

Common toxicants, such as herbicides, are considered as potential threats for fertility. The aim of the present study was to investigate the impact of a low concentration of the herbicide Roundup® (1ppm of Roundup®, -Monsanto- corresponding to 360ng/ml of the active ingredient, glyphosate), on sperm motility of frozen-thawed bull semen and on the subsequent *in vitro* bovine embryo production.

In experiment 1, frozen semen samples, from the same bull and ejaculation, were allotted in two groups [treated (R) and controls (C), each n=3, in three replicates)] and separated by swim–up. In group R, the swim-up medium was modified with the addition of 1ppm of Roundup®. After 1 hour, all semen samples were centrifuged for 5 min at 10000r/min, the supernatant was discharged, 800 μl of new swim-up medium was used to reconstitute the semen pellet, and samples were evaluated for kinetics by CASA [(progressive, immotile, rapid, medium, slow moving spermatozoa, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), beat cross-frequency (BCF), amplitude of lateral head displacement (ALH) and wobble (WOB)].

In experiment 2, immature cumulus oocyte complexes (COCs n=494, 4 replicates) were obtained by aspirating small-medium size follicles (2-6mm) from ovaries of slaughtered cows. COCs were matured in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml EGF at 39^{o}C under an atmosphere of 5% CO_2 in air with maximum humidity. Matured oocytes were divided in two groups, (control (C) n=162 and treated (R) n=332) and were inseminated using frozen-thawed swim-up separated bull sperm (as in exp 1) at a concentration of 1 x 10^6 spermatozoa/ml. Treated sperm was exposed to the herbicide only for 1 hour; this was during the swim-up process. Gametes were co-incubated at 39^{o}C under an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 h post insemination (hpi), presumptive zygotes were denuded, transferred to 25 ml culture droplets under mineral oil, and cultured in SOF supplemented with 5% (v/v) FCS at 39^{o}C in 5% CO_2, 5% O_2 and max. humidity. Cleavage and blastocyst formation rate were evaluated at 48 hpi and on days 7, 8 and 9 respectively. Comparisons on semen kinetics were analyzed by t-test, while cleavage and blastocysts formation rates were carried out by χ^2; in all cases significance was set at 0.05 level.

Sperm evaluation by CASA revealed no difference in any of the parameters studied. Cleavage rate was similar between groups (C: 88.3%, R: 90,9%, p=0.5) while in group R blastocyst formation rates were steadily lower than that of group C (day 7, C: 24.7 ± 3.6% , R:14.5±4.8%; day 8 C:29.7±3.5%, R:18.1±5.8%; day 9 C: 32.8±3.5% , R:18.1±5.8%; in all cases p<0.02) .

These results imply that short exposure to roundup brings about alterations to sperm that are expressed during early embryo development. Further research is underway to evaluate sperm of DNA integrity and to assess embryo quality

Evaluation of exogenous DNA integration and fertilization ability of transfected boar spermatozoa

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Keywords: sperm, transfection, embryo

The use of boar spermatozoa as vectors for introduction of exogenous DNA into the oocyte during fertilization could be an alternative and simple method for generation of transgenic pigs. So far, the results of using sperm as DNA vectors are very controversial. The aim of this study was to evaluate the integration of gene construct and the ability of fertilization by transfected boar spermatozoa. The boar sperm was transfected by direct incubation with the p12hGH-GFPBsd gene construct labeled with rhodamine. We investigated sperm of 92 ejaculates collected from 30 boars of which 16 ejaculates from 4 boars were selected for transfection. Sperm motility was evaluated after centrifugation and transfection. The presence of the gene construct in transfected boar spermatozoa was detected by fluorescence *in situ* hybridization (FISH) by assessment of a positive fluorescence signal for rhodamine. The control group consisted of non-transfected boar spermatozoa hybridized with labeled construct. Boar sperm after transfection was used for *in vitro* fertilization of pig oocytes. For this purpose, sperm motility was evaluated before and after capacitation. The control group consisted of capacitated, non- transfected sperm. Selected semen from 3 boars after transfection and capacitation, exhibiting the best motility parameters was used for *in vitro* fertilization (IVF) of oocytes. Presumptive zygotes were cultured in the NCSU-23 medium up to the blastocyst stage. The percentage of potential cleaved zygotes, morulae and blastocysts was evaluated and the presence of the gene construct in embryos was assessed by observing a positive fluorescence signal for rhodamine. After transfection a decrease of sperm motility was observed. The gene construct was detected in 7138 (47.1%) out of 15981 analyzed transfected spermatozoa. Two types of transgene fluorescence were observed: a single fluorescence signal specific to a given transgene, and a fluorescence signal indicating transgene-coated sperm. Among the control group, the signal was present in 2082 (15.3%) analysed cells, which should be regarded as a false-positive result obtained from the fluorochrome attached to the probe. After capacitation of transfected boar sperm, slight differences in seminal motility were observed in comparison to the control group. As a result of IVF with transfected sperm, out of 77 (59.68%) presumptive zygotes 19 (24.64%) and 9 (11.68%) developed to the morula and blastocyst stage, respectively. The development of the other presumptive zygotes have been stopped at the stage of 3-4 blastomers. The presence of the gene construct in evaluated embryos was not detected. Our results confirm the ability of boar transfected spermatozoa to bind exogenous DNA. However, in embryos obtained after in vitro fertilization with DNA transfected spermatozoa no gene expression was detected. Further studies are required to determine the transfection efficiency using ICSI technique.

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Effect of foodborne contaminants on sperm fertilization competence and embryonic development

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Keywords: AFB1, ATZ, sperm

Background: There is growing concern about the effects of environmental toxins on human and domestic animal reproduction. Among the toxin sources, food products can potentially be contaminated with mycotoxins and/or pesticides. Aflatoxins are poisonous by-products of the soilborne fungus *Aspergillus*, found in food products such as maize, rice and wheat. Of these, aflatoxin B1 (AFB1) is the most toxic. Atrazine (ATZ) is an herbicide, that is extensively used to control weeds in broadleaf and grassy crops. ATZ is considered a ubiquitous environmental contaminant and is frequently detected in the ground, surface water and various types of crops. In the body, ATZ is metabolized to diaminochlorotriazine (DACT), which is further detected in the urine, serum and tissues. Exposure to ATZ and DACT impaired sperm viability, acrosome reaction and mitochondrial function (Komsky-Elbaz, Reprod Toxicol 67:15, 2017). Furthermore, exposure to AFB1, ATZ or DACT resulted in DNA fragmentation in sperm. However, these toxins' effects on fertilization have never been examined.

Objectives: Examine the effects of AFB1, ATZ and DACT on sperm fertilization competence and early embryonic development.

Materials and methods: Cumulus oocyte complexes (n=30–60/group; 6 replicates) were aspirated from bovine ovaries, in-vitro matured (22 h) and fertilized (18 h) with fresh semen. Before fertilization, sperm was capacitated in the absence (control) or presence of AFB1 (10 μM; Cayman Chemical, MI, USA), ATZ (0.1 or 1 μM) or DACT (1 or 10 μM; Chem Service Inc., PA, USA). Cleavage into 2- to 4-cell-stage embryos and blastocyst-formation rates were evaluated 42 h and 7 days post-fertilization, respectively, using JMP-7 software (SAS Institute Inc., 2004, Cary, NC, USA). Cleavage and blastocyst-formation rates, and distribution of blastocysts to various developmental stages were compared by chi-square test followed by Fisher's exact test. Data are presented as means.

Results: Exposing sperm to 10 μM AFB1 pre-fertilization reduced the proportion of embryos that cleaved to the 2- to 4-cell stage relative to controls (70.9 vs. 85.1%). Similar findings were noted when sperm was exposed to 1 μM ATZ (60.7 vs. 91.3%), 1 μM DACT (72.6 vs. 85.1%), or 10 μM DACT (64.6 vs. 85.1%) relative to controls, respectively ($P < 0.005$). Blastocyst-formation rate was significantly lower when oocytes were fertilized with sperm exposed to ATZ (0.1 or 1 μM; 7.7 and 8.7%, respectively) or DACT (1 or 10 μM; 20.5 and 16.6%, respectively) relative to controls (30.4%; $P < 0.04$). Blastocyst-formation rate did not differ from controls after fertilization with sperm exposed to AFB1. Distribution into different embryonic stages differed among groups, with significantly lower rates of development to the blastocyst stage for ATZ-treated sperm (0.1 or 1 μM; 6.6 and 8.7%, respectively) relative to controls (16.1%; $P < 0.05$).

Conclusions: The findings reveal risk associated with exposure of sperm to foodborne contaminants. Even relatively low doses of AFB1, ATZ or DACT and short time exposure impair fertilization and blastocyst formation.

Heat stress effects on reactive oxygen species production and lipid peroxidation in bovine spermatozoa

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Keywords: oxidative stress, semen analysis, heat stress

The aim of this study is to elucidate the effects of heat stress on oxidative status in bovine spermatozoa by quantifying reactive oxygen species (ROS) and lipid peroxidation (LPO). Heat stressed (HS) and non-heat stressed (NHS) frozen bovine semen samples were examined. HS semen, collected 14 to 42 days after artificial scrotal insulation, showed lower protamination and motility, and changes in the methylation of paternal pronuclei (Rahman et al., Theriogenology, 76, 1246–1257, 2011). Spermatozoa were passed through a discontinuous Percoll gradient (45/90% (v/v); VWR International) and adjusted to a final concentration of $2.5x10^6$ cells/ml in PBS.

100mM (final concentration) of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, USA) was added to the sperm samples to stain ROS and incubated at 37ºC for 15 minutes. Dead cells were stained with 1.5µM (final concentration) of propidium iodide (PI) and analyzed using a Cytoflex flow cytometer (Beckman Coulter, Brea, USA) (n=4). 10µM (final concentration) of BODIPY 581/591 C11 (Thermo Fisher, Waltham, USA) was added and incubated at 37ºC for 15 minutes before Cytoflex analysis (n=4). It emits red fluorescence in the non-oxidized state, shifting to orange and green after LPO.

5mM (final concentration) of Luminol sodium salt (Sigma-Aldrich, St. Louis, USA) was added to measure chemiluminescence for 15 and 30 minutes at 37ºC using a luminometer (n=3). PI, DCFH-DA and BODIPY signals were acquired screening a minimum of 5000 spermatozoa per sample. Data were analyzed using the Student's t-test ($p \leq 0.05$) and Spearman correlation. A significantly higher percentage of PI^+ dead cells was present in HS (30.7%) than in NHS semen (21.9%). No differences were observed in the percentage of DCFH-DA^+ cells between HS and NHS semen. However, a higher mean fluorescence intensity (MFI) was observed in HS compared to NHS semen. Significant differences were observed in BODIPY between red stained HS (83.6%) and NHS (91.3%) sperm cells, although no significant differences were observed in the percentage of green stained cells. This shift towards green fluorescence was higher in HS compared to NHS semen. Although no significantly higher ROS production in HS compared to NHS semen was observed after luminol quantification, there was a positive correlation between ROS production (luminol) and LPO (BODIPY green) ($r=0.82$, $p=0.01$).

The survival rate of sperm cells was higher in NHS than in HS semen, while a higher LPO and ROS production were observed in HS compared to NHS semen. Further evaluations are needed to better understand these effects of heat stress on the oxidative status of bovine spermatozoa.

Effect of antioxidants and thawing rates on the quality of cryopreserved camel sperm

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Keywords: Camel sperm, cryopreservation, antioxidants

The viscous seminal plasma of camel semen may prevent penetration of cryoprotectants into spermatozoa, making cryopreservation difficult. This study examined whether addition of antioxidants catalase (CAT), carnitine (CARN) and glutathione (GSH) pre-freezing could improve post-thaw semen quality, and/or increasing thawing temperature from 30°C (30 sec) to 60°C (10 sec).

Two ejaculates were collected from each of four fertile dromedary males. Spermatozoa were separated from seminal plasma by single layer centrifugation (SLC) and sperm pellets were resuspended in freezing extender supplemented with individual antioxidants or control without antioxidants for freezing in liquid nitrogen.

One straw from each treatment was thawed at 60°C for 10 sec and the other at 30°C for 30 sec; sperm quality was evaluated at 0, 1 and 2h post thawing. Parameters evaluated were total motility (TM), progressive motility (PM) and kinematics: ALH (lateral head displacement), BCF (beat cross frequency), LIN (linearity), STR (straightness), VAP (average path velocity), VCL (curvilinear velocity), VSL (straight line velocity) using computer-assisted semen analysis, membrane integrity and acrosome integrity (eosin-nigrosin), and membrane functionality (HOST-test). Normality of the samples was analysed by Saphiro-Wilk test. Normally distributed samples were analysed using ANOVA (Antioxidants) or t-Student (Thawing rates). Non-normally distributed data were analysed using Kruskal-Wallis (Antioxidants) or Mann-Whitney (Thawing rates). Values are means ± SEM. There were no differences among treatments at 0h for any parameters. A significantly higher TM was observed at 1h post thawing (P=0.009) for CAT (37%) and CARN (32%) compared to control (26%). After 2 h, TM was significantly higher (P=0.001) for CAT, CARN and GSH (27%, 25%, 23%, respectively) compared to control (15%). VAP 1h post thawing was increased for CAT (72 µm/s) and CARN (67 µm/s) compared to control (62 µm/s) (P=0.014). At 2h post thawing the following parameters were significantly higher for CAT, GSH and CARN: PM (9%, 11%, 9%, respectively; P=0.004), ALH (7 µm for each; P=0.015), VAP (65 µm/s, 66 µm/s, 62 µm/s; P=0.014), VCL (136 µm/s, 139 µm/s, 134 µm/s; P=0.019), VSL (43 µm/s, 46 µm/s, 42 µm/s; P=0.013) compared to control (4%, 6 µm, 54 µm/s, 117 µm/s, 36 µm/s, respectively).

Thawing at 60°C for 10 sec gave significantly higher TM at all time points (P<0.01), 40%, 35%, 26% at 0, 1 and 2 h compared to 37ºC for 30 sec (34%, 29%, 19%, respectively). Greater values were also observed at 60ºC for PM at 0 h (18%; P=0.002), 1h (P=0.007; 16%) and 2h (10%; P=0.013) compared to 37ºC (13%, 12%, 7% respectively). Thawing at 60º C increased the values of ALH (7.9 µm; p=0.033), STR (68%; P=0.034) and VCL (149 µm/s; P=0.004) at 1h compared to 37ºC (7.6 µm, 67% and 137 µm/s, respectively). No differences were observed in membrane or acrosome status for the different thawing rates.These results suggest that antioxidants exert a protective effect during cryopreservation of camel spermatozoa; it is better to thaw camel semen at 60°C for 10 sec compared to 37°C for 30 sec.

Mouflon *(Ovis musimon)* sperm cryosurvival is better at the end of the rutting season coinciding with low plasma testosterone concentrations

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Keywords: semen, cryopreservation, seasonality

Cryosurvival of spermatozoa in Iberian ibex (*Capra pyrenaica*) is poorer at the onset and in the middle of the rutting season, when plasma testosterone levels are the highest, than at the end of the rutting season coinciding with fall of testosterones levels. We hypothesized that high plasma testosterone concentration might have a negative effect on sperm cryosurvival, and thus a similar situation may be found in other wild ruminants, such as the mouflon (*Ovis musimon*). Sperm samples were obtained from 22 mouflons, using the transrectal ultrasound-guided massage of the accessory sex glands technique. Samples were collected during autumn (October) when plasma testosterone concentrations are high, and at the end of the rutting season (January), when levels of testosterone tend to decrease to basal levels. Sperm motility was assessed with a computer-aided sperm analysis system. Membrane integrity and acrosomal status were evaluated by fluorescence and by the eosin-nigrosin technique. Morphological abnormalities and acrosome integrity were evaluated in samples fixed in buffered 2% glutaraldehyde. Each sample was cryopreserved following two different protocols. Ejaculates were diluted using a Tris-TES-glucose-based medium with 6% egg yolk and two different cryoprotectants: glycerol 5% for the traditional freezing protocol in straws in liquid nitrogen vapors (frozen sperm), and sucrose 100 mM for the ultrarapid freezing protocol in pellets (vitrified sperm). Plasma testosterone concentrations were measured by radioimmunoassay. Statistical analysis was performed by one-way ANOVA. Sperm quality of frozen-thawed and vitrified-warmed samples was higher in January, when levels of testosterone are decreasing, than in October. Plasma testosterone concentration was higher ($P<0.01$) in October (5.49 ± 1.33 ng/ml) than in January (1.02 ± 0.55 ng/ml). There were no differences in fresh sperm variables between samples collected in October and in January. Frozen-thawed sperm cryopreserved in January had a total sperm motility, curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement and beat-cross frequency greater than samples collected in October ($P<0.05$). Sperm viability was also higher in frozen-thawed samples collected in January than in October ($P<0.05$). Vitrified-warmed sperm had a VCL ($P<0.01$), VAP ($P<0.01$), straight-line velocity ($P<0.05$) and viability ($P<0.05$) higher in samples collected in January than in October. These results confirmed the hypothesis that the pick of plasma testosterone concentration that occurs in October, could affect negatively to mouflon sperm cryosurvival.

Melatonin has a protective role against cryocapacitation of ram spermatozoa

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Keywords: melatonin, sperm cryopreservation, apoptosis

Melatonin is present in the ram seminal plasma and in the male reproductive tract. In vitro treatments with melatonin have shown a direct effect on ram spermatozoa by decreasing apoptosis-like changes, affecting capacitation, and increasing the IVF results. Therefore, this study raises the hypothesis that melatonin could have a protective effect against cold-shock in ram spermatozoa, avoiding changes related to cryocapacitation such as the increase in apoptosis indicators that sperm suffer at low temperatures. Briefly, two successive ejaculates were collected three times per week (from February until June) from nine healthy *Rasa Aragonesa* rams (2-6 years old) of proven fertility with the aid of an artificial vagina. Second ejaculates were pooled and processed together in order to eliminate individual differences. After sperm selection by a swim-up method, aliquots of 300 µl were incubated with 100 pM, 10 nM and 1 µM melatonin (Sigma-Aldrich Co., dissolved in PBS with 0.1 ‰ DMSO) for 30 min at room temperature. Then, samples with melatonin plus a control sample with 0.1 ‰ DMSO were directly cooled at 5 °C on ice-bath for 10 min followed by 5 min at 37 °C (cold-shock treatment). The following sperm functionality parameters were analyzed in the swim-up sample and in cooled samples (with and without melatonin): motility, using a CASA system (ISAS 1.04; Proiser SL, Valencia, Spain); membrane integrity (CFDA/PI stain); capacitation state related to intracellular calcium distribution by chlorotetracycline (CTC) staining, and tyrosine phosphorylation of membrane proteins by SDS-PAGE immunoblotting. The apoptotic markers assessed were phosphatidylserine (PS) translocation (Annexin V/PI stain); DNA damage by TUNEL (In situ cell death detection kit, ROCHE), and caspase activity (Vibrant® FAM™ Caspase-3 and -7 Assay, Invitrogen). Eight replicates were performed and data were compared by Chi-squared test. The obtained results indicate that cold-shock produced a significant ($P<0.001$) decrease in all the seminal quality parameters, except in DNA damage. The addition of 100 pM and 10 nM melatonin before cooling decreased significantly ($P<0.05$) the percentage of capacitated spermatozoa (51.71% ±2.55 and 49.86% ±2.83 respectively) comparing to the control sample (57.43% ±2.88). The lowest dose of melatonin (100 pM) reduced the PS translocation and caspase activation compared to the control sample without hormone. The addition of melatonin did not result in significant differences in motility, membrane integrity or tyrosine phosphorylation. It can be concluded that melatonin at low doses (100 pM) is able to prevent, at least partially, the cold-associated apoptosis and premature capacitation-related changes.

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Effect of seminal plasma on cytokine production from bovine endometrial epithelial cells in culture

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Keywords: Bovine seminal plasma, Uterine cell, Cytokine response

Seminal plasma (SP) is involved with immune-regulation in the female reproductive tract through specific cytokines. Variations in fertility among bulls could be due to SP. The objective was to investigate the type and level of cytokine response bovine endometrial epithelial cells (bEEC) in culture (passage 5) after challenge with SP. Donor bulls were categorized as below average (L) or above average fertility (H) according to an index based on the 56-day non-return rate from at least 1,000 artificial inseminations. Bulls of average fertility scored 100. The L-bulls had a score of ≤ 92 (n=2) and H-bulls > 104 (n=3). Approximately 5 – 13 x 10^5 bEECs per flask were challenged with 1% or 4% SP from L- or H-fertility bulls (L1, L4, H1, H4, respectively) or 1% or 4% PBS as control (C1, C4) in 13 replications with cells from 8 uteri. After 72h, the total number of cells, stained with trypan blue, was counted in a Burker hemocytometer. The supernatant was analysed for transforming growth factor beta (TGF-β1, TGF-β2 and TGF-β3) by Luminex (MILLIPLEX™ MAP, Merck Millipore, USA) and Interleukin 8 (IL-8) by ELISA (Bovine IL-8, MABTECH, Sweden). The concentration of each cytokine was calculated (pg/million cells). Data were analysed using the mixed model in SAS® (Proc Mixed, SAS® 9.3, USA). Fertility of bull, concentration of SP, and their interaction were fixed parts of the model, with cytokine response as variable parameter. Cow and cow interaction with replication were used as random factors. Post-hoc comparisons were adjusted for multiplicity using Tukey's, and the Contrast option was used to analyze individual differences. All values are presented as LSMEAN ± SEM. Challenge had significant effects on cytokine production (TGF- β1, TGF- β2 and IL-8) due to fertility of bull (*p<0.0001*), concentration of SP (*p<0.0001*) and the interaction between both factors (*p<0.0001*). There were no differences in TGF- β1, TGF- β2 and IL-8 production after challenge with L1 (4.8±3.2, 52.2±19.7, 18.0±5.1; x10^3 respectively) and H1 (7.1±3.0, 38.9±19.0, 18.4±4.7 x10^3 respectively) compared to C1 (3.5±3.0, 27.1±18.9, 17.5±4.7 x10^3 respectively) and C4 (3.2±3.1, 25.3±19.5, 17.1±4.9 x10^3 respectively). A higher production of TGF-β1, TGF-β2 and IL-8 (*p<0.0001*) resulted from challenge with L4 (20.6±3.1, 136.3±20.5, 54.8±4.9 x10^3 respectively) or H4 (18.6±3.2, 106.6±19.9, 44.6±5.1 x10^3, respectively); challenge with L4 SP was differed from H4 (*p<0.05*). For TGF-β3, fertility of bull (*p<0.05*), concentration of SP (*p>0.05*) and the interaction between factors were significant (*p<0.01*). The highest production of TGF-β3 was found in L4 (2.4±0.4 x10^3) than H4 (1.2±0.4 x10^3) (*p<0.05)*, L1 (0.6±0.4 x10^3) (*p<0.01*), H1 (0.4±0.4 x10^3) (*p<0.01*), also C1 (0.5±0.4 x10^3) and C4 (0.2±0.4 x10^3) (*p<0.01*). In conclusion, higher concentrations of SP stimulated more cytokine production; 4% SP from L-bulls stimulated more TGF-β1, TGF-β2, TGF-β3 and IL-8 production than SP from H-bulls, which could be associated with impaired cell adhesion or cell damage.

Comparison of the apoptotic like-changes in boar semen before and after in vitro capacitation

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Keywords: boar, apoptosis, spermatozoa

Boar semen is very sensitive to all biotechnical modifications, including *in vitro* capacitation and *in vitro* fertilization. Moreover, the quality of boar semen may affect the efficiency of *in vitro* fertilization. However, the process of capacitation required by spermatozoa to acquire the fertilization ability is impaired in the IVF system. Presence of the apoptotic-like changes (ALC) in capacitated spermatozoa can lead to decreased fertility. The assessment of ALC can be useful for estimating sperm ability for fertilization. The purpose of this study was to determine the ALC in boar spermatozoa before and after *in vitro* capacitation. Semen was obtained from 11 boars of different breeds with normal fertility from the AI Center in Klecza Dolna. The sperm capacitation took place during incubation in a medium based on TCM-199 (Sigma, Germany) for 1 hour, at 39^{o}C and 5% CO_2 in the air. For assessment of ALC, semen was incubated with the fluorophore YO-PRO-1 (Vybrant Apoptosis Assay Kit≠4, Molecular Probes, USA) (Trzcinska et al, Anim Reprod Sci, 124, p 90-97, 2011) in the dark for 20 – 30 minutes, in room temperature before and after *in vitro* capacitation. After incubation, the semen was analysed under a fluorescent microscope. Statistical analysis was performed using the t-test. The mean percentage of viable spermatozoa before capacitation ranged from 65 to 91% (mean 78.4%; P>0.01), while after capacitation from 44 to 88% (mean 61.6%; P>0.01). The viable spermatozoa with ALC before capacitation oscillated between 0.5 and 9% (mean 4.4%) and after capacitation 0 and 21% (mean 5.9%). The percentage of nonviable spermatozoa before capacitation ranged from 4 to 32% (mean 16.5%; P>0.01), while after capacitation from 8 to 48% (mean 32.6%; P>0.01). When boar semen before *in vitro* capacitation was compared with semen after capacitation, an increase in the percentage of cells with ALC was detected accompanied by a significant decrease in nonviable spermatozoa. In conclusion in vitro capacitation of boar semen resulted in an increase in the percentage of apoptotic-like changes which may result in a decrease in fertility.

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Progesterone-induced changes in the ram sperm acrosome reaction are inhibited by the antagonist mifepristone

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Keywords: Mifepristone, acrosome reaction, ram spermatozoa

Progesterone (P4) is an important hormone regulating the reproductive functions and may exert rapid non-genomic effects on sperm functionality. P4 may stimulate sperm hyperactivation, chemotaxis, in vitro capacitation and the acrosome reaction in several species. Most of these rapid actions could be mediated by P4 binding to membrane receptors on the sperm surfaces. The aim of this study was to investigate the effect of progesterone on ram sperm functionality and to elucidate whether these actions are mediated by its binding to sperm membrane receptors. For this purpose, sperm samples were incubated in capacitating conditions in the presence of P4 with or without mifepristone, a progesterone receptor antagonist. Briefly, two successive ejaculates were collected three times per week from nine males (2-6 years old) with the aid of an artificial vagina, and second ejaculates were pooled and processed together in order to eliminate individual differences. After sperm selection by a swim-up-dextran method, samples were incubated for 3 h at 39 °C, 5% CO_2 and 100% humidity in a high-cAMP medium (cocktail), already successfully demonstrated for capacitating ram spermatozoa. After pre-incubation with 4 or 40 µM mifepristone (dissolved in PBS with 0,1‰ DMSO), 1 µM P4 was added to samples. Two samples without P4 were included as controls, the cocktail-sample and another one incubated in TALP medium. Analyzed parameters were sperm motility (CASA), plasma membrane integrity (CFDA/PI stain), capacitation status by chlortetracycline (CTC) staining and tyrosine phosphorylation of membrane proteins (by western blotting), and phosphatidylserine (PS) translocation (Annexin V/PI stain) as an apoptotic marker. Data were compared by Chi-squared test. The results obtained showed that the presence of P4 in capacitating conditions led to a significantly higher percentage of acrosome-reacted spermatozoa compared with the sample without hormone (43.3±3.5% vs. 24.0±1.1). Pre-incubation with mifepristone significantly inhibited the increment in rate acrosome-reacted sperm rate (17.0±1.5 and 16.0±1.5 for 40 µM and 4 µM mifepristone, respectively) and also decreased the percentage of sperm with phosphatidylserine translocation (20.7±3.4, 24.7±2.9 vs. 35.7± 2.4 for 40 µM, 4 µM and without mifepristone, respectively). However, mifepristone did not affect the percentage of motile and with integral plasma membrane spermatozoa. In conclusion, this study showed that P4 induces the acrosome reaction and apoptotic changes in ram spermatozoa and that these actions are mediated by sperm progesterone receptors, given that mifepristone is able to decrease both the acrosome reaction and apoptotic markers.

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Effect of 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ2) on sperm motility and binding to *in vitro* cultured oviductal epithelial cells (OEC). A preliminary study in porcine.

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Keywords: Spermatozoa, boar, prostaglandin J2

The effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) on its receptor, the sperm peroxisome proliferation-activated receptor gamma (PPARγ), has been linked to numerous biological activities, including boar sperm capacitation (Soriano-Úbeda, 2013, The 1st EPICONCEPT Annual Meeting, Antalya, Turkey). The aim of this work was to evaluate the effect of PGJ2 at 10 µM [Santoro, J Exp Biol, 216(6), 1085-1092, 2012] on sperm viability, motility and binding to *in vitro* cultured oviductal epithelial cells (OEC). Spermatozoa samples were obtained from 4 ejaculates of 2 to 3 years-old boars of proven fertile. OEC were obtained from oviducts of 6 to 7 month-old sows slaughtered at an abattoir and cultured in monolayer (López-Úbeda, Asian J Androl, 18, 1-18, 2016). For viability and motility analysis, spermatozoa were incubated in a capacitation medium (TALP) in presence (PGJ2) or absence (ø) of 10 µM of PGJ2 (D8440, Sigma-Aldrich®, Madrid, Spain). The viability was analyzed at 1 and 30 min of incubation using the eosin-nigrosin staining. The motility parameters were determined by computer-assisted sperm analysis (CASA) at 1, 5 and 30 min of incubation. The binding to OEC was evaluated after insemination with $1x10^5$ spermatozoa/ml stained with bisbenzimide and 30 min of co-culture in TALP in PGJ2 and ø groups. After co-culture, inseminated monolayers were fixed with paraformaldehyde and analysed under fluorescence microscopy at 10x determining the number of attached spermatozoa to OEC per square millimeter (spz/mm^2). Independent t-test ($p<0.05$) were performed and the results were expressed as mean ± SD for viability and motility parameters or SEM for binding to OEC. The viability of spermatozoa was not affected by the presence of PGJ2 both at 1 and 30 min of incubation. Several CASA parameters were influenced by PGJ2 presence throughout the incubation. At 1 min, PGJ2 produced a higher percentage of motile spermatozoa (%Mot: PGJ2: 93.9%; ø: 90.7%), progressive spermatozoa (%Prog: PGJ2: 46.6%; ø: 43.6%), curvilinear velocity (PGJ2: 146.3±89.6 µm/s; ø: 131.3±81.2 µm/s) and amplitude of lateral head displacement (ALH: PGJ2: 3.1±1.9 µm; ø: 2.8±1.6 µm). At 5 min, PGJ2 showed a higher linearity (LIN: PGJ2: 38.5±25.9%; ø: 32.7±24.0%) and wobble (WOB: PGJ2: 56.3±22.7%; ø: 53.8±21.8%), whereas at 30 min, none of the CASA parameters showed statistical differences between groups. When PGJ2 was added to OEC-spermatozoa co-culture, the number of bound spermatozoa was 27.5% lower than in ø (PGJ2: 85.2±3.4 spz/mm^2; ø: 117.5±3.6 spz/mm^2). Concluding, PGJ2 did not affect sperm viability, however it produced an immediate effect on motility, which probably increased the energy expenditure of the spermatozoa via activation of PPARγ, allowing a lower binding of spermatozoa to OEC.

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NOS/NO modulate the protein phosphorylation on serine and threonine residues during boar sperm capacitation

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Keywords: nitric oxide, sperm, capacitation

Spermatozoa need to undergo a series of functional changes before they can fertilize, which constitute the process known as capacitation. This involves the early activation of protein kinases and the inactivation of protein phosphatases [Battistone MA, Mol Hum Reprod. 19(9):570-80; 2013]. It has been reported that NO can be generated by spermatozoa during capacitation and it can modulate this process through protein S-nitrosylation [Lefièvre L, Proteomics. 7(17):3066-84; 2007], activation of the cAMP/PKA pathway [Belén Herrero M, Free Radic Biol Med. 29(6):522-36; 2000], but also by increasing the cGMP concentration [Murad F, Recent Prog Horm Res. 49:239-48; 1994; Wiesner B, J Cell Biol. 142(2):473-84; 1998]. High levels of cGMP may inhibit cAMP degradation, which leads in turn to PKA activation [Kurtz A, Proc Natl Acad Sci U S A. 95(8):4743-7; 1998].

The aim of this study was to further investigate the NO's involvement in PKA activation during the *in vitro* capacitation of boar spermatozoa.

For this purpose, ejaculated sperm were incubated for 1 hour in capacitating and non-capacitating conditions (TALP medium [Rath D, J Anim Sci. 77(12):3346–52; 1999] and PBS (D1408, Sigma-Aldrich, St. Louis, USA), respectively). The media were supplemented with 100 µM S-Nitrosoglutathione (N4148, Sigma-Aldrich, St. Louis, USA), a NO donor, and two NOS inhibitors: 10 mM N^G-Nitro-L-arginine Methyl Ester Hydrochloride (483125, Merck, USA) and 10 mM Aminoguanidine hemisulfate salt (A7009, Sigma-Aldrich, St. Louis, USA).

The pattern of protein phosphorylation on Ser and Thr residues was evaluated by Western blotting. The antibodies used in this study were: rabbit monoclonal antibody anti-protein kinase A (9624, Cell Signaling Technology, Beverly, USA, 1:2000) and goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology, USA, 1:10000). The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK).

Our results indicated that when capacitated in the presence of NOS inhibitors, spermatozoa showed a lower Ser and Thr phosphorylation pattern than those capacitated with or without the NO donor. This effect was not observed under non-capacitating conditions.

In conclusion, this study provides additional evidence that NOS/NO plays a role in regulating the phosphorylation of Ser and Thr residues during sperm capacitation in porcine.

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Embryology, Developmental Biology, and Physiology of Reproduction

Possibilities of preserving the local cow breeds in Latvia

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Keywords: cow endometrium, reproduction, embryo transfer Latvian Brown (LB) and Latvian

Blue (LZ) are local Latvian breeds of the bovine gene-fund (GF) and therefore unique and characteristic only to Latvia. In 2016, only 152 LB and 313 LZ cows were registered as GF animals. So far, natural breeding and artificial insemination (AI) have been the methods of conserving GF in Latvia. Majority of the GF animals are at advanced age, they live on small farms without calculated feeding ration, and therefore may suffer from reproductive disorders. In 2017, through ERAF project Nr. 1.1.1.1/16/A/025 „Latvian Brown and Latvian Blue cow gene pool conservation using embryo transfer (ET) and related biotechnologies", ET will be restarted in Latvia after 35 years of interruption. The aim of this investigation was to consider possibilities of using ET in LB and LZ cows that could be classified as inferior embryo donors. The analysed data was obtained in Latvia in 1984-85. Thirty Holstein-Friesian cows of which 22 (73.3%) were 9-10 years old were removed from their herds due to reproductive problems (83%) or mastitis (17%) and used as embryo donors. Uterine biopsies were taken from 30 cows on Days 8-9 i.e.1-3 days before superovulatory treatment initiated. Results are presented as average ± standard error and Student's t-test was used to compare two independent samples. Histological investigation revealed different types of endometrial alterations in 91.7% of the cows. A different degree of endometrial oedema and hyperaemia were observed in 23 (76.7%) cows. Despite the fact that vascular fibrosis, hyalinosis concurrently with lymphoid-histiocytic infiltration in stratum compactum of the endometrium were established in 12 (40%) cows, superovulation was reached in 9 (75%) and the amount of corpora lutea (CL) was 6.3±1.42. Embryos of excellent or good quality (0.9±0.45) were obtained from 5 (41.7%) cows. Eighteen cows without strongly expressed endometrial pathologies had successful superovulatory response (CL 8.6±0.82) with 4.5±0.96 embryos of which 3.5±0.66 were viable. In cows with or without serious endometrial pathologies, the number of CL was equal ($p>0.05$), but the number of good quality embryos differed significantly ($p<0.05$). Also, in recipients receiving embryos from relatively healthy cows versus embryos from cows suffering from serious endometrial pathologies, the viability of transferred embryos significantly differed ($p<0.05$). In general, a successful superovulatory response occurred in 22/30 (73.3%) cows. No embryos were obtained from 9 (30.0%) cows. After 2 months, pregnancy was approved in 49% of recipient heifers. Lymphoid-histiocytic, plasmatic and mast cells infiltration in endometrial subepithelium and stroma as well as vascular hyalinosis and fibrosis had a significantly negative influence on embryo recovery. It is profitable to conduct a histological investigation of uterine tissue alongside with other analyses if a cow is considered as an inferior embryo donor due to its health and general condition.

Artificial activation of ovine oocytes is required after ICSI with freeze-dried spermatozoa.

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Keywords: freeze-drying, ICSI, spermatozoa.

Objectives. Freeze-drying allows to store the biological samples in a dry state and represents an interesting alternative low-cost strategy of semen biobanking to save the endangered species. Here, we have established a dry sperm biobank from an endangered Italian sheep breed (Pagliarola) and tested its efficiency through ICSI.

Materials and methods. The motile spermatozoa from ram ejaculates collected with artificial vagina was selected by swim-up in TRIS-based medium (2.42g TRIS, 1.36g citric acid, 1.00g fructose, 100.000 U.I. penicillin G, 100mg streptomycin, in 67.20ml bidistilled water (ddH_2O); pH was adjusted to 6.7) for 20 minutes at 38.5°C. The motile spermatozoa were frozen in freeze-drying medium (10mM EGTA and 50mM NaCl in 10mM Tris–HCl buffer; pH was adjusted to 8.4) in a -80°C freezer for 75 minutes and subsequently lyophilized by the freeze-drying apparatus SP Scientific-VirTis, Freeze-dryer 2.0 BenchTop, 20 hours with a condenser temperature of -58°C and vacuum of 20 mTorr). The vials were sealed in glass vials under vacuum and stored in the dark at 4°C for 1-2 months.

Just before the ICSI, the freeze-dried spermatozoa were rehydrated by adding 100µl ddH_2O. To evaluate the fertilizing capability of freeze-dried spermatozoa, 108 MII sheep oocytes were subjected to ICSI and allocated to two groups: 56 oocytes were activated by incubation with 5µM ionomycin (ICSI-FDS*a*); 52 were left un-activated (ICSI-FDS*na*). Forty-four oocytes injected with frozen spermatozoa (ICSI-FS) and left un-activated, served as control. Pronuclear formation (2PN) and blastocyst development were investigated at 14-16 hours and 7-8 days after ICSI, respectively. Differences were considered statistically significant for $p<0.05$ (*Chi-square* test). Data were analyzed using PRISM, software version 5.0; GraphPad.

Results. The freeze-dried spermatozoa were completely immotile after rehydration, however they maintained the capacity to fecund oocytes after ICSI. Two PN were found in 83.3% of ICSI-FDS*a,* 81.4% of ICSI-FS while only in 14.3% of ICSI-FDS*na* ($p<0.05$ ICSI-FDS*na* vs ICSI-FDS*a;* $p<0.01$ ICSI-FDS*na* vs ICSI-FS). Likewise, the ICSI freeze-dried spermatozoa yielded blastocysts only following artificial activation (ICSI-FDS*a*: 10.2%; ICSI-FS: 31%; ICSI-FDS*na*: 0%; $p<0.05$ ICSI-FDS*a* vs ICSI-FDS*na* and ICSI-FS; $p<0.0001$ ICSI-FDS*na* vs ICSI-FS).

Conclusions. Our finding show that freeze-dried spermatozoa have lost the capacity to trigger oocyte activation but maintained their nuclear viability, whose developmental potential was fully released following artificial activation. Our results support the evidence that freeze-drying effective approach of spermatozoa storage to save endangered species.

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AVEN and BCL-xL expression pattern and protein-protein interaction assessment through bovine early embryo development

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Keywords: Oocyte, Embryo, Apoptosis

Apoptosis in embryonic cells is important for embryo development; stabilizing cell numbers and playing a role in cell quality control. However, it is also associated with embryonic loss and cellular response to suboptimal developmental conditions and stress. AVEN, a novel P4-regulated protein, inhibits the mitochondrial apoptosis pathway by binding to and enhancing antiapoptotic BCL-xL activity. The objective of this study was evaluate the protein expression profile and protein-protein interaction of AVEN and BCL-xL during early embryo development in cattle. Briefly, cumulus oocyte complexes were recovered from abattoir derived ovaries and submitted to in vitro embryo production (IVP). Six replicate sets of samples were retrieved from IVP at 7 different developmental stages (Germinal vesicle (GV), Metaphase II (MII), zygote (16 h), 2-cell (44 h), 8-cell (72 h), compact morula (Day 5) and blastocyst (Day 7), -post insemination (pi)), and fixed in 4% paraformaldehyde. Samples were then processed for Proximity Ligation Assay (PLA) or whole-mount fluorescent immunocytochemistry: PLA detection of Aven-BCL-xL interaction was achieved using the DUOLink™ In Situ Red Starter Kit reagents according to the manufacturers (Sigma-Aldrich) instructions. Whole-mount immunofluorescence: Following washing and blocking, samples were incubated overnight with a single: monoclonal mouse (m) anti-AVEN (1:400, Abcam, ab77014), or polyclonal rabbit (r) anti-BCL-xL (1:400, Abcam, ab2568); dual: (mAven & rBCL-xL) and negative control: none, primary antibodies. Secondary antibodies Alexafluors- 594 goat anti-mouse and 488 goat anti rabbit were employed at a dilution of 1:400, for immunofluorescent labelling. Fluorescent labelling was observed under epifluorescent and confocal laser scanning -microscopy. The number of PLA Aven-BCL-xL interaction foci was counted per unit area of oocyte or embryo sample at each developmental stage (n= >14, per stage), using ImageJ software. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's test. There were significantly more foci in blastocysts compared to GV oocytes, 2-cell, 8-cell and morula -stage embryos ($p<0.05$, mean and SEM = 332,8 ± 56,04 vs. 158 ± 27,94, 162,7 ± 16,98, 160,1 ± 14,02 and 80,34 ± 13,67, respectively). The lowest number of interaction foci was detected in compact morulas (80,34 ± 13,67), this was significantly lower than MII oocytes (213 ± 22,12), zygotes (273,5 ± 41,68) and Blastocysts (332,8 ± 56,04). A distinct labelling pattern was observed during development; foci were evenly distributed in the cytoplasm of GV and MII oocytes, zygotes, 2-cell and 8-cell -embryos, but restricted mainly to peripheral cells in compact morulas and to the trophoblast cells of blastocysts. These results were corroborated by the co-localization pattern of AVEN and BCL-xL in the whole-mount samples. This is the first study to employ PLA for the analysis of protein to protein interaction in bovine oocytes and embryos and shows a very precisely regulated interaction of AVEN and BCL-xL during bovine embryo development.

Caspases and TNFα activation in sperm storage tubules is correlated with hen's fertility

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Keywords: Sperm storage tubes, Caspase, TNFa

Blebbing is a basic event of apoptotic cell death mediated by caspase proteins. Used by cells to package cytoplasm portions, organelles or DNA, this biochemical cascade can also be used by secreting cells to release vesicles by Actin–Myosin II contraction (Mills et al., J. Cell Biol., 146(4):703–707, 1999). Blebbing does not occur in some cells lacking caspase 3 (Janicke et al., J. Biol Chem. 273:9357–9360, 1998; Zheng et al., Proc. Natl. Acad. Sci. USA. 95:13618–13623, 1998). Avian sperm storage tubules (SST) are apocrinal tubular glands located in the hen's utero-vaginal junction (UVJ) that store spermatozoa for long period (Fujii and Tamura, J. Fac. Fish. Anim. Husb. 5(1): 145-163, 1963). In this work, we investigated the correlation between capases activation in SST cells and hen's fertility. Hens from two divergent fertility lineages with high (DF+=21 days) and low (DF-=10 days) period of sperm storage (Beaumont C., J. An. Sci. 72:193-201, 1992.) were artificially inseminated (AI) (200 x 10^6 sperm). Animals (3 hens per condition) were sacrificed without insemination (control), and at 24 hours, 1 wk, 2 wks and 3 wks after AI. Paraffined UVJ tissue was prepared for immunohistochemistry (IHC) against Tumor Necrosis Factor a (TNFa), Caspase-3 (CASP-3), and -8 (CASP-8). The same antibodies were used for Western blotting (WB) quantitative analysis. Statistical analysis was performed by Kruskal-Wallis test and Turkey post-test, the significance threshold was set at $p<0.001$. Protein quantitative analysis (WB) reveals that CASP-3 was 1.3-fold lower in control DF+ than in DF-. At 24 h after AI, CASP-3 was 1.7-fold higher in DF+ than in DF-. CASP-8 was 6.4-fold higher in control DF+ than in DF-, 5.3- and 10.4-fold lower after 1 wk and 2 wks of insemination, respectively, and 1.6-fold higher after 3 wks of insemination. TNFa was 3.4-fold lower in control DF+ than DF-, similarly to caspases, and 2.5- and 1.3- and 3.6- fold higher after 24 hours, 2 wks and 3 wks of insemination, respectively. TNFa, CASP-3, and -8 were observed (IHC) inside UVJ in all moments in both lineages. CASP-3 and CASP-8 were detected with high intensity in external UVJ epithelial surface, and in SST cells of control DF+ and DF- hens. After 24 hours of insemination, CASP-3 and CASP-8 were observed specifically in DF- SSTs and DPF+ SSTs, respectively. TNFa was strongly distributed in connective tissue in association with SST cells as well as in surface epithelia at all moments. These results suggest that caspase activation in SST cells can be correlated with hen's fertility. Rapid caspase activation (within 24 hours) correlated to TNF pathway after insemination could allow longer period of sperm storage.

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Effect of non-esterified fatty acids during in vitro oocyte maturation on the development of bovine embryos after transfer

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Keywords: NEFA, oocyte, embryo transfer

Metabolic disorders, as in negative energy balance (NEB) dairy cows, are associated with elevated non-esterified fatty acid (NEFA) concentrations, predominantly palmitic acid (PA), in the follicular fluid. These NEFAs are known to jeopardize oocyte *in vitro* maturation and elicit altered blastocyst quality and physiology. Lipotoxic conditions during final oocyte maturation also influence epigenetic reprogramming in the resultant day (D) 7 embryo and may thus affect subsequent development, potentially imprinting lasting marks during later stages of life. Therefore, we hypothesized that exposure of oocytes to high NEFA concentrations during IVM affects post-hatching development of D7 blastocysts after embryo transfer.

Bovine oocytes were matured for 24h under 2 conditions: 1) physiological NEFA conditions (28µM stearic acid (SA), 21µM oleic acid (OA), 23µM PA (BAS) and 2) elevated PA concentration as present in follicular fluid during NEB (150µM) with physiological concentrations of SA (28µM) and OA (21µM) (HPA). Matured oocytes were routinely fertilized and cultured in SOF with serum until D7. Cleavage (D2) and blastocyst rate (D7) were compared among treatments using a binary logistic regression model. Eight blastocysts (normal and expanded, equally distributed per treatment and per replicate) were transferred per cow (n=8, 5 replicates). Four cows were attributed to HPA or BAS per replicate and were crossed over for the next replicate. Embryos were recovered at D14 and morphologically assessed (n=46). Glucose, lactate and pyruvate turn-over and interferon-tau (IFNT) secretion were measured in extra-embryonic tissue (EXT) after 24h culture (n=62). Morphological, metabolic and IFNT data were tested for normality with a Kolmogorov-Smirnov test and differences between treatment were analysed with a T-test. Data are presented as mean ± SEM.

Developmental competence at D7 was not significantly different between treatments (blastocyst rate of 26 vs. 29.6% for HPA and BAS, resp.). Recovery rate at D14 was 30% and 36% for HPA and BAS, resp. (P>0.05). HPA during IVM significantly reduced embryo elongation (3.7±1.5 vs. 8.6±1.7mm, P=0.001) but did not affect diameter of embryonic disc compared to BAS. EXT from HPA group consumed similar amount of glucose but tended to produce less lactate compared to EXT from BAS group (1732±211 vs. 2428±355pmol/mm^2/h, P=0.073). IFNT secretion was significantly lower in HPA group (0.47±0.71pg/ml) compared to BAS group (3.79±1.16pg/ml, P=0.018).

In conclusion, exposure to elevated PA during *in vitro* oocyte maturation affected post-hatching development at D14. Embryos were less elongated, were metabolically altered and produced less IFNT, a major signal of pregnancy recognition, than their physiological counterparts. This suggests that metabolic stress during oocyte maturation may have long-lasting effects on embryo development that may lead to higher pregnancy loss and reduced fertility in high yielding dairy cows. More research is ongoing to investigate underlying mechanisms through genome wide transcriptome pathway mapping.

Hypothermic storage (4°C) of ovine embryos with different medium and duration

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Keywords: ovine, embryo, hypothermic storage

Generally, sheep embryos are transferred immediately after collection, however developing of a culture media for successful cooling and short-term storage would have enormous theoretical and practical implications in sheep embryo transfer programs (Romao et al., 2016). Differences in species' cryosensitivity of embryos are responsible for different approaches in their cryopreservation. It was reported that, one of the major concerns in embryo cryopreservation is its lipid content that can hamper the process (Dattena et al., 2000), as it happens in species such as sheep or pigs. Embryo lipid content effect on chilling sensitivity is not totally elucidated at the moment. In cattle, Ideta et al., (2013) introduced a medium that bovine embryos can be held for up to 7 days at 4°C and pregnancy rate of 75% was obtained for embryos held in this medium and transferred to primed recipients.

The objective of this study was to determine if the commercial holding media (SYNGRO, S) and hypothermic medium (199 plus 50% FBS supplemented with 25 mM HEPES) could maintain viability of ovine embryos during extended hypothermic storage at 4°C in vitro as assessed by transfer to synchronized recipients.

Donor ewes from prolific breeds (n=26) were superovulated to recover embryos on day 6 and embryos with grade 1 to 2, morula and blastocyst-stage embryos were sorted in holding media (SYNGRO, S) at room temperature (22°C). The embryos were then allocated at random to different treatment groups within 2 h of collection. T1 embryos (n=80) were washed three times in hypothermic medium (199 plus 50% FBS supplemented with 25 mM HEPES) and loaded into a plastic straw (1/4 cm3 clear straw; 4-6 embryos/straw). T2 embryos (n=20) were stored in commercial holding media (SYNGRO, S) in a plastic straw (1/4 cm3 clear straw; 4-6 embryos/straw). Then a water jacket was prepared by placing a number of 10mm goblets into a 65mm goblet filled with room temperature tap water and with wet cotton or gauze to keep the small goblets from floating/tipping. The holding container was placed in a 4°C fridge and kept adding straws of embryos for at least 24h. Then, 65mm goblet bath was be placed in a Styrofoam box with ice packs for air transportation. Upon the arrival, embryos were placed in a 4°C fridge for 168h in T1 group and 48h in T2 group.

For transfer, the straws were kept at 4°C until needed and were them emptied into a dish of fresh holding media at room temp and then loaded into IVF catheters as they are transferred twin into the recipients (number of recipients for T1 embryos=40; number of recipients for T2 embryos=10). Following hypothermic storage for 7 d (T1) and 2 d (T2), embryos were transferred into recipients by laparoscopy to the uterine horn ipsilateral to a corpus luteum on day 6, and survival was determined on day 50 by ultrasound. There was no pregnancy established with either T1 or T2 group of embryos. It was concluded that these two techniques were not found successful for enabling liquid nitrogen-free storage and air transportation of embryos.

Bovine endometrial cells are responsive to embryonic sex in vitro

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Keywords: endometrial cells, embryonic sex

Bovine endometrium recognizes embryonic sex at early stages with multiple embryos in vivo (Gómez et al, J Proteome Res 12:1199-210). In this work, we investigated the influence of embryonic sex on carbohydrate, protein and gene expression of endometrial cells co-cultured in vitro (EC) with a single embryo.

Day-6 in vitro-produced morulae were individually cultured for 48h on the epithelial side of ECs. EC consisted of epithelial cells grown in inserts plated on stromal cell cultured in wells with TCM-199. Day-8 expanded blastocysts were collected and sexed by amelogenin gene amplification. Samples of EC and conditioned media were cultured with one male embryo (ME), one female embryo (FE) and no embryo (negative control; C). Samples were collected from 4 uterus as follows: uterus-1, 2 ME, 1 FE, 1 C; uterus-2, 2ME, 1 FE, 1 C; Uterus-3, 2 ME, 0 FE, 1 C; Uterus-4, 0 ME, 2 FE, 1 C. Expression of genes (N=13) coding for growth factors, receptors for hormones that regulate estrus cycle, receptors that bind embryonic signals, and metabolism, were analyzed in epithelial and stromal cells. Concentrations of glucose, fructose, lactate, artemin protein and total protein were determined in conditioned medium from the epithelial side. Data were analyzed using GLM and REGWQ Test and Principal Component Analysis (PCA). The relative mRNA abundances for candidate genes were compared using ANOVA y All Pairwise Multiple Comparison (Student-Newman-Keuls Method). Embryos altered transcription only in epithelial cells, not in stromal ones. Thus, expressions induced by ME were lower ($P<0.01$) than FE and controls (C) in hexose transporters solute carrier family 2 member 1 (*SLC2A1*: M=1.00±0.12, FE=6.05±0.07, C=7.19±0.10) and member 5 (*SLC2A5*: M=1.00±0.13, FE=5.58±0.08, C=7.17±0.11), connective tissue growth factor (*CTGF*: ME=1.00±0.22, FE=3.13±0.15, C=2.85±0.09), interferon alpha and beta receptors subunit (*IFNAR1*: ME=1.00±0.18, FE=2.46±0.29, C=2.86±0.15; *IFNAR2*: ME=1.00±0.17, FE=2.30±0.10, C=2.24±0.29). Male embryos elicited lower expression of artemin (*ARTN*) than FE (ME=1.00±0.26, FE=4.53±0.15, $P<0.05$) and controls (ME=1.00±0.26, C=3.74±0.30; $P<0.01$). Female embryos reduced ($P<0.01$) *SLC2A1* and *SLC2A5*, and increased ($P<0.05$) *ARTN* expression with respect to controls (*SLC2A1:* FE=6.05±0.07, C=7.19±0.10; *SLC2A5:* FE=5.58±0.08, C=7.17±0.11; *ARTN*: FE=4.53±0.15, C=3.74±0.30). ARTN protein and gene expressions strongly correlated ($R>0.90$; $P<0.05$) in the group of ME or FE, but not in controls. Embryonic sex did not alter hexoses or lactate concentrations in EC-conditioned medium. The concentrations of carbohydrates and expressions of genes that showed sexual dimorphism covariated significantly (|0.429971|; PCA). In contrast total protein mainly covariated with expressions of estrogen and progesterone receptors. Isolated male and female embryos may differentially release signaling factors that induce sexually dimorphic responses in endometrial cells.

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Transcriptomic response of bovine oviduct epithelial cells to the early embryo

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Keywords: BOEC, Transcriptome, Embryo

Previous data from our group and others indicate that the early bovine embryo may be more than just a passive structure during its journey through the oviduct, and may in fact elicit a transcriptomic response from the oviduct epithelial cells. However, such an effect is likely to be very local in nature, making it challenging to detect *in vivo*. In order to examine the possible embryo effect on BOEC transcriptome and whether it is local or not, we used an *in vitro* model involving co-culture of early embryos with a monolayer of bovine oviduct epithelial cells (BOEC). Oviducts corresponding to the early luteal phase were collected from the slaughterhouse and BOEC were mechanically harvested from the isthmus and cultured in 500 µl of Tissue Culture Medium-199 supplemented with 10% fetal calf serum (FCS) in four-well culture plates in a humidified atmosphere at 5% CO_2 in air at 38.5°C during 6 days until confluence. A day before co-culture the medium was replaced with synthetic oviduct fluid (SOF) supplemented with 10% FCS. Embryos (n=50) at the 2- to 4-cell stage (Experiment 1) or at the 8-cell stage (Experiment 2) were cultured on BOEC in a polyester mesh to maintain the position of the embryos on top of the cells. After 48 h of co-culture, the cells directly beneath the embryos and those in the same well but located away from the embryos (i.e., not in direct contact) were recovered as well as cells from a control well without embryos. BOEC were snap frozen from 5 replicates and they were analyzed by qPCR to assess the expression of 12 candidate genes. These included oviduct genes previously shown to be affected by the presence of an embryo *in vivo* (Maillo et al. Biol Reprod. 2015. 92: 144) and *in vitro* (Schmaltz-Panneau *et al.* Anim Reprod Sci. 2014. 149(3-4):103-106). Statistical differences were assessed by ANOVA. Regardless of being in direct contact or not with 2- to 4-cell embryos, BOEC displayed a decreased abundance of *ARG3;* a gene implicated in the regulation of intracellular calcium and cytoskeleton organization; compared to control cells ($P<0.05$). Co-culture with 8-cell embryos also lead to an increased abundance of *ARG3* besides to others genes involved in BMP signaling pathway (*SMAD6*, *TDGF1*) and a decreased abundance of oxidative stress gene (*GPX4*) in BOEC whatever they have been in direct or indirect contact with embryos. While *SOCS3* a gene related to the inflammatory response was decreased in cells in direct contact with the embryos compared to both other groups ($P<0.05$). In conclusion, under our experimental conditions, the transcriptomic response of BOEC is embryo–stage dependent. For one gene, *SOCS3*, expression was only altered in BOEC in direct contact with the embryo. This may be due to a non-diffusible embryo-secreted factor.

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Effect of nutritional level on the onset of puberty in the Sardi ewe lamb: relationships with FSH, GH and Leptin

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Keywords: Ewe lamb, Nutrition, Puberty

The aim of this study was to investigate the possibility of a compensatory growth following starving and re-feeding of ewe lambs, their puberty onset and the related endocrine events in Sardi sheep.Thirty lambs born in autumn were assigned to 3 groups (10 animals per group): HH fed a high-level diet, LL a low-level diet and LH a low-level diet during 2 months (period 1) followed with a high level diet during 4 months (period 2). The low-level diet consisted of straw (500g) and concentrate (200g) while in high-level diet the concentrate (1kg per animal per day) and straw were fed ad libitum. The assay started on July the 1st when the lambs were 7 months old and an average weight of 22 kg, and finished on December the 30th.The lambs were weighed at birth, at weaning and at 2 week intervals thereafter until the end of the experiment. Blood samples were taken three times per week for measuring progesterone, FSH, GH and leptin concentration. Plasma P4 level > 1 ng/ml is a reliable indicator for an active corpus luteum and is related to cyclic ovarian activity.The main results showed a significant difference between growth rates of lambs on low or high diet but the average daily gain remained low 87 ± 9 g/d. The mean live weight at the end of experiment of HH lambs was significantly higher compared to LL and LH lambs (37.4±0.5 vs 24.8±2.7 vs 33.3± 3.3 kg respectively, $p< 0.05$) with HH lambs being 12kg heavier than those of LL group. Similarly, LH lambs were significantly heavier than LL lambs (33.3± 3.3 vs 24.8±2.7 kg, $p<0.05$). There was a partial compensatory growth in fasted-reefed lambs.The onset of puberty was mainly related to the live-weight in all ewe lambs. Thus, the average live weight at puberty was 31.4 ±0.7 kg and 29.8 ± 1.2 kg in HH and LH groups, respectively. In the LL group, however, no ewe lamb reached the puberty at 400 days, but the average LW was then only 25 kg. Before first ovulation, an increase in FSH plasma level was observed (2.5±1.2 vs 0.20 ng/ml) in HH group and (3.4±2.2 vs 0.23 ng/ml) in LH group.The average plasma GH concentrations in the HH group seemed to be lower (20.7±1.03 ng/ml) than in the other groups (25.5±2.6 vs 33.4±3.6 ng/ml, respectively, in the LH and LL)./>No significant relationship was found between the age at puberty (320 ±18 vs 372 ± 9 days) and the plasma leptin levels (1.8±1.1 vs1.9±1.3 ng/ml) in the HH and LH groups, respectively. These results confirm the importance of body development on the appearance of puberty, and raise the possibility of a compensatory growth in the lambs. Elsewhere, FSH concentrations were not significantly altered by feed restriction. The observed absence of relationship between the age at puberty and the plasma leptin levels may reflect the presence of an other metabolite, rather than leptin, to signal the nutritional status to the reproductive axis in lamb.

Elevated non-esterified fatty acid concentrations during bovine oviduct epithelial cell and zygote coculture hamper early embryo development

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Keywords: NEFAs, BOEC/zygote coculture, polarized cell culture system

Maternal lipolytic disorders and the associated systemic rise of non-esterified fatty acids (NEFAs) have been suggested to affect oviduct physiology and functionality. An altered oviduct micro-environment may influence early embryo development, however its consequences remain largely unknown. Therefore, we hypothesize that elevated NEFAs in a polarized cell culture system hamper early embryo development. Furthermore, we state that effects will depend on the presence of bovine oviduct epithelial cells (BOECs) and the direction of NEFA exposure.

In 4 repeats, early luteal BOECs were seeded at $1x10^6$ cells/mL in a polarized cell culture system. After reaching 100% confluency (day 7) monolayers were cocultured with 25 zygotes per insert in 100µL SOF with 10% FBS and 0.75% BSA for 96h. Hereto, bovine oocytes were matured and fertilized *in vitro* following standard procedures. During subsequent BOEC/zygote coculture in SOF, NEFA exposure (720µM containing 210µM oleic acid + 230µM palmitic acid + 280µM stearic acid) was implemented in 3 groups: 1) [APICAL NEFA] i.e. 720µM NEFA + 0.45% EtOH in the apical compartment, 2) [BASAL NEFA] i.e. 720µM NEFA + 0.45%EtOH in the basal compartment, 3) [A/B NEFA+] i.e. 720µM NEFA + 0.45%EtOH in both compartments. Treatments were compared to [SOLVENT+] i.e. 0.45% EtOH in both compartments with BOEC coculture, [A/B NEFA-] i.e. 720µM NEFA + 0.45%EtOH in both compartments but without BOEC coculture, and [SOLVENT-] i.e. 0.45%EtOH in both compartment without BOEC coculture. After 96h, all morulae were transferred to SOFmedium in a 96-well plate without BOEC. Embryo development was assessed using cleavage- (48h pi), morula- (120-126h pi), and blastocyst rates (192h pi).

Data were analysed using binary logistic regression with Bonferroni correction in SPSS, and were considered statistically different when $P<0.05$.

Total cleavage in A/B NEFA+ (51.63%) and A/B NEFA- (43.19%) differed significantly ($P=0.02$), and were lower compared to other treatments. From the cleaved oocytes APICAL NEFA showed an increased percentage of zygotes in 3-cell stage (17.61%; $P=0.032$). Morula rates were on average 28.05% out of total oocytes and 47% out of cleaved oocytes, and similar between all treatments ($P>0.05$). Blastocyst rates (out of total oocytes) were significantly higher in SOLVENT+ and SOLVENT- (26.11% and 22.67% resp) compared to NEFA treatments (12.59%; $P<0.001$). In all treatments, day 8 blastocysts were mostly in expanded stage (55.06%), except for APICAL NEFA which showed 48.14% young blastocysts.

In conclusion, NEFAs negatively affect embryo developmental competence. During cleavage, but not at blastocyst level, these effects are limited to bidirectionally exposed groups, and the cocultivation with BOECs seemed to have beneficial effects. Data suggest that elevated NEFAs in the oviduct may attribute to the complex pathogenesis of sub- and infertility during lipolytic disorders, however, more research is required to further elaborate on potential compensatory effects mediated by the oviduct.

Functional activity of actin cytoskeleton in porcine oocytes during in vitro maturationT.I. Kuzmina*[1], A.V. Molchanov[2], T.I. Stanislavovich[1], and D.N. Tatarskaya[3],
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Keywords: oocyte, cytoskeleton, IVM

Actin is an abundant protein with well established roles in fundamental processes ranging from cell migration to membrane transport (Sun Q. &Schatten H. Reprod. 131, 193–205, 2006). Brilliant cresyl blue (BCB) staining has been used for selection of the functional status of oocytes. BCB^+ oocytes (oocytes that have finished growth phase in vivo) had significantly higher development competence than BCB^- oocytes (oocytes that have not finished growth phase in vivo, Ishizaki C. et al., Theriogenology, 72(1): 72–80, 2009). The aim of the present study was to compare the functional activity of the actin cytoskeleton [the intensity of fluorescence of rhodamine-phalloidin (IFRF) conjugated with actin filaments] in dynamics of meiosis of BCB^- and BCB^+ porcine oocytes. Before IVM compact cumulus oocyte complexes (COCs) were incubated in BCB solution(13 μM) for 60 min. at 38.5°C in 5% CO_2. Then oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored) oocytes. COCs were cultured in maturation medium (NCSU 23) supplemented with 10% follicle fluid (FF), 0.1 mg/ml cysteine,10 IU/ml eCG and 10 IU/ml hCG at 38.5°C in a humidified atmosphere containing 5% CO_2. FF was collected from follicles with 3 - 6 mm in diameter. COCs cultured in maturation medium with pieces of follicle's wall (600-900 μm in length, Abeydeera L, et al., Biol Reprod. 58:213-218.1998). After 22 h of culture COCs and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for next 22 h of culture. For assessment of chromatin and IFRF fixed oocytes were incubated sequentially in rhodamine-phalloidin (RF, R415 Invitrogen, Moscow, Russia), 1 IU/ml, for 30 min to label actin. Then oocytes were incubated in 4',6-diamidino-2-phenylindole, 10 |μg/ml, for 10 min to label chromatin. Oocytes were examined using confocal laser scanning system Leica TCS SP5 with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543 nm laser lines were used for fluorochrome excitation. IFRF were expressed in arbitrary units. All chemicals used in this study, except for RF, were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test**.** Chromatin status and IFRF of 159 BCB^+ and 101 BCB^- oocytes (total 260 oocytes, in 3 replicates, 10-26 oocytes/group) were evaluated before and after 22 and 44 h of cultivation. Significant differences in IFRF of BCB^+ (33.2 ± 2.2) and BCB^- oocytes (42.6 ± 2.1) were identified before cultivation ($P < 0.05$). There were no differences between the IFRF in BCB^+ and BCB^- oocytes on metaphase-I stage(49.1 ± 6.9 and 51.2 ± 4.8, respectively). The decrease of IFRF in BCB+ oocytes was found after 44 h of IVM (49.1 ± 6.9 and 35.8 ± 6.3 ,respectively, $P < 0.01$). There were no differences between the IFRF in BCB^- oocytes on metaphase-I (51.2 ± 4.8) and methaphase II (49.1 ± 6.9) stages. Overall our data clearly showed that actin cytoskeleton actively involves in maturation of porcine oocytes in vitro. Features of the functional activity of actin cytoskeleton in BCB^+ and BCB^- oocytes during IVM have been identified.

Modifying the fastness to age-related alterations of in vitro maturing bovine oocytes by luteotrophic factors and granulosa cells

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Keywords: progesterone, prolactin, oocyte aging

The quality of mammalian oocytes determines their ability to embryonic development. Once the oocyte matures to the metaphase-II (M-II) stage, it undergoes accelerated senescence processes leading to an impairment of its quality. The objective of this work was to study effects of prolactin (PRL), progesterone (P4), and granulosa cells during the completion of in vitro maturation of bovine oocytes on their subsequent resistance to age-related functional alterations. Bovine cumulus-enclosed oocytes (CEOs) were cultured for 12 or 24 h in the first IVM medium (TCM 199 supplemented with 10% fetal calf serum (FCS), 10 μg/ml FSH, and 10 μg/ml LH) at 38.5°C and 5% CO_2. After the 12 h-culture, the CEOs were transferred to the second IVM medium (TCM 199 supplemented with 10% FCS) and matured for next 12 h in the absence and in the presence of granulosa cells (GCs) preliminary cultured under the same conditions for 12 h. The following additives to the second IVM medium were applied: (1) no additives (Control), (2) 25 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia), (3) 50 ng/ml PRL, and (4) 50 ng/ml P4. Then CEOs were cultured for additional 24 h in the aging medium (TCM 199 containing 10% FCS). At the end of culture, the state of the oocyte nuclear material was evaluated by the Tarkowski's method. Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA). The data for the nuclear status (n=4, 80-91 oocytes per treatment) and apoptosis (n=5-6, 91-121 oocytes per treatment) were analyzed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). During 24 h aging of CEOs matured in the first IVM medium without transfer, the rates of M-II oocytes with destructive changes of chromosomes (decondensation, adherence, clumping) and apoptotic oocytes rose ($P < 0.001$) from 28.6 ± 2.1% and 10.1 ± 1.4% (prior to aging) to 67.1 ± 2.0% and 24.3 ± 0.4%, respectively. Similar age-related increases in these rates were observed in case of CEOs matured during two-step culture. In the absence of GCs, P4 decreased the frequency of chromosome abnormalities in aged oocytes from 68.5 ± 1.9 (Control) to 51.2 ± 2.9% ($P < 0.01$), whereas PRL did not. Maturation of CEOs in the presence of GCs and PRL resulted in a reduction (at least $P < 0.01$) in the rate of oocytes with abnormal chromosome modifications following aging (from 67.9 ± 2.3% (Control) to 50.6 ± 3.9% (25 ng/ml of PRL) and 46.5 ± 5.0% (50 ng/ml of PRL)). By contrast, the addition of GCs to the second IVM medium abolished the positive effect of P4 ($P < 0.001$). Furthermore, P4 caused a decline ($P < 0.05$) in the rate of aged CEOs with apoptotic signs matured in the absence of GCs (from 24.3 ± 0.4% (Control) to 17.6 ± 1.6%). Thus, during the completion of bovine oocyte maturation, PRL and P4 can raise the subsequent resistance of aging ova to age-related changes in their quality, with GCs being able to modulate the hormonal effects.

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Effect of different estrous sheep serum batches on sperm capacitation and *in vitro* embryo development

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Keywords: sperm capacitation, *in vitro* fertilization, estrous sheep serum

Nowadays, the estrous sheep serum (ESS) is the additive used to capacitate ram spermatozoa in *in vitro* fertilization systems. This non-defined substance is present during the co-incubation of spermatozoa and oocytes. The main problem of using non-defined substances is the need to test their validity and the effect of these substances on future embryo (García-Álvarez et al., Theriogenology 84:948-956 2015). The aim of this work was to assess the effect to capacitate thawed ram spermatozoa with different batches of estrous sheep serum on tyrosine phosphorylation, key event in capacitation, and on embryo yield. A pool of thawed semen from three rams was used to carry out the analysis of tyrosine phosphorylation by western blot and an *in vitro* fertilization trial. After discontinuous density gradient on Percoll, spermatozoa were incubated for 15 min in synthetic oviductal fluid (SOF) with 10% of three ESS batches (ESS1, ESS2 and ESS3). A negative control (SOF without ESS) was also used (NCap). The intensity signal of different bands was analysed with C-Digit® Blot Scanner from LI-COR and relativized to Tubulin. For the *in vitro* fertilization 395 oocytes were used and was performed according to García-Álvarez et al. (García-Álvarez et al., Theriogenology 84:948-956 2015). General lineal models were used with the following dependent variables: signal intensity, cleavage rate at 48 h post insemination (cleavage rate) and percentage of blastocyst at 9 days (embryo rate), and fixed variables: treatment (NCap, ESS1, ESS2 and ESS3) and replicate. There were no differences in the band intensity between treatment. However, cleavage and embryo rates were different for NCap and ESS1, ESS2 and ESS3 (0±9, 41±5, 46±6, 56±5, and 0±7, 27±5, 29±5, 31±5, respectively) but were similar between the ESS batches. In conclusion, the ESS batches do not influence sperm capacitation, cleavage and embryo rates although the presence of ESS is necessary to fertilize oocytes. Nevertheless, more studies of the quality of these embryos are necessary.

Improvement of *in vitro* produced bovine embryo quality using Charcoal:Dextran Stripped Fetal Bovine Serum on culture media

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Keywords: CDS FBS, HI FBS, bovine embryo

This study investigated the effect of Charcoal:Dextran Stripped fetal bovine serum (CDS FBS) and heat-inactivated FBS (HI FBS) in embryo culture medium (SOF-BE1 medium supplemented with 10% of serum) on their ability to support *in vitro* development of bovine embryos. Charcoal:Dextran treatment of FBS removes lipophilic chemicals, certain steroid hormones and certain growth factors. The developmental ability and quality of bovine embryos were determined by assessing their cell number, lipid content, mitochondrial activity, gene expression, and cryo-tolerance. The differences in embryo development (350 oocytes per each group were cultured in six replicates), integrated optical intensity, and expression levels of the various genes between experimental groups were analyzed by one-way ANOVA. The percentages of embryos that underwent cleavage and formed a blastocyst were significantly ($P < 0.05$) higher in medium containing CDS FBS than in medium containing HI FBS (42.84 ± 0.78% vs. 36.85 ± 0.89%, respectively). The total number of cells per day 8 blastocyst was ($P > 0.05$) higher in the CDS FBS group (208.40 ± 14.77) than in the HI FBS group (195.11 ± 19.15), however, this difference was non-significant. Furthermore, the beneficial effects of CDS FBS on embryos were associated with a significantly increased mitochondrial activity, as identified by MitoTracker Green, and reduced intracellular lipid content, as identified by Nile red staining, which increased their cryo-tolerance. The post-thaw survival rate of blastocysts was significantly ($P < 0.05$) higher in the CDS FBS than in the HI FBS group (85.33 ± 4.84% vs. 68.67 ± 1.20%). Quantitative real-time PCR showed that the mRNA levels of acyl-CoA synthetase long-chain family member 3, acyl-coenzyme A dehydrogenase long-chain, hydroxymethylglutaryl-CoA reductase, and insulin-like growth factor 2 receptor were significantly increased upon culture with CDS FBS. Moreover, the mRNA levels of sirtuin 1, superoxide dismutase 2, and anti-apoptotic associated gene B-cell lymphoma 2 in frozen-thawed blastocysts were significantly ($P < 0.05$) higher in the CDS FBS group than in the HI FBS group, however, the mRNA level of the pro-apoptotic gene BCL2-associated X protein was significantly reduced. Taken together, these data suggest that supplementation of medium with CDS FBS improves *in vitro* bovine embryo developmental competence and cryo-tolerance.

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Novel approach for measuring mitochondrial function in bovine oocytes and embryos

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Keywords: Oxygen consumption, Oocytes, Metabolism

Oxygen consumption is an established marker for cellular energy metabolism and an indicator of mitochondrial function. In reproductive biology, it has been correlated to a range of outcomes including oocyte viability and maturation, embryo development, implantation potential and pregnancy rate. However, measuring oxygen consumption is technically challenging, requiring specialist equipment. The recent availability of Seahorse Bioanalysers has transformed the study of cellular metabolism in a range of systems, however to date, this technology has not been applied to oocytes and embryos. We have therefore examined whether Seahorse XFp is capable of measuring oxygen consumption of small groups of oocytes and embryos.

Bovine oocytes were collected from abattoir-derived ovaries and cultured overnight in maturation media (nutrient and hormone-supplemented M199). Oocytes were either allowed to mature or treated with cycloheximide to maintain their immature state. 2-cell embryos generated by IVF were selected after culture for ~24 hours in synthetic oviduct fluid (SOF). Media used was made up in-house as has been reported previously (Guerif et al., PloS One, Volume 8, e67834, 2013). Oocytes or embryos were loaded into Seahorse XFp bioanalyser plates in groups of 6 and oxygen consumption rate (OCR) was measured non-invasively. To further probe the constituents of oxidative function in bovine oocytes, mitochondrial uncoupler FCCP, and electron transport chain inhibitors oligomycin and Antimycin A/rotenone were serially injected (Sigma Aldrich). Assays were repeated on three independent occasions. Data was analysed using one way ANOVA with Tukey's post-hoc.

Using this approach, we were able to generate reproducible OCR values for bovine oocytes and embryos. 2-cell embryos were significantly different ($p<0.01$) to germinal vesicle (GV) stage and metaphase-II (met-II) stage oocytes – 0.62 ± 0.15 pmol/min/embryo, compared to 2.36 ± 0.22 and 1.83 ± 0.31 pmol/min/oocyte respectively (mean ± SEM). Crucially, fertilisation rates for oocytes having undertaken the assay were not significantly different to controls ($p>0.05$). The response to mitochondrial inhibitors, shown in real-time, indicated that the approximately 60% of oocyte OCR was coupled to ATP synthesis, 20% was non-mitochondrial with the remaining being proton leak. Furthermore, oocytes have the capacity to increase OCR by approximately 60% spare capacity.

These data demonstrate the use of the Agilent Seahorse XFp as a technique for the direct assessment of mitochondrial function in bovine oocytes and embryos. Importantly, we demonstrate that oocytes are competent to undergo fertilisation after this assay, indicating the non-invasive nature of the test. Compared to previously applied assays for oxygen consumption, Seahorse is fast, simple and automated, allowing investigations of higher throughput. With increasing recognition of the critical role mitochondria play in supporting healthy reproduction; this tool facilitates investigation into mitochondrial function which has extensive scope for applications within reproductive biology.

Bovine embryos release extracellular vesicles into the medium during group culture *in vitro*

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Keywords: embryo-embryo communication, extracellular vesicles, embryo culture

Efficient communication between cells and tissues is paramount in many physiological process, including embryo development. Inside the genital tract, embryos usually communicate with the mother and vice versa through autocrine, paracrine and endocrine signaling. From our previous research (Wydooghe et al. Reprod. Fertil. Dev, 26, 115, 2013) it has been clearly demonstrated that, in the absence of maternal genital tract, preimplantation embryos cultured in group are able to promote their own development *in vitro* by the production of autocrine embryotropins. Recent studies indicate that among autocrine secreted factors extracellular vesicles play a prominent role in communication. Extracellular vesicles are membrane bound vesicles that are found in biological fluids and in culture media conditioned by embryos or cells. They carry and transfer regulatory molecules, such as microRNAs, mRNAs, lipids and proteins. Here we show that preimplantation bovine embryos cultured in group can release extracellular vesicles into the medium, as novel way of embryo communication. The aim of the current study is to standardize a protocol for isolation and quantification of extracellular vesicles from culture medium conditioned by bovine embryos. Since BSA(Sigma A9647) may contain extracellular vesicles, for optimization of this protocol, bovine presumed zygotes (n = 1140, 4 replicates) produced *in vitro* were allocated to two culture media (SOF with insulin, transferrin and selenium supplemented with either 0.4% BSA (Sigma A9647) or with 0.1 % PVP(Sigma P5288)) and were cultured until Day 8. Media conditioned by embryos were pooled at day 8 until 1ml was obtained, and subjected to density gradient ultracentrifugation (Van Deun et al., J. Extracell. Vesicles. 3, 2014) to extract extracellular vesicles. Extracted suspension with extracellular vesicles was analyzed with Nano particle tracking for quantification. For identification, negative staining electron microscopy was performed, and specific antibodies CD9 (CST), CD63(Serotec) and TSG101(Abcam) were tested for further confirmation of extracellular vesicles presence in the extracted suspension. Blastocyst development rate on day 8, was analyzed by using Student t test (Statistical Analysis System (SAS) for Windows) had showed no significant difference between both media's (40±3.43 % vs 38.64±2.88 %; in SOF+BSA vs SOF+PVP respectively). Results obtained from extracellular vesicles quantification and identification analysis provided evidence that bovine embryos can release extracellular vesicles with a size ranging from 40~200nm into the culture medium. The concentration of extracellular vesicles extracted from 1ml of conditioned medium was 9.18×10^7 +/- 4.52×10^7 particles/ml. Further experiments will be performed to extract a higher concentration of extracellular vesicles from a limited amount of medium conditioned by embryos, by following different isolation techniques, such as size exclusion chromatography and ultra-centrifugation.

The effect of diet on fatty acid composition of elongated bovine conceptuses

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Keywords: dairy cattle, CLA, milk fat depression

Conceptus elongation is crucial for establishment of pregnancy in ruminants. During the elongation step, endometrium secreted lipids are required as a source of energy and for the remodeling and proliferation of cellular membranes (Ribeiro et al., Reprod 2016;152:R115-R126). The present study investigated the effects of diets designed to cause 15 % reduction in milk fat synthesis (milk fat depression) on fatty acid (FA) composition of elongated bovine conceptuses. The day-14 conceptuses were collected from 22 Nordic Red multiparous dairy cows during two sequential indoor housing periods. Treatments comprised a grass silage based basal diet (CO; 10 cows), basal diet supplemented with 12 g/d of rumen protected *trans*-10, *cis*-12 CLA (Lutrell Pure, BASF, Germany) (CLA; 8 cows), and grass silage based diet containing high-starch concentrate components and supplemented with 26.7 g/kg diet dry matter of sunflower oil and 13.3 g/kg diet dry matter of fish oil (MFD; 4 cows). CLA supplement was mixed in total mixed ration and administered in two equal proportions per day. Oil supplements replaced concentrate ingredients. Cows were randomly allocated to the treatments immediately after parturitions. The diets were total mixed rations with 55:45 forage:concentrate ratio on dry matter basis. In total of 45, 35 and 13 conceptuses having visible embryonic discs were used after recovery from the superovulated CO, CLA and MFD donors 130 days after parturitions, respectively. Lipids were extracted separately from each cryopreserved conceptus using a mixture of hexane and 2-propanol (3:2, vol/vol). FA were transesterified to methyl esters using methanolic sodium methoxide and analyzed with a gas chromatograph equipped with a flame ionization detector (Shingfield et al., Anim Sci 2003;77:165–179). Data were analyzed using linear mixed models with MIXED procedure in SAS 9.4. The proportions of the most abundant FA in conceptuses, *cis*-9 18:1 (30-32 g/100g FA, %), 16:0 (25-26 %), and 18:0 (12-14 %), did not differ between treatments ($P > 0.10$). In addition, total proportions of *cis* unsaturated FA (53-54 %) and saturated FA (43-45 %) and ratio of *cis* unsaturated FA to saturated FA was not different among treatments ($P > 0.10$). CLA had no effect on the conceptus' FA composition compared with CO. However, MFD induced higher ($P < 0.01$) 22:6n-3, and lower ($P < 0.05$) 22:4n-6 and 22:5n-6 proportions compared with CLA and CO. The proportion of *cis*-12 18:1 and *trans*-9, *cis*-12 18:2, which are biohydrogenation products of 18:2n-6 found in rich amounts in sunflower oil, tended to be higher ($P < 0.10$) in MFD compared with other treatments. In conclusion, although CLA and MDF caused changes in lipogenesis in the mammary gland and milk fat depression, a substantial effect on the FA composition of conceptuses was not observed. However, unsaturated FA deriving from the MFD diet and metabolized in the rumen biohydrogenation processes had a specific impact on conceptus FA profiles during elongation stage.

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Expression profile of genes involved in sex determination in cattle

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Keywords: sex determination, cattle, gonad

Sex determination is the process by which a bipotential gonad develops into a testis or ovary depending on the genetic background of the individual. There is a paucity of information about this process in large mammalian species. Bovine *SOX9* (a critical gene for sex determination in mice and humans) has lost the two transactivation motifs that are essential for sex determination in mammals; therefore, an alternative sex determination pathway could be responsible for sex determination in cattle. In order to clarify the genes involved in gonad differentiation in cattle we performed quantitative analysis of RNA expression in the genital ridges of bovine foetuses collected at the abattoir. The age in days from conception was estimated based on the crown-rump length (CRL). We collected 14 female foetuses ranging in CRL from 18 mm (Day 38 of development) to 57 mm (Day 59) and 19 male foetuses ranging from 13 mm (Day 33) to 48 mm (Day 56). The sex of the embryos was determined by PCR with primers for bovine amelogenin. RNA was extracted and converted to cDNA using a reverse transcription kit (Applied Biosystems, Carlsbad, CA). All qPCR reactions were carried out using a PCR mix (GoTaq® qPCR Master Mix, Promega Corporation, Madison, USA) containing the primers selected for the bovine genes *SF1* and *WT1* (related with gonadal formation)*; FOG2*, *GATA4*, *SOX9*, *SRY*, *DMRT1* (involved in sex determination and testis development); *WNT4*, *FOXL2* (participating in ovary formation and maintenance, respectively); and *ZRSRY2*, *SOX8*, *SOX10* (candidates genes potentially involved in testis determination in cattle). The reference gene used was H2AFZ. In addition, the location of the primordial germ cells (PGC) was evaluated by immunohistochemistry to identify testis and ovary formation differences between the sexes after SRY peaks. Genital ridges were fixed and stored in Bouin's solution for immunohistochemistry of cell marker OCT4.

We found that *SRY* expression peaked at a CRL of 18 mm (Day 38). We detected expression of *SOX8* and *SOX10* in male foetuses after the SRY peak (earlier than observed in mice and humans), and *ZRSR2Y* (a splicing factor related to RNA processing and RNA splicing) expression along all the stages analysed showing an increasing pattern from Day 33 to Day 56. Regarding immunohistochemistry, we identified that PGC follow two distinct patterns in males and females. Before SRY peak, PGC localize along the genital ridges of both sexes. After SRY peak, testis cords begin to be distinguishable at a CRL of 25 mm (Day 42) in males, with one to three PGC within each of the developing tubules. In the case of females, PGC tend to distribute along the periphery of the developing ovary at a CRL of 36 mm. Overall, these results indicate that sex determination in bovine genital ridges present characteristic features with SOX8 and SOX10 showing early expression after SRY peak, and ZRSR2Y as a splicing factor that could be involved in sex determination.

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Nuclei number and DNA fragmentation in pig embryos derived from IVF, in vivo-IVC and in vivo-derived blastocysts evaluated by TUNEL assay

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Keywords: pig, embryos, quality

Embryo quality is assessed on the basis of the rate of development, morphology, number of cell nuclei and the degree of apoptosis of the embryonic DNA. Detection of apoptosis in embryos is possible due to the occurrence of DNA fragmentation, the degree of which can be determined using the TUNEL assay. The aim of the study was to compare the quality of pig embryos obtained after IVF (IVP), *in vivo*-IVC (*in vivo* zygote and *in vitro* culture) with *in vivo*-derived blastocysts (control) by TUNEL assay. IVP embryos were obtained from *in vitro*-matured, *in vitro* fertilized oocytes. The putative zygotes were cultured in NCSU-23 medium at 39^{o}C and 5% CO_2 in the air up to the blastocyst stage. *In vivo*-IVC zygotes were obtained surgically from superovulated and inseminated donor gilts and cultured in NCSU-23 medium up to the blastocyst stage. *In vivo*-derived blastocysts were obtained surgically on the sixth day after insemination from superovulated and inseminated donor gilts by flushing the uterus with PBS-BSA solution. The IVP, *in vivo*-IVC and *in vivo*-derived blastocysts were subjected to TUNEL assay according to the manufacture protocol (TUNEL reagent In Situ Cell Detection Kit, Roche Diagnostic, Germany). The analysis was carried out under an epifluorescence microscope using the following filters: 520 nm (TUNEL) and 358-461 (DAPI). Statistical analysis was performed using the t-test. It was observed that the mean number of cell nuclei was statistically significantly higher in *in vivo* embryos compared to *in vivo*-IVC and IVP embryos (106.47; 39.20 and 38.73; respectively, $P<0.01$). In turn, the mean number of apoptotic nuclei was significantly higher in embryos derived *in vivo*-IVC compared to IVP and *in vivo* embryos (2.56; 1.63 and 0.06 respectively, $P<0.01$). The TUNEL index was 4.20% for IVP, 0.06% for *in vivo*-derived blastocysts and 6.53% for *in vivo*-IVC blastocysts. The study showed that quality of IVP and *in vivo*-IVC embryos was lower compared to the quality of embryos derived *in vivo*. The quality of the embryos thus obtained is mainly affected by the *in vitro* culture conditions.

Investigating the impact of hyperglycaemia on bovine oviduct epithelial cell physiology and secretions *in vitro*

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Keywords: oviduct, hyperglycaemia, epithelium

A key role of the oviduct, or Fallopian tube, is the creation of the environment where fundamental developmental processes take place, including gamete activation, fertilisation and early embryo development. Previous studies have partially determined the composition of oviduct fluid. However, the impact of maternal physiology on the oviduct environment is unknown.

The aim of this study was to investigate the impact of a hyperglycaemic challenge on the physiology of oviduct epithelial cells in vitro as well as the biochemical and physical properties of oviduct-derived fluid, using an air:liquid model of the oviduct.
Bovine oviduct epithelial cells, harvested from slaughterhouse-derived tissues (mainly stage II reproductive tracts), were cultured in DMEM-F12, at 39oC and 5%CO2 for 6 days. Cell identity was confirmed using confocal and optical microscopy. The cells were grown to confluence on a permeable membrane, allowing selective transportation of nutrients between apical and basal chamber. TransEpithelial Electrical Resistance (TEER) measurements, >700Ohm.cm2 were used to indicate the barrier properties of the epithelial monolayer(n=4). This was independently confirmed by observing that fluorescein was unable to cross the monolayer(n=3), when the cells were determined confluent by TEER measurements. Once confluence was achieved, the apical medium was discarded and cells cultured in an air:liquid interphase, mimicking the in vivo environment. After 24h, a thin film of fluid accumulated, which was collected for biochemical analysis. In Experiment 1, physiological (7.3mM) and hyperglycaemic (8.5mM,11mM) concentrations of glucose were added together with 20ng/ml of insulin to the basal compartment for 24h. In Experiment 2, cells were exposed to the same conditions but for 7 days. Data were analysed using Kruskal-Wallis test with Dunn's post-hoc. Chemicals and consumables were used as previously (Simintiras et al, Reproduction, 153,23–33,2017).

Our data revealed that acute hyperglycaemia in the basolateral compartment did not change the luminal concentrations of glucose, pyruvate or lactate. However, the presence of insulin reduced glucose in the lumen when cells were exposed to hyperglycaemia. By contact, 7-day basolateral exposure to hyperglycaemia in the absence of insulin increased luminal concentrations of glucose (1.09mM for normoglycaemia compared to 8.9mM for chronic hyperglycaemia). Notably, the presence of insulin reduced the volumes of oviduct-derived fluid (6.24µl in hyperglycaemia compared to 75.5µl for normoglycaemia ($p<0.05$).

Using an in vitro oviduct model we have shown that long term exposure to hyperglycaemia induces glucose transport in oviduct secretions and that insulin appears to reduce fluid flow across the oviduct monolayer. Future work will focus on investigating differences in gene expression in response to hyperglycaemia, as well as a detailed evaluation of how insulin affects ion transport. Furthermore, we will determine the impact of hyperglycaemic-conditioned oviduct fluid on gamete maturation and early embryo development.

Effect of oxygen tension on the development of *in vitro* embryos from Iberian red deer (*Cervus elaphus hispanicus*).

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Keywords: 'Oxygen tension', 'Iberian red deer', '*In vitro* fertilization'.

Culture conditions during *in vitro* oocyte maturation and fertilization, such as exposure to different oxygen concentrations, have been shown to affect in the developmental ability and the generation of reactive oxygen species (ROS). Low oxygen concentrations may significantly improve the developmental potential of cleavage stage embryos, thus resulting in a positive effect on subsequent blastocyst formation in different species (Leoni et al., Reprod Domest Anim 42(3):299–304. 2007). With the aim to improve the *in vitro* production of embryos in the Iberian red deer (*Cervus elaphus hispanicus*), we evaluated the influence of two oxygen tensions (5 and 21%) during *in vitro* maturation (M) and fertilization (F) on developed blastocysts. The *in vitro* embryo production was performed as García-Álvarez et al., Theriogenology 75:65-72, 2011 and Berg et al., Anim Reprod Sci 70:85-98. 2002. Similarly, we analysed differences in gene expression of the resulting expanded blastocysts. To assess embryo production, a total of 588 COCs were divided into four experimental groups that were evaluated according to the oxygen tension used (M5F5, M5F21, M21F5 and M21F21). Relative poly(A) mRNA abundance of *GAPDH*, *G6PH*, *HPRT*, *SOD2*, *BAX*, *SHC1*, *AKR1B*, *PLAC8*, *GJA1* and *SOX2* was analyzed using quantitative real-time RT-PCR (qRT-PCR). General linear models were used with the independent variable being percentage of total blastocysts at 9 days (embryo rate=96 blastocysts); and the fixed variables being treatment (M5F5, M5F21, M21F5 and M21F21) and replicate (n=7). Additionally, relative mRNA abundance differences in blastocysts were analyzed by one way ANOVA. Results showed that regardless of the oxygen concentration, blastocyst rates did not differ ($P \geq 0.05$) (M5F5=21.98±6.26; M5F21=12.46±5.78; M21F5=18.21±2.90; M21F21=20.54±5.75). With regard to gene expression, *SOD2* was up-regulated ($P<0.05$) in oocytes matured in low oxygen, independently of the tension used during fertilization, whereas *SOX2* was down-regulated ($P<0.05$) in oocytes that were also matured in low oxygen but fertilized in high oxygen tension ($P<0.05$). Likewise, *AKR1B* and *PLAC8* were up-regulated ($P<0.05$) when oocytes were matured and fertilized under high tensions. To our knowledge, this is the first study that demonstrates that Iberian red deer embryos can be produced *in vitro* using different oxygen tensions. Although the four groups compared do not reflect significant differences in terms of embryo production, the use of different oxygen tensions during *in vitro* maturation and fertilization significantly alters the expression of genes related to oxidative stress (*SOD2*), implantation (*AKR1B1* and *PLAC8*) and transcription factors involved in the regulation of embryonic development and determination of cell fate (*SOX2*). In conclusion, both oxygen tensions (5 and 21%) result in similar embryonic development and therefore are feasible for *in vitro* production of Iberian red deer embryos, but more studies are necessary to determine blastocyst quality.

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Regulatory actions of progesterone and luteotrophic hormones on bovine oocyte apoptosis during the terminal phase of in vitro maturation

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Keywords: progesterone, luteotrophic hormones, oocyte apoptosis

The currently available evidence points out the cumulus-derived progesterone (P4) as a key antiapoptotic signal involved in maintaining the bovine oocyte viability during in vitro maturation (O'Shea et al., Biol Reprod, 89:146, 2013). However, effects of exogenous P4 on the oocyte quality are not quite clear. The aim of the present research was to compare actions of P4 and two luteotropic hormones, prolactin (PRL) and LH, on apoptosis of bovine oocytes during the second phase of in vitro maturation (from M-I to M-II). Bovine cumulus-oocyte complexes (COCs) were cultured for 12 h in TCM 199 containing 10% fetal calf serum (FCS), 10 μg/ml of porcine FSH, and 10 μg/ml of ovine LH at 38.5°C and 5% CO_2. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Then COCs were transferred to the following culture systems: (1) TCM 199 containing 10% FCS (Control 1) and (2) a monolayer of granulosa cells (GCs) precultured for 12 h in TCM 199 containing 10% FCS (Control 2). Just before the oocyte transfer, the medium of experimental groups was supplemented with either 50 ng/ml of P4 or 50 ng/ml of bovine PRL (Research Center for Endocrinology, Moscow, Russia) or 10 μg/ml of ovine LH. At the end of culture, the nuclear status of oocytes was assessed by staining with DAPI. Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA). The content of P4 and estradiol-17β (E2) in culture media was determined by ELISA. All data (n=5, 87-99 oocytes per treatment) were analyzed by ANOVA, with percentage data being arcsine transformed. After 24 h of culture, the rate of M-II oocytes was similar in the compared groups and reached 80.3-89.2%. The addition of P4 to the control medium of both systems resulted in the reduction (P<0.05) of the apoptosis frequency in matured oocytes from 11.7±1.2 to 5.9±1.7% (System 1) and from 13.8±1.6 to 7.2±0.9% (System 2). In the absence of GCs, PRL and LH did not affect oocyte apoptosis. When COCs were cocultured with GCs, the apoptosis rates increased (P<0.05) from 9.4±1.6 (without GCs) to 16.3±1.8% for the PRL groups and from 13.0±1.5 (without GCs) to 17.6±2.2% for the LH groups. Meanwhile, in the presence of GCs, these rates were higher than that for the P4 group (P<0.01). At the end of oocyte culture in both systems, the content of P4 in the medium was 1.2-1.3 times lower (P<0.05) in groups treated with PRL or LH than in the group treated with P4, whereas the content of E2 did not differ between groups tested. Furthermore, concentrations of P4 and E2 were increased 1.2-1.3 times (at least P<0.05) in the presence of GCs regardless of the hormonal treatment. Our findings indicate that exogenous P4 can exert granulosa-independent antiapoptotic action on bovine oocytes during the second phase of in vitro maturation. At the same time granulosa cells are able to cause proapoptotic effects of PRL and LH on the oocytes that complete maturation.

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Effect of prolactin and dithiothreitol during prolonged culture of aging oocytes on the development potential of parthenogenetic bovine embryos

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Keywords: oocyte aging. parthenogenetic embryos

The evaluation of factors responsible for the protection of the oocytes attained the metaphase-II stage from aging is important for successful in vitro embryo development. The aim of the present research was to study dose-dependent effects of two potential regulators of oocyte quality, prolactin (PRL) and dithiothreitol (DTT), during the prolonged culture of bovine oocytes on their developmental potential after artificial activation. Slaughterhouse-derived cumulus-oocyte complexes (COCs) were matured for 22 h in TCM-199 supplemented with 10 % fetal calf serum (FCS), 0.2 mM sodium pyruvate, 10 μg mL^{-1} porcine FSH, and 10 μg mL^{-1} ovine LH. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 22 h maturation, the part of COCs were transferred to the fresh medium consisting of TCM 199 supplemented with 10 % FCS and cultured for 10 h in the absence (Control) or in presence of bovine PRL (20 and 50 ng mL^{-1}; Research Center for Endocrinology, Moscow, Russia) or DTT (2.5, 5 and 10 μM). After maturation (22 h) or the prolonged culture (10 h), oocytes were activated by culturing in 5 μM ionomycin solution during for 5 min followed by 4 h in 2 mM 6-dimethylaminopurine. Activated oocytes were cultured in CR1aa medium (Rosenkrans, First, J Anim Sci 1994, 72:434-7) until Day 5 and then transferred to the same medium supplemented with 5 % FCS and cultured up to Day 7. All the cultures were performed in at 38.5°C and 5% CO_2 in humidified air. At Days 2 and 7 after activation, the cleavage and blastocyst rates were determined. In addition, obtained blastocysts were fixed with 4% paraformaldehyde, and the total cell number was determined by DAPI staining. The data from 4 replicates (111-122 oocytes per treatment) were analyzed by ANOVA. For oocytes activated just after IVM, the cleavage and blastocyst rates, and total blastocyst cell number were 74.1±3.5 and 20.6±2.8 %, and 54,0±1,8, respectively. The prolonged culture of matured COCs in the aging medium (10 h) (Control) had no effect on the cleavage rate (74.7±2.9 %) and the total number of cells in embryos (49.1±2.0), but caused the blastocyst yield to decline to 9.8±1.2 % ($p<0.05$). At the same time, the addition of both PRL (50 ng mL^{-1}) and DTT (5 μM) to the aging medium raised the blastocyst rate to 18.0±3.1 and 18.8±2.5 % ($p<0.05$), respectively. Cleavage rates of aging oocytes after their activation and total cell number in blastocyst produced from aging oocytes were unaffected by both PRL and DTT (except DTT 10 μM group). In the case of DTT 10 μM, the blastocyst rate and total blastocyst cell number (9.6±1.3 % and 35.4±1.1, respectively) was lower than in the DTT 5 μM group ($P<0.05$). Thus, PRL and DTT are able to maintain competence for parthenogenetic development of bovine COC during their prolonged in vitro culture.

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Initial characterization of bovine embryos developing at the air-liquid interphase on oviductal epithelial cells

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Keywords: Air-liquid interphase, oviductal epithelial cells, embryo development

Air-liquid interphase cultures of bovine oviductal epithelial cells (ALI-BOEC) have recently been reported to support embryo development in co-culture up to the blastocyst stage without the addition of embryo culture medium.To initially characterize such ALI-BOEC produced embryos, we assessed the expression of 41 target genes in 8-cell embryos and blastocysts. For comparison, we analyzed embryos produced in standard IVP media (IVP-S) and in a commercial serum-free media suit (IVP-SF; IVF Bioscience, Falmouth, UK). A total of fifteen 8-cell embryos (randomly divided into 3 pools of 5 embryos) and six single blastocysts were collected under each culture condition. Gene expression was analyzed by means of a 48.48 Dynamic Array™ on a Biomark HD instrument. To identify genes differentially expressed in 8-cell embryos and blastocysts, we applied one-way ANOVA with Tukey post-hoc test in SPSS. Furthermore, we re-analyzed previously published transcriptomics data from *in vivo* embryos (GSE12327).

The cleavage rate in ALI-BOEC co-culture (70.71%) was comparable to the standard IVP procedure (74.75%), and lower than the cleavage rate reached with the commercial media suit (90.79%). However, the blastocyst rate in ALI-BOEC co-culture (9.1%) was much lower than in either IVP-S (33.1%) or the commercial IVP-SF system (54.7%). Re-analysis of the *in vivo* data set revealed that sixteen of the chosen target genes were significantly regulated between the 8-cell and blastocyst stage embryos *in vivo*. The *in vitro* embryos showed expression patterns similar to the *in vivo* embryos. The culture conditions lead to differential gene expression in both 8-cell embryos (CDH1, NOS2, OVGP1, APEX1, REX1, PLAGL, BAX, SREBP1, SMPD2) and blastocysts (CCL26, CDH1, NID2, IFNAR1, SLC2A5, SREBP1, SERPINE1, LDLR, CYP51A1), respectively. Five of the genes differentially expressed in blastocysts from different culture conditions (LDLR, CDH1, NID2, SLC2A5 and CYP51A1) were previously reported to also be differentially expressed between *in vivo* and *in vitro* blastocysts. Embryos produced in the ALI-BOEC co-culture system followed the *in vivo* expression pattern for all five genes.

The present study confirmed that the ALI-BOEC co-culture system is much less efficient in supporting blastocyst formation than conventional IVP procedures. Given the lack of a direct comparison to *in vivo* embryos, interpretation of the biological relevance of the differentially expressed genes warrants caution. However, our results indicate that blastocysts produced on ALI-BOEC may have an improved *in vivo*-like gene expression signature. The establishment of a sequential culture system of oviductal and uterine epithelial cells including a hormonal stimulation protocol might further increase the efficiency of the co-culture both quantitatively and qualitatively.

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Maternal metabolic disorders and early embryonic loss: pathways to bridge the gap between bovine embryo quality and endometrial receptivity

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Keywords: nutrient sensing, blastomere differentiation, BEEC responses

The pre-implantation embryo is very sensitive to perturbations in its micro-environment and, therefore, a tight regulation of the embryonic milieu is essential. Such an environment is not assured in females suffering metabolic disorders. Our previous data show that altered nutrient abundance in the embryonic micro-environment results in suboptimal embryonic cell differentiation patterns. Here, we hypothesize that suboptimal nutrient conditions during embryo culture can affect the blastocyst's capacity to participate in the first maternal-embryonic interactions.
Earliest preimplantation phases of embryo development were studied as 'window' for nutrient sensitive manipulations. Embryos (4 repeats; 890 zygotes) were cultured during the first 4 days after fertilization (p.i.) in distinct nutrient conditions: [control] based on serum-free SOF medium; [HIGHGLUC] with 3.5mM glucose; [LOWAA] with 10% lower amino acid concentrations as presented in control. At morula stage, embryos were transferred to monolayers of bovine luminal epithelial endometrial cells (BEEC; subculture 1), in SOF medium + 5% serum, till D8 p.i.. In D8 blastocysts, mRNA expression of 12 genes involved in nutrient sensing, pluripotency and differentiation was analyzed by qRT-PCR. Differently expressed genes (DEG) were identified using (mixed model) ANOVA. Using NGS, transcriptomes of BEEC (4 repeats) exposed to distinct groups of embryos were sequenced and data were normalized by EdgeR.
Blastocysts originating from HIGHGLUC morulae displayed a tendency for increased transcript levels of *PDK1* (P=0.075), a key gene in nutrient sensing regulation. Also a down-regulated expression of the pluripotency marker, *OCT4 (P=0.002),* was observed compared to controls. Transcriptome reaction of **BEEC exposed to the HIGHGLUC embryos** was rather limited. Only 27 DEG genes were identified, of which 20 down- and 7 up-regulated in BEEC exposed to HIGHGLUC embryos compared to control embryos ($Padj$<0.1). Enriched genes involved endoplasmic reticulum activities, whereas cell-cell signalling pathways were down-regulated. **Blastocysts from LOWAA conditions** showed tendencies (P≤0.1) for decreased transcript levels of *SIRT1*, *mTOR*, *GLUT1* and *LDHA*, all involved in mTOR pathways. Also a down-regulated mRNA expression was observed for *OCT4* (P<0.0001) and *SOX2* (P<0.1), both genes involved in pluripotency, and for *ITGB5* (P<0.05) and *CTNN1* (P≤0.1), two blastomere differentiation markers. **BEEC exposed to LOWAA embryos** revealed 120 DEG compared to BEEC exposed to controls ($Padj$<0.1). Here, 63 of the 120 DEG were down- and 57 were up-regulated in the LOWAA condition. Up-regulated genes involved transcription regulation and down-regulated genes concerned inhibition of both Notch and immune responses.
Overall, suboptimal metabolite conditions during the first 4 days of embryo culture can impact on resultant blastocyst cell proliferation and differentiation pathways. Furthermore, BEEC genes were differently regulated when placed in contact with the three distinct groups of embryos.

Cloning, Transgenesis, and Stem Cells

Subfertility and zona pellucida alterations in ZP4 KO rabbits produced by CRISPR

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Keywords: CRISPR, Zona pellucida, rabbit

Mammalian zona pellucida, the glycoprotein layer that surrounds oocytes and embryos up to the blastocyst stage, may be composed by 4 different glycoproteins. One of these proteins, ZP4, is present in the zona pellucida of rabbits, cattle and women, among others, but it is absent in the only species where Knock-out (KO) models were readily available: the laboratory mouse. For this reason, the function of ZP4 remains elusive. CRISPR technology greatly simplifies the generation of KO models in livestock species such as rabbits. In this experiment, we have generated ZP4 KO rabbits, i.e. rabbits lacking ZP4 protein, by CRISPR technology and have compared their reproductive performance to that of heterozygous (Hz) and wild type (wt) rabbits. Delivery rates following natural breeding with males of proven fertility were analysed in 5 animals per experimental group (wt, Hz and KO). Pregnancy was clearly impaired in KO animals, with only one female producing a litter of 4 pups, resulting in a significant reduction in litter size compared to wt or Hz groups (pups delivered: wt 9.2±0.6; Hz 10.6±0.5; KO 0.8±0.8; mean±s.e.m., Kruskal-Wallis ($p<0.05$)). Aiming to elucidate the possible causes of subfertility, ovulation and cleavage rates were assessed following natural mating. Surprisingly, neither ovulation (oocytes ovulated: wt 11.7±1; Hz 15±2.9; KO 13.3±2.9), nor cleavage rates (% of cleavage: wt 81.7±0.1; Hz 95.5±0.1; KO 87.3±0.1) showed significant differences between groups. However, clear morphological differences were noted on the zona pellucida from oocytes ovulated by KO rabbits compared to those produced by Hz or wt rabbits. Zona pellucida thickness was significantly reduced in KO compared to Hz or wt (thickness μm: wt 15.2±1.5; Hz 15.3±1.4; KO 10.9±0.7, ANOVA ($p<0.05$)). Besides, KO rabbits produced irregular zonae pellucida, i.e. not perfectly spherical as in Hz of wt animals, and noticeable less elastic and easier to deform. These results suggest that the impaired fertility in ZP4 KO rabbits is not due to reduced ovulation or cleavage, and that ZP4 may act as a crosslinker of other ZP proteins, conferring mechanical properties to the zona pellucida which are important for embryo survival.

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Does scriptaid-dependent epigenomic modulation of peripheral blood-derived fibroblast-like cells affect the *ex vivo* developmental abilities of caprine□porcine nuclear-transferred embryos to reach blastocyst stage?

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Keywords: SCPT-dependent epigenomic modulation, adult goat peripheral blood-derived fibroblast-like cell, inter-species (caprine□porcine) NT embryo

The present research was carried out to ascertain whether inter-family and inter-genus (caprine→porcine) nuclear-transferred (NT) embryos can acquire and retain the competences to complete their extracorporeal development to the blastocyst stage. To generate inter-species (caprine→porcine) cloned embryos, enucleated *in vitro*-matured pig oocytes were subzonally microinjected and subsequently electrofused with adult goat peripheral blood-derived fibroblast-like cells (AGPB-FLCs) that either had been epigenetically transformed by exposure to 350 nM scriptaid (SCPT) during their 24-h contact inhibition (Group I) or had not been exposed to SCPT (Group II). Efficiently electroactivated caprine→porcine nuclear-ooplasmic hybrids were cultured to the morula and blastocyst stages for 7 to 8 days. Among 231 inter-species NT embryos assigned to Group I, 172 (74.5%)^{a} underwent cleavage divisions. The percentages of embryos that progressed to the morula and blastocyst stages were 65/231 (28.1%)^{a} and 26/231 (11.3%)^{a}, respectively. In Group II, out of 217 hybrid NT embryos, 147 (67.7%)^{a} were able to divide *ex vivo* (^{a,a} $P\geq0.05$; χ^2 test), but 36 (16.6%)^{b} and 0 (0.0%)^{b} developed to the morula and blastocyst stages, respectively (^{a,b} $P<0.01$; χ^2 test). Summing up, inter-species (caprine→porcine) NT embryos that had been reconstructed with porcine enucleated oocytes and SCPT-treated AGPB-FLC nuclei exhibited developmental capabilities to reach the blastocyst stage. In contrast, their counterparts originating from porcine enucleated oocytes and SCPT-untreated AGPB-FLC nuclei were not developmentally competent to progress to the blastocyst stage. Additionally, due to desirable enhancement of donor cell nuclear reprogrammability, the strategy of SCPT-mediated epigenomic modulation of AGPB-FLCs resulted in not only remarkable improving morula formation rate of hybrid (caprine→porcine) NT embryos, but also acquiring and maintaining capacities to complete the *in vitro* development to the blastocyst stage.

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Effect of Estradiol and Progesterone on ovine Amniotic Epithelial Cells.

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Keywords: oAEC, Estradiol, Progesterone

Objective.

This study was designed to clarify Estradiol (E2) and Progesterone (P4) steroid effects on ovine Amniotic Epithelial Cells (oAECs) that has a conserved plasticity and highly self-renewable capacity (Parolini et al., Stem Cells, 26(2), 300–311, 2008; Barboni et al., Stem Cell Rev Rep, 10:725–741, 2014). Based on their conserved immunomodulatory properties, oAECs are suitable for allo and xeno-transplantation (Barboni et al., Cell Transplant, 21(11), 2377–2395, 2012; Muttini et al., Res Vet Sci,94(1),158–169, 2013). To date, no information is present on the effects of prolonged steroid exposition on AECs.

Materials and Methods.

Treatments and differentiation. oAECs were cultured as previously reported (Barboni et al., Cell Transplant. 21(11), 2377–2395, 2012) and treated with 12.5µM and 25µM of E2 or P4 (Sigma-Aldrich, Milan, Italy), alone and in both combinations, for three passages. Untreated cells were marked control (CTR). At 70% confluency, cells were detached for doubling time (DT) evaluation. Cells at fourth passage were differentiated for 21 days in osteogenic media (DM) (Mattioli et al., Cell Biol Int 36(1):7-19, 2012) without steroid. Alizarin Red and Alcian-Blue (Sigma-Aldrich, Milano, Italy) stainings were performed.

Real Time PCR. RNA and cDNA were obtained as previously reported (Barboni et al., Cell Transplant. 21(11), 2377–2395, 2012). Real Time for *NANOG*, *SOX2* ,*OCT4* stemness genes expression were performed by SensiFast SYBR (Bioline, Aurogene, Rome, Italy) using specific primers (Mattioli et al., Cell Biol Int. 36(1):7-19, 2012).The protocol was: 5 min at 95°C, 30 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec. Comparative Ct $2^{-\Delta\Delta C(t)}$ normalization to *GAPDH* was applied.

Immunohistochemical (IHC) Analysis. IHC analyses were carried out for Cytokeratin 8 and αSMA expression as previously reported (Barboni et al. PLoS ONE 7(2): e30974, 2012).

Statistical analysis. Data expressed as mean (±SD), compared by one-way ANOVA followed by Tukey's test (GraphPad Prism 5). Significant values for $p< 0.05$.

Results.

Steroids treated ovine AECs proliferate with significant differences between concentrations. While P4 treated cells showed cuboidal shape and Cytokeratin expression until third passage, CTR and E2 treated cells showed a rapid downregulation of Cytokeratin and increased αSMA expression. oAECs with E2+P4 showed both cell type morphology. Steroids modified stemness genes based on the concentration. 12.5 µM E2, 25µM P4 and 25µM of both E2+P4 treatments maintained higher *OCT4*, *NANOG* and *SOX2* expressions in treated cells despite their progressive downregulation in the CTR. Moreover, compared to CTR, after Alizarin staining, steroid pretreated cells suffered morphological changes under DM acquiring Alcian Blue-positive chondrogenic-like morphology.

Conclusion.

AECs stemness properties and plasticity can be modified by prolonged steroidal treatment. These data improve our knowledge, opening new prospective on oAEC use in stem cell-based therapy.

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Intra-family and inter-genus (caprine/bovine) cloned embryos do not fail to complete their *in vitro* development to blastocyst stage

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Keywords: intra-family and inter-genus (caprine/bovine) cloned embryo, intra-species (caprine) cloned embryo, *ex vivo* developmental capacity

The current study was undertaken to comparatively analyze the *ex vivo* developmental outcomes of inter-species (caprine→bovine) nuclear transfer (NT)-derived embryos (Group I) and intra-species (caprine) NT-derived embryos (Group II). In Group I, to create inter-species clonal cytoplasmic hybrids (cybrids), enucleated extracorporeally matured heifer/cow oocytes were reconstituted with the cell nuclei of adult goat peripheral blood-retrieved fibroblast-like cells (AGPB-FLCs) that had undergone the *in vitro* synchronization of mitotic cycle at the G1/G0 phases by contact inhibition. In Group II, to produce intra-species clonal cybrids, enucleated metaphase II-stage doe oocytes were reconstituted with the cell nuclei of contact-inhibited AGPB-FLCs. The inter- or intra-species clonal cybrids that had been successfully electrofused and then were subjected to calcium ionomycin- and 6-dimethylaminopurine (6-DMAP)-mediated activation were classified for *in vitro* culture. In Group I, from among 212 cultured inter-species NT-derived embryos, 168 (79.2%)[a] were cleaved. The proportions of embryos that developed to morula and blastocyst stages were 69/212 (32.5%)[a] and 41/212 (19.3%)[a], respectively. In Group II, out of 203 cultured intra-species NT-derived embryos, 172 (84.7%)[a] were able to divide, but 75 (36.9%)[a] and 48 (23.6%)[a] reached the morula and blastocyst stages, respectively ([a,a] $P \geq 0.05$; χ^2 test). To summarize, the *ex vivo* developmental capacities of inter-species (caprine→bovine) cloned embryos to progress to the morula and blastocyst stages did not differ considerably from those indicated among intra-species (caprine) cloned embryos. This seems to result from close taxonomic distance and phylogenetic consanguinity between donor specimens of somatic cells (*Capra aegagrus hircus*) and donor specimens of nuclear recipient oocytes (*Bos primigenius taurus*). Such symptomatic relationships undoubtedly encompass intra-family (*Bovidae*) and inter-genus (*Capra-Bos*) model of inter-species cloning of domestic goats by somatic cell nuclear transfer (SCNT).

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

Spindle configuration of *in vitro* matured bovine oocytes vitrified and warmed in media supplemented with a biopolymer produced by an Antarctic bacterium

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Keywords: Vitrification, Chromosome, Microtubule

Biological molecules isolated from organisms that live under subfreezing conditions could be used to protect oocytes from cryoinjuries suffered during cryopreservation. Bacterial exopolysaccharides (EPS) constitute a common class of molecules that interact with ice in nature either by triggering ice nucleation or by inhibition of ice nucleation and growth. The aim of this work was to evaluate the spindle configuration of in vitro matured bovine oocytes vitrified/warmed in media supplemented with exopolysaccharide (M1) produced by *Pseudomonas* sp ID1 (Carrión et al., Carbohydr Polym 117:1028. 2015). After 22 h of *in vitro* maturation, a total of 546 oocytes form prepubertal (3 replicates) and 405 oocytes from adult cows (4 replicates) were vitrified/warmed in media supplemented with various concentrations of EPS M1 (0, 0.001, 0.01, 0.1 and 1 mg/ml). After warming, oocytes were allowed to recover for 2 additional hours in IVM medium. Fresh, non-vitrified oocytes were used as a control. At 24 h of IVM, oocytes from all treatments were fixed and immunostained with the Alexa-fluor 488 antibody and DAPI. Microtubule and chromosome distribution was analyzed by immunocytochemistry under a fluorescent microscope. ANOVA was performed to analyze differences in meiotic spindle configuration ($p< 0.05$). When cow oocytes were vitrified, similar percentages of normal spindle configuration were observed when compared to fresh control oocytes, except for the 0.1 mg/ml EPS M1 group that showed significantly lower rates compared to the fresh control group. Significantly higher rates of prepubertal oocytes exhibiting a normal spindle configuration were recorded in the non-vitrified group compared to all vitrified/warmed groups, regardless of the EPS M1 supplementation. However, the addition of EPS M1 to the vitrification/warming media decreased the ratio of decondensation or absence of chromosomes and microtubules in prepubertal oocytes. Although percentages of normal spindle configuration after vitrification were lower for prepubertal than for cow oocytes, no significant differences were observed when oocytes were vitrified with 0.001, 0.1 and 1 mg/ml EPS M1. In conclusion, supplementation with EPS M1 concentrations during vitrification and warming did not induce adverse changes in the spindle of bovine oocytes, regardless of the concentration used. Although a more severe damage on spindle configuration could be observed after vitrification of prepubertal oocytes, EPS supplementation during vitrification and warming seems to have a greater benefit during vitrification of prepubertal than adult bovine oocytes. Further experiments are required to investigate if *in vitro*-matured oocytes vitrified/warmed in presence EPS M1 can improve their development competence after being vitrified/warmed.

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Comparison of lipid profiles and gene expression in granulosa and cumulus cells in bovine.

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Keywords: *cumulus, granulosa, lipid metabolism*

Cumulus cells (CC) derive from granulosa cells (GC) during follicular growth and antrum formation and are coupled with an oocyte. Fatty acid (FA) synthesis and oxidation in GC impact cell proliferation and steroidogenesis (Elis et al, 2015 Theriogenology. 2015, 83(5):840-53) whereas in CC these processes are crucial for oocyte maturation (Sanchez-Lazo et al, 2014, Mol Endocrinol. 2014 28(9):1502-21). Both GC and CC contribute to oogenesis and reflect oocyte quality.

The objective of our study was to compare intracellular lipids and lipid related transcripts between these compartments. Lipid profiles obtained using MALDI-TOF mass spectrometry were compared between GC from individual follicles (n=12) and pools of CC (n=12) aspirated from 4-5 mm follicles of slaughtered cows. Freshly isolated cells were analysed using UltrafleXtreme MALDI-TOF/TOF instrument (Bruker) in positive (+) and negative (-) reflector mode, with 2,5-dihydroxyacetophenone matrix. Peaks were detected in 100 -1000 m/z range and values of the normalized peak heights (NPH) were quantified using Progenesis MALDI™ (Nonlinear Dynamics). Student's t-test was applied to NPH values for hunting lipid content variations between GC and CC. Peaks were annotated using MS/MS fragmentation confronted to lipid databases.

Lipid fingerprints from CC and GC gathered 462 peaks in (+) and 486 peaks in (-) modes, with coefficients of variation = 27% for CC and 18% for GC. 143 species were significantly upregulated in CC ($p<0.01$, fold change >2.0). Among them, 2 lyso-phosphatidylcholines (LPC 20:4 and 20:3) 12 phosphatidylcholines (PC), and 12 sphingomyelins (SM) were identified. Among 44 molecular forms which were more abundant in GC, we identified LPC (14:0), 4 PC, ceramide (22:1), SM (15:1), phosphatidylethanolamines (28:0 and 38:7) and phosphatidylserine (29:0). 4 peaks were annotated as triglycerides.

Gene expression in pools of CC (n=4) and GC (n=4) was analyzed using a customized 60K bovine microarray (Agilent technology, 61326 probes). Differential analysis revealed 2009 differentially expressed genes (DEG) which were up-regulated in CC and 694 in GC ($p<0.05$, Benjamini-Hochberg correction). DEG showed significant enrichment in the pathways related to carbon metabolism, glycolysis /gluconeogenesis, ATP-binding cassette transporters, amino acid and O-glycan biosynthesis, thyroid hormone, PI3K-Akt signaling, p53 and PPAR signaling pathway (corrected $p < 0.05$). Among the DEG related to lipid metabolism and regulated by PPARs, genes *ACOX2, LPL, SCD, PPARG, FABP3, FADS2, ACADL, SLC27A2* were up-regulated in CC and *CPT1A, CPT1B, SCD5, PLTP* were more expressed in GC. Ten sphingolipid metabolism genes were over-expressed in CC.

In conclusion, numerous genes related to lipid metabolism were differently expressed in CC and GC. This corroborates differences in GC and CC lipids and may reflect different involvement of GC and CC in glyceroneogenesis, lipogenesis, oxidation and steroid production.

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Reproductive Pixel Grey-Intensity Score with Image-J and Freezability in Angora Bucks

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Keywords: Angora goat, Pixel gray intensity, Ultrasound

Objective: We aim to evaluate male reproductive tract ultrasonography and its relation with sperm quality and freezability in Angora bucks.

Material and Methods: A total of five Angora bucks were selected with age range (2-3 years). The reproductive monitoring of testicular and accessory glands was assessed with ultrasound (US) (Esaote® MyLab One, Italy). Scrotal circumference, total testicular volume, epididymal area, vesicular gland area and bulbourethral gland area were calculated. Semen collection through the Trans-rectal massage (TM) and collection time were recorded. Semen is frozen with Tris egg-yolk diluent and post-thaw Delta (Δ) motility, Δ progressivity and kinetic parameters with The Hamilton-Thorn computer-aided semen analyzer, version 10 Ivos (HTR analyzer, Hamilton-Thorn Research, Beverly, MA, USA), validated for buck semen analysis. Mean differences between PGI values and post-thaw motility were evaluated by paired Student's t-test. All the US images collected were analyzed for their pixel gray intensities (PGI) using Image-J software and classified depending on the difference between the average group value. Regression analysis was carried out among all the parameters collected.

Results: Post-thaw Delta (Δ) Medians of motility, progressivity, VAP, VSL, VCL, Lateral Amplitude, Straightness and Linearity were -23.22, -20.22, -0.04, -0.92, -10.21, 0.13, 1.13, and 0.36 respectively. High levels of relationship ($P<0.05$) were identified among ejaculate volume, PGI and area selected of the epididymis. Besides, a high relationship was identified between Total testicular volume and pixel gray intensity. Delta score and collection time was also positively correlated.

Conclusion: We concluded that extreme PGI levels seem to be related to the worst sperm quality in terms of motility. Ultrasound monitoring represents an innovative technology, which may give a high impulse to the field application of the BSE in small ruminants. PGI of the reproductive tract result as useful diagnostic tools for sperm quality assessment and genetic material use.

Cell-signalling metabolites predominate among small molecules differently released by male and female bovine embryos cultured in vitro

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Keywords: bovine, embryo, sex

The cow uterus recognizes embryonic sex, and male and female early bovine embryos show dimorphic transcription that impacts metabolism. Most metabolites are small molecules that may exert a role within early embryo maternal interactions.

Individual release of metabolites was examined in a 24h single culture medium from Day-6 male and female morulae that developed to Day-7 expanded blastocysts. Embryos were produced in vitro from slaughterhouse oocytes, fertilized with a single bull and cultured in SOFaaci+6g/L BSA. Prior to metabolomics analysis, embryos had their sex identified (amelogenin gene amplification). Embryos (N=10 males and N=10 females) and N=6 blank samples (i.e. SOFaaci+6g/L BSA incubated with no embryos) were collected from 3 replicates. Metabolome was analysed by UHPLC-TOF-MS in spent culture medium as described allowing identification of 5 sex biomarkers (Gómez et al, J Chromatogr A 2016; 1474:138–144). The remaining output data were submitted to Principal Component Analysis (PCA) to detect outliers, Kolmogorov-Smirnov test to evaluate normality and Levene's test to assess the equality of the variances. Thereafter, analysis of variance by one-way ANOVA was performed to detect the different peak area averages ($p<0.05$). We found 1,720 metabolite signals showed significant differences between male and female embryos. Potential metabolites were tentatively identified by matching the m/z to those published in the Human Metabolome Database within a mass accuracy window of 10 ppm. In addition, Molecular Formula Generator algorithm of MassHunter software (Agilent) was used to support the tentative identification considering their isotopic distribution. N=13 metabolites were differentially identified. LysoPC(15:0) was the only metabolite found at higher concentration in females (fold change [FC] male to female = 0.766). FC of metabolites more abundant in male (12) varied from 1.069 to 1.604. Chemical taxonomy grouped metabolites as amino-acids and related compounds (DL-2 aminooctanoic acid, arginine, 5-hydroxy-L-tryptophan, and palmitoylglycine); lipids (2-hexenoylcarnitine; Lauroyl diethanolamide; 5,6 dihydroxyprostaglandin F1a; LysoPC(15:0); DG(14:0/14:1(9Z)/0:0) and triterpenoid); endogenous amine ((S)-N-Methylsalsolinol/(R)-N-Methylsalsolinol); n-acyl-alpha-hexosamine (N-acetyl-alpha-D-galactosamine 1-phosphate); and dUMP, a product of pyrimidine metabolism. Among the compounds originally contained in CM, female embryos significantly depleted more arginine than males and blank controls ($P<0.001$). Male and female embryos induce different concentrations of metabolites with potential signalling effects that may facilitate sex recognition in the uterus. The increased abundance of metabolites released from males is consistent with the higher metabolic activity attributed to such blastocysts.

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In vitro assessment of acrosomal status of boar sperm bound to beads conjugated to ZP proteins

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Keywords: gamete interaction, ZP conjugated-beads, 3D model

The oocyte's zona pellucida (ZP), composed by 3 or 4 glycoproteins, mediates the interaction with sperm. An *in vitro* 3D model mimicking the oocyte's shape based on studies showing the relevance of the processing of ZP2 at N terminal position in sperm-oocyte interaction (LADEN) (Avella, Sci Transl Med 8:336ra60, 2016) is being used to study the molecular mechanisms involved on gamete interaction in pigs (Hamze, Animal Reprod 13: 647, 2016). The model consists of magnetic beads (His Mag Sepharose™ Excel) conjugated with porcine ZP2, ZP3 and ZP4 recombinant proteins.

The objective was to study the binding kinetics and acrosomal status of boar spermatozoa bound to beads conjugated with ZP2, ZP3 or ZP4. ZP2 and ZP4 models were produced as previously described (Hamze, Animal Reprod 13: 647, 2016) and ZP3 was identified by electrophoresis and western blot with anti-ZPC polyclonal antibody showing a molecular weight of 55kDa. Once produced, 45-50 ZP proteins conjugated-beads were incubated in TALP medium (500 ul) with 200.000 boar spermatozoa/ml for 30, 60 and 120 min. At each time, an aliquot of beads was washed (PBS), fixed (0.5% glutaraldehyde, 30 min) and stained for 15 min with bisbenzimide (0.01mM) and Peanut agglutinin (PNA, 4µg/µL) to evaluate the number and acrosomal status of sperm bound to the beads . Three replicates with a blind analysis were done. We calculated the rate of beads with at least one sperm bound (BSB), the mean number of sperm per bead (S/B) and the acrosomal reaction of bound sperm. Results were analysed by one-way ANOVA considering statistical differences when P-value <0.05.

After 30 min of coincubation, the BSB was higher for ZP3 and ZP4 (71.54 ± 3.67%, n=158 and 75.56 ± 3.53%, n=155) than for ZP2 (56.52 ± 4.01%, n= 154) and the S/B was higher for ZP4 (3.87 ± 0.31) than ZP2 (2.58 ± 0.18) and ZP3 (2.69 ± 0.17). No differences were observed at 60 min for BSB but S/B was higher for ZP2 (6.66 ± 0.43) than ZP3 (4.87 ± 0.31) and ZP4 (4.23 ±0.24). Finally, at 120 min both BSB and S/B were higher for ZP2 (93.5 ± 2.0%, 9.00 ± 0.45) and ZP3 (93.6 ± 2.0%, 8.54 ± 0.49) than ZP4 (81.0 ± 3.2%, 6.68 ±0.61). After 30 min of incubation the ZP2 model induced a higher acrosome reaction since 77.50 ± 3.76% of the bound sperm had reacted whereas for ZP3 and ZP4 models rates were 69.84 ± 3.84 and 65.04 ± 3.43, respectively. No differences were found at 60 and 120 min probably due to the high capacitation ability of TALP medium.
In conclusion, ZP2 conjugated beads bound a higher number of reacted spermatozoa at 30 min. Regarding binding kinetics, differences between groups were observed through time, being ZP2 and ZP3 conjugated beads the models with a higher S/B at 120 min. The full development of this 3D model will permit in the future a better and deeper understanding of gametes interaction in pigs and the reduction of female gametes in gametes interaction studies.

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Efficiency of Preimplantation Genetic Diagnosis (PGD) of bovine IVP embryos using blastocoele fluid or embryonic cells

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Keywords: embryo sexing, PGD, blastocoele fluid

Preimplantation Genetic Diagnosis (PGD) involves collecting a few cells from a preimplantation embryo, which will then be used for genetic testing. Palini et al. were able to diagnose the sex human embryos using only the blastocoele fluid as a source of DNA [Palini, Reprod Biomed Online, 26(6):603-10, 2013]. Recently, we demonstrated that the DNA in the blastocoele fluid of bovine IVP embryos can be amplified by PCR to diagnose the sex of the embryos [Herrera, Proceedings in 42° Veterinär-Humanmedizinische Gemeinschaftstagung, Abstract 51, 2017]. The aim of our work was to compare the efficiency of PGD of bovine IVP embryos using blastocoele fluid or embryonic cells as a source of DNA for sexing the embryos by PCR. Bovine embryos were produced in vitro and all expanded blastocysts were randomly assigned to one of three experimental groups: 1) Collapsed Embryos (CE): blastocoele fluid was collected from blastocysts, 2) Biopsied Embryos (BE): 1 to 5 cells were collected form blastocysts by aspiration and 3) Intact Embryos (IE): blastocysts were left intact. In 1) and 2) blastocyst stage embryos were placed under an inverted microscope equipped with a micromanipulation system for the collection of blastocoele fluid or embryonic cells, as decribed previously for equine embryos [Herrera, Theriogenology 81(5):758-63, 2014; Herrera Theriogenology 83(3):415-20, 2015]. Collapsed, biopsied or intact blastocysts were vitrified and warmed using the vitrification method described by Vajta et al. (1999), except a hemistraw instead of an Open Pulled Straw (OPS) was used as a carrier. After warming, embryos were cultured in vitro and observed for 48 h to detect reexpansion and hatching. The DNA from the blastocoele fluid or from the embryonic cells was amplified by PCR as decribed previously [Herrera, Proceedings in 42° Veterinär - Humanmedizinische Gemeinschaftstagung, Abstract 51, 2017]. The survival rates after warming and in vitro culture for 48 h and the efficiency of amplification after PCR were compared by ANOVA and Fisher's exact test between the experimental groups. The post-warming survival rates of blastocysts did not differ significantly between CE, BE or IE (93.1%, 96.8% and 95.6% respectively) ($P>0.05$). The hatching rates after warming and 48 h of IVC, did not differ between BE or IE (75% vs. 47.8%), was significantly higher for CE when compared to IE (79.3% vs. 47.8%) and did not differ between CE and BE. The amplification rates after PCR was significantly higher for blastocoele fluid samples (41/41, 100%) that for biopsied cells (30/34, 80.3%) ($P<0.05$). The present results demonstrate that blastocoele fluid can be collected from IVP blastocysts and used as a source of DNA for PCR, without impairing the viability of the embryo. In our hands, the use of blastocoele fluid was more efficient than cells after PCR.

Bovine embryo lipid metabolism is affected by perfluorononanoic acid (PFNA) exposure during oocyte maturation *in vitro*

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Keywords: cow, reproductive toxicology, *in vitro* embryo production

The final maturation of the oocyte, fertilization and the early embryo development are sensitive processes that can be affected by chemicals. In reproductive toxicology studies, bovine *in vitro* production (IVP) of embryos provides a controlled setting where testing of chemicals is possible without the use of laboratory animals. Bovine IVP is a good model for humans.

PFNA is used in consumer products such as water and stain/oil repellent products. The substance is highly persistent and found in both wildlife and nature, but also in human follicle fluid (0.2-2.1 ng/ml (Petro et al., Sci Total Environ 496, 282–288, 2014)). Studies regarding PFNAs potential toxicological effects, especially developmental toxicity, are limited. The aim of this study was to explore the bovine IVP model (Abraham et al., Acta Vet Scand 54:36, 2012) and examine effects of PFNA exposure on oocytes during *in vitro* maturation by evaluation of blastocyst mitochondrial and lipid status since these are suspected to be involved in the mode of action via oxidative stress and peroxisome proliferation-activated receptor α.

Abattoir-derived bovine ovaries were used to collect cumulus oocyte complexes (n = 440). The oocytes were matured *in vitro* under PFNA exposure (100 ng/ml to include a safety margin) or non-exposed controls. *In vitro* fertilization and culture were done according to standard protocols. Embryo development was assessed by cleavage rate and blastocyst development and morphology. Day 8 blastocysts were stained for visualization of active mitochondria (MitoTracker® Orange CMTMRos, ThermoFisher Scientific, Waltham, USA) and fixed in paraformaldehyde. Additional staining was done with nuclear stain (Deep Red Anthraquinone 5, BioNordika, Stockholm, Sweden) and neutral lipid stain (HCS LipidTOX™ Green Neutral Lipid Stain, ThermoFisher Scientific, Waltham, USA). For analysis of the embryos confocal laser scanning microscope was used (LSM 510, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The embryos were manually evaluated. Mitochondrial staining was evaluated with regards to distribution (even distribution to uneven distribution with areas devoid of mitochondria or distinct aggregations of mitochondria). Neutral lipid staining was evaluated with regards to dominating size of lipid droplets.

Statistical analyses were performed by linear mixed models and generalized linear mixed models, with replicate as random factor and observations on day 7 and 8 as repeated measures.

There was no significant difference (p>0.05) between treated and control group regarding, cleavage rate, blastocyst development day 7 and 8, quality grade of blastocysts, stage of blastocysts, number of nuclei or mitochondrial distribution scoring. However, there was a significant difference in distribution of lipid droplet size where the treated group had an increased amount of large lipid droplets (p = 0.048).

To conclude, the bovine IVP model suggests a disturbance in lipid metabolism but the exact working mechanism of PFNA must be further explored.

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Bull spermatozoa have better membrane integrity and mitochondrial membrane potential when cryopreserved with a liposome-based extender

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Keywords: semen, single layer centrifugation, liposome-based extender

Functional, robust spermatozoa are needed for assisted reproduction biotechnologies. Good quality spermatozoa can be separated from the rest of the ejaculate using colloid centrifugation, especially Single Layer Centrifugation (SLC) (Morrell et al., Animal Reproduction 13, 340-345; 2016). To avoid including material of animal origin, such as egg yolk, commercial extenders have been developed containing soy lecithin or liposomes. The aim of this study was to evaluate the effects of SLC on bull sperm quality when spermatozoa were frozen in these extenders. Semen was collected from 12 bulls at a commercial bull station (Viking Genetics, Skara, Sweden). Immediately after collection, each semen sample was split into control and SLC samples. Controls were extended to provide a sperm concentration of 69×10^6 spermatozoa/mL, in AndroMed® (Minitube, Tiefenbach, Germany - soy lecithin based extender - control A) or OptiXcell® (IMV Technologies, L´Aigle, France - liposomes based extender - control O). SLC samples were extended to 50×10^6 /mL in Tris-egg yolk prior to centrifugation through the colloid Bovicoll; after SLC the sperm pellet was resuspended in AndroMed® (SLC A) or OptiXcell® (SLC O) to the same concentration as controls. All samples were frozen in 0.25 mL plastic straws. After 3-10 days storage in liquid nitrogen, the straws were thawed at 37°C for 12 s for sperm quality evaluation. Analyses of membrane integrity (MI) and mitochondrial membrane potential (MMP) were made by flow cytometer. Means were analysed using the proc mixed procedure for linear mixed models. Correlations were calculated using Pearson´s correlation test; Scheffe's adjustment was used for multiple-post ANOVA comparisons. Results are presented as LSMeans ± Standard error of means (SEM); the differences were considered significant at $p<0.05$. The samples cryopreserved with OptiXcell® showed better MI and MMP ($p<0.05$) than those cryopreserved with AndroMed®, both in control groups (control O versus control A, MI: 48.5+3.2 vs. 38.8+3.2; MMP: 55.8+3.0 vs 34.1+3.0) and SLC groups (SLC O versus SLC A, MI: 45.5+3.2 vs. 30.8+3.2; MMP: 66.4+3.0 vs. 41.4+3.0). Within extender, no differences were observed between control and SLC for MI ($p>0.05$) (control A versus SLC A, 38.8+3.2 vs. 30.8+3.2; control O versus SLC O: 48.5+3.2 vs. 55.8+3.0) or MMP (control A versus SLC A: 34.1+3.0 vs. 41.4+3.0; control O versus SLC O: 55.8+3.0 vs. 66.4+3.0). In conclusion, bull spermatozoa, selected or not by SLC, have increased MI and MMP when the liposome-based extender was used.

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Comparison of the survival rates of ovarian tissue after slow freezing and vitrification by assessing histological structure and estradiol production during in-vitro culture

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Keywords: Cryopreservation, Ovarian tissue

Objectives

Our study was designed to identify a cryopreservation technique, which ensures better surviving of ovarian tissue. The aim is to compare effectiveness of slow freezing (SF) and vitrification (VIT), followed by in-vitro culture and histological analysis.

Materials and methods

All reagents were purchased from Sigma Aldrich SRL (Milan, Italy), unless other specified.

Cortical tissue was isolated from pubertal ovine ovaries, transported from the local slaughterhouse. Slivers (1*5*5 mm) were randomly allocated into six groups (n=5 in each): 1-non frozen control, 2-SF protocol, 3-VIT protocol, 4-non frozen control for in-vitro culture (IVC), 5-SF protocol for IVC, 6-VIT protocol for IVC.

The cryoprotectants used in SF protocol were 1.5M ethylene glycol (EG) and 0.1M sucrose (SUC). Vials with samples were thawed in a water bath at 37ºC and then washed in phosphate buffered saline containing 0.75M EG and 0.25M SUC. In VIT protocol cryoprotectants were 2.5M dimethyl sulfoxide, 2.5M EG and 0.5M SUC. Warming performedn at 37ºC in McCoy's 5a medium contained 0.5M SUC and then washed in the medium with 0.25M SUC.

For the histological analysis pieces of tissue were fixed in 4% paraformaldehyde, then dehydrated in series of ethanol and embedded in paraffin. The samples were sectioned (5 μm) and stained with hematoxylin and eosin. Follicles in the tissue were assessed by criteria established in our laboratory (Martelli et al., J Mol Endocrinol, 2006) and classified into three quality groups: intact, partially damaged and degenerated. Cortical strips were cultured in McCoy's 5a medium for 6 days at 37ºC and 5% CO2 with medium changed every 2 days. Then culture medium was analysed for the content of estradiol (E2) by ELISA assay (DRG, Marburg, Germany).

Results

The proportion of normal follicles showed significant difference between SF (total number of follicles counted=177) and VIT groups (total number of follicles=223): 27,96% vs 19,36% ($p<0,001$, χ^2 test). After the in-vitro culture, 84 and 69 follicles in total were counted for the SF and VIT groups, respectively. In this case, a higher percentage of intact follicles after slow freezing also has been shown: 21,87% vs 16,52% ($0<0,001$, χ^2 test).

The mean E2 concentrations for days 1,3 and 6 of in-vitro culture after SF protocol were 3,1 pg/ml; 11,4 pg/ml and 12,1 pg/ml, which were 20% lower, than values for non-frozen control (3,7 pg/ml; 13,6 pg/ml and 14,6 pg/ml). However, the difference of E2 concentration from the non-frozen control was even greater for the VIT group, where the values were more than 50% lower: 1,8 pg/ml; 5,9 pg/ml and 7,6 pg/ml. An increase of E2 concentrations during the in-vitro culture was observed, which proved tissue recovering after cryopreservation.

Conclusions

Slow freezing is ensuring better morphological structure of ovarian cortex than vitrification. More specifically, a higher number of morphologically healthy follicles could be seen and a better production of estradiol during in-vitro culture of ovarian slivers was present.

Annual control chart of bull semen freezability

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Keywords: Freezability, bull semen, CASA.

Objective: Optimization of the center bull production is depending on the application of a fine control chart creation. The use of CASA system improved the precision and accuracy on the early identification of production problems.

Material and Methods: Aim of the present work was the modelling of a control chart that considered the monthly variations during a whole year in terms of semen freezability and number of straws produced. A total of 536 ejaculates were immediately evaluated, diluted with Andromed® and frozen using an automatized freezer (Digitcoo5300 ZV, IMV). Thereafter the straws were plugged in liquid nitrogen. Fresh and post-thawed motilities where evaluated. Computer assisted sperm analysis (CASA) parameters were analyzed considering the monthly variations of individual kinetic characteristics as average pathway velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL) and hyperactive % (HYP).

Results: The variations were scored in terms of number of SD from the average value for each parameter. Delta motility (difference between post-thawed and fresh semen motility) was higher during the summer and autumn period and in Holstein bulls (up to -30%). Collected and elaborated data were analyzed through ANOVA for repeated measures using month and season as independent variables while breed, semen quality parameters and number of straws as dependent variables.

Conclusions: The number of produced straws were significantly changed between the spring and the summer months indicating the lower resistance of the bulls to the hot environment. CASA parameters as VAP, VCL, VSL and HYP reinforce the indication that heat stress can influence up to three months the freezability of bull semen.

Effect of conceptus size on embryo-maternal communication during early pregnancy in cattle

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Keywords: Uterine-explant, conceptus, transcriptome

Conceptus elongation is an essential prerequisite to maternal recognition of pregnancy and implantation in cattle. During elongation, the trophoblast cells secrete interferon-tau (IFNT), which prevents the upregulation of oxytocin receptors in the endometrium required for prostaglandin-induced luteolysis. Large variation exists in the length of conceptuses recovered on the same day of gestation, which may reflect an inherent lack of developmental competency. For example, larger conceptuses produce more IFNT, but the underlying factors that regulate conceptus-maternal crosstalk between advanced (large) or retarded (small) conceptuses and the endometrium are unknown. Thus, the aim of this study was to interrogate the response of the endometrium to Day 15 conceptuses of different sizes by examining the global transcriptome profiles of uterine explants exposed to large vs small conceptuses. First, 10 grade 1 *in vitro* produced blastocysts were transferred into synchronized recipient heifers on Day 7 for further development. The resulting conceptuses were recovered on Day 15 by *post-mortem* uterine flushing. Seven large (mean length ± SEM 25.4 ± 5.7 mm) and six small conceptuses (1.8 ± 0.3 mm) were individually placed on top of endometrial explants that had been collected from uteri during the late luteal phase of the estrous cycle, and co-cultured for 6 h in one mL of RPMI media. Additional explants were cultured with media containing 100 ng/mL of recombinant ovine IFNT (IFNT; n=6) to identify endometrial responses dependent and independent of IFNT or in media alone (Control; n=6). Total RNA was isolated from explant cultures and analysed by RNA-Seq. Exposure of endometrium to IFNT, a large conceptus or a small conceptus altered ($P < 0.05$) expression of 491, 498 and 230 transcripts, respectively, compared with control endometrium. Further, 223 differentially expressed transcripts were common between conceptus-treated and IFNT-treated explants, and classical interferon-stimulated genes (e.g., RSAD2 and ISG15) were amongst the most upregulated transcripts compared to control endometrium. In addition, 369 transcripts were uniquely altered in explants exposed to large conceptuses and IFNT. Of these transcripts, 101 and 100 were specific to large conceptuses and IFNT-treated endometrium, respectively, while 168 were common to both groups. Only 6 of 108 conceptus-induced differentially expressed genes were shared between small- and large conceptuses. Interestingly, 101 transcripts were exclusively regulated by large conceptuses; of these, *PCSK1*, *TNNI3K*, *MPV17L*, and *IL17* were the most upregulated and *TEPP*, *CACNA1L*, *AQP1*, and *HIP1* the most downregulated. This study provides new knowledge of differences in gene expression in endometrial tissue induced by large and small conceptuses. The results provide a better understanding of the underlying molecular pathways involved in embryo survival and maternal recognition of pregnancy in cattle.

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Development competence of bovine oocytes selected by brilliant cresyl blue before vitrification

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Keywords: oocyte, vitrification, cow

Numerous factors influence *oocyte cryoresistance*, including diameter of follicle, meiosis stage, functional status of oocytes etc. (Papis K., Slovak J. Anim. Sci., 48, 2015 (4): 163-171). It was shown that bovine oocytes selected by brilliant cresyl blue (BCB) had different developmental competence. Native BCB^+ oocytes (oocytes that have finished growth phase in vivo) had significantly higher development competence than oocytes that have not finished growth phase in vivo (BCB^- oocytes, Heleil B. et al., J. Reprod. & Infertility 1 (1):01-07, 2010).In previous studies we demonstrated that pretreatment of bovine oocytes with follicular fluid from follicle 3 mm before vitrification improves nuclear and cytoplasmic maturation and development of vitrified bovine oocytes (Kuzmina T.I. et al., *Reprod Biomed online,* 20(3): S38–S39, 2010). The aim of the present study was to evaluate the development competence of devitrified BCB^+ and BCB^- oocytes. Before vitrification cumulus oocyte complexes (COCs) were incubated in BCB solution (26 μM) for 90 min. Oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored) and then COCs were incubated 40 min in follicle fluid (d of follicles ≤ 3 mm). Vitrification was performed by equilibration of oocytes in CPA (Cryoprotective Additive) - 1: 0.7 M dimethylsulphoxide (Me2SO) + 0.9 M ethylene glycol (EG) (30 sec); CPA-2: 1.4 M Me2SO + 1.8 M EG (30 sec); CPA-3: 2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose (20 sec) and loading into straws. After thawing COCs washed by step-wise dilution in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199 alone. COCs were cultured in TCM 199 + 10% (v/v) heat-treated FCS + 50 ng/ml PRL with 10^6/ml granulosa cells. COCs were cultured in this medium 15 h. Then medium were supplemented by 10 IU/ml hCG. The time of cultivation for BCB^+ and BCB^- oocytes were 24 h. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols. Cleavage and development rates were examined on days 2 and 7 after fertilization, respectively. All chemicals used in this study were purchased from Sigma – Aldrich (Moscow, Russia). A total of 604 COCs were vitrified**,** 399 COCs were treated by BCB. Cleavage was significantly higher in BCB^+ oocyte in compared to BCB^- oocytes [51% (103/201) vs. 31 %(61/198), respectively, $P<0.05$, χ^2 test]. Blastocyst development rate was significantly higher in BCB^+ vs. BCB- oocytes [9% (18/201) vs. 1% (2/198), respectively, $P<0.05$, χ^2 test]. In the control group (oocytes have not treated by BCB) the cleavage and blastocyst development rates were significantly low in comparison to the BCB^+oocytes [39% (80/205) vs 51% (103/201) and 3(6/205) vs 9(18/201), respectively, $P<0.05$, χ^2 test]. We have not find differences in cleavage and blastocyst development rate between control group of oocytes and group of BCB^- oocytes. In conclusion, BCB test is an effective method for selection of more competent bovine GV- oocytes for vitrification.

SIMPLIFYING THE OVIDUCTAL CELL ADHESION TEST FOR BOVINE SPERM QUALITY ASSESSMENT

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Keywords: Oviductal adhesion test, sperm quality, endangered breed

Classic procedures for semen evaluation in breeding soundness evaluation require time and high cost. The evaluation techniques with CASA has limits due to the lack of tools standardization in different laboratories and to data analysis methods. Recent studies cast doubts on the actual predictive ability of this method regarding semen fertility at field level. Therefore, many studies have focused on finding a simple and objective test which can give the maximum correlation between in vitro and in vivo results. In this study, we aim to develop a repeatable protocol of sperm adhesion test using oviductal explant (AOC) and comparing the results with CASA parameters and field fertility (ERCR). In this test, the interaction between spermatozoa and oviduct was assessed by incubation in co-culture oviductal explants, in order to calculate the number of spermatozoa adhered per unit area or adhesion index (AI) and create a correlation between this index and field fertility. Oviductal explant cells on the glass slide were exposed to 5uL of diluted semen, containing approximately 35000 motile sperms. Slides were incubated for the 20 mins. Subsequently, reading of the slide by means of optical microscope (Olympus CX41) and without any staining with magnification of 400 X was performed. A mobile field in which the explant cells of oviductal rectilinearly ran through the major diameter of the field was considered and only motile sperm still adhering to one side of explant cell were counted. Three optic fields (OF) for each slide and the average of the three counts are evaluated. Class 1 (ERCR> 1.00): high fertility; Class 2 (ERCR -1 / + 1): medium fertility; Class 3 (ERCR <1.00): low fertility. Five Frisona breed bulls within each different class have been selected randomly and for each bull three doses of semen belonging to the production lots that have contributed to the definition ERCR were considered. Three doses of semen were considered and for each sample were counted sperm immobilized on three microscopic fields. AI was different ($P<0.05$) among the ERCRs with 26.22±2.34, 17.9±1.44 and 12.64±1.68 sperms/OF for Class 1, Class 2 and Class 3 respectively. After the developmental phase, AOC was applied to a group of endangered Burlina breed bulls (N:8) where the effective field results are difficult to obtain due to the small size of population. Burlina bulls resulted with an AI lower then Class 1 with 15.21±1.14 sperms/OF. AOC test provides a prediction on bull semen fertility. Counting the sperms adhered in three microscopic fields after a co-incubation in PBS can give useful information on the field fertility level. AOC gives additional information to the standard of semen evaluation methods applied to endangered breeds.

Effects of polyvinyl alcohol on fresh and post-thawed physiological motion characteristics of Angora goat semen

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Keywords: Angora Goat, Freezability, Polyvinyl alcohol

Small concentrations of the synthetic polymer polyvinyl alcohol (PVA) were found to inhibit the formation of ice in water/cryoprotectant solutions. Therefore, we aim to define a methodology to evaluate the freezability of Angora goat semen diluted with 3 different molecular weighted polyvinyl alcohol (PVA; 9, 18 and 100 kDa) with computer-assisted sperm analysis (CASA) before freezing in which each sperm head trajectory is reconstructed. In total, 30 ejaculates from seven mature Angora bucks (2 years old) were collected twice a week by artificial vagina. Immediately after collection, sperm samples were diluted with three different PVA co-polymers PVA 9, 18 and 100 kDa in with five different concentration 0,001 %, 0,01%, 0,1 %, 1% and %2 added to Tris-egg yolk diluent with 7% glycerol in three experimental groups respectively. After dilution semen was loaded into 0,25 ml French straws and cooled down to + 4C in three hours, frozen in a programmable freezing machine (Digitcool 5300, IMV, France). After thawing, following sperm motion characteristics were evaluated: Progressive motility and kinetic parameters with Hamilton-Thorn CASA, validated for buck semen analysis. Data collected and elaborated were analyzed using through ANOVA with PVA type and concentration as independent variable while CASA parameters as dependent variables. General post-thaw average of motility, progressivity, average pathway velocity (VAP), Straight line velocity (VSL), curvilinear velocity (VCL) and Lateral Amplitude were 55.76, 29.26, 121.84, 97.72 and 8.55 respectively. The mean differences (Δ) of motile percentages between the pre and post-thawed semen were 9.07, -9.73 and 14.58 and Δprogressive motility percentages were -24.71, -22 and -8.90 for three groups of PVA as 9 kDa, 18 kDa, and 100 kDa respectively. Lowest progressivity loss gained with group PVA 100 kDa along with ΔVAP, ΔVSL, ΔVCL and concordantly with ΔTotal rapid percentage. However, post-thaw motion trajectory/characteristics were better in PVA 18 kDa group average considering the lowest loss of ΔBeat cross frequency, ΔStraightness, and Δ Linearity. Δtotal static percentage was highest with PVA 9 kDa group ($P<0,05$)

In conclusion, the addition of PVA 100 kDa was beneficial for sperm kinetic parameters. PVA 18 kDa group shown more proper motion characteristics. PVA addition to semen extender can decrease the glycerol concentration and thus can decrease the deleterious toxic effect. Synthetic PVA-derived ice blocking agents can be produced much less expensively than antifreeze proteins, offering new opportunities to improve the sperm cryopreservation.

Ultrastructure of porcine embryos after cryopreservation

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Keywords: pig, embryo, cryopreservation

In this study we examined the effect of cryopreservation on the ultrastructure of porcine embryos. It is considered that low survival of pig embryos after cryopreservation is related to a high content of lipid droplets (LDs) in their cytoplasm. In porcine zygotes these organelles occupy up to 60% of their volume (Romek et al., Reprod Domest Anim 44, 24-32, 2009). Cryopreservation can cause damages in the structure of LDs and mitochondria, and microsurgical LD removal enhances survival after cryopreservation (Kawakami M., Animal Reproduction Science 106, 402-411, 2008). Therefore, in the present study we focused on the morphology of mitochondria and LDs, which play a crucial role in embryo metabolism. Under a transmission electron microscope (TEM) we analyzed *in vivo* and *in vitro* derived embryos at three developmental stages: zygote, morula and blastocyst. Polish Large White gilts were artificially inseminated and embryos were collected surgically after flushing oviducts (zygotes) or uteri (morulae and late blastocysts). Additionally, part of the zygotes were cultured up to the morula and late blastocyst stage in the NCSU–23 medium supplemented with 4 mg/ml BSA at 39^{o} C in atmosphere containing 5% CO_2 in air. *In vivo* and *in vitro* derived control embryos were fixed immediately after acquisition with 2.5% glutaraldehyde in 0.67 M cacodylate buffer at 4 ^{o} C for 24 h, post-fixed in 1% osmium tetroxide, dehydrated in graded series of ethanol and embedded in PolyBed 812 epoxy resin (Polysciences Inc., Warrington, USA). The embryos were then cut into ultra-thin sections, contrasted with uranyl acetate and lead citrate and examined under the TEM. Experimental groups of embryos were vitrified using the Open Pulled Straw technique, thawed and then processed for TEM. All chemicals, unless otherwise stated, were from Sigma-Aldrich Co. (St. Louis, USA). In non-cryopreserved embryos differences in morphology of mitochondria between developmental stages were evident. In zygotes they were round shaped, contained a small number of cristae and a dark matrix. In morulae and blastocysts mitochondria were elongated, contained more cristae and a bright matrix. LDs were not disturbed. However, *in vivo* and *in vitro* embryos showed damages in ultrastructure after cryopreservation. Mitochondria contained bright vesicles and disturbed inner membranes, while in LDs we observed long cracks, often reaching the surface of organelles. Moreover, vitrification caused changes in LD surface, which was less homogeneous and contained bright areas with irregular edges. Mitochondrial functions including ATP production and calcium homeostasis can be disturbed due to the inner mitochondrial membranes disruption. Furthermore, LD cracks break the continuity of the lipid monolayer on the surface of LDs, which plays a crucial role in regulation of embryo lipid metabolism including lipolysis. Therefore, damages in embryo ultrastructure following cryopreservation may impair its developmental potential, leading to embryo death .

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