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Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

34ème COLLOQUE SCIENTIFIQUE

34th SCIENTIFIC MEETING

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Dr. Patrice Humblot

Special Celebration

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Nantes, France, 7th and 8th September 2018

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SUPPORT BIOTECHNOLOGIES: CRYOPRESERVATION and CRYOBIOLOGY, DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, and "OMICS"

Dr. Patrice Humblot

A.E.T.E. Medalist 2018

Dr. Patrice Humblot

A.E.T.E. Medalist 2018

Commendation of Dr. Patrice Humblot for AETE Pioneer Award 2018

Firstly, I should explain how I first got to know Patrice. In 1980, before the fall of the Iron Curtain, four Western Europeans were invited to Warsaw to participate in a meeting about measurement of progesterone in cows' milk as a means of pregnancy diagnosis and clinical surveillance. We had to have special visas to enter Poland and I knew only one other participant, Jean Saumande from France. Our hosts were very grateful to us for sharing our scientific experiences so openly, and we were invited to the home of Romeck Stupniski for dinner one evening – a brave thing for him to do in those days (on leaving the country I was asked by the Polish border guards where I had been during my stay in Warsaw….and they reminded me that I had been to his home). I digress – to get to Romeck's apartment, there wasn't enough room in the cars, so Patrice and I agreed to travel by public transport. Ask Patrice about waiting for Tram number 8! This educative experience cemented a life-long friendship.

Patrice Humblot was very young when he was born 64 years ago.

Patrice is the younger of two brothers raised in suburban Paris by two of the most wonderfully generous people I have had the honour to meet. Over the years, the whole family very politely corrected my attempts at trying to converse in French, usually quickly reverting to English.

Patrice graduated from the National Veterinary School in Maisons Alfort (Paris) in 1978, with a particular interest in reproductive physiology (…mainly of cows). He was clearly quite astute and took further specialised training in statistics within 5 years of graduating. This was a decision that has paid dividends for him – and all those with whom he has worked and interacted since.

Immediately after graduation, Patrice joined UNCEIA (the French National Union of Breeding Companies) becoming responsible for programmes to improve cow fertility. This enabled him to apply his theoretical education to very practical issues with the outcomes being of direct benefit to farmers. This typifies his scientific character – pinpointing the cause of problems, working out solutions, then providing strategies to avoid such difficulties in the future. But his work has also been pro-active – while understanding contemporary advances in most complex aspects of science, he often 'thinks out of the box' to identify ways of applying this knowledge in very practical ways. Thus, it was no surprise when he was appointed Scientific Director of UNCEIA R&D Department in 2002, a role that involved supporting research and development of reproductive technologies such as ET, embryo freezing, embryo sexing, IVF/IVP and genomics. He was also very actively involved in the successful preparation of several patents in the field of pregnancy diagnosis, bull sperm production and embryo cryopreservation.

It may seem strange to some younger scientists that a person could work for the same commercial employer for ~30 years but Patrice did this in dedicated fashion. He didn't stay hidden away in his office or lab. He made sure he could get out and about. He talked to farmers to understand their businesses so he could ensure that UNCEIA delivered what was required – this encompassed genetic progress through sensible genetic selection and then provision of elite high-performance bulls; always seeking to improve AI and ET techniques to be delivered for use in the field; developing ways to monitor embryonic death by methods that could be applied easily on a large scale; as well as considering the effect of other environmental factors, such as nutrition, on the efficiency of cow fertility (beef and dairy). He also will be the first to admit that all his work has benefitted from interaction with other scientists – whether he visits their labs, they visit UNCEIA or Uppsala, or he meets them at conferences. He has had long-term fruitful collaborations and friendly support of many at UNCEIA, INRA (Nouzilly, Jouy en Josas and Rennes), different teams in Europe including more recently SLU, and colleagues further away, especially in Brazil and Thailand.

In February 2010, Patrice was attracted to Sweden, becoming Professor in Domestic Animal Reproduction at the Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala.

During both his working appointments, he has produced an impressive number of cutting edge scientific papers (more than 500), popular science articles, and technical notes. He has achieved this as well as supervising PhD students many from different countries which helped to establish international collaborations. In addition, he is sought after as a specialist lecturer on many international conferences and post-graduate courses (including in the UK at the University of Liverpool). In parallel, Patrice has developed internationally recognized expertise in the field of animal reproduction and reproductive technologies through numerous aid-missions in developing countries. He has developed a wide network of research collaborations having inspired many scientists in his spheres of research throughout the world.

With this kind of experience, it is obvious that he has been invited to serve on many Evaluation Panels, for example for INRA (France) and their research units, ANR (Agencie Nationale pour la Recherche, France) and other European consortia (Ireland and Estonia).

Patrice has handsomely contributed back to the scientific community by acting as a reviewer for many international scientific journals, and especially as acting as a co-editor of Livestock Production Science. He is also an active member of the European Society for Reproduction in Domestic Animals (ESDAR), of the European Embryo Transfer Association (AETE), of the International Embryo Transfer Society (IETS) and of World Buiatrics Congress (WBC), having himself been on the Organising Committee of at least 6 international conferences. Indeed, early in his career he was involved in arranging the first of many successful joint meetings between the Society for Study of Fertility (SSF, as it was then) and the French Society for Study of Fertility. For the first meeting in France, the English contingent arrived very late (after midnight due to bad weather in the English/French Channel) but Patrice remained unflustered. He delayed the start of a 7-course banquet until we arrived, but I admit he was surprised by the amount of wine consumed by the assembled scientists from both Societies on the second evening.

I have known Patrice for many years, as a scientific colleague and as a soul-mate. His family (including his three daughters) have been closely associated with mine as we have all grown up (?). If we disagree, we have another glass of wine (French, of course), and all is resolved.

With many demands on his time, Patrice never forgets his main raison d'etre: to make the world a better place, and where better to target than the oocyte, sperm and embryo?

In short, Patrice Humblot is a most worthy recipient of the AETE Pioneer Award 2018.

Hilary Dobson,
University of Liverpool, UK
Sept 2018

From clinics to (cow)mics: a reproductive journey

Patrice Humblot[1]

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Abstract

This manuscript describes the different topics I have been involved in the fields of reproductive physiology and embryo biotechnologies with attempts to address practical issues raised mainly by the breeding industry. The journey started with phenotyping work in the field of reproductive physio-pathology. Other issues were related to the optimization of reproductive biotechnologies to favorize genetic selection. The implementation of genomic selection raised opportunities to develop the use embryo biotechnologies and showed the interest of combining them in the case of embryo genotyping. There is still a need to refine phenotyping for reproductive traits especially for the identification of markers of uterine dysfunction. It is believed that new knowledge generated by combining different molecular approaches will be the source of applications that may benefit AI practice and embryo technologies.

Keywords: cattle, genomics, reproductive phenotypes, reproductive technologies.

Introduction
Warning!

Working most of my life for the breeding industry had two major consequences. This led to develop research based on application driven approaches. In addition, although working mainly in the field of animal reproduction, I have been almost permanently at the border of different domains, endocrinology at first, embryology and embryo technologies, genetic selection and more recently reproductive genomics. I started with clinical medicine, dealing with reproductive problems in high producing dairy cows and approach today the mechanisms underlying the development of inflammation and resilience to stress, using cow, dog and cat endometrium as models.

The following text is an attempt to describe the context in the field of reproduction at the beginning of my working life, the major developments in reproductive physiology, veterinary medicine and genetic selection, I have been witness too and their promising applications followed or not by real development. The environment of present research providing extremely powerful tools, especially for genomics, stresses out the need for Bioinformatics to integrate information when approaching reproductive physiology or diseases with concepts referring to precision medicine.

Hence, this text should be seen as just an overview produced by a "generalist" who approached too many topics. Despite the associated limitations, I hope that the description of existing gaps in knowledge and/or some of the perspectives drawn from it may be the source of research ideas for future adventurers discovering by mistake this text on a dusty shelve.

AI and the birth of phenotyping for fertility

Clinical approaches for the control of fertility and oestrus synchronization

I joined the world of Artificial Insemination (AI) in 1977, soon after the end of its golden age. Following a rapid growth after the creation of the first French AI centre in 1946 the number of AI's reached a plateau in the 70's and then started to decrease, due to the decrease in cow numbers associated with the increase in cow productivity. This technique is still widely used in France with a total of 7 millions of AI's in 2017 (Grimard *et al.*, 2018) and represents the major way of reproduction in dairy cows (in 2017, 80% of calves issued from AI) whereas it's development has been limited in beef cows (only 13% of calves issued from AI). Due to genetic selection oriented essentially to improve milk traits, fertility after AI decreased regularly between the 70's and year 2000 (Barbat *et al.*, 2010). The above trend was not specific to France, and was observed in all dairy producing countries (Royal *et al.*, 2000; Lucy, 2001; Bousquet *et al.*, 2004). The need for a better characterization of reproduction and treatment of reproductive disorders emerged from this situation. In the 70's major progresses in the mechanisms regulating corpus luteum and pituitary function and the commercialization of hormones such as PGF2α and GnRH combined with the development of accessible progesterone Radio-Immuno assays offered new opportunities for the treatment of reproductive disorders (Thibier *et al.*, 1977; Humblot and Thibier 1980, 1981). Progesterone in plasma then milk allowed the characterization of ovarian activity and the different types of postpartum reproductive disorders. Achieving this was "the birth of precise reproductive phenotypes"

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(As Mr Jourdain in "Le Bourgeois gentilhomme" [Molière] who did speak "prose" without knowing it, we were establishing the first reproductive phenotypes…) and gave opportunities to develop targeted treatment protocols depending on ovarian cyclicity (Humblot and Thibier 1981, Thibier *et al*., 1985). These studies were followed by the wave of systematic synchronisation treatments followed by fixed time AI(s) known today as FTAIs (see for review Sartori *et al*., 2016). All these first steps together with the development of efficient protocols to synchronize oestrus in dairy and beef cattle (Chupin *et al*., 1974; Deletang, 1975; Grimard *et al*., 1995; Humblot *et al*., 1996) were crucial for the subsequent emergence of embryo based technologies.

Characterization of reproductive disorders Post AI / embryo mortality:

In ruminants, the access to progesterone assays, the discovery of pregnancy specific proteins from the conceptus (Martal *et al*., 1979; Thatcher *et al*., 1989) and later produced by placental cells (Butler *et al*.,1982; Sasser *et al*.,1986; Beckers *et al*., 1999; Perenyi *et al*., 2002) allowed deciphering the mechanisms by which pregnancy was maintained or leading to embryo mortality. In the cow, the consequences of embryonic losses on luteal function were determined (Northey and French, 1980; Humblot and Dalla Porta, 1984) showing that contrary to later losses, embryonic mortality before day 14 post-AI do not induce any change in oestrus cycle length. This information associated with the characterization of the Pregnancy Associated Glycoproteins (PSPB/PAGs) profiles were the basis to determine the relative weight of early and late embryonic losses (Humblot *et al*., 1988; Humblot 2001). The results, obtained from thousands of cows (both dairy and beef breeds) showing the higher frequencies of very early losses (either non fertilization or early embryonic mortality) when compared to later losses were further confirmed with other methods and in a different environment and breeds (Diskin *et al*., 2006). In addition, PAG measurements when repeated sequentially allowed the precise characterisation of the time of embryo death or later abortions. While revealing the strong gap between the time at which embryo death occurred and clinical abortion (reaching often 2 months or more) they represented a much better source of information to identify the causes of pregnancy failures and were the source of more precise phenotypes for pregnancy failures (Dobson *et al*., 1993; Wallace *et al*., 1997; Humblot, 2001). More recently, new systems have been successfully developed for assaying PAGs in cow blood or milk (Ricci *et al*., 2015). At the same time the PAGs family has been enriched with some new members allowing their measurement at an earlier stage of pregnancy (Touzard *et al*., 2013), but today there is still a need for a specific and reliable marker of "non pregnancy" which being predictive of return in oestrus, would allow the planning of a new AI.

The bull as a major source of variation of fertility

When the decrease in fertility following AI was ascertain, investigating differences in fertility between AI bulls became a concern. At the beginning of the 80's, we analysed data from AI centres reputed for their proper management of information, gathering at this occasion the results of several millions of AIs and the available sources of variation which represented huge data sets. The procedures for the transfer of data, their validation and statistical analysis had to be customized and computing time and resources appeared as limitations. Although the bull factor was found significant, the results revealed that differences originated from a few extreme individuals representing less than 10% of the population (either with a very high or a very low fertility). The sequential analysis of non return (absence of oestrus following AI, being predictive of pregnancy) rates recorded at different times after AI did show that the bull used for AI influenced almost exclusively non fertilization or early embryonic mortality (before 14 days of pregnancy in the cow), subsequent impacts on fertility (late embryonic mortality or abortions) being very marginal (Humblot *et al*., 1991). These results were further confirmed in one field trial where fertility phenotypes were defined with more precision from few thousands of AIs (Grimard *et al*., 2006). Beyond the results obtained, the above work was an excellent opportunity to develop a fruitful collaboration with bioinformaticians and biostatisticians. This challenging experience, as a young reproductive physiologist, strongly influenced my education and way to approach research.

Changes in AI practices and sperm processing; consequences for reproductive performances and the environment

In cattle, the landscape of AI has changed considerably since the 70's. The changes are essentially linked to increased herd size, improved automatization and recordings associated to a relative reduction in man-power. This combination was not necessarily favourable to fertility as AI success is still related to the quality of heat detection in the absence of FTAIs. Two major changes in AI practice and technology occurred during the past decades (Grimard *et al*., 2018). i) The number of AI performed by farmers instead of specialized AI technicians increases regularly especially in herds >100 cows (for instance +12% in France between 2015 and 2016). The impact of this practice on fertility is difficult to evaluate as non-return rates are evaluated from records deviating from the usual standards. ii) Semen sexing

became a commercial reality with patent advantages for individual farmers and breeding companies. There is still no alternative to flow cytometry and related logistics (Galli, 2017). Today, there is still differences in fertility (from 8 to 15% lower) in cows and even heifers following AI's performed with sexed and conventional semen (Le Mezec, 2018 cf review from Grimard *et al*., 2018) showing that the unfavourable consequences of sperm processing through flow cytometry are not fully controlled.

The advantages of encapsulation of sperm giving more flexibility in timing of AI have been put forward for a while (Ghidoni *et al*., 2008), and improvements of sperm quality have been reported in an *in vitro* study (Alm-Kristiansen *et al*., 2018). However so far field results are not so demonstrative (Standerholen *et al*., 2015). Other issues relate to the environmental impact of adding antibiotics during sperm processing. Due to the large amounts of extenders to be prepared, inducing antibiotic resistance may be of critical importance especially in the pig (Morrell 2016; Morrell and Humblot, 2016). With this perspective, all alternative solutions lowering the potential impact of sperm handling on the environment would be most useful.

For each of these fields related to semen processing and AI practice, new technological developments are awaited to improve fertility results while limiting at the same time possible impact of AI technology on the environment. In the near future, profit may be taken from the evaluation of seminal plasma (SP). Effectively, protein patterns in SP, which are representative of individuals, have been related to resistance to freezing, survival of sperm in the genital tract (Soleilhavoup *et al*., 2014; Rickard *et al*., 2015), fertility (Morrell *et al*., 2018) and also to be involved in immune-tolerance mechanisms which may be of importance especially for the success of implantation (Robertson, 2005).

Changes in genetic selection objectives and consequences for reproductive performances

In the seventies inheritance of fertility and its relationship with dairy production was already a concern (Foote, 1970; Maijala, 1976). However, reproductive performances, still acceptable by this time declined steadily and even more during and after the 80's (Royal *et al*., 2000; Barbat *et al*., 2010). Following studies developed initially in Nordic countries, mostly Sweden (Maijala, 1976), studies on the heritability of fertility traits (Humblot and Denis, 1986) suggested that genetic selection for milk yield could be partly responsible for the decline in reproductive performance. These results were confirmed and much documented from further studies demonstrating strong negative genetic links between milk production traits and reproductive traits in all French dairy breeds (Boichard and Manfredi, 1994; Ducrocq *et al*., 2008). This led to develop a genetic evaluation based on fertility and other functional traits, which was routinely used in France since year 2000 and helped to adjust breed selection objectives.

In most European countries, genomic selection has now been implemented for about 10 years. It is well established that genomic selection especially due to increased precision is much more efficient than former selection from quantitative genetics to orientate favourably reproductive traits or other traits with low heritability (Barbat *et al*., 2010). Considering selection objectives which are more balanced than in the past, this could lower considerably the decrease in reproductive performance observed these last decades in most dairy breeds or even lead to some recovery (Barbat *et al*., 2010, Le Mezec, 2017). However, in countries where the use of FTIA protocols is very frequent, there is also a risk to select cows for their responsiveness to oestrus synchronization treatments instead of selecting for more physiological fertility traits (Lucy, 2001). Despite a more balanced selection, dairy farmers will have to deal with individuals producing more and more (Britt *et al*., 2018) and all problems related with high production are far from being solved. Although improvements in diets and management of feeding takes place there is still strong individual variations in the way the cows are dealing with the metabolic challenge they are faced too (Bedere *et al*., 2017; Mellouk *et al*., 2017; Ntallaris *et al*., 2017). Responses to lactation and feeding are associated to huge differences between individuals in changes in Negative Energy Balance, body condition and fat mobilization (Mellouk *et al*., 2017; Ntallaris *et al*., 2017). Effects of energy restriction on reproductive performance due to excessive fat mobilization can be even more pronounced in suckled beef cows (Grimard *et al*., 1995, 1997). Understanding these issues may help to find solutions to lower the amplitude of these changes during the postpartum period. This may lead in turn to a better control of the reestablishment of ovarian activity and overall reproductive performance. In this perspective, the impacts of fat mobilization on inflammatory processes and sensitivity to post-partum diseases such as endometritis (Wathes *et al*., 2009; Valour *et al*., 2013) are major issues but practical diagnostic tools are still missing. Such tools are needed to diagnose cows with sub-clinical inflammation, decide if AI is appropriate or not and define alternative therapies. In addition, such markers will give the basis for new phenotypes to be used in future selection programs with the objective to produce more robust animals, not only for reproductive traits but also for resistance to diseases.

Embryo technologies

The development of embryo based biotechnologies started in the 70's. The use of MOET's (Multiple Ovulation Embryo Transfer) programs became very popular and the proportion of bulls favourably tested issued from embryo transfer and used massively as AI sires, increased very quickly to reach about 90% before the development of IVF-IVP (*in vitro* fertilization- *in vitro* production) occurring in the 90's.

The technical developments of *in vivo* embryo transfer, which took place during these 20 years have been reviewed by Ponsart *et al*., 2004. Superovulation protocols included initially the use of eCG (equine Chorionic Gonadotrophin), which has been replaced successfully by FSH in the 80's (Nibart and Humblot, 1997a). Crucial improvements occurred with the use of non-surgical techniques for collection and subsequent transfer of embryos together with the optimization of freezing protocols ultimately allowing routine use of direct embryo transfer which was thus performed as an ordinary AI. The development of procedures insuring the quality of field work and the safety of conditioning fresh and frozen embryos made embryo transfer the safest way to exchange genes as reviewed by Thibier 2001, 2011. I contributed marginally to these first improvements, the corresponding work from our group being performed mainly by M Nibart who was coordinating the activities of embryo transfer technicians in France and established first strong connections with Brasil (Nibart *et al*., 1997) where the success of the technique became exponential. In commercial groups in France, as well as in other countries in Europe mean pregnancy rate close to 60% were easily achieved after on-farm non surgical transfer of single fresh embryos (Nibart and Humblot, 1997b; Ponsart *et al*., 2004). Embryo sexing became considered, to better target the use of embryo transfer either for the benefit of the farmer who wanted new female calves of a high genetic merit or to provide male calves as future sire candidates for breeding companies (Thibier and Nibart, 1995). The success of embryo development following biopsy and achieving pregnancies from frozen and biopsied embryos became critical (Lopes *et al*., 2001). Pregnancy rates over 60% were rapidly obtained by different groups following the transfer on farm of fresh biopsied *in vivo* produced embryos (Lacaze *et al*., 2008; Ponsart *et al*., 2008) and later on similar percentages were reported following use of frozen biopsied embryos either on farm or in station (Gonzalez *et al*., 2008).

IVF-IVP and subsequent embryo based technologies such as cloning have been reviewed very nicely and extensively by C. Galli (AETE pioneer award 2017). As it would be inappropriate and vain to develop the matter with a similar approach, we will focus here on the practical issues we did try to address with the group in the Research and Development department of UNCEIA (Union Nationale des Coopératives d'Insémination Animale). The advantages and some of the questions raised by the use of these technologies and emerging ones in selection schemes especially in relation with genetic variability will be discussed.

Improving the quality of oocytes and embryos

While postpartum dairy cows meet a more or less pronounced status of negative energy balance (NEB), investigations performed in donor cow and heifers revealed that these do not usually suffer from energy deficit (Humblot *et al*., 1998). On the contrary, embryo donors were very frequently overfed and present high concentrations of glucose and insulin associated to a high Body Condition Score (BCS). Due to positive effects on follicular growth, these characteristics may be favourable to the superovulatory response but not necessarily to fertilization and early embryo survival. In donor cows with high BCS, the number of unfertilized oocytes was increased (Humblot *et al*., 1998). Superovulated dairy heifers submitted to a high growth rate presented high concentrations of insulin (Freret *et al*., 2004) and blastocyst development following repeated OPU (Ovum Pick up) and IVF was decreased when compared to restricted ones (Freret *et al*., 2006). This led to the concept that a transient increase of energy could be favourable to follicular growth and superovulatory response whereas constant exposure to high energy may affect negatively fertilization and early embryonic development (Humblot *et al*., 2008; Garnsworthy *et al*., 2009).

It was confirmed later on, that exposure of restricted donor heifers to a transient increase in energy brought by propylene glycol which increased insulin levels, improved the superovulatory response and the production of high quality embryos (Gamarra *et al*., 2015). Although the full mechanisms by which such effects are induced is still to be deciphered, the favourable changes observed could be related to restoration of critical gene expression of the IGF system in follicles associated to epigenetic effects in blastocysts (Gamarra *et al*., 2018). There is still issues to be solved while making the above diets attractive for donors and their use practical. If successful, they may be applied also more extensively in dairy or beef cows for which energy supply is often a limitation (Grimard *et al*., 1995, 1997). The positive effects of improved diets could be used to optimize the results of MOETs or OPU-IVP programs through more "personalized approaches" when implementing superovulation protocols or even before AI. Similarly, the measurement of anti-Müllerian

hormone (AMH) which helps to predict an animal's response to superovulation (Rico *et al*., 2009; Mossa *et al*., 2017), may be used to individualize treatment protocols. This is probably more promising than implementing selection on this phenotype that would result in a drastic reduction of families in selection schemes detrimental to genetic variability.

Embryo technologies and selection

At the same time we tried to improve reproductive technologies and control better the factors influencing the success of superovulation, *in vivo* and *vitro* production, pregnancy rates after embryo transfer as fresh or frozen, work was done on the concept of assembling different techniques for the sake of genetic selection and later on in the emerging context of genomic selection. These efforts were both, technically and politically driven. Our work was supported by breeding companies, thus, demonstrating the advantages of the different embryo based biotechnologies for selection purposes was a major concern mixed with the necessity of making embryo based techniques economically sustainable. Although being reproductive physiologists, our "*Credo*" was; Genetic progress:

$$\Delta g = \frac{\text{(selection pressure x precision x genetic variability)}}{\text{Generation interval}}$$

We had to consider how reproductive biotechnologies could serve each of the terms of this basic equation. This was not so simple as for instance selection pressure and the possible resulting genetic variability of a given trait are antagonistic. Also, as mentioned before, due to negative genetic correlations, selecting exclusively for a given trait would be detrimental to other traits in a very "efficient" way (this has been demonstrated from the example of milk production and reproductive traits). The practical cost of the techniques was an additional parameter conditioning our activities and the respective development of each embryo based technologies. Due to this combination of constraints, the different techniques were more or less affected by the evolution of genetic selection and the environment of the milk market. In France and more generally in Europe, AI and *in vivo* embryo transfer were well implanted, considered as robust and not too expensive under well established routines. Implemented by AI technicians/or specialized ones their application by the breeding companies was not put into question. On the contrary, although significant improvements in embryo production related to oocyte maturation (Humblot *et al*., 2005; Lequarre *et al*., 2005), culture systems (Menck *et al*., 1997; Guyader Joly *et al*., 1998; Holm *et al*., 1999, 2002) and embryo freezing (Vajta *et al*., 1997, 1999; Guyader Joly *et al*., 1999; Diez *et al*., 2001) have been achieved, IVF-IVP technologies were chronically and sometimes acutely seen as expensive, not always reliable or not efficient enough. In the context of genetic schemes, using males of a high genetic merit, not necessarily among the most fertile ones was mandatory. Despite efforts were made to customize the *in vitro* production system, especially fertilization steps (Marquant Le Guienne *et al*., 1990; Marquant Le Guienne and Humblot, 1998), the direct effect of the bull on fertilization and early development rates as evoked above in the context of AI, represented often (and still represents) an additional limitation for the production of viable embryos. In addition, the need for consistent investments, both in terms of facilities (laboratory and station) and personnel, made them regularly criticized. Despite strong advantages especially in terms of generation interval and genetic variability were seen (Humblot *et al*., 2010; Humblot, 2011) they did not balanced sufficiently the above limitations in the hands of European "genetic drivers". However, the economical situation and bases for marketing genetics were totally different in other parts of the world, especially South America and most particularly Brazil, where the growth of IVF-IVP techniques became exponential at the same time these were confronted to limitations in their development in Europe (the number of transfers with *in vitro* produced embryos were reduced by -33% between years 2003 and 2004; Lonergan, 2004; Merton, 2005).

Things started to change and a new era opened for embryo based biotechnologies with the emergence of the first generation of genomic selection. In the bovine species, the discovery of DNA regions where polymorphism was associated with phenotypic performance for traits of interest (QTL; Quantitative Trait Loci) was at the origin of the present revolution in the selection process. From 2000 to 2005 a few QTL of interest were available and the idea emerged to genotype embryos for those markers before transferring them with the main objective to increase selection pressure. This was the birth of the programme "TYPAGENAE" in which we planned to combine different embryo biotechnologies to perform genotyping on embryonic material (Le Bourhis *et al*., 2008, 2010; Humblot *et al*., 2010; Humblot, 2011). Due to the very limited amount of biological material available from the biopsy, it was planned to use biopsy culture techniques and cloning of blastomeres to satisfy the DNA requirements for typing. Although possible, such procedures where quite heavy to set up for a routine use and fortunately very quick improvements in DNA typing techniques allow bypass these steps. All the work previously done to improve the freezability of biopsed *in vivo* or *in vitro* produced blastocysts was valorized in this application (Guyader Joly *et al*., 2008). By the time the programme was initiated some advantages were found in terms of genetic progress (Humblot *et al*., 2010). Surprisingly we observed that the efficiency of

the technical steps were not among the major variables influencing genetic gain. The need for a high selection pressure and economical factors such as the price of heifers had more weight than any of the reproductive steps involved in the process. However this was found in the context of selection for a single trait. As selection for multiple trait is much more demanding in terms of genetic resources, it is likely that the efficiency of reproductive techniques would be more important in this context. By this time, it was clear that the progresses made in genomic selection (genomic tools, number of markers, genomic knowledge from parents/former generations, precision of the genetic estimation related to the size of reference populations) increased the potential advantages of using embryo genotyping. The technique is now used by the major breeding companies in Europe and worldwide (Le Bourhis *et al*., 2010; Shojaei Saadi *et al*., 2014). Two years ago, the workshop organized on this topic (Association of Embryo Technology in Europe - AETE, 2016) revealed that the major limitations were related to logistics i.e access to a typing center and delay of response. This technique is also easier to handle for breeding companies running field stations with OPU donors and recipients. In this context, the percentage of genotyped embryos is no more marginal, and reach today 40% of the total number of transfer performed by some selection units in Europe (S Lacaze, 2018; Auriva, Denguin, France; personal communication, AETE activity statistics 2017). Other benefits results from the systematic eradication of known genetic defects at an early stage. In the future, embryo genotyping may favours also the management of genetic variability through the optimization of the use of available recipients which is still a limiting factor in European conditions.

There are many discussions today about emerging technologies susceptible to change completely the practices in genetic selection. Using methods derived from rodents (Brinster and Avarbock, 1994), advances in culturing cattle and pig spermatogonial stem cells (SSCs) have occurred over the past few years (Oatley, 2018). These cells have the capacity to regenerate spermatogenesis following transplantation into testes of a recipient male that lacks endogenous germline. There is still limitations in the proliferation of SSCs to provide sufficient numbers of cells for transfer into multiple recipient males. If successful, this ability could be exploited in livestock production as a breeding tool to shorten generation interval then enhancing genetic gain. Another possibility raised from the recent work of Bogliotti *et al*. (2018) would be to use embryonic stem cells as donors for nuclear transfer to produce blastocysts. Both types of techniques could be combined with gene editing to produce animals with close specific characteristics. The potential interest of Parental Allele Gene Editing (PAGE) for selection has been put forward a few years ago (Jenko *et al*., 2015). On the contrary, recent reports showed that the genetic gain allowed by gene editing would be quite low (especially if causal mutations corresponding to a given trait are not perfectly identified) and extremely costly (Simianer *et al*., 2018). It may be possible to overcome all technical issues one day or another. However it is very difficult to see how the intensive production from a limited set of donor animals induced by these technologies would not be detrimental to genetic variability. As its maintenance in the main dairy breeds is of a crucial importance today to insure the sustainability of dairy cattle productions (Colleau and Sargolzaei, 2011; Colleau *et al*., 2017; V. Ducrocq, 2018; INRA, Jouy en Josas, France; personal communication), it is unlikely, due also to the limitations of PAGE in the context of complex traits (Gao et al., 2017), that use of these new technologies will develop quickly in dairy cattle selection schemes. Nevertheless, it will be interesting to follow the development of these techniques and the place they may find for other types of productions and in other species. The social acceptability of these techniques should also be discussed in the future when considering the growing concern related to a more natural approach of breeding practices.

Functional genomics in reproductive tissues

Relationships between reproductive phenotypes and gene expression

By the end of the 90's, methods based on use of a limited set of informative genomic regions (initially a few QTLs, quantitative trait loci) were implemented in selection schemes (Meuwissen and Goddard, 1999). The promising results obtained in terms of genetic progress raised the need to enrich and refine the set of markers available. An agreement between breeding companies and the French national research funding agencies created a favourable environment to run genomic studies aiming at developing new methods and at identifying new markers for genomic selection. As other functional traits, reproductive traits were among the most difficult to select with conventional methods and thus were susceptible to benefit largely from genomic selection. This gave us the opportunity to initiate projects to relate phenotypic and genomic information in reproductive tissues. A QTL approach based on the registration of precise phenotypic information obtained in young bulls allowed the identification of 15 markers for sperm quality (Druet *et al*., 2009). As early embryonic mortality or lack of fertilization were major sources of poor fertility (see above #1) and due to the strong relationships existing between oocyte growth, maturation, the first cleavages and the success of subsequent embryonic development and maintenance of pregnancy (Lonergan *et al*., 1999; Sirard, 2001; Sirard

et al., 2006; Lequarré *et al*., 2005; Humblot *et al*., 2005), several projects aimed at studying oocyte quality and related gene expression (Pennetier *et al*., 2005). Putative markers were identified from extreme phenotypes (Guyader Joly *et al*., 2007) and differential gene expression in relation with oocyte maturation (Angulo *et al*., 2015). Benefits have been taken also from the experience obtained from the study of the sources of variation of reproductive performance (Grimard *et al*., 2006) to approach differences in fertility between progeny groups from a large data base and relate them to the existence of candidate mutations in Holstein cows (Ledoux *et al*., 2015). This work allowed the identification of one QTL for early embryonic mortality in the Prim'Holstein cow (Lefebre *et al*., 2011).

The results from these first functional studies on reproductive genomics had so far a marginal impact on genomic selection progressing mainly today from the use of a very large set of markers with whole genome approaches (V. Ducrocq, 2018; INRA, Jouy en Josas, France; personal communication). However, these projects helped to clarify the specific impacts of genetic variants on reproductive function (Coyral-Castel *et al*., 2011; Ledoux *et al*., 2015). In other projects, attention was paid on the relationships between reproduction and metabolism. The ability of cows to be fertilized and sustain pregnancy was investigated through the study of the effects of diet on gene expression in the genital tract (Valour *et al*., 2013). As a continuation, we use the cow endometrium and *in vitro* models to study the impacts of metabolic and infectious stress on gene expression and pro-inflammatory response in the endometrium (Chanrot *et al*., 2017a, b; Guo *et al*., 2016; Piras *et al*., 2017; Chankeaw *et al*., 2018). Together with others (Oguejiofor *et al*., 2015a,b; Salilew-Wondim *et al*., 2016), these studies reveal that infectious stress alters a very large number of genes belonging to pro-inflammatory, proliferative, metabolic and oxidative stress (over-expressed) and to cell structure and cell adhesion (under-expressed) pathways. Some of these changes have been documented in different cow models, but the above studies show that alterations of endometrial function concerns also a large number of genes involved specifically in maternal recognition of pregnancy (Cheng *et al*., 2017), immune-tolerance and implantation (Guo *et al*., 2016; Piras *et al*., 2017). Such studies bringing a more complete view of alterations induced by pathogens, pave the way for *in vivo* work and will probably be the source of alternative therapies in the future.

In addition, some of the above studies confirm the links between metabolic imbalance and increased sensitivity to infectious stress through increased gene expression promoting pro-inflammatory reactions. Together with other approaches based on metabolomics (Munoz *et al*., 2014a, b) measurement of NEFA's in milk (Martin *et al*., 2015) or other biomarkers (Adnane *et al*., 2017) they may allow developing predictive tools to evaluate the ability of cows to re-establish ovarian activity, be able to sustain pregnancy following AI or embryo transfer and increase success rates.

The "renaissance" of Epigenetics and its potential for selection and precision medicine

Different theories have been put forward with an evolutionary perspective. Among those the theory of Lamark (1744-1829) proposed a "soft adaptation" of species to their environment and possible transmission of induced changes to next generations. This theory has been debated for long but has taken over former criticism with the accumulation of scientific evidence obtained from examples showing the importance of transgenerational epigenetics (Haig, 2007). The concept that gene expression is controlled by epigenetic mechanisms and that DNA associated molecular patterns can be transferred to next generations is now well established (Segars and Aagaard-Tillery, 2009). The knowledge accumulated in that field based on the development of next generation sequencing technologies raises numerous common challenges to be addressed for public health and animal health. Humans and animals share a large number of diseases, for instance either metabolic diseases or those induced by pathogens. The occurrence and severity of diseases are often determined by the environment humans and animals are exposed to. There is now evidence that the development/severity of many diseases is linked to epigenetic mechanisms controlling for instance DNA accessibility to pathogens and improper immune response of host (Doherty *et al*., 2016). The fact that some of the mechanisms initiating the development of metabolic, cardio-vascular or neurological diseases are taking place during the peri-conception period is now largely documented (Van Soom and Fazeli, 2015; Fazeli and Holt, 2017; Ord *et al*., 2017). This put the maternal environment and more generally reproduction in a central place and associated knowledge particularly critical for public and animal health. The involvement of epigenetic mechanisms in the development of diseases represents already a huge field of research in the human species. Animals are intensively used as experimental models or often as sentinels in the case of wild life to evaluate the impact of the environment on diseases (Guillette *et al*., 2016). However, there are many fields where specific research made in animals (including livestock species), can contribute to improve animal productions and welfare. Progresses made in the description of animal genomes and reduction of the costs, offers new opportunities to perform these studies and it can be foreseen that epigenetic studies will be the basis for new developments for reproductive physiology and biotechnologies. Obtained from models or taken

from the environment, the identification of extreme phenotypes combined with use of genetic and epigenetic information allowed already the identification of critical set of genes and epigenetic marks associated to cell processes as altered responses to disease (Jhamat *et al*., 2016). Although more complex such links can be established between the embryo and its environment either metabolic (Laskowski *et al*., 2018) and or induced by use of embryo technologies like cloning (Beaujean, 2014; Sepulveda-Rincon *et al*., 2016) or embryo culture (Salvaing *et al.,* 2016). Further steps include functional studies to validate the differences observed at the genetic and epigenetic levels and eventually the role of critical genetic variants that would be associated with increased risk to suffer from diseases. Due to the complexity of the mechanisms involved, especially the multiplicity of epigenetic marks and their respective roles, it will take time to integrate epigenetics in selection schemes but its association with genomic information can be anticipated (Britt *et al*., 2018; V. Ducrocq, 2018; INRA. Jouy en Josas, France; personal communication) especially in the field of resistance to disease. There are also emerging fields such as inter-cellular communication through extra-cellular vesicles (Hwang, 2013). New knowledge about the molecular and especially the epigenetic signals they vehicle, will bring substantial improvements in the diagnostic and therapy of diseases (Giebel *et al*., 2017) and probably be the source of critical information for embryo survival (Rizos *et al*., 2017).

Conclusions

This retrospective and present developments in genetics biology, developmental biology and reproductive techniques raise a lot of questions in many different fields. It is not possible at the moment to give the right answers for most of them. However there is no doubt that these questions are very critical for future generations especially in Livestock species due to the weight of human decisions and the acceleration in the selection process associated to genomic selection. Reproduction and reproductive technologies will go on to play a central role as their efficiency is a key element to preserve genetic variability. New technologies such as genome editing or reproduction techniques based on germinal cells of a few individuals to shorten generation interval are tempting for the breeding industry. On top of technical limitations and high costs, there is a big risk that they will increase dramatically consanguinity thus altering many other traits such as reproductive efficiency. It is not risky to predict that phenotyping will go on to progress and this will be very positive to facilitate the selection for new traits important for animal health and or the environment. As part of the picture, although still rather complex to obtain due to limitations of the bovine genome, the integration of epigenetic data in selection schemes is promising especially for traits such as resistance to diseases. Reproduction and especially embryo developmental biology stands in the central place to approach the epigenetic mechanisms translating the impact of the environment on individuals especially at time of peri-conception which induce the development of physio-pathological processes leading to diseases. Strong limitations still exist when approaching for instance reproductive diseases or the impact of the environment on reproduction from molecular studies generating huge amounts of data to be integrated. The end of this reproductive journey revealed the need to reinforce the tiny links between the communities of reproductive physiologists, molecular geneticists, bio-informaticians and bio-statisticians to investigate arising problems with common translational approaches.

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Commercial Embryo Transfer Activity in Europe 2017

Collated by Marja Mikkola

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	Istvan Pentek
Ireland	Patrick Lonergan
Israel	Amir Shifman, Yoel Zeron
Italy	Giovanna Lazzari
Latvia	Vita Antane
Lithuania	Rasa Nainiene
Luxembourg	Marianne Vaessen
Macedonia	Toni Dovenski
The Netherlands	Helga Flapper, Hilde Aardema
Norway	Eiliv Kummen
Poland	Jędrzej 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, Brian Graham

Bovine *In vivo* embryo production

Country	Collections	Viable embryos	Embryos/ collection	Dairy breeds (% collections)	Sexed semen (% collections)
Austria	271	2014	7,4	93,0 %	3,7 %
Belgium	1160	5973	5,1	12,5 %	0,3 %
Bosnia and Herzegovina	0	0			
Croatia	0	0			
Denmark	759	4785	6,3	92,4 %	0,0 %
Estonia	0	0			
Finland	310	1949	6,3	99,4 %	2,9 %
France	6729	35277	5,2	79,7 %	14,0 %
Germany	3412	22882	6,7	88,8 %	0,0 %
Greece	0	0			
Hungary	60	371	6,2	30,0 %	28,3 %
Ireland	785	4328	5,5	100,0 %	0,0 %
Israel	60	212	3,5	100,0 %	3,3 %
Italy	2500	19883	8,0	94,0 %	40,0 %
Latvia	6	0	0,0	100,0 %	0,0 %
Lithuania	4	23	5,8	100,0 %	0,0 %
Luxembourg	212	1151	5,4	95,3 %	10,8 %
Macedonia	0	0			
Netherlands	2493	21910	8,8	100,0 %	0,0 %
Norway	60	370	6,2	61,7 %	0,0 %
Poland	226	1177	5,2	93,8 %	42,0 %
Portugal	102	510	5,0	69,6 %	26,5 %
Russian Federation	1855	12832	6,9	30,8 %	26,4 %
Serbia	6	25	4,2	100,0 %	0,0 %
Slovakia	0	0			
Slovenia	11	33	3,0	100,0 %	0,0 %
Spain	551	2930	5,3	67,0 %	45,0 %
Sweden	130	564	4,3	97,7 %	0,0 %
Switzerland	543	3911	7,2	94,5 %	49,2 %
Ukraine	10	21	2,1	100,0 %	0,0 %
United Kingdom	61	327	5,4	11,5 %	4,9 %
Total	**22316**	**143458**	**6,4**	**79,1 %**	**14,1 %**

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

Country	Sessions	Oocytes	Oocytes/ Session	Embryos	Embryos/ Session	Stimulated sessions (%)	Sexed semen (%)	Dairy beeds (%)
Finland	455	3748	8,24	1211	2,66	77,4 %	17,8 %	100,0 %
France	691	6171	8,93	1756	2,54	99,0 %	18,4 %	95,7 %
Germany	1020	13880	13,61	1794	1,76	55,2 %	0,0 %	98,3 %
Italy	482	4819	10,00	818	1,70	21,6 %	40,2 %	100,0 %
Netherlands	7345	83421	11,36	16695	2,27	80,0 %	0,0 %	100,0 %
Poland	14	89	6,36	34	2,43	0,0 %	78,6 %	100,0 %
Russian Federation	7758	87120	11,23	26762	3,45	0,0 %	73,6 %	42,2 %
Spain	839	11122	13,26	2746	3,27	12,0 %	65,8 %	86,1 %
Switzerland	41	312	7,61	81	1,98	0,0 %	56,1 %	100,0 %
United Kingdom	39	415	10,64	74	1,90	0,0 %	7,7 %	7,7 %
Total	**18684**	**211097**	**11,30**	**51971**	**2,78**	**49,0 %**	**35,8 %**	**74,9 %**

Bovine *In vitro* embryo production - abattoir

Country	Donors	Oocytes	Embryos	Embryos/ Donor
Italy	16	566	107	6,7
Netherlands	21	1041	177	8,4
Russian Federation	34	654	105	3,1
Spain	235	1912	480	2,0
Switzerland	3	103	39	13,0
Total	**309**	**4276**	**908**	**2,9**

Bovine embryo technologies

Country	Sexed embryos		Genotyped embryos	
	In vivo	In vitro	In vivo	In vitro
France	2491	0	2388	0
Germany	768	73	367	19
Netherlands	0	0	164	1080
Total	**3259**	**503**	**2919**	**1099**

Embryo exports

Country	Embryos exported		
	Bovine, dairy	Bovine, beef	Bovine, non-separated
Austria	274	5	0
Belgium	35	58	0
Denmark	51	0	0
Finland	53	0	0
France	250	304	0
Luxembourg	50	0	0
Netherlands	1701	0	0
Norway	0	40	0
Spain	43	0	160
Switzerland	97	0	0
United Kingdom	0	119	0
Grand Total	**2554**	**526**	**160**

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	640	824	77	12	95	27	0	0	0	1675
Belgium	173	493	1616	995	3017	34	0	0	0	6328
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	2494	1253	0	129	124	0	0	0	0	4000
Estonia	0	0	0	0	0	0	0	0	0	0
Finland	722	1484	212	9	1	33	0	0	0	2461
France	14216	12668	1615	2630	4548	289	56	0	0	36022
Germany	8047	11078	0	717	1351	0	0	0	0	21193
Greece	0	0	2	0	0	0	0	0	0	2
Hungary	60	58	0	29	129	0	0	0	0	276
Ireland	1698	2155	0	0	0	0	0	0	0	3853
Israel	192	8	0	0	0	0	0	0	0	200
Italy	7400	0	0	0	0	0	0	0	0	7400
Latvia	0	0	0	0	0	0	0	0	0	0
Lithuania	0	0	0	0	0	0	0	0	0	0
Luxembourg	280	800	35	20	70	0	0	0	0	1205
Macedonia	0	0	0	0	0	0	0	0	0	0
Netherlands	6303	17867	0	0	0	0	0	0	0	24170
Norway	35	142	0	25	28	113	0	0	0	343
Poland	760	288	123	20	3	0	0	0	0	1194
Portugal	92	339	0	31	32	13	0	0	0	507
Russian Federation	823	1484	24	45	11334	0	0	0	0	13710
Serbia	10	15	0	0	0	0	0	0	0	25
Slovakia	0	0	0	0	0	0	0	0	0	0
Slovenia	13	5	0	0	0	8	0	0	0	26
Spain	453	485	59	403	147	21	664	471	56	2759
Sweden	205	412	0	2	0	0	0	0	0	619
Switzerland	1012	2152	622	32	69	32	0	0	0	3919
Ukraine	21	0	0	0	0	0	0	0	0	21
United Kingdom	21	23	8	57	116	37	0	0	0	262
Total	**45670**	**54033**	**4393**	**5156**	**21064**	**607**	**720**	**471**	**56**	**132170**

Bovine *In vitro* embryo transfer

Country	OPU			Abattoir			Total transfers
	Fresh	Frozen domestic	Frozen foreign	Fresh	Frozen domestic	Frozen foreign	
Finland	49	798	0	0	0	0	847
France	815	329	49	19	3	0	1215
Germany	1508	174	0	0	0	0	1682
Hungary	0	0	105	0	0	0	105
Italy	212	503	0	0	42	0	757
Netherlands	10196	4731	0	63	114	0	15104
Poland	16	7	0	0	0	0	23
Russian Federation	20302	7658	0	0	0	0	27960
Spain	1407	448	12	28	20	3	1918
Switzerland	2	7	117	0	0	0	126
United Kingdom	0	15	0	0	0	0	15
Total	**34507**	**14670**	**283**	**110**	**179**	**3**	**49752**

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

Country	Embryo collection		Embryo transfer		
	Collections	Viable embryos	Fresh embryos	Frozen domestic	Frozen foreign
SHEEP					
France	10	33	0	0	0
Hungary	5	39	0	0	45
Sweden	20	67	0	67	365
United Kingdom	557	2717	217	116	0
Total	**592**	**2856**	**217**	**183**	**410**
GOAT					
France	9	76	0	0	0
Total	**9**	**76**	**0**	**0**	**0**
HORSE					
Finland	4	1	1	0	0
France	1476	776	776	0	0
Italy	289	197	197	0	0
Netherlands	284	164	164	0	0
Poland	7	7	7	0	0
Russian Federation	3	2	2	4	0
Spain	15	12	12	0	0
Sweden	23	15	15	0	0
Switzerland	72	37	30	2	0
Total	**2173**	**1211**	**1204**	**6**	**0**
OTHER					
Slovakia	10	20	0	0	0
Total	**10**	**20**	**0**	**0**	**0**

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

Country	Embryo collection			Embryo transfer	
	OPU sessions	Oocytes	Embryos	Fresh embryos	Frozen embyos
BUFFALO					
Italy	3	66	26	0	0
Total	**3**	**66**	**26**	**0**	**0**
HORSE					
Italy	934	10866	885	82	203
Netherlands	213	2807	241	0	140
Switzerland	56	215	16	0	3
Total	**1203**	**13888**	**1142**	**83**	**346**

INVITED LECTURES

Ovarian antral follicle populations and embryo production in cattle

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Abstract

Reproductive biotechniques such as embryo production are important tools to increase the reproductive performance in cattle in a short time. In this context, the antral follicle count (AFC), which reflects the population of antral follicles present in an ovary, has been indicated as an important phenotypic characteristic related to female fertility and closely correlated to the performance of *in vivo* and *in vitro* embryo production (IVEP). A positive correlation was evidenced between AFC and oocyte retrieval by ovum pick up (OPU) sessions and and with the number of embryos produced. Several studies have reported that females with a high AFC had greater embryo yields compared to those with medium and low AFC. However, controversial results were obtained by studies conducted in different bovine breeds. Many conflicting data may be due to the differences in the experimental design, particularly regarding the classification of animals in AFC groups, subspecies particularities, herd aptitude or even issues related to animal management. Therefore, aspects such as the choice of donor, type of aspirated follicles and the stage of follicular wave need to be clarified. Thus, this text aims to discuss the use of AFC as a reproductive tool and its applications in the *in vivo* and *in vitro* production of embryos, besides describing consistent results and new challenges regarding AFC and embryo production.

Keywords: antral follicle count, *Bos taurus*, *Bos indicus*, cow, fertility.

Introduction

In the world scenario for bovine embryos, in the last decade, the *in vitro* embryo production (IVEP) has expanded remarkably when compared to *in vivo* embryo production (Watanabe *et al*., 2017). In the year 2016, a total of 666,215 *in vitro* embryos were produced, exceeding for the first time the volume of embryos produced *in vivo* (Perry, 2017). In this context, Brazil has contributed to the consolidation of large-scale application of this biotechnique and many challenges have been faced to improve the IVEP (Perry, 2016).

Several studies have been conducted to maximize the reproductive efficiency of the herd. In this way, the ovarian antral follicular count (AFC) as a tool to evaluate the ovarian reserve, has been positively correlated with parameters such as the number of viable oocytes, blastocyst (Santos *et al*., 2016) and conception rate after AI (Mossa *et al*., 2012). Different research groups investigated the embryo production performance of females with different AFC. This parameter is variable among females but highly constant in the same female (Burns *et al*., 2005; Morotti *et al*., 2017). For both *indicus* and *taurus* subspecies and *indicus* x *taurus* crossbred, a positive correlation was found between AFC and oocyte retrieval in the ovum pick up (OPU) sessions and the number of embryos produced *in vitro* (Ireland *et al*., 2008; Monteiro *et al*., 2017).

Considering embryonic production, females with high AFC presented a larger number of *in vitro* embryos when compared to those with low AFC (Ireland *et al*., 2008; Monteiro *et al*., 2017). Such information is quite predictable, considering that more follicles provide more oocytes to the *in vitro* embryo production. On other hand, the influence of AFC on the efficiency of embryonic production remains not well understood. For example, Rosa *et al*. (2018), reported no differences in both cleavage and blastocyst rates of oocytes that came from ovaries with different AFC. Also evaluating different AFC patterns, Rosa *et al*. (2018) evaluated the genes involved in oogenesis and folliculogenesis, which were differentially expressed in granulosa (*progesterone receptor - PGR* and *Anti-müllerian hormone receptor type II - AMHR2*) and cumulus cells (*natriuretic peptide receptor 2 and 3 - NPR2/ NPR3, fibroblast growth factor 10 - FGF10* and *signal transducer and activator of transcription 3 - STAT3*) from high versus low AFC cows. Mossa *et al*. (2010) reported lower abundance of *cytochrome P450 family 17 subfamily A member 1 - CYP17A1* mRNA in thecal cells of low versus high AFC. Moreover, the study by Ireland *et al*. (2009) reported that the abundance of mRNAs for *cytochrome P450 family 19 subfamily A member 1 - CYP19A1* in granulosal cells and *estrogen receptor 1 and 2 - ESR1/ ESR2*, and *cathepsin B - CTSB* in cumulus cells were greater, whereas mRNAs for AMH in granulosal cells and *TBC1 domain family member 1 - TBC1D1* in thecal cells were lower for animals with low compared to the high AFC group during follicle waves.

Considering a general view on AFC, many points need to be addressed. For example, there is no standard to the classification of AFC according to the number of follicles. Comparing all the reports at the literature, it is easy to recognize several patters of AFC groups. In this way, it is quite difficult to compare data among the articles. Therefore, considering the

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importance of AFC on the embryo production this text aims to discuss: i) The use of AFC as a reproductive tool and the relationship between different fertility parameters (i.e. blastocyst rate and pregnancy, AMH concentration, among others); ii) applications of AFC on the *in vivo* and *in vitro* production of embryos and, iii) controversial data and new challenges regarding to the practical use of AFC as a reproductive tool for cattle.

Use of AFC as a reproductive tool

The association between reproductive biotechniques and genetic improvement programs in cattle has contributed to the development of the milk and food production (Hansen, 2014). The selection of females suitable for breeding is of great importance for reproductive efficiency, mainly because some ovarian physiological characteristics can directly influence the number and quality of oocytes (Lonergan *et al*., 1994). In this context, cows can be classified as low, intermediate or high AFC, according to the number of antral follicles (of 3 mm in diameter) detected via ovarian ultrasonography.

The AFC has been related to fertility parameters such as ovarian size, corpus luteum diameter, number of healthy oocytes, endometrial thickness, progesterone concentration, pregnancy rate and calving interval (Jimenez-Krassel *et al*., 2009, Martinez *et al*., 2016). Thus, considering the relationship between AFC and several characteristics linked to fertility, the selection of donors could be performed using a single ultrasound examination at the beginning of reproductive life (Silva-Santos *et al*., 2014; Morotti *et al*., 2017). Also, the Anti-mullerian hormone (AMH), has been recognized as an indicator of ovarian response to superovulation protocols (Souza *et al*., 2015), being AMH also highly correlated to the AFC and viability of the oocytes (Baruselli *et al*., 2016). It was also observed a positive association between AMH and fertility in dairy cows (Jimenez-Krassel *et al*., 2015).

The AFC is a characteristic of low to moderate heritability, and it was verified that there is no correlation between AFC and milk production during lactation in Holstein cows (Walsh *et al*., 2014). Similarly, for Braford (beef) cattle, some parameters related to meat production showed low correlation with AFC (Morotti *et al*., 2017). Then, based on these studies, it can be suggested that AFC selection does not cause genetic demerit for the progeny.

In general, a greater number of antral follicles results in better OPU/IVEP quantitative efficiency. Many reports suggest that AFC is positively associated with the number of embryos produced by the donor in different breeds (Ireland *et al*., 2008; Silva-Santos *et al*., 2014, Santos *et al*., 2016). Although, more recently, Monteiro *et al*. (2017) did not find any advantage for *Bos indicus* females with high AFC relative to IVEP rates (Blastocyst rate 33.9% and 34.2% for low and high AFC animals, respectively).

In taurine females submitted to fixed time artificial insemination (FTAI) presenting high AFC, it was also found a higher pregnancy rate when compared to those with low AFC (94% and 84%, respectively; Mossa *et al*., 2012). In contrast, studies with indicine cattle did not find a positive correlation between AFC and pregnancy rates with artificial insemination (Mendonça *et al*., 2013; Santos *et al*., 2016). Surprisingly, an evaluation of AFC and timed artificial insemination showed better reproductive performance in low AFC Nelore cows than high AFC females (conception rate 61.7% and 49.5%, respectively; Morotti *et al*., 2018). Recently, another unexpected result came from New Zealand with *Bos taurus* dairy cattle. The results showed that females with high AFC (≥ 25 follicles) had suboptimal fertility and shorter productive life when compared to those with low AFC (≤ 15 follicles) (Jimenez-Krassel *et al*., 2017). In contrast, also working with dairy cattle, Modina *et al*. (2014) had reported that those cows with intermediate number of antral follicles (n < 10) were identified with reduction in the fertility parameters, when compared to females with > 10 follicles. Once again, the comparison between the articles is quite difficult considering that AFC was not the single condition that was different in the experimental design. As pointed by Martinez *et al*. (2016), the associations of AFC with other fertility parameters need for further evaluations to ensure the best way to use this strategy on reproductive programs.

In conclusion, the relationship between AFC and fertility needs to be clarified. Both in *Bos taurus* and *Bos indicus* cattle have shown conflicting data, mainly when different approaches, embryo transfer - ET or artificial insemination – AI, have been considered.

Applications of AFC for *in vitro* and *in vivo* production of embryos

The embryo yield is highly variable in both *in vitro* and *in vivo* embryo production systems (Pontes *et al*., 2010). The IVP may be affected by oocyte recovery rate on OPU, as well as ET is affected by the response of the donor afterthe superovulation protocol (SOV) - (Ireland *et al*., 2007; Silva-Santos *et al*., 2014). Therefore, it is interesting to consider that the success of these techniques is dependent on the individual ovarian characteristics of the donor, which may affect the number and quality of the oocytes / embryos that are recovered (Stojsin-Carter *et al*., 2016).

In 2007, Ireland and collaborators performed a study with *Bos taurus* cattle and observed that the number of IVF embryos per animal in the high AFC group (≥ 25 follicles) was four times higher (P < 0.05) than the low AFC group (≤ 15 follicles). In the multiple ovulation and embryo transfer (MOET), the number of embryos recovered after artificial insemination was 10.6 ± 2.7 *vs.* 4.7 ± 0.7 for the high and low AFC group, respectively (P < 0.05).

Similarly, *indicus-taurus* donors with high AFC (≥ 40 follicles), produced a higher (P < 0.05) number of blastocysts than animals of low AFC group (≤ 10 follicles) both in IVF and MOET approaches (Silva-Santos *et al*., 2014). However, the same team reported less consistent results for *Bos indicus* cattle. The high AFC showed better embryo production performance, but the cleavage rate was similar among

low (≤ 7 follicles), intermediate (18 to 25 follicles) and high AFC (≥ 40 follicles) (Santos *et al*., 2016).

The relationship between of the ovarian pool of follicles and the response to the superovulation has been previously evaluated (Cushman *et al*., 1999). However, possible applications of AFC and specific protocols to SOV is topic that remains to be better understood.

Considering AFC and genes related to folliculogenesis and oogenesis, it was reported differences in gene expression in cumulus and granulosa cells collected from Nelore cows with low and high AFC. Interestingly, in the study by Rosa *et al*. (2018), animals com high AFC had genes upregulated in granulosa cells while cows with a low AFC presented genes upregulated only in cumulus cells. Taking together with data from other studies, these findings suggest that AFC may influence the molecular network that controls ovarian function (Ireland *et al*., 2009; Mossa *et al*., 2010). Considering the importance of communications between oocyte and cumulus cells, as well as the influence of gonadotropins on the cAMP in the two cell types (Luciano *et al*., 2004), it is quite predictable that AFC may really interfere on the rates of embryonic production. However, a precise experiment to consider only AFC and embryo production remains a challenge, since multiple aspects need to be isolated in the experimental design.

As described in Table 1, the main problem for comparing the AFC data is the classification of animals in groups of high and low AFC. Each team considered a specific strategy. Thus, it is quite difficult to compare the results.

Controversial data among studies in AFC classification also are present in both *in vivo* and *in vitro* embryonic production, as shown in Table 2.

Considering data of pregnancy rate and AFC after artificial insemination, it is also interesting to identify the clear differences among the groups, as shown in the Table 3.

Table 1. Results (mean ± SD) from studies comparing *in vitro* embryo production in *Bos taurus*, *Bos indicus* and *Bos indicus-taurus* (crossbred) between high and low AFC groups.

Author	type	AFC	Animals n	PU n	COC's n	Blastocyst n	Blastocyst rate %
Ireland *et al*., 2007	*Bos taurus*	Low (≤ 15 follicles)	68***	---	7.5^{a}	1.3^{a}	29.6
		High (≥ 25 follicles)	37***	---	29.5^{b}	4.9^{b}	30.9
Silva-Santos *et al*., 2014	*Bos indicus-taurus*	Low (≤ 10 follicles)	20*	20	5.8 ± 3.4^{A}	0.5 ± 0.8^{A}	9.5^{A}
		High (≥ 40 follicles)	20*	20	36.9 ± 13.7^{B}	6.1 ± 4.5^{B}	16.5^{B}
Santos *et al*., 2016	*Bos indicus*	Low (≤ 7 COC's)	19*	19	$3.8 \pm 1.1^{\alpha}$	$0.6 \pm 0.6^{\alpha}$	13.0^{α}
		High (≥ 40 COC's)	22*	22	$40.4 \pm 10.6^{\beta}$	$18.4 \pm 6.7^{\beta}$	41.9^{β}
Monteiro *et al*., 2017	*Bos indicus*	Low (< 15 COC's)	18**	216	10.8 ± 0.4	3.6 ± 0.2	33.9
		High (≥ 15 COC's)	18**	216	21.2 ± 1.0	7.1 ± 0.4	34.2
Rosa *et al*., 2018	*Bos indicus*	Low (≤ 31 follicles)	356***	---	536 (total)	203 (total)	38.6
		High (≥ 92 follicles)	356***	---	617 (total)	251 (total)	40.6

*Animals submitted to OPU only once. **Animals submitted to OPU more than once. ***Animals submitted to postmortem ovarian aspiration. $^{a,b/A,B/\alpha,\beta}$For the same author and variable were different ($P \leq 0.05$) between the AFCs groups. Adapted from Ireland *et al*. (2007); Silva-Santos *et al*. (2014); Santos *et al*. (2016); Monteiro *et al*. (2017) and Rosa *et al*. (2018).

Table 2. Results (mean ± SD) from studies comparing *in vivo* embryo production in *Bos taurus* and *Bos indicus-taurus* (crossbred) between high and low AFC groups.

Author	Animals	Number of follicles	Number of flushes	Transferable embryos/ animals
Ireland *et al*., 2007	*Bos taurus*	Low (≤ 15 follicles)	21*	3.8 ± 0.8[a]
		High (≥ 25 follicles)	19*	5.4 ± 1.3[b]
Silva-Santos *et al*., 2014	*Bos indicus-taurus*	Low (≤ 10 follicles)	20**	1.9 ± 2.1[A]
		High (≥ 40 follicles)	20**	6.9 ± 5.3[B]

*The same female may have been superovulated and collected up to twice. **One single collection per animal. [a,b/A,B]For the same author were different (P ≤ 0.05) between the AFCs groups. Adapted from Ireland *et al*. (2007) and Silva-Santos *et al*. (2014).

Table 3. Reproductive performance of females with high or low follicle count (AFC) after artificial insemination.

Author	Group	Pregnancy rate (%)
Mossa *et al*., 2012	Low AFC (≤ 15 follicles)	84
	High AFC (≥ 25 follicles)	94
Mendonça *et al*., 2013	Low (≤ 12 follicles)	51.85
	High AFC (≥ 30 follicles)	44.73
Santos *et al*., 2016	Low AFC (≤ 10 follicles)	58,6
	High AFC (≥ 25 folículos)	51,9

Adapted from Mossa *et al.* (2012); Mendonça *et al.* (2013) and Santos *et al*. (2016).

AFC: consistent data versus new challenges

The strategy used to the classification of females in AFC categories is very different in each author. Each one exhibited a distinct experimental design; some studies did not consider the AFC intermediate group or, for example, the oocytes were recovered in different ways - by OPU or ovaries from a slaughterhouse (Ireland *et al*., 2008; Monteiro *et al*., 2017). In some cases, the parameters used to classify females in AFC categories differed considerably among authors (Morotti *et al*., 2015). Therefore, when comparing studies concerning AFC, we should consider how the separation of animals into the categories was made, trying to establish a more balanced comparison.

Moreover, there are differences between the studied subspecies inherent to the physiology of the estrous cycle, which encompass divergences from follicular wave number per cycle to the number of follicles recruited per growth wave (Baruselli *et al*., 2007). For example, Holstein cows tend to present the predominance of two or three waves of follicular growth per estrus cycle (Ginther *et al*., 1989; Wolfenson *et al*., 2004), whereas Zebu cows are more related to three to four waves per estrus cycle (Rhodes *et al*., 1995; Nelore - Figueiredo *et al*., 1997). Also, *Bos indicus* females recruit more follicles per follicular growth wave than *Bos taurus* females (33,4 ± 3,2 *vs* 25,4 ± 2,5; Carvalho *et al*., 2008). Taking those data together, it is easy to realize the challenge to compare studies that present results of distinct subspecies. So, the comparison of AFC with fertility in different breeds should be studied individually.

Furthermore, for a better precision of the factors that interfere with AFC, it is necessary to consider the development phase in which the follicles are aspirated since there is a direct interference on the oocyte competence for IVEP. In this context, Cavalieri *et al*. (2018), showed that cows submitted to follicular wave synchronization had a better IVEP rate and pregnancy rate after ET when compared to females aspirated on a random day of estrous cycle. Because of that, the strategy for oocyte recovery must be considered when comparing different AFC cows.

In addition to the subspecies, it is also important to consider the influence of the age of the females in the AFC studies, since there are indications, that the ovarian follicular reserve decreases after the female reaches five years, suggesting a decrease in fertility (Cushman *et al*., 2009). The same authors also reported the importance of birth weight as a parameter that influences AFC, but the stage of estrous cycle seems to not interfere in the AFC classification, which facilitates the establishment of pattern (high, intermediate or low AFC) to each cow (Cushman *et al*., 2009).

In summary, according to the literature and data cited above, AFC seems to be correlated with

several fertility parameters, and it may be a tool that can contribute to the success of embryo production both *in vivo* and *in vitro*. However, there is a great need to study the real long term impact of AFC on fertility, to establish specific parameters of AFC classification and to understand the physiological causes of the variation in the AFC among individual female cattle.

Final comments

Despite many studies on antral follicle population, the relationship between AFC, fertility, and efficiency of biotechniques are not fully understood. However, in the context of embryo production, the AFC can be used as an auxiliary tool for the selection of animals with the greater quantitative potential of embryos. Finally, a better understanding of the factors linked to AFC and of the reproductive characteristics from *Bos indicus* and *Bos taurus* may provide adjustments in cattle management and to improve the efficiency of reproductive biotechniques.

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Preservation of female fertility in humans and animal species

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Abstract

A detailed understanding of the cryobiology of gametes and complex tissues has led to the development of methods that facilitate the successful low temperature banking of isolated mature human oocytes, or immature oocytes *in situ* within fragments of human ovarian cortex. Although many outstanding research challenges remain to be addressed, the successful development of new treatments to preserve female fertility for a range of clinical indications has largely been underpinned by the conduct of extensive, fundamental research on oocytes and ovarian tissues from a number of laboratory and commercially important farm species. Indeed, the most recent evidence from large animals suggests that it is also possible to cryopreserve intact whole ovaries along with their supporting vasculature for later auto-transplantation and restoration of natural fertility. This review will explore how the methods developed to preserve human oocytes and ovarian tissues can now be used strategically to support the development of conservation strategies aimed at safeguarding the genetic diversity of commercially important domestic animals and also of preserving the female germplasm for wild animals and endangered species.

Key words: cryopreservation, fertility preservation, oocyte, ovary.

Introduction

In the last few decades, advances in cryobiology have been combined with the development of new assisted reproduction technologies (ARTs) and used as a means to cryopreserve the structural integrity and biological function of key reproductive cells. Translation of these research advances has resulted in the development of the capacity to cryopreserve and long-term store isolated gametes, embryos, complex gonadal tissues and even whole reproductive organs in humans and laboratory species as well as commercially important farm animal breeds and a limited number of exotic or endangered species. Indeed, the issue of fertility preservation is particularly relevant in animals as over the last two decades, some 300 of 6000 farm animal breeds have become extinct and a further 1350 domestic breeds are being threatened with extinction as a result of aggressive animal breeding strategies using limited genetic stocks of animals of high merit for a range of economically valuable traits (Taberlet *et al*., 2008).

The standard method of fertility preservation in males whether humans or animals is the cryopreservation and storage of sperm (for review see Ehmcke and Schlatt, 2008). Similarly, since the first successful cryopreservation of embryos in laboratory species in the early 1970s (Whittingham *et al*., 1972) thousands of animal and human offspring have been born following embryo cryopreservation. With regard to oocytes, the recent development of improved methods and devices for the vitrification of isolated cells has increased the success rates of metaphase II (MII), oocyte preservation in humans (Rienzi *et al*., 2017). This technological advance has only had limited application to the oocyte preservation in animals due to a general lack of basic understanding of oocyte biology in different target species (Comizzoli, 2017). While sperm, oocytes and embryo preservation using ART are clearly effective, recent research focus has shifted to the preservation of gametes *in situ* either within fragments of ovarian and testicular tissue or following whole organ cryopreservation. Indeed, the banking of gonadal tissue is particularly valuable as a means of preserving fertility as in the case of the ovary the banked tissue may contain thousands of the earliest staged primordial follicles and oocytes. In contrast testicular tissue cryopreservation facilitates the banking of spermatogonial stem cells that can subsequently can be harvested and used to repopulate the testis and restart spermatogenesis (Picton *et al*., 2015). Although no standardized protocols exist for fertility preservation in any species, the preservation of gonadal tissues offers near unlimited potential for fertility preservation and restoration in humans as well as domestic and wild animal species. This review aims to provide an overview of the recent advances in human oocyte and ovarian tissue banking and how these clinical reproductive technologies can be used to support fertility conservation strategies in animals.

Cryobiology and fertility preservation

Cryopreservation is a multi-step procedure that requires that cells tolerate exposure to: molar concentrations of penetrating and non-penetrating cryoprotective agents (CPAs); cooling to subzero temperatures; and either removal or conversion of the greater majority of liquid water within the cells into a solid state. The reverse occurs during thawing or warming. Any of these elements can inflict damage on the cells to be preserved although the level and nature of the damage is dependent on the cryobiological properties of each individual cell type. With regards to

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the cryopreservation of complex tissues containing multiple cell types, such as the ovary, the success of cryopreservation is dependent on the need to balance the freezing optima for a range of different cell types which are influenced by cell number and size and, in the case of oocytes, maturational status, as well as the requirement to preserve the structural integrity of the tissue. Furthermore, the nature of the cryopreservation methodology, whether slow freezing or vitrification, the cooling and warming rates and the containment vessel used for tissue preservation and storage will all influence the efficacy of the preservation method and hence impact on the subsequent viability of the preserved tissue. Finally, the unique biological properties of oocytes challenge our potential to freeze-store these important cells. Mammalian oocytes are very large cells of approximately 120 µm diameter with a small surface area to volume ratio and high lipid and water content. The latter confers a high sensitivity to chilling injury and intracellular ice formation. In MII oocytes these parameters are further confounded by the presence of a fragile cytoskeleton that is resistant to the volumetric excursions commonly associated with equilibrium freezing and a highly temperature sensitive meiotic spindle apparatus (Saragusty and Arav, 2011). These time-dependent sensitivities to chilling to 0°C are evident in MII oocytes from a range of species including mouse (Pickering and Johnson, 1987) rhesus monkey (Songsasen *et al*., 2002) and human (Pickering *et al*., 1990; Zenzes *et al*., 2001). Indeed, cooling oocytes to room temperature for even as little as 10 minutes can cause irreversible damage to the meiotic spindle. Furthermore, chilling oocytes to 0°C in the presence of a CPA appears to exacerbate these abnormalities (Mullen *et al*., 2004).

Despite these difficulties, significant advances have been made in our ability to cryopreserve MII oocytes in a range of species. The most convincing data on the efficacy of MII oocyte preservation is undoubtedly derived from human ART. Initial attempts to slow freezing human MII oocytes (Picton and Chambers, 2009) met with only limited success as evidenced by the key statistic of live birth rates per oocyte thawed (Oktay *et al*., 2006). Since then the efficacy of MII oocyte cryopreservation has increased dramatically with live birth rates following human MII oocyte vitrification by the best IVF clinics now being equivalent to those achieved during embryo freezing (Glujovsky *et al*., 2014; Rienzi *et al*., 2017). The improved efficiency of human MII preservation can be attributed to the development of optimized vitrification protocols that minimize the cells exposure to the cytotoxic impact of high concentrations of usually two or more CPAs including penetrating CPAs such as ethylene glycol and propanediol and non-penetrating CPAs such as sucrose or trehalose. Oocyte cooling rates during vitrification have also been maximized by the commercial development of specialized vitrification devices for use in ART that support the preservation of individual oocytes or small groups of cells within minimal volumes of cryopreservation reagents. As a result of these advances MII oocyte vitrification is no longer considered an experimental technique in human ART (Rienzi *et al*., 2017) and the approach is regularly used to preserve oocytes for young women who are undergoing infertility treatment, teenage girls or young women who need to safeguard their future fertility before exposure to the ablative cancer treatments, or more controversially, young women who wish to delay childbearing and elect to safeguard their future fertility by oocyte banking (Cobo *et al*., 2016). Regardless of species, it is clear that the success of oocyte cryopreservation is dependent on oocyte quality, a key parameter that is profoundly and negatively influenced by advancing maternal age (Goldman *et al*., 2017).

While significant improvements have been made in human MII oocyte vitrification these methods have had only a limited impact on oocyte banking in animals over and above the laboratory and livestock species that are used as models for the development and testing of the human ART protocols (Wildt *et al*., 2010). Never-the-less encouraging results for MII vitrification have been obtained in some domestic species such as the cat (Fernandez-Gonzalez and Jewgenow, 2017) as well as some exotic species including for example the Tasmanian devil (Czarny and Roger, 2010*)* and Mexican gray wolf (Boutelle *et al*., 2011). While commonalities in the biophysical traits associated with cell freezing between taxonomically-related species may be useful to inform protocol development and support improved post-thaw viability across species, in general the similarities between taxa tend to be overshadowed by the magnitude of species specific differences in the ability of their oocytes to tolerate osmotic and toxic effects of CPA exposure and chilling injury (Woods *et al*., 2004). For example, felid oocytes appear far more tolerant to osmotic changes and cold temperatures than oocytes from bovids, cervids or equids that appear to be more sensitive to cold shock (Comizzoli *et al*., 2010, 2012). Overall the ability of MII oocytes to survive the freezing and thawing, or cooling and warming processes is highly variable and difficult to predict across species (for review see Comizzoli *et al*., 2012). Thus despite the advances that have been made with MII oocyte cryopreservation in humans, fundamental cryobiological investigations and further optimization of oocyte cryopreservation protocols combined with comparative studies across species are needed before MII oocytes cryopreservation can be used efficiently to preserve the fertility of wild or endangered animals, especially where the numbers of oocytes available for preservation are limited.

Some of the difficulties associated with the cryopreservation of MII oocytes may potentially be overcome by the preservation of fully grown, but nuclear immature, germinal vesicle (GV) staged oocytes. However, this option has proved inconsistent and like the MII oocyte preservation detailed earlier, success rates appear to be influenced by species. In addition to rodent and ruminants, successful GV oocyte vitrification has recently been reported in equids (Ortiz-Escribano *et al*., 2018) and domestic cats. The evidence

suggests that GV oocytes appear to be more cryo-resistant than their MII counterparts (Comizzoli *et al*., 2008). Regardless of species, GV oocytes cryopreservation requires the freeze-storage of both the gamete and its supporting compliment of cumulus granulosa cells. The success of GV oocyte cryopreservation is critically dependent on the post-thaw/warming maintenance of the functional integrity of the heterologous gap junctional contacts connecting the cumulus cells to the oocyte. This network of cumulus cells supply the oocyte with vital nutrients and signaling molecules that are essential to drive the cytoplasmic and nuclear maturation of the oocyte to the MII stage. Furthermore, cumulus cells have a discretely different cryopreservation optima compared to oocytes. The loss of gap junctional contacts between the cumulus cell compartment and the GV oocyte is a common casualty of the cryopreservation process such that both the capacity of the oocyte to under go *in vitro* maturation and its subsequent fertile potential and developmental competence are severely compromised. Finally, GV oocyte preservation must be supported by the provision of robust culture environments that support oocyte maturation *in vitro*. Insight into the discrete, species-specific differences in the composition of the culture environment required to drive oocyte maturation will be needed to maximize oocyte quality after cryopreservation.

Regardless of their stage of nuclear maturity secondary oocytes can be harvested in humans and other mammalian species by ultrasound-guided laparoscopy or by laparotomy. Controlled ovarian stimulation with exogenous hormones is frequently used to increase the number of appropriately staged antral follicles at the time of oocyte aspiration. Alternatively, secondary oocytes can be harvested by needle aspiration of antral follicles at any stage of the reproductive cycle following ovariectomy, or as a byproduct of surgical procedures or following the natural mortality of an animal.

Ovarian tissue preservation

Many of the biophysical challenges associated with preserving secondary oocytes can be avoided by the banking of immature primordial oocytes through ovarian tissue cryopreservation. Indeed, the biological characteristics of primordial oocytes are well suited to preservation since primordial oocytes are relatively undifferentiated and, in the case of humans, are 3-4 fold smaller in diameter than their secondary oocyte counterparts. Importantly, primordial oocytes represent the building blocks of female reproduction and, depending on age, the mammalian ovarian cortex is endowed with thousands to millions of primordial oocytes enclosed within primordial follicles (Picton, 2001). The quantity and quality of the primordial follicles within this ovarian reserve ultimately defines the reproductive longevity and fertile potential of an individual. While our understanding of the mechanisms that regulate the activation of primordial follicle growth and hence depletion of the ovarian reserve is limited, especially in wild animals and there is considerable variability between species, ultimately the ovarian reserve represents the major germplasm resource that can be consistently and safely banked by wildlife reproductive biologists, veterinarians and clinicians alike as a means of safeguarding future fertility and maintaining genetic diversity.

Strategies for ovarian tissue cryopreservation are predominantly centered around the preservation of primordial follicles *in situ* either within fragments of ovarian cortex or by banking the whole ovary. It is possible to freeze-store isolated primordial oocytes but in the absence of supporting ovarian stromal cells these presumptive gametes have only limited viability post thaw/warming. The efficacy of ovarian cryopreservation is dependent on: optimization of the cryopreservation protocol for the species of interest; the individual's age at the time of tissue harvest and storage; the requirement to successfully preserve both the structural integrity and biological complexity of the tissue. Ovarian cortical tissue is comprised of functionally diverse cell types including immature GV oocytes, follicular somatic cells, stromal cells, blood vessels and the ovarian surface epithelium (Picton *et al*., 2000; Picton, 2001). All of these cell types have different cryopreservation optima and all play different but important functional roles in supporting the activation and completion of follicle and oocyte growth and as well as the maintenance of the ovarian reserve, whether *in vivo* or *in vitro*. Finally, strategies designed to successfully preserve the integrity and fertile potential of ovarian tissues and, conversely, to limit cryo-damage need to be developed in parallel with the methods to use the tissue to restore fertility following banking. Despite these challenges ovarian tissue cryopreservation have been optimized and used successfully for a range of species including mice, rats, rabbits, cats, dogs, sheep, goats, cows, pigs, horses, camels, wallabies, wombats, dasyurids, elephants, monkeys and humans (for review see Devi and Goel, 2016).

The majority of methods used to cryopreserve ovarian tissue are based on the original validated slow freezing protocols developed for the successful cryopreservation of human ovarian cortex as published by Newton *et al*. (1996) and the associated publications and live births recorded in sheep by Gosden *et al*. (1994) and Baird *et al*. (1999). In general, ovarian cortex is harvested following ovary removal or ovarian biopsy and thin fragments of cortex are equilibrated in CPA before undergoing controlled rate cooling with manual ice seeding before the temperatures are brought down to -196^{o}C and the tissue is stored at liquid nitrogen temperatures. There is now a substantial clinical evidence base to substantiate these early successes. Several thousand girls and young women worldwide have safeguarded their future fertility using ovarian tissue preservation methods. The most common indication for ovarian tissue banking is the need to preserve the future fertility of girls and women: who are at risk of premature ovarian failure (POF) following exposure to the ovotoxic impact of chemotherapy agents

for the treatment of cancer (Gellert *et al*., 2018; Oktay *et al*., 2018) or other haematological pathologies such as sickle cell anemia (Demeestere *et al*., 2015) or beta thalassemia (Matthews *et al*., 2018); or who carry a high risk of POF for genetic conditions such as Turners syndrome (Oktay *et al*., 2016); or who will suffer ovarian tissue damage and loss following abdominal trauma.

The potential of the stored ovarian tissue to support fertility restoration when the individual wishes to start her family is demonstrated by the delivery of more than 130 healthy babies world wide after autotransplantation of the frozen-thawed tissue and the restoration of endocrine and ovarian function (Pacheco and Oktay, 2017) with more ongoing pregnancies being recorded. Although the overall efficiency of fertility restoration following ovarian tissue preservation and transplantation is unknown, recent evidence from defined cohorts of patients suggests a ~30% live birth rate after autotransplantation of slow frozen-thawed ovarian cortex, (Jadoul *et al*., 2017; Keden *et al*., 2018). When fertility preservation strategies require the removal of the whole ovary and it is age appropriate to do so, ovarian cortex harvest and cryopreservation can be most effectively combined with methods for *in vitro* maturation and vitrification of MII oocytes to maximize the likelihood of a future successful pregnancy outcome for the patient. Although significant progress has been made in the development and use of ovarian tissue cryopreservation as a means to safeguard the future fertility of girls and young women at risk of POF, further optimization of the cryopreservation and transplantation protocols are likely to be beneficial as the longevity of ovarian autograft function following transplant remains unclear. The latter is likely to be determined by patient age at tissue harvest and by the degree of follicle loss that results from ischaemia and reperfusion injury following ovarian tissue transplantation. Importantly, further research is also needed to define and mitigate against any potential risk of reseeding cancer cells through the transplanted ovarian tissues (Dolmans and Masciangelo, 2018).

While the majority of successful live births have occurred following the slow freezing and autotransplantation of human ovarian tissues, recent evidence from a number of species, including women, suggests that it may also possible to preserve ovarian tissue by vitrification. Histological evaluations and assessment of the viability of oocytes grown *in vitro* from vitrified ovarian tissues have been published for a range of species including, for example, mouse (Tokieda *et al*., 2002; Migishima *et al*., 2003), sheep (Bordes *et al*., 2005) cow (Kagawa *et al*., 2009); goat (Santos *et al*., 2007, Carvalho *et al*., 2013); camel (Madboly *et al*., 2017) dog (Ishijima *et al*., 2006), non-human primate (Santana *et al*., 2012) and human (Suzuki *et al.*, 2015). Collectively these results suggest that it is possible to maintain tissue integrity and oocyte health following super cooling and warming. However, other studies are more skeptical about the suitability of vitrification for ovarian cryopreservation and have recorded increased necrosis in vitrified ovarian tissues (Rahimi *et al*., 2004; Gandolfi *et al*., 2006). Overall, at the time of writing it appears that the outcome of ovarian tissue vitrification is far more variable than slow freezing methods and considerable further research effort is needed to optimize and validate vitrification protocols before this approach can be considered a reliable method for the preservation for complex ovarian tissues. Furthermore, the risk of freeze fracture of vitrified samples during long-term tissue storage and compatibility/ potential risk of de-vitrification of samples during tissue bank audits and/or transportation remains to be proven.

An alternative approach than can be used to maximize the preserved ovarian reserve whilst minimizing the potential for ischemic follicle loss following autografting is to cryopreserve and re-transplant the whole ovary. This fertility preservation strategy involves the cryopreservation of not only the intact, whole ovary but also its supporting blood vessels (Martinez-Madrid *et al*., 2004; Baudot *et al*., 2007). Evidence to support the concept of the slow freezing and transplantation of whole ovaries and their supporting vasculature has been provided in sheep with the total restoration of natural fertility in all of the treated animals with 64% live birth rate following whole organ preservation and transplantation (Onions *et al*., 2009, 2013; Campbell *et al*., 2014).

Lessons from human ART and tissue cryopreservation

The development of strategies for human fertility preservation are informing the development of an equivalent agenda in animals. Valuable lessons can be learned from clinical ARTs and human fertility preservation programmes that can be applied to domestic livestock and also be used for the conservation of wild or endangered animals by the cryopreservation of sperm, oocytes and embryos and the preservation of intact gonadal tissues (Paulson and Comizzoli, 2018). Examples of exactly how the potential of bio banks and ARTs such as artificial insemination with cryopreserved sperm have been used successfully to save endangered animals are provided by giant pandas (Huang *et al*., 2012), domestic and wild carnivores (Comizzoli *et al*., 2009; Comizzoli and Wildt, 2012) and the black footed ferret (Howard and Wildt, 2009). In contrast to the successful use of sperm freezing, the recent progress made with human oocyte cryopreservation by vitrification has been far more difficult to translate to conservation programmes in animals due to a general lack of basic understanding of the complexity of mature oocyte biology and their cryo-sensitivity in different, target species (Comizzoli, 2017). Further collaborative research effort and cross species comparisons are clearly needed.

The practice of ovarian tissue preservation is highly relevant to the maintenance of the genetic diversity in domestic animals including bovine, ovine, caprine, swine and equine species as well as in wild

animals. Indeed, the development of ovarian tissue cryopreservation and fertility restoration strategies in humans have been underpinned by animal models of ovarian freezing. For example, the original proof of principle studies used to establish CPA toxicity and penetrability into ovarian tissues and to determine if the histology and function of these tissues could be retained following slow freezing were conducted in mice (Harp *et al*., 1994) and marmosets (Candy *et al*., 1995) as well as humans (Newton *et al*., 1998). The efficacy of ovarian tissue preservation methods have been evaluated by histological analysis of frozen-thawed tissue and by assessment of follicle and oocyte morphology post thaw/warming in animal tissues, e.g. in cattle (Gandolfi *et al*., 2006) and sheep (Chambers *et al*., 2010) or after short term culture and staining for cell viability and/or apoptotic markers (Chambers *et al*., 2010). Furthermore, while sheep models of ovarian tissue cryopreservation and autografting provided the first definitive proof that cryopreserved ovarian tissue could be used to restore natural fertility with the production of live young in large animals (Gosden *et al*., 1994), live births has previously been reported in laboratory species including mouse (Parrot, 1960), rat (Wang *et al*., 2002) and rabbit (Almodin *et al*., 2004). Similarly, as detailed above the concept of the slow freezing and transplantation of whole ovaries and the supporting ovarian vasculature with the complete restoration of natural fertility has been demonstrated in sheep (Onions *et al*., 2009, 2013; Campbell *et al*., 2014). Other comparisons of the efficacy of whole ovary preservation by slow freezing *vs.* vitrification methods are ongoing across a range of species including mice, goat and cow in the anticipation that these experimental findings can in the future be translated for human use. Finally, xenografting was originally established as model to test the viability (Oktay *et al*., 1998), quality, integrity (Kim *et al*., 2005) and safety of the ovarian tissue transplantation following the preservation of tissue for cancer patients (Kim *et al*., 2001).

While ovarian tissue preservation can clearly be used to preserve and exploit the germplasm of domestic animals of high genetic merit, this technology is also particularly relevant to the preservation and long-term storage of the germplasm of rare or endangered species and/or indigenous native animal breeds where the creation of genetic resource banks is urgently required to maintain future genetic diversity. Over recent years there has been an increase in the number of *ex situ* conservation programmes involving both *in vivo* and *in vitro* preservation and the establishment of germplasm banks (Silva *et al*., 2012). The latter provides a means of connecting *in situ* and *ex situ* conservation programmes (Andrabi and Maxwell, 2007). However it will be difficult to optimize ovarian cryopreservation methods for rare or wild animals which may have highly specialized reproductive adaptations and/or for which only a limited amount of tissue can be safely harvested and cryopreserved. For this group ovarian cryopreservation protocols that have been developed for closely related domestic animals have been adapted and applied to preserve the germplasm of their endangered counterparts (Leibo and Songsasen, 2002). The avoidance of the impact of seasonality on mature oocyte quality and the lack of need for fresh or cryopreserved sperm for IVF at the time of oocyte harvest is a further significant practical advantage of ovarian tissue banking for animals species compared to oocyte or even embryo banking. For occasions where serious damage occurs that results in the unexpected slaughter of a valuable or particularly rare animal (e.g. after limb fracture) multiple ovarian cortical tissue fragments and/or whole ovary(s) can be harvested for storage. Thus cryopreservation strategies developed for, for example, cattle, sheep, goats, horses or pigs, can be used as models for tissue preservation in non-domestic ungulates, while domestic cats can provide valuable models for tissue preservation in wild felids (Luvoni, 2006), dogs for rare canids, and capuchin monkeys for new world primates etc (Comizzoli and Wildt, 2012). Ovarian tissue banking may therefore prove to be a practical and effective tool for the conservation of rare species.

Fertility restoration following ovarian tissue cryopreservation

A significant and outstanding challenge that needs to be addressed in parallel with the development and optimization of protocols for the banking and long-term storage of the germplasm for humans and domestic animals or wild species, is how best to use the stored tissue to restore fertility and/or generate offspring. Regardless of the origin of the stored ovarian tissue, our ability to realize its potential and produce viable offspring is rigidly linked to our depth of understanding of the reproductive characteristics of the species involved. Fertility restoration and live birth has been achieved in sheep and women where, in both cases, it is possible to autotransplant cryopreserved tissues. The issue of how to realize the potential of preserved ovarian tissues in wild animals or endangered species is far more challenging as autografting may not be feasible. Evidence from a number of species indicate that xenografting of cryopreserved ovarian tissue into mice with severe combined immunodeficiency may provide a temporary solution to this problem. Indeed, xenografting has been shown to support the activation of primordial follicle growth and antral follicle production in cryopreserved ovarian tissues from a range of rodent and domestic species including mouse (Snow *et al*., 2002), cat (Bosch *et al*., 2004), and cow as well as a variety of wild animals including tammar wallaby (Mattiske *et al*., 2002), common wombat (Cleary *et al*., 2003), African elephant, (Gunasena *et al*., 1998) and lion (Wiedemann *et al*., 2012). The combined approach of ovarian tissue banking followed by xenografting may in the future be used in conjunction with follicle culture technologies to produce full sized, MII oocytes for *in vitro* fertilisation and embryo transfer into a suitable recipient animal.

Multiphase culture strategies are being developed to support the complete *in vitro* growth and maturation (IVGM) of oocytes from the primordial follicle stage to maturity in the laboratory (Picton *et al*., 2008). This goal is very ambitious but never-the-less significant advances have been made in our ability to support the IVGM of oocytes in mice (Eppig and O'Brien 1996), sheep (Newton *et al*., 1999; Picton *et al*., 2003) cow, (McLaughlin and Telfer, 2010), non-human primates, (Xu *et al*., 2011) and humans (McLaughlin *et al*., 2018). To date the production of healthy, live offspring from primordial follicles has only been achieved in mice (Eppig and O'Brien, 1996; Hikabe *et al*., 2016; Hayashi *et al*., 2017). It is important to note that on the basis of the published literature it is highly likely that the culture timelines, nutrient requirements needed to support follicle and oocyte growth *in vitro*, the subsequent fertile capacity of the *in vitro* derived oocytes and, ultimately, the efficiency of IVGM strategies for cryobanked tissues will be species specific. Furthermore, it is likely that no single IVGM strategy will fit all species and IVGM strategies for rare or wild species will likely have to be used in conjunction with xenografting in order to realize the fertile potential of the stored germplasm.

Conclusion

In conclusion although many questions remain to be answered, considerable recent progress in cryobiology, reproductive science, and IVGM technology have led to therapeutic advances in clinical ART that have significantly improved our ability to cryopreserve female fertility by banking primordial oocytes *in situ* within ovarian tissues or by the vitrification of MII oocytes. The stage is now set to translate these clinical advances for animal conservation and to use them to develop comprehensive strategies that will not only safeguard the future genetic diversity of commercially important domestic species but will also facilitate germplasm preservation for animals at risk of extinction.

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Directions and applications of CRISPR technology in livestock research

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Abstract

The ablation (KO) or targeted insertion (KI) of specific genes or sequences has been essential to test their roles on a particular biological process. Unfortunately, such genome modifications have been largely limited to the mouse model, as the only way to achieve targeted mutagenesis in other mammals required from somatic cell nuclear transfer, a time- and resource-consuming technique. This difficulty has left research in livestock species largely devoided of KO and targeted KI models, crucial tools to uncover the molecular roots of any physiological or pathological process. Luckily, the eruption of site-specific endonucleases, and particularly CRISPR technology, has empowered farm animal scientists to consider projects that could not develop before. In this sense, the availability of genome modification in livestock species is meant to change the way research is performed on many fields, switching from descriptive and correlational approaches to experimental research. In this review we will provide some guidance about how the genome can be edited by CRISPR and the possible strategies to achieve KO or KI, paying special attention to an initially overlooked phenomenon: mosaicism. Mosaicism is produced when the zygote´s genome edition occurs after its DNA has replicated, and is characterized by the presence of more than two alleles in the same individual, an undesirable outcome when attempting direct KO generation. Finally, the possible applications on different fields of livestock research, such as reproduction or infectious diseases are discussed.

Introduction

Genome modification has been crucial to understand the molecular root of physiological or pathological processes. The ablation (knock-out, KO) or insertion (knock-in, KI) of specific genes or sequences have allowed to unequivocally assess the role of a specific gene product on a particular process, to assess the spatial and temporal expression of a gene or to modify its expression pattern, among other applications. KO generation requires targeted mutagenesis (i.e., the modification of the genome at a specific locus), and targeted KI (i.e., the insertion of a sequence at a specific locus) is also preferred to random KI. Most experiments involving KO or KI models have been carried out in the only mammalian species where targeted genome modification was easily achievable: the laboratory mouse. In this sense, although non-targeted mutagenesis, achieved by different means such as such as pronuclear injection (Hammer *et al*., 1985), transduction (Chan *et al*., 1998) or mediated by intracytoplasmic sperm injection (Shemesh *et al*., 2000) have been applied to farm animals, the only available method to achieve targeted mutagenesis, homologous recombination, was difficult to apply to livestock species.

Homologous recombination (HR) is a genome modification technique based on an homonymous DNA repair mechanism that can be directed to insert a given sequence in a specific genomic locus. The main drawback of this technique is that the proccess is extremely inefficient, resulting in insertion rates below 0.1 % (Brinster *et al*., 1989). This handicap can be bypassed by performing HR in Cell Cultures, where the few cells containing the intended modification after HR can be selected by introducing a selection cassette for resistance to a cytotoxic agent (Doetschman *et al*., 1988). Once the genetic modification has been introduced into the cell genome, there are only two possible strategies to obtain a genetically modified animal. The first method to be developed was the use of genetically modified Embryonic Stem Cells (ESCs) for embryonic aggregation. This strategy generates chimeric animals partly composed of genetically modified cells derived from the ESCs. By this approach, if the genetically-modified ESCs-derived cells have formed germinal cells, the genetic modification could be transmitted to the offspring (Evans *et al*., 1985). The main limitation of this strategy was that it could only be applied to mice, as truly pluripotent ESCs –hence able to derive into germinal cells- could not be obtained in other species.

The second approach to produce genetically modified offspring from genetically modified cells is to perform Somatic Cell Nuclear Transfer (SCNT). In this case, the genetically modified nucleus of a somatic cell (usually a fibroblast) is reprogrammed by the ooplasm of an enucleated oocyte, resulting in an individual entirely composed by cells containing the genetic modification (Schnieke *et al*., 1997). This method allowed site-specific genome modifications in livestock species, but its application was highly restricted due to several technical limitations. SCNT is a technique difficult to master and very inefficient, resulting in less than 5 % delivery rates (Wilmut *et al*., 1997; Kato *et al*., 2000) and often yielding to developmental defects associated with deffective epigenetic reprogramming of the donor genome. Furthermore, the donor somatic cells used for HR are mortal, unlike ESCs, so they can senescence over the multiple passages required to perform the genetic modification, leading to the loss of the transgenic cellular line or in even lower embryo

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developmental rates following SCNT.

The technical constraints associated to targeted mutagenesis in farm animals have restricted the myriad of applications of genome modification in these species. Luckily, the advent of targeted mutagenesis techniques based on site-specific endonucleases has unleased the potential of genome editing in livestock species. Genome edited animals have been produced by different site-specific endonucleases such as Zinc-Finger Nucleases (ZFN) (Geurts *et al.*, 2009; Whyte *et al.*, 2011), Transcription Activator-Like Effector Nucleases (TALEN) (Tesson *et al.*, 2011; Carlson *et al*., 2012) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Shen *et al*., 2013; Wang *et al*., 2013), but due to the ease of use and flexibility, CRISPR has become the most popular method.

Mutagenesis induction by CRISPR

CRISPR technology has its origin on an adaptive immune system from prokaryotes which retain memory of past viral exposures by storing short fragments of the viral DNA (Mojica *et al*., 2005). Between the diverse CRISPR system existing in nature, several class II systems have been adapted for genome editing in eukaryotes (Ran *et al*., 2015). The most commonly used system derives from the type II CRISPR system of the bacteria *Streptococcus pyogenes*, and it is composed by a Cas9 protein (CRISPR associated nuclease) and a sgRNA (single-guided RNA, which directs Cas9 to the target site, composed by 20 nucleotides followed by –NGG) (Jinek *et al*., 2012).

CRISPR, as other site-specific endonucleases, is able to find its particular target across the genome and induce a DNA double stranded break (DSB) at that locus. In this sense, CRISPR *per se* does not generate any mutation, the mutation is actually generated by the endogenous DSB repair mechanisms of the eukaryotic cell. Eukaryotic cells mainly repair DSB by one of two mechanisms: Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR). The editing process is dynamic, as CRISPR remains active after one repair mechanism has fixed the DSB (Figure 1). In this sense, if the repair mechanism has reconstituted the CRISPR target site or it has only slightly modified it, CRISPR will recognize the repaired site and generate a DSB again. The cycle will continue until CRISPR activity ceases or a modification in the target site impedes CRISPR recognition and thereby DSB generation. NHEJ is an error prone mechanism that often introduces or deletes bases (insertion/deletion, known as indel) at the DSB in the repair process (Moore and Haber, 1996), thereby producing mutated sequences that are not recognized by CRISPR. In contrast, HR uses another DNA molecule as template (Orr-Weaver *et al*., 1981) and thus, in the absence of any exogenous DNA, it reconstitutes CRISPR target site. Therefore, if CRISPR remains active after HR repair, it will reproduce the DSB on the repaired site. In contrast, if a template for homologous recombination able to modify CRISPR target site is provided (Capecchi, 1989), this mechanism could be used to introduce DNA sequences at specific loci (KI).

CRISPR for KO generation

The indels generated by NHEJ are the most common way to generate a KO by CRISPR. For this aim, CRISPR components are directly injected into a zygote, and CRISPR target site should be located at the beginning of the Open Reading Frame (ORF) of the target gene. On that region, if the indel generated is not multiple of three, it will originate a disruption of the ORF (frame-shift mutation), leading to a truncated and non-functional peptide (i.e., a KO allele). However, as indels are randomly generated, some will be multiple of three, resulting in the insertion or deletion of few aminoacids, but leading to a probably functional protein (Figure 2). In other words, although virtually 100 % gene editing efficiency can be achieved, 100 % KO generation is statistically unachievable, as some of the indels generated will be multiples of 3 and thereby will not disrupt gene translation. In this context, genotyping strategy should be able to detect all indels (alleles) generated on a given individual, as solely individuals containing only frame-disrupting indels can be considered as KO.

A strategy to increase the percentage of KO out of edited embryos may be the use of multiple guides for the same gene (Wang *et al*., 2015b; Chuang *et al*., 2016; Wang *et al*., 2016a; Wang *et al*., 2016b; Vilarino *et al*., 2017). Multiple guides lead to multiple DSB that may result in either the deletion of a large fragment within them, which may include the start codon, or in the alteration of the ORF at different points. However, this strategy holds several drawbacks: 1) the indel generated on downstream DSB may reconstitute again the ORF disrupted by a first indel, resulting only in an alteration of the fragment between both DSBs, leading to a partially modified protein with unpredictable functionality, which contrasts with the neat and simple alleles generated with a single target; 2) for the same reason, the genotyping is more complicated and difficult to interpret; and 3) it increases the chances of offtarget.

As it will be discussed below, one of the main advantages of CRISPR over HR is that it allows the direct generation of KO individuals by direct injection of CRISPR components into a zygote. Due to the low efficiency of the technique, HR achieves monoallelic modifications in either ES or fibroblasts, resulting in heterozygote founders that need to be crossed to obtain a homozygous KO. The generation of a KO individual in one step (i.e. homozygous KO on F0 generation) is particularly useful to understand the role of specific genes during embryo development and it is extremely important to reduce the number of generations required to produce a KO animal in livestock species, where, in contrast to mice, generation times can be counted by years, rather than by months.

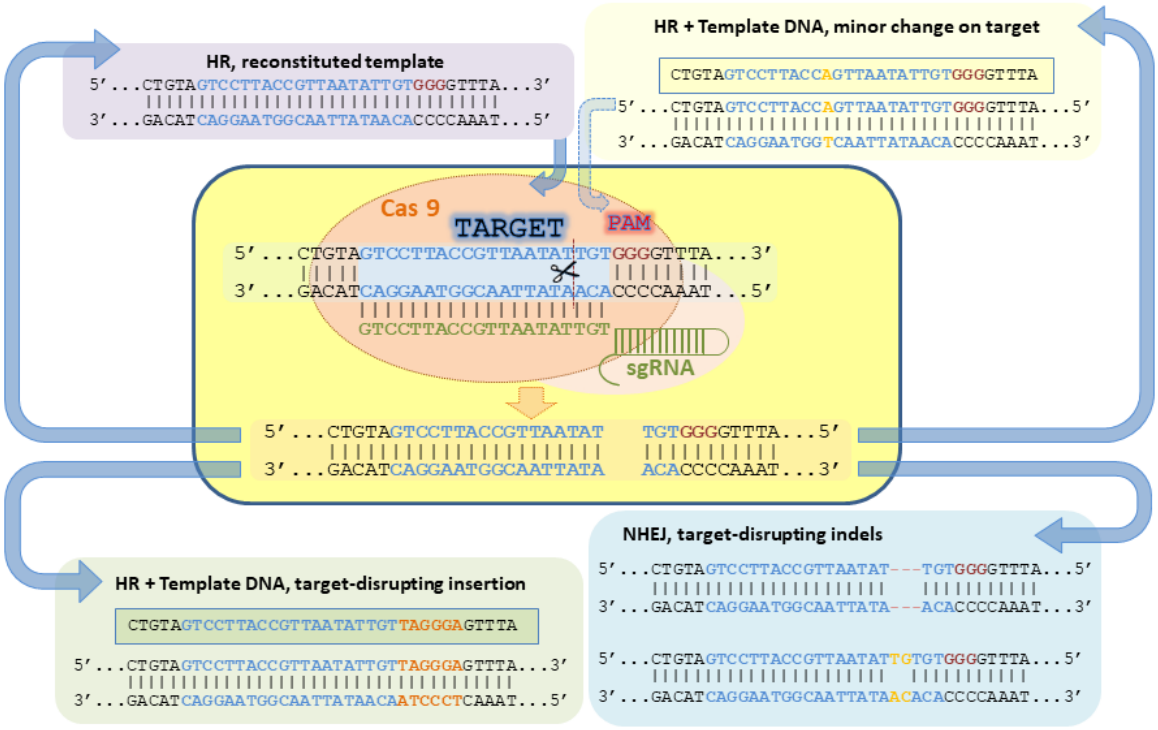

Figure 1. Dynamics of DSB repair by endogenous eukaryotic mechanisms (NHEJ or HR). Repairment by NHEJ often results in indels at the target site that impair CRISPR recognition. In contrast, repairment by HR reconstitutes the CRISPR target site unless a recombination template containing a target-disrupting insertion is provided. The reconstitution of the CRISPR target site leads to a new DSB at the repaired target unless CRISPR activity has ceased.

Figure 2. Examples of indels generated by CRISPR at the beginning of the coding region of rabbit *ZP4* gene. Wild-type, frame-disrupted and in frame alleles are shown. For each allele, aminoacid sequence is depicted in big letters that match the codons situated below, start codon (ATG) is underlined and CRISPR target site is marked in bold letters. On the frame-disrupted allele, a insertion of a single base (red T) disrupt the aminoacid sequence beyond that point. In contrast, a in frame indel consisting in a 9 bp deletion only eliminates 3 aminoacids, leaving the rest of the sequence unaltered.

Mosaicism impairs direct KO generation by CRISPR

In the context of random generation of indels by NHEJ, a reduction in the number of alleles generated in a given individual is desired to obtain KO individuals: the more alleles an individual harbours, the less probable will be that all of them are frame-disrupting. Ideally, indels should be generated at the 2n2c stage, resulting in 2 alleles. However, DNA replication occurs soon after fertilization in most species and thus genome edition may occur after DNA replication (2n4c), resulting in more than 2 alleles (Figure 3). This is phenomenon is called mosaicism, as it results in mosaic individuals composed by more than one cell population. Mosaicism was initially overlooked, as it is not a common problem in the generation of murine KO models (Bermejo-Alvarez *et al*., 2015), but most of the publications that have performed allele screening following CRISPR direct injection in zygotes have observed mosaicism in different species such as pigs (Hai *et al*., 2014; Sato *et al*., 2015; Wang *et al*., 2015c; Chuang *et al*., 2016; Kang *et al*., 2016; Petersen *et al*., 2016; Yu *et al*., 2016; Zhou *et al*., 2016; Burkard *et al*., 2017; Park *et al*., 2017; Whitworth *et al*., 2017), goats (Wang *et al*., 2016a), sheep (Crispo *et al*., 2015; Wang *et al*., 2016c; Vilarino *et al*., 2017; Zhang *et al*., 2017), cattle (Bevacqua *et al*., 2016) and rabbits (Yan *et al*., 2014; Honda *et al*., 2015; Guo *et al*., 2016; Lv *et al*., 2016; Song *et al*., 2016a; Song *et al*., 2016b; Sui *et al*., 2016; Yang *et al*., 2016; Yuan *et al*., 2016).

Although it was initially overlooked, the appearance of mosaicism is not surprising given that in most of the cases, conventional IVF or *in vivo* protocols used to collect zygotes for microinjection obtain them at or close to the 2c4n state, which obviously results in at least 4 alleles following edition. In the case of bovine, conventional IVF co-incubates oocytes and spermatozoa for ~20 h (Parrish *et al*., 1986), while DNA replication has been reported to occur between 8 and 18 hours post-insemination (Eid *et al*., 1994). The time of gamete co-incubation used in bovine is roughly similar to those employed in sheep and goats, where pronuclear formation, which precedes DNA replication, occurs even earlier than in bovine (Mogas *et al*., 1997; Gomez *et al*., 1998). Pig IVF zygotes are usually obtained after a short 5-6 h gametes co-incubation aiming to reduce polyspermy, whereas according to studies performing sperm injection (ICSI) the onset of S-phase occurs ~10 h after injection (Kim *et al*., 2003). However, pronuclei formation is delayed about 4 h in ICSI-derived embryos (Kim *et al*., 2003) compared to IVF-derived counterparts (Matas *et al*., 2003), and thus porcine zygotes may be at or very close the onset of DNA replication right after IVF. Similarly, *in vivo* porcine zygotes are usually collected at 52-60 hours post-hCG and DNA replication has been reported to occur between 56-60 h post hCG (Laurincik *et al*., 1995). Rabbit zygotes are fertilized at ~14 hours post-mating (Pincus and Enzmann, 1932) and replicate its DNA 3-6 h after sperm penetration (Oprescu and Thibault, 1965; Szollosi, 1966). Although it is unclear how long the genome editing mediated by CRISPR combined to the definitive repair of the DSB lasts, it seems that strategies focussed on an earlier delivery of CRISPR components may help to reduce mosaicism.

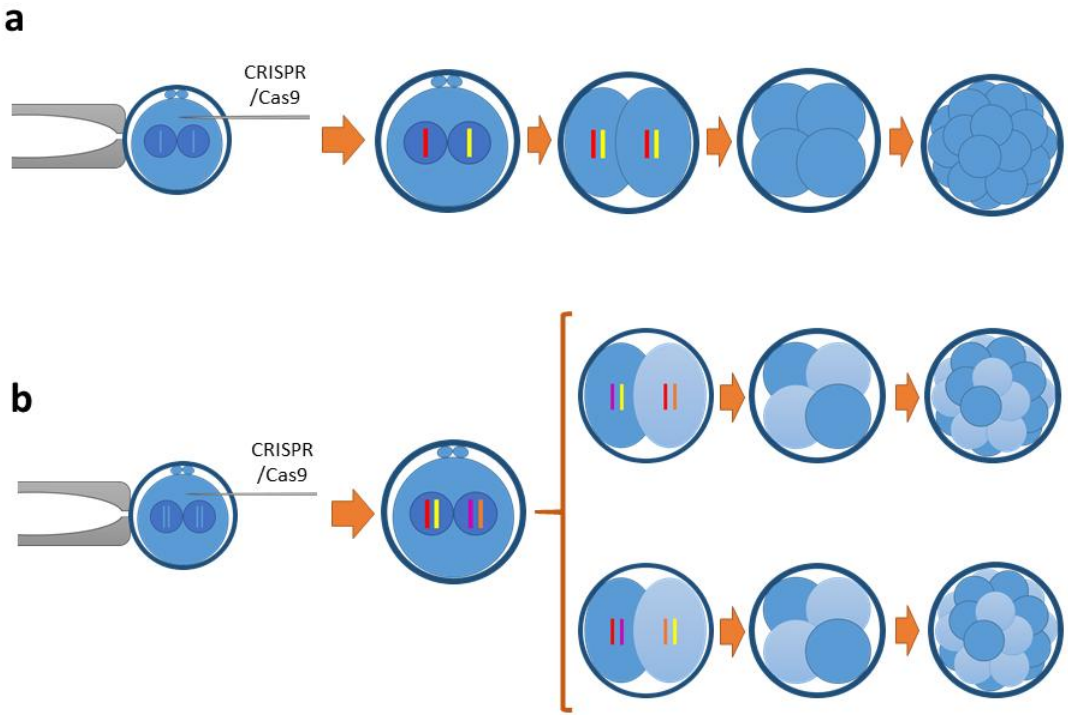

Figure 3. Possible outcomes following CRISPR microinjection into zygotes and NHEJ repair: a) If the DSBs and their definite repairs occur before DNA replication, at the 2n2c stage, 2 indels (alleles) are generated; or b) If DNA has been already replicated (4n4c), CRISPR edition generates 4 alleles that seggregate following first cleavage, leading to two populations of blastomeres harbouring 2 alleles each.

CRISPR for KI generation

Targeted insertion of a given sequence can be achieved by homologous recombination (Orr-Weaver *et al*., 1981), which alone (i.e. limited to the incorporation of a sequence containing homology arms) is a very inefficient technique (Brinster *et al*., 1989) that requires the use of an intermediary (ESCs or fibroblasts cultures) to generate a genetically modified animal (Doetschman *et al*., 1988; Schnieke *et al*., 1997). However, when a DSB is produced at the HR target locus, the efficiency of HR is improved by >1000 fold (Moehle *et al*., 2007). Under this improved efficiency, the insertion can be directly achieved by co-injecting a HR template and CRISPR components in zygotes, especially when the insert size is small (Yang *et al*., 2014). However, in farm animals the use of fibroblasts as intermediaries followed by SCNT remains being the most commonly used strategy to generate KI animals, as it ensures that all animals generated will carry the intended mutation. For this purpose, the combination of CRISPR+HR template has become the method of choice over HR alone, as the boosted HR efficiency also facilitates genome modification in cell cultures.

The repair template can be double or single-strand DNA (dsDNA or ssDNA). ssDNA often result in higher editing efficiency with reduced random insertions (Ran *et al.*, 2013b), but circular vectors are also effective and convenient to introduce long inserts and homology arms (Yang *et al.*, 2014). As previously mentioned, an essential requisite of the HR template to be used combined with CRISPR is that the insertion should disrupt CRISPR recognition site, as otherwise, CRISPR will reproduce the DSB at the reconstituted target site. This can be difficult to achieve when single nucleotide modification is intended, as it can be the case for the introgression of a SNP.

A strategy employed for KI generation is the use of nickase, a mutant form of Cas9 that only produces a break in one strand (Ran *et al.*, 2013a). For this purpose, nickase should be co-injected with two sgRNAs (one for each strand), which, in contrast to Cas9, leaves long 5´overhangs that may benefit HR, although not clear consensus has been reached about its putative increased efficiency over conventional Cas9. Another aspect that can be modified from the KO generation protocol is that, as double insertion may be difficult to achieve, the generation of mosaics may be beneficial, as it increases the chances of generating a founder with at least 1 allele harbouring the insertion. Other strategies to improve HR efficiency include the use of NHEJ inhibitors such as SCR-7 (Singh *et al*., 2015) or HR activators as RS-1 (Song *et al*., 2016a).

The insertion of a particular sequence at a specific locus allows precise reporter experiments using the endogenous promoter/s and enhancer/s or the endogenously controlled expression of a transgene, among others, but can also be used to generate KO models. For this purpose, a stop codon can be inserted at the beginning of the ORF of a gene. This strategy holds the advantage over conventional KO generation by the random NHEJ-created indels of being easier to genotype, as a restriction enzyme site can be introduced along the stop codon, which allows a sequencing-free identification of the founder offspring. HR can also be used to introduce loxP or FRT sites flanking a target exon for the conditional ablation of genes by Cre-lox (Orban *et al*., 1992) or FLP-FRT (Buchholz *et al*., 1998) recombination systems.

Applications of CRISPR in livestock research

Genome modification in farm animals holds a myriad of applications on different fields, including the production of therapeutic proteins (Spencer *et al*., 2005; van Veen *et al*., 2012; Sheridan, 2016), the generation of biomodels for human diseases (reviewed by Whitelaw *et al*., 2016), the creation of animal organs less prone to rejection after transplantation (reviewed by Whyte and Prather, 2011), the development of human organs generated into an animal host (Wu *et al*., 2017), or, maybe the closest applications to the farm: the improvement of productive rates, animal products, animal health or the environmental impact of farming via genetically modified livestock (reviewed by Lamas-Toranzo *et al.*, 2017). However, the latter applications are currently stopped by a ban (or lack of approval) of animal products derived from any genetically modified animals (GMAs) for human consumption. Legislation of different countries is slowly adapting to the new scenario created by genome editing (reviewed recently by Van Eenennaam, 2018), and the classifications of GMAs into different types depending on the kind of genetic modifications performed could lead to different sets of requirements for approval (discussed in Lamas-Toranzo *et al.*, 2017). In any case, today CRISPR constitutes a powerful tool for research in livestock species, being readily able to generate knowledge applicable to non-edited livestock.

As it has been previously explained, the benefits of genome modification in research have been largely limited to the mouse model, leaving livestock research devoid of KO or KI models. Although the knowledge generated by some KO or KI murine models can and has been applicable to some aspect of the physiology of livestock species, some processes such as some involved in reproduction, embryo development or infectious disease are highly species specific, impeding the extrapolation of data between species. Besides, CRISPR technology allows to unequivocally prove the role of a particular allele detected on a Genome-wide Association Study (GWAS) on productive traits, which may be helpful when population size or allele frequency is too small to drawn proper conclusions or to test whether such allele will produce a similar phenotype in other genetic background or species. Examples of alleles known to affect production that have been generated by CRISPR include myostatin KOs, which enhance muscular development in CRISPR-edited pigs (Wang *et al*., 2015a), goats (Wang *et al*., 2015b), sheep (Crispo *et al*.,

2015) and rabbits (Lv *et al*., 2016); FGF5 KO in goats (Wang *et al*., 2016a), which improves cashmere production; and the POLLED allele introduced in horned bovine genetic lines (Tan *et al*., 2013).

The direct generation of KO by CRISPR is particularly advantageous for its use on experiments aiming to elucidate the molecular aspects of embryo development, as it allows to restrict the ablation from the zygote stage onwards. This contrasts to the approach commonly used in murine KO models, where the low efficiency of HR alone or the lethal phenotype of the homozygous KO force the generation of homozygous KO embryos by the cross of heterozygous (wt/KO) parents (Evans *et al*., 1985). In this context, the gametes originating the KO embryos have been developed in haploinsuficiency (they are wt/KO and then wt or KO as meiosis progresses; Pattabiraman *et al*., 2015), which may lead to confusing conclusions about whether the gene disruption exerted its effect during gametogenesis or during early development. This is especially relevant when the gene of study is involved in stable and long term alterations such as epigenome remodelling (de Frutos *et al*., 2016). Apart from this advantage, which also applies to the mouse model, the direct generation of a KO embryo circumvents the need of genetically modified animals, as only wt gametes are required to produce KO embryos. Embryonic development in farm animals is known to greatly differ in terms of epigenetic events (Bermejo-Alvarez *et al*., 2010) and early lineage segregation determinants (Berg *et al*., 2011) to the mouse model. Particularly in ungulates, which accounts for the most relevant mammalian livestock species worldwide, the blastocysts does not attach after hatching as it occurs in rodents or humans. Instead, it undergoes a series of developmental events including early and late gastrulation in a period termed embryo elongation. These developmental processes are poorly understood and research on this area is particularly relevant to improve reproductive rates, as failures during this period account for most reproductive losses in pigs (Bennett and Leymaster, 1989) and cattle (Dunne *et al*., 2000; Santos *et al*., 2004; Berg *et al*., 2010).

Another field that can be greatly benefited from the use of GMA is the research on infectious diseases, especially given the high species specificity of several pathogens. Cattle with increased resistance to tuberculosis have been generated by CRISPR-mediated insertion of natural resistance-associated macrophage protein-1 (*NRAMP1*) (Gao *et al*., 2017). CRISPR has also been used to generate pigs resistant to African Swine Fever by the substitution of the porcine gene RELA for its orthologue from a closely related species that is resistant to the infection: the warthog (Lillico *et al*., 2016). Pigs resistant to the infection of the porcine reproductive and respiratory syndrome virus (PRRVS), a viral disease difficult to eradicate and responsible for major losses in the pig industry, have been generated by CRISPR (Whitworth *et al*., 2016). Although these models were generated thinking about a future use for human consumption, they already provide insights about the pathogenesis and entry ways of infectious agents that can be used to develop therapeutic or prophylactic treatments in conventional non-edited animals.

Concluding remarks

Genome editing in farm animals has been hampered by the inefficiency and difficulty of early techniques, based on HR combined with SCNT. This obstacle has deprived research in livestock species of the definite answers provided by KO models. The advent of site-specific endonucleases and particularly CRISPR, the easiest to tailor between them, is meant to inaugurate a new era in livestock research. This technology allows direct targeted genome modification in one step by a simple microinjection in zygotes, allowing to unequivocally know the role of a particular gene product on a given process. The novel affordability of KO and KI models for livestock research can improve the quality of scientific results, as it grants the exchange of descriptive and correlational approaches by experimental ones.

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Oocyte related factors impacting on embryo quality: relevance for *in vitro* embryo production

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Abstract

The outcome of pregnancy is closely linked to early events that occur during the onset of embryogenesis. The first stages in embryonic development are mainly governed by the storage of maternal factors present in the oocyte at the time of fertilisation. In this review, we outline the different classes of oocyte transcripts that may be involved in activation of the embryonic genome as well as those associated with epigenetic reprogramming, imprinting maintenance or the control of transposon mobilisation during preimplantation development. We also report the influence of cumulus-oocyte crosstalk during the maturation process on the oocyte transcriptome and how *in vitro* procedures can affect these interactions.

Keywords: cumulus cells, germinal-somatic interactions, maternal RNAs, oocyte maturation, preimplantation development.

Introduction

The notion of embryo quality refers to the capacity of an embryo to develop and support successful pregnancy to full term. In cattle, most failed pregnancies result from embryonic mortality that occurs before implantation, during the first two weeks after fertilisation (Berg *et al*., 2010; Lonergan *et al*., 2016). During this window, the fertilised oocyte undergoes profound morphological changes. Embryonic cell divisions and cavitation lead to blastocyst formation. The blastomeres segregate between the inner cell mass and trophectoderm which gives rise to initiation of the first lineages, namely the embryonic epiblast, extraembryonic hypoblast and trophoblast (Artus and Chazaud, 2014). The embryonic epiblast will give rise to all future intraembryonic tissues while extraembryonic tissues will be involved in forming the placenta (Ribeiro *et al*., 2016; Schroder *et al*., 2016). Cellular differentiation and lineage specification require prior resetting of the epigenetic information that is contained in each gamete to enable a return to totipotency. It also necessitates processes to restrict further expression to lineage-appropriate subsets of genes. Epigenetic information participates in mechanisms that preside over genetic information. This consists in a combination of marks such as DNA methylation and posttranslational histone tail modifications (Kouzarides, 2007). The extensive modifications of parental epigenetic marks that occur soon after fertilisation and are referred to as early epigenetic reprograming are involved in regulating gene expression throughout early development (Canovas and Ross 2016; Sepulveda-Rincon *et al*., 2016; Zheng *et al*., 2016). A small fraction of genes within the whole genome escapes from the extensive DNA methylation erasure that occurs in the zygote. These genes include imprinted genes that display different types of DNA methylation depending on their parental origin, leading to parental-allele-specific gene expression during development. Genome imprinting plays a key role in maintaining normal embryogenesis (Elhamamsy, 2017). In cattle, genomic reprogramming has been shown to be associated with an increase in the expression of transposable elements (TEs), specifically from a subclass of these elements called retrotransposons (Bui *et al*., 2009). Because the potential proliferation of TEs within the genome due to autonomous copy-and-paste mechanisms can result in genome instability and may damage the embryo, this transposon mobilisation needs to be constrained to permit normal embryo development.

Mammalian embryos are transcriptionally quiescent at the start of development. Early embryogenesis is mainly governed by post-transcriptional and post-translational events. The stockpile of maternal RNAs and proteins, which are stored within the oocyte during oogenesis, sustains the first stages of development until embryonic genome activation (EGA; Vigneault *et al*., 2009; Deutsch *et al*., 2014). Inherited factors from the oocyte contribute to epigenetic reprogramming and imprinting maintenance during early development (Canovas and Ross, 2016; Lodde *et al*., 2017). In cattle, factors that are likely to be involved in protecting the embryo against the deleterious effects of transposon mobilisation have been evidenced in the oocyte at the time of fertilisation (Russell *et al*., 2017). The time frame for the embryo transcription machinery to be fully functional varies between species (Telford *et al*., 1990). In cattle and sheep, the major EGA that conveys the transition from maternal to the embryonic control of embryo development occurs gradually from the 8- to the 16-cell stage (Graf *et al*., 2014). This process is considered to be one of the most critical events governing bovine embryo viability during preimplantation development and is closely associated with the nuclear and cytoplasmic characteristics of oocytes at the time of fertilisation (Gad *et al*., 2012).

Throughout this manuscript, we shall focus on several aspects of the oocyte transcriptome at the time of fertilization that are known to be related to a successful course of preimplantation development, and particularly with the achievement of EGA. We shall also discuss how cumulus-oocyte interactions can refine the oocyte transcriptome during the maturation process and how *in vitro* procedures may potentially affect these interactions and subsequent embryonic development in cattle.

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Impact of oocyte transcriptome on early embryo development

The storage of maternal RNA enclosed in the fertilised oocyte is involved in the control of early embryogenesis (Tadros and Lipshitz, 2009). In cattle, several maternal transcripts (also known as maternal effect genes) have been shown to play an essential role during the first embryonic cleavage cycles, activation of the embryonic transcription machinery, pluripotency gene expression and blastocyst cell allocation (Bettegowda *et al*., 2007; Tejomurtula *et al*., 2009; Tripurani *et al*., 2011a). During oogenesis, the oocyte actively synthetises and accumulates a large collection of coding and non-coding RNAs (Susor *et al*., 2016). Oocyte transcription gradually decreases during terminal differentiation within the preovulatory follicle, ceasing finally with the resumption of meiosis. Maternal RNAs are stabilised and stored so that they will be available over a period of several days that include embryo transcription silencing. RNA stabilisation mechanisms such as the de-adenylation and capping processes have been shown to control the translation of several key mRNAs in embryos in a timely manner (Kim and Richter 2007; Richter, 2007; Weill *et al*., 2012). The extent of maternal transcript polyadenylation is positively correlated with translation efficiency. For example, JY-1, a maternal effect factor identified in the bovine oocyte, is required to reach the embryonic 8- to 16-cell stage and complete EGA (Bettegowda *et al*., 2007; Lee *et al*., 2014). JY-1 transcripts display temporal variations in their adenylation status throughout the initial cleavages. The abundance of polyadenylated JY-1 mRNA is low during oocyte maturation; it increases at the pronuclear and 4-cell stages, and then decreases to almost undetectable levels after the 16-cell stage of embryo development. In contrast, the amount of total (de-adenylated + polyadenylated) JY-1 mRNA, which is highest in the immature oocyte, gradually decreases to become undetectable after the 16-cell stage. These changes to transcript adenylation lead to dynamically regulated JY-1 mRNA translation during early development.

Deadenylation and readenylation mechanisms are not always involved in regulating mRNA translation. The expression of Jumonji domain-containing protein 3 (JMJD3) during the early development of bovine embryos is a paradigm. JMJD3 belongs to the Jumonji family of genes that are epigenetic regulators. JMJD3 is a lysine demethylase associated with histone demethylation. Its activity is required for the removal of trimethylated histone 3 lysine 27 (H3K27me3) marks during the reprogramming process (Canovas *et al*., 2012). The histone H3K27me3 mark is involved in the silencing of gene expression. At fertilisation, H3K27me3 marks have to be removed from the gametic chromatin in order to reactivate silenced genes, thus enabling EGA and development to the blastocyst stage (Bogliotti and Ross, 2012). The level of JMJD3 mRNA, which is high in MII-stage oocytes, decreases from the zygote to the 16-cell stage. JMJD3 protein, which is undetectable in the oocyte, is translated soon after fertilisation and persists throughout the first cleavage cycles. Although JMJD3 protein expression is dynamically regulated during the window from fertilization to EGA, maternally inherited JMJD3 mRNA do not display changes to patterns of transcript abundance between the total and polyadenylated fractions, suggesting that other mechanisms are involved in the regulation of mRNA translation (Canovas *et al.*, 2012).

While tight temporal activation of the translation of dormant maternal mRNAs is required for successful progress through the first cleavages, destabilisation and degradation of the maternal RNA pool is a major determinant for the start of EGA (Tesfaye *et al*., 2017). Inherited maternal micro RNAs (miRNAs) seem to be critical players in the control of maternal transcripts during early development in cattle (Mondou *et al*., 2012). miRNAs are a large class of small non-coding RNAs (less than 200 nucleotides) that play important gene-regulatory roles in repression of the mRNAs of protein-coding genes (Guo *et al*., 2010). miRNAs derive from long primary microRNA transcripts that are successively processed by the nuclear microprocessor complex Drosha-DGCR8 and by the cytoplasmic RNAse III enzyme Dicer. miRNA-mediated repression leads to a reduction in translational efficiency and/or decreased mRNA levels (Dallaire and Simard, 2016). Decreased mRNA levels are associated with the mRNA de-adenylation and de-capping processes that trigger mRNA destabilisation and degradation. For example, NOBOX (Newborn ovary homeobox gene) is a maternal-derived transcription factor that is stage-specifically expressed during oocyte maturation and early embryonic development in cattle (Tripurani *et al*., 2011a). NOBOX is required for embryonic development to the blastocyst stage. It is involved in regulating POU5FI/OCT4 and NANOG pluripotency gene expression, blastocyst cell allocation and embryonic transcriptional activity. The 3'-untranslated region (3'-UTR) sequence of NOBOX mRNA exhibits a binding site for miR-196a. After fertilisation, NOBOX mRNA and protein expression are gradually suppressed from the 2-cell to 8-cell stages as the expression of mature miR-196a increases (Tripurani *et al*., 2011b). The highest abundance of mature miR-196a near the 8-cell stage of embryogenesis supports its involvement in maternal NOBOX mRNA degradation at the onset of EGA in cattle.

Piwi-interacting RNAs (piRNAs), another class of small non coding RNAs, are potentially involved in the control of maternal mRNA translation and decay as well as in that of transposon activity during early embryogenesis (Barckmann and Simonelig, 2013; Russell *et al*., 2017; Zhang *et al*., 2017). These piRNAs associate with PIWI proteins to form ribonucleoprotein complexes referred to as piRNA-induced silencing complexes (piRISCs), which bind to RNA targets with complementary nucleotide sequences, leading to splicing activity, transcriptional repression and/or degradation. A recent study using bovine, macaque, and human material showed that most small RNAs that are

present into MII-stage oocytes are represented by a piRNA-like population (approximately 26 nucleotides in length) whereas miRNAs account for less than 1% (Roovers *et al*., 2015). A pool of piRNAs similar to that observed in oocytes can still be detected at the 2- to 4-cell-stage in bovine embryos. Testis-derived piRNAs with a length preference of approximately 30 nucleotides are not obvious after fertilisation, suggesting that embryonic piRNAs mainly derive from the oocyte. The oocyte-derived piRNAs pool is strongly enriched with transposon-derived sequences and may help to prevent transposon activity during genome reprogramming in cattle (Russell *et al*., 2017). To be functional, the PIWI pathway requires the presence of both piRNAs and their associated PIWI proteins. A recent study in cattle evidenced the dynamically regulated expression of a transcript coding for the PIWI protein PIWI1during early development (Russell *et al*., 2016). PIWI1mRNA expression displays a peak at the 2-cell stage, after which the levels fall through to the blastocyst stage. The PIWI1 mRNA expression profile, as well as the ability of PIWI1 protein to bind piRNAs, suggests its involvement in transposon control during embryonic reprogramming.

Long non-coding RNAs (lncRNAs) are increasingly being recognised as modulators of gene expression. These lncRNAs are a class of transcripts longer than 200 nucleotides that do not usually code for a protein (Ruiz-Orera *et al*., 2014; Mattick and Rinn, 2015). Recent studies in mammals suggested functional roles for maternal lncRNAs during early embryonic development (Taylor *et al*., 2015; Bouckenheimer *et al*., 2016; Svoboda, 2017). A transcriptomic analysis performed in human embryos evidenced the dynamic expression of oocyte-inherited lncRNAs which included TUBB8P7 (tubulin beta 8 class VIII pseudogene 7), BCAR4 (breast cancer anti-oestrogen resistance 4), WEE2-AS1 (WEE2 antisense RNA 1) and TUNAR (TCL1 upstream neural differentiation-associated RNA) during preimplantation development (Bouckenheimer *et al*., 2018). Their expression remains stable during the first two cleavages. It then declines during progression to the blastocyst stage, specifically between the 4-cell and 8-cell stage, suggesting a role in embryonic cell division as well as in the control of gene activation at the onset of EGA. The clearance of lncRNAs may involve the post-transcriptional adenylation process (Mattick and Rinn, 2015). A recent review reported the involvement of several lncRNA in control of the mono-allelic expression of imprinted genes at almost all stages of mammalian development, including preimplantation stages (Saha *et al*., 2017).

Effect of periconceptional cumulus-oocyte crosstalk on the oocyte transcriptome

The capacity of the fertilised egg to support successful embryonic development is reliant upon the content of the oocyte at the time of fertilisation. The phenotype of a mature oocyte is the culmination of continuous and highly coordinated interactions between the germinal and somatic compartments of the ovarian follicle that occur throughout folliculogenesis, and particularly during terminal differentiation of the cumulus-oocyte complex (COC) (Li and Albertini, 2013). The LH surge induces cell signalling cascades within the preovulatory follicle leading to oocyte maturation, cumulus expansion and ovulation of the COC. Oocyte maturation consists of a stream of events that occur at both the nuclear and cytoplasmic levels and are often referred to as meiotic and cytoplasmic maturation (Sirard *et al*., 2006). While the oocyte resumes meiosis and progresses to the metaphase II (MII) stage, cytoplasmic organelles (including mitochondria, endoplasmic reticulum and the Golgi apparatus) undergo important changes affecting their structure, function and/or distribution (May-Panloup *et al*., 2007). There is a growing body of studies that have provided molecular explanations (concerning oocyte-somatic cell communication) for the underlying mechanisms of several cytoplasmic maturation features (Cakmak *et al*., 2016; Sousa Martins *et al*., 2016). Epidermal growth factor (EGF)-like peptides have been shown to play a central role in transmission of the preovulatory LH signal from mural granulosa cells to cumulus cells (CCs) in several mammalian species, including humans (Prochazka *et al*., 2017; Richani and Gilchrist, 2018). In turn, CCs exchange signals with the oocyte enabling both of them to reciprocally modulate the transcriptional and/or translational events that occur in preparation for fertilisation (Conti *et al*., 2012; Conti and Franciosi, 2018). In mice, the interaction between EGF-like growth factors and cumulus EGFRs leads to activation of the phosphatityl-inositol-3-kinase-AKT-mechanistic target of rapamycin (PI3-AKT-mTOR) pathway in the oocyte. The mTOR signalling pathway is involved in controlling the translation of several maternal transcripts that are critical to embryonic development (Chen *et al*., 2013). A recent study in mice showed that mTOR-dependent pathways in growing oocytes are also involved in the control of oocyte translation during the maturation process (Guo *et al*., 2018). Up-regulated expression of the prostaglandin G/H synthase-2 (PTGS2) pathway in granulosa cells and cumulus cells following the LH signal has been reported in several mammalian species, including bovines (Sirois, 1994; Nuttinck *et al*., 2002). Prostaglandin E2 (PGE2) is the main PTGS2-related prostaglandin secreted by follicular somatic cells. Autocrine and paracrine PGE2 signalling is required to intensify and propagate the EGF-like peptide signal within the preovulatory follicle (Shimada *et al*., 2016). In turn, EGF-like peptides produced by CCs promote the mRNA expression of genes such as PTGS2 that are involved in the cumulus expansion process. Bovine oocytes express PGE2 binding sites, suggesting that PGE2 is involved in cumulus-oocyte coupling in cattle (Nuttinck *et al*., 2011). The modalities of information exchanges between somatic and germinal compartments of the COC vary as oocyte maturation progresses (McGinnis *et al*., 2013). Some hours before ovulation, gap junction communications between the oocyte and CCs are promptly down-regulated while the pattern of oocyte secreted factors continues to be regulated dynamically by

feedback loops between the gamete and expanding CCs (Coticchio *et al*., 2015; Cakmak *et al*., 2016).

Novel mediators of the intercellular communication that is involved in building the oocyte transcriptome during the preovulatory period have recently been highlighted. Studies in cattle have revealed that cumulus cells contribute to oocyte transcript stores by way of active RNA transfer. Before the initiation of meiosis resumes, large cargos including mRNAs and lncRNAs appear to move from the CCs to the oocyte through transzonal projections (TZP; Macaulay *et al*., 2014, 2016). The abundance of several specific transcripts such as RASL11B (RAS like family11 member B), KIF5B (kinesin family member 5B) and AFF4 (AF4/FMR2 family member 4) mRNAs increases in the oocyte during transition from the immature to the mature stage, while endogenous transcription of the oocyte becomes silenced. In bovines, the potential for large cargo transfer from the CCs to the oocyte via TZPs terminates around 9 h after the induction of meiosis resumption when both compartments of the COC gradually dissociate and CC projections are released. This exogenous source of RNA may potentially enrich the pool of maternal factors that are involved in the control of early embryogenesis. It has been suggested that other classes of cumulus transcripts may be exchanged with the oocyte. The transcriptional profiles of human MII oocytes and their surrounding CCs indicate that many genes expressed in oocytes are potential targets of CC miRNAs, suggesting that oocyte-CC crosstalk might also be mediated via miRNAs (Assou *et al*., 2013). Among the oocyte mRNA targets of CC miRNAs are transcripts coding for factors associated with chromatin remodelling such as the DNA methyltransferases DNMT1, DNMT3A, DNMT3B and SMARCA5 (SWI/SNF related, matrix associated, actin regulator of chromatin, subfamily a, member 5). An *in vitro* study using bovine model shown that modulation of miRNA-130b expression in maturing oocyte affects the meiosis progression as well as the proliferation rate and the glucose metabolism activity of surrounding CCs (Sinha *et al*., 2017).

The *in vitro* maturation process may alter cumulus-oocyte crosstalk

In cattle, *in vitro* embryos are produced through successive steps which include *in vitro* maturation (IVM) and fertilisation (IVF). The resulting zygotes undergo a 7-day culture period that permits them to reach the blastocyst stage. Embryo quality is assessed morphologically at the end of this culture period in order to select embryos that are compatible with the transfer procedure (Rocha *et al*., 2016). IVM implies that the COC achieves its terminal differentiation in the absence of a follicular environment, and thus when LH-induced granulosa cell cues are absent. While nuclear oocyte maturation appears to be progressing normally, other aspects of COC terminal differentiation may be altered. A comparison of CC transcriptomes obtained from *in vitro* matured COCs with those obtained from *in vivo* counterparts highlighted critical deficiencies affecting several cumulus molecular pathways known to support developmental potential of the oocyte in mice, humans and cattle (Brown *et al*., 2017). The expression of genes involved in several CC functions such as epidermal growth factor (EGF)-like signalling, extracellular matrix production, glucose metabolism, fatty acid metabolism and immune-like processes, were seen to be impaired during the *in vitro* procedure. The alteration of terminal molecular events in CCs before fertilisation could compromise cumulus-oocyte dialogue and hence the full development of oocyte competence. Studies using a bovine model have shown that the rise in PTGS2-related PGE2 production that occurs in CCs concomitantly with the resumption of meiosis is affected by IVM conditions and the presence of exogenous EGF (Nuttinck *et al*., 2008). However, PTGS2 expression remains weaker than that which is observed after *in vivo* maturation (Nuttinck *et al*., 2002). Several aspects of terminal differentiation of the bovine COC are affected by the level of PGE2 present in the oocyte environment during IVM (Nuttinck *et al*., 2011). The inhibition of cumulus PGE2 production reduces the kinetic of meiosis progression, oocyte MAPK activation and cumulus expansion. Recent publications have recommended the supplementation of IVM media with bioactive molecules that are involved in cumulus-oocyte interplay in order to counterbalance the alterations induced by the *in vitro* procedure (Richani and Gilchrist, 2018). We previously reported that the addition of PGE2 to IVM/IVF media promotes embryonic cell survival and cell lineage development during the two weeks of the preimplantation period in cattle (Nuttinck *et al*., 2017).

Conclusion

Disruption or deregulation of the interactions between a maturing oocyte and surrounding CCs can affect the final stages of the storage of maternal factors and consequently of subsequent embryonic development. One of the most promising options to improve the viability of *in vitro* produced embryos is to optimise the *in vitro* maturation conditions in order to preserve the integrity of cumulus-oocyte coupling, which will contribute to achieving the full developmental competence of the oocyte.

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SHORT COMMUNICATIONS

TAI/FTET/AI

A set of conditions that could enhance fertility rates and litter size in Sardi Morrocan sheep after exo-cervical insemination

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Keywords: Artificial insemination, fertility and letter size, semen quality

Artificial insemination (AI) with fresh semen has been used in sheep breeding programmers around the world. In Morocco, this technique has been limited due to low lambing rates (ranging from 34 to 54%) while performed with exo-cervical AI. The present work aimed to study the effect of ewe ages, rams, rank of ejaculate (first *vs.* second) and extenders (Skim milk *vs.* Duragen) on fertility rates and litter size of Sardi Moroccan Sheep. One hundred ewes have been chosen in the same herd based on their age. Only ewes of 4-tooth (4T) having 18-24 months old (n=50) and 6-tooth (6T) having 23-36 months old (n=50) were used. They were synchronized using sponges (20 mg cronolone, Pharmavet, Morocco) and PMSG at fixed dose (300 IU). Semen was collected from five rams (n=9 ejaculates) using an artificial vagina and diluted with Skim milk (SM) (n=6 ejaculates) or Duragen (n=3 ejaculates) (DR) (claimed as long term extender, Magapor S.L., Zaragoza, Spain) and maintained at 15°C. The statistical analyses were performed using the software JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA). Fertility data was assessed by chi square analysis of contingency tables. The liter size was assessed by ANOVA followed by student t test. There was no effect (P>0.05) of age on fertility (78.75% vs. 70.21%) nor on litter size (1.24. vs 1.15). When using semen with high characteristics (a concentration>$2x10^9$ spermatozoa/ml, mass motility>3 and individual motility>70) no difference on fertility rate (74.19% *vs.* 74.14%) and litter size (1.23±0.09 *vs.* 1.21±0.07) has been recorded (P>0.05). In addition, the results revealed that fertility rates were higher with the first ejaculate (81.25%) compared to the second ejaculate (55.56%) in ewes inseminated with SM (P<0.05). No difference was found between ejaculate regarding the litter size (P>0.05). No difference on fertility rates was recorded comparing SM to DR (73.47 *vs.* 75%) (P>0.05). The litter size was significantly higher in ewes inseminated with SM (1.26±0.06) than DR (1.16±0.85) SM (P<0.05). In conclusion, the set of conditions gathered in the present study (4T and 6T as age of ewes, Skimmed milk, duragen) were proven to enhance fertility rate in Sardi Moroccan sheep. The study is in progress and the whole set will be tested on a large number of ewes from different herds.

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Timed embryo transfer program from charolais heifers to holstein heifers

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Keywords: Charolais heifers, embryo, fixed time embryo transfer

The objective of this work was to transfer fresh charolais embryos to holstein dairy heifers for profitable beef production. This study describes embryo flushings from charolais heifers and fixed-time embryo transfer to holstein heifers. In this experiment, twelve clinically healthy charolais heifers, 14-16 months old, 500-600 kg body weight were used as donors and clinically healthy holstein heifers 14-16 months old, 350-400 kg body weight were used as recipients. In donors; estrous was synchronised with 5-day Co-Synch + CIDR protocol. In 5-day Co-Synch-CIDR protocol, CIDR (1,38 gr progesteron, Zoetis, Turkey) device was inserted and GnRH (Acegon®, Gonadorelin acetate, Zoetis, Turkey) was administered on day 0. Five days later CIDR was removed and $PGF_{2\alpha}$ (Dinolytic®, dinoprost tromethamine, Zoetis, Turkey) was administered followed by GnRH administration 72 hours later without artificial insemination (AI). Ten days after the 2nd GnRH administration, superovulatory treatment was initiated. Superovulatory treatment consisted of eight FSH (Stimufol; Reprobiol, Soiron-Pepinster, Belgium) injections twice daily (12 hours apart) for four days with decreasing doses (as decribed by pharmaceutic company). During third day of superovulatory protocol, $PGF_{2\alpha}$ was administered twice daily concurrent with FSH injections. Donors were artificially inseminated 12 and 24 hours after the last FSH injection. Conventional frozen charolais semen was used with proven fertility. Seven days after AI, uterus was flushed and embryos were recovered and graded according to the IETS standards. Donors were collected in 9 replicates of 2 to 5 heifers. Embryo recovery rate was 35.7 % (84/235) based on flushed embryos with respect to visible corpus luteum (CL) at flushing time. In total 84 embryos were recovered from 28 flushings. In average, three embryos were recovered per donor. Among collected embryos (n=84), 27% (23/84); 21% (18/84); 36 % (31/84); 14 % (12/84) were compact morula, early blastocyst, blastocyst, expanded blastocyst respectively. Recipient heifers were synchronised as describied for donors. Seven days later from the 2nd GnRH administration, fresh embryos transfered to recipient heifers as fixed-time embryo transfer program. Due to technical problems only 61 embryos were transferred. Among recipient heifers (n=61), seven have recieved two embryos. Blood samples were collected 21 days after fixed-time embryo transfer program to determine pregnancies by PAG technique. In general, pregnancy rate was 24,6 % (15/61). Pregnancy rate for recipient heifers transferred with double embryos was 14% (1/7). Although inadequate pregnancy rates were obtained following fixed-time embryo transfer program, it could be acceptable without the need of estrous detection in embryo transfer programs.

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OPU - IVF and ET

Nobiletin supplementation in maturation media enhances in vitro oocyte maturation and subsequent embryo development

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Keywords: Nobiletin, mitochondria, cortical granule

Nobiletin is a polymethoxylated flavonoid isolated from citrus fruits exhibiting a wide biological effect in cell adhesion, cell migration, cell cycle regulation and inhibition of reactive oxygen species (ROS) production (Huang et al. Evid Based Complement Alternat Med. 2016); important factors for oocyte *in vitro* maturation (IVM). However, there is no information available on the effect of nobiletin in *in vitro* embryo production (IVP). The aim of this study was to evaluate the effect of nobiletin supplementation in IVM of bovine oocytes on nuclear and cytoplasmatic maturation and their developmental competence. Immature cumulus oocytes complexes (COCs) were aspirated from ovaries of slaughtered heifers. Selected COCs were *in vitro* matured in TCM-199+10% FCS and 10 ng/mL epidermal growth factor (Control) supplemented either with 10, 25, 50 and 100 µM of nobiletin (MedChemExpress, MCE, Sweden) (Nob10, Nob25, Nob50 and Nob100 respectively) or 0.01% dimethyl sulfoxide (DMSO), vehicle for nobiletin dilution. After 24 h of IVM at 5% CO_2 in air at 38.5 °C, a representative number of oocytes from each group were fixed and stained with Hoëscht-LCA-FITC or Hoëscht-MitoTracker DeepRed to evaluate nuclear and cytoplasmic maturation (n=60/group/treatment). Also, 50 oocytes/group were stained with CellROX Deep Red Reagent and CellTracker Fluorescent to measure oocyte metabolism in terms of ROS and glutathione (GSH) content. The remaining oocytes were fertilized (D0) and cultured *in vitro* to evaluate their developmental competence by cleavage rate (D2) and blastocyst yield (D7-8). Data from eight replicates were analyzed by one-way ANOVA. Significantly higher percentage of matured oocytes ($P<0.05$) were observed in metaphase II when Nob25 (87±0.6%) or Nob50 (89.3±0.3%) were added to the IVM medium compared to Nob10 (72.9±0.3%), Nob100 (71.5±0.8%), control (71.7±0.7%) and DMSO (70.5±0.5%) groups. Furthermore, Nob25 and Nob50 showed higher rate of oocytes with peripheral migration of cortical granules (85.7±0.3% and 89.9±2.2% respectively) and mitochondria (86.7±0.6% and 88.9±1.2% respectively) compared to the remaining groups ($P<0.05$). In addition, the supplementation of Nob25 and Nob50 showed a significant reduction ($P<0.05$) in the ROS (2.53±0.8; 2.62±1.2 a.u. respectively), and GSH (2.84±0.4; 3.09±0.1 a.u. respectively) content in comparison with all other groups. Cleavage rate was significantly higher ($P<0.05$) for Nob25 (89.9±0.3%) and Nob50 (91.3±0.3%) compared to all other groups (Nob10: 75.6±0.3%; Nob100: 74.0±0.6%; control: 74.2±0.4%; and DMSO: 73.6±0.4%). Similarly, cumulative blastocyst yield at D8 was significantly higher ($P<0.05$) for Nob25 (32.1±0.8%) and Nob50 (35.5±0.8%) compared to Nob10 (23.1±0.7%), Nob100 (24.5±0.9%), control (25.9±0.4%) and DMSO (26.1±0.6%) groups. In conclusion, supplementation of 25 µM or 50 µM of nobiletin to the IVM medium improves oocyte nuclear and cytoplasmic maturation, reduces oxidative stress and improve embryo development.

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Nuclear maturation rate of caprine oocytes after *in vitro* maturation in three different media with base of TCM 199

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Keywords: Polar Body, Antioxidant, EGF.

The goats are economically important animals as a source of meat and milk especially in the Northeast of Brazil where most of the national herd is reared. The necessity of In vitro production of caprine embryo (IVP) increases due to the importance of genetic selection for high milk production and the importance of the goat as transgenic pattern. IVP results have not yet met neither the scientific objective nor the commercial one. The low IVP rates are mostly caused by the low efficiency of in vitro maturation (IVM). Objective: Verify three different IVM media for caprine oocytes; Materials and Methods: Groups of caprine oocytes that were aspirated from the ovaries of recently slaughtered goats were matured in vitro in three different media for 26 hours in a CO2 incubator (38.5C°, 5% CO2, and Saturated humidity) as follows: T1 [Tissue culture medium 199 (TCM199) + LH (50µg/ml) + FSH (1µg/ml) + Pyruvate (0.2 mM) + 10% FBS (Fetal Bovine Serum)], T2: [TCM 199 + EGF (Epidermal growth factor) (10ng/ml) + Cysteamine (1 mM/ml)], and T3 [TCM 199 + EGF (10 ng/ml) + Cysteamine (1 mM) + Cysteine (1 mM) + Ascorbic Acid (1 mM)]; 64, 62, and 64 oocytes were matured in T1, T2, and T3 respectively. After the maturation, the oocytes of each group were denuded in 100 µl of PBS (phosphate saline buffering solution) and the first polar body was observed under a phase-contrast microscopy (*40); the oocytes that showed the first polar body were considered matured (Metaphase II oocytes); the results were analyzed with One Way ANOVA with P-Value = 0.05. Results: There was no significant difference in maturation rate (P>0.05) among the groups (T1, T2, and T3 were 35.9%, 48.38%, and 50%, respectively). Conclusion: The nuclear maturation rate was not affected by the different media with TCM 199 base. The presence of the exogenous hormones (FSH and LH) did not improve the nuclear maturation rate, as well as the addition of an antioxidant (Ascorbic Acid), and the precursor of glutathione (Cysteamine) did not improve the nuclear maturation rate of caprine oocytes too, however we can conclude that a simple in vitro maturation medium of caprine oocytes may be enough for the nuclear maturation of caprine oocytes, and the in vitro maturation of goat oocytes can be developed in the absence of a protein source in TCM-199 medium. The maturation can be performed in a medium with absence of gonadotrophins, FSH and LH, reaching stages of nuclear maturation (Metaphase II) and finally; the maturation of goat oocytes can occur in the absence of antioxidants, but more researches have to be conducted to improve the nuclear maturation rate of caprine oocytes.

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Effects of an enriched n-3 polyunsaturated fatty acid diet on in vitro embryo production in dairy cowsEffects of an enriched n-3 polyunsaturated fatty acid diet on in vitro embryo production in dairy cows

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Keywords: OPU, oocyte, n-3 PUFA

High-yielding dairy cows fertility remains relatively low, with first service mean success rate between 35 and 40%. In a previous study (Elis s. Animal Reproduction Science 164: 121-132, 2016), long chain n-3 polyunsaturated fatty acids (n-3 PUFA LC) supplementation of the diet of dairy cows tended to decrease the non-fertilization – early embryo mortality rate after first service (n3 PUFA LC: 13.5% (n=22) vs control 38.8% (n=23), p=0.09), suggesting a potential effect on oocyte quality. In this study, we evaluated the effects of n-3 PUFA supplementation on in vitro embryo production in dairy cows, after hormonal ovarian stimulation. 37 primiparous Holstein cows were supplemented with n-3 PUFA (n=18, micro encapsulated fish oil, 1% DM, OMG750®, Kemin) or n-6 PUFA (n=19, micro encapsulated soy oil, 1% DM, OMG Soy®, Kemin). Three ovum-pick up sessions were performed on cows every two weeks (5 groups of 6 to 9 cows), after 2, 5 or 7 weeks of supplemented diet (between 92.0 0 ± 2.4 and 127.0 ± 2.4 day postpartum). Fatty acid composition in plasma was measured to assess the efficiency of the diet. Plasma anti mullerian hormone (AMH) assay was performed on the first day of diet supplementation to evaluate potential response of cows to hormonal ovarian stimulation. After follicular puncture, oocyte-cumulus complex (OCC) underwent in vitro maturation, fertilization (IVF) and development. Fertilization rate was determined 48 hours after IVF by counting cleaved embryos. Embryo development rate and embryo quality were determined 7 days after IVF by counting blastocysts. To compare n-3 and n-6 cows, multifactorial linear repression (quantitative parameters) or logistic regression (rates) models were used (fixed effects: diet, supplementation duration and interaction, cow as a random effect). Fatty acid composition showed a significant 1.62-fold increase in plasma EPA after 2 weeks of n-3 supplementation ($p<0.0001$) while the increase in plasma DHA became significant (1.46-fold, $p<0.0001$) only after 7 weeks diet. A total of 1462 follicles were punctured on n-3 cows (54 puncture sessions) and 1538 follicles on n-6 cows (57 puncture sessions). OCC recover rate was significantly increased in n-3 cows (41.6% vs 36.2% in n-6 cows, p=0.0035). No significant difference was reported on cleavage rate (p= 0.1033) between n-3 cows (77%) and n-6 cows (85%). Nevertheless, blastocyst rate (relative to cleaved embryo) tended to increase (p= 0.0865) in n-3 cows (48.2%) compared to n-6 cows (38.7%). A significant increase in good quality blastocysts (grades 1 and 2, relative to cleaved embryo) was observed (p= 0.0217) in n-3 cows (42.7%) compared to n-6 cows (33.3%). The number of total blastocysts and of grade 1 and 2 blastocysts produced per OPU session was 2.87 ± 0.34 and 2.48 ± 0.31 in n-3 cows versus 2.28 ± 0.31 and 1.88 ± 0.28, respectively, in n-6 cows. These results suggest that n-3 supplementation in the diet could improve embryo quality.

Follicular wave synchronization and superstimulation prior to ovum pick-up for improving *in vitro* embryo production in non-lactating Holstein cows

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Keywords: OPU/IVP, synchronization, FSH stimulation

Transvaginal ultrasound needle-guided ovum pick-up (OPU) and IVP represent powerful tools to multiply selected females in a short period of time. Maximizing the quantity and quality of retrieved oocytes is key for obtaining the best embryo development and implantation rates. The aim of this study was to compare OPU yields and developmental competence of oocyte-cumulus complexes (COCs). Holstein (>5 year-old) pluriparous open dry cows (n=25), handled under the same feeding and environmental conditions, were used for OPU as oocyte donors. Control and treatment groups were organized as follows: G1: no synchronization (Synch) (n=5), G2: Synch with no superstimulation (SOV) (n=5), G3: Synch with SOV (n=5) and OPU 36 h after the last FSH injection, G4: Synch with SOV (n=5) and OPU 48 h after the last FSH injection, and G5: Synch with SOV (n=5) and OPU 72 h after the last FSH injection. G1 received saline solution intramuscularly (i.m.). Follicular waves in all groups were synchronized by GnRH, PGF, and CIDR followed by SOV treatments 48 h later. FSH injections (pFSH=180 mg, Folltropin, Bioniche) were performed i.m. twice a day, for three days. The OPU procedures were performed using an ultrasound device (Mindray DP-30 Vet) equipped with a micro-convex transducer 5.0-8.5 MHz probe, disposable 20G needle and a flow rate of 15 mL/min. The 50 mL collection tube with aspiration media (PBS + BSA + heparin) was maintained at 36°C. Retrieved oocytes were classified according to IETS guidelines. Only viable COCs containing compact and complete cumulus cell layers were selected and matured. The IVP protocol followed Ferré *et al.*, Reproduction, Fertility and Development 29, 132-132, 2017. On Days 3 and 7 cleaved zygotes and blastocysts, respectively, were evaluated according to IETS standards. Cows were arranged in a crossover design and data analyzed using ANOVA and logistic regression with α= 0.05. Fisher's LSD test with Bonferroni correction was used to determine treatment differences. Synch versus no Synch, respectively, resulted in significantly more follicles/OPU (12.88±0.96 vs. 9.60±0.96), oocytes/OPU (10.92±0.80 vs. 7.72±0.80) and embryos/OPU (2.24±0.20 vs. 1.40±0.20) but not for cleavage rate (65.39±3.13% vs. 63.03±3.76%) and embryo rate (24.14±2.81% vs. 21.21±3.18%). Synch combined with SOV showed: follicles/OPU (18.00±0.96*, 19.44±0.96* and 22.12±0.9**), oocytes/OPU (15.24±0.80*, 17.44±0.80** and 14.32±0.80*), cleavage rate (67.38±2.60%*, 72.70±2.25%** and 75.25±2.48%***), embryo rate (26.46±2.45%*, 29.85±2.31%** and 33.66±2.71%***) and embryos/OPU (3.44±0.20*, 4.68±0.20** and 4.08±0.20***) for 36 h, 48 h and 72 h FSH coasting, respectively (different superscripts = P<0.05). In conclusion, Synch and FSH stimulation prior to OPU in non-lactating Holstein cows increased the number of collected and viable oocytes, cleavage, and embryo development rates. More transferrable embryos were obtained using 48 h FSH SOV coasting which may justify the extra cost associated with a Synch protocol and FSH treatment.

Risk of *Coxiella burnetii* transmission via embryo transfer using *in vitro* early caprine embryos

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Keywords:

Previous experiments using *in vitro* infection have shown that *Coxiella burnetii* has a strong tendency to adhere to the zona pellucida (ZP) of *in vivo* derived goat embryos, and the washing procedure recommended by the IETS for bovine embryos failed to remove it (Alsaleh et al., 2013). The aim of this study was, for *in-vitro* produced caprine embryos infected *in-vitro*, to (i) evaluate the ability of *C. burnetii* to adhere to intact *zona pellucida,* (ii) test the efficacy of IETS recommended rules for the washing of bovine embryos to eliminate *C. burnetii,* and (iii) determine by confocal microscopy the bacteria location.

One hundred ZP-intact caprine embryos, produced *in vitro*, at the 8 to 16 cell stage, were randomly allocated into 11 batches of eight to nine embryos. Nine batches were incubated for 18 hours with 10^9 *Coxiella*/ml of CbB1 strain (ISP, INRA Val de Loire). The embryos were then recovered and washed in batches in 10 successive baths following the IETS guidelines. In parallel, two batches of embryos were subjected to similar procedures but without exposure to *C. burnetii* to serve as the control group. One of the nine batches of infected embryos and one of the two non-infected control batches were used to perform immunolabeling to localize the bacteria. *C. burnetii* DNA was detected by C-PCR in all eight batches of infected embryos after 10 successive washings. However, bacterial DNA was not detected in the embryos of the control group. The first five washing media of the infected groups were consistently positive and *Coxiella* DNA was detected up to the 10^{th} wash in two batches. After immunolabeling, the observation of embryos under confocal microscopy allowed to localize C. *burnetti* on the external part of the *zona pellucida* without deep penetration. The presence of *C. burnetii* was seen on the surface of the *zona pellucida*, with bacterial loads differing from one embryo to another in the same batch. This study clearly demonstrates that *C. burnetii*, after *in vitro* infection at 10^9 *Coxiella*/mL, stick strongly to the external part of the *zona pellucida* of *in vitro* produced early caprine embryos without profound penetration. The 10 washings protocol recommended by IETS to eliminate the pathogenic agents of bovine embryos is unable to eliminate these bacteria in caprine embryos. Nevertheless, the finding of *C. burnetii* DNA by C-PCR does not imply that the bacteria found are still infective. Further studies are required to investigate whether enzymatic and/or antibiotic treatment of caprine embryos infected by *C. burnetii* would eliminate or inactivate the bacteria from the *zona pellucida* of *in vitro* produced goat embryos.

A retrospective study on influence of weight of heifer's donors at 12 months in relation to age at first estrous, age at first embryo flushing and number of viable embryos in a breeding program

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Keywords: MOET, Heifers donnors, Nutrition.

It has been known that calves nutrition plan influence the onset of puberty and the age of first estrous in heifers. In AURIVA Holstein breeding Program heifers come from different farms (in different breeding conditions). They arrive at AURIVA station (Denguin South West, France) with the aim to start in vivo embryo production as early as possible to maximize gain genetic and minimize the costs. This retrospective study from 2010 to 2017 aimed to evaluate the impact of heifers weight at 12 months on age of first estrous, first in vivo embryo collection and number of viable embryos under breeding program.

Holstein heifers animals (n=174) were used to analyze these parameters under a breeding MOET program. Animals were grouped according to their weight at 12 months of age as Low (L; <320 kg; n= 57), Normal (N; 320 to 370 kg; n= 81) or High (H; >370 kg; n=36). Superovulation was induced by eight intramuscular injections of follicle-stimulating hormone (FSH), Folltropin (Bioniche Teo, Inverin, Co., Galway, Ireland) or Stimufol (Stimufol; Reprobiol, Liège, Belgium), at 12-hour intervals over 4 days, involving decreasing doses, 500 IU (Folltropin) or 350 mg (Stimufol) on 9 to 12 days after the onset of standing estrus. Our unpublished data showed no difference in superovulatory response to these two FSH preparations. The donors were treated with 500µg of cloprostenol (PGF) with the 5th FSH treatment. First insemination was performed 12 hours after the onset of standing estrus. The donors were inseminated twice 12 hours apart with conventional semen. Embryo flushing was performed 7 days after AI. Recovered ova/embryos were evaluated according to the International Embryo Transfer Society classification system. Age of first estrous, first embryo flushing and number of viable embryos were analyzed by ANOVA test.

First heat (days ± SD) was observed significantly earlier ($p<0.05$) in H animals (391.2 ± 47.7) compared to L animals (417.5 ± 41.18), $P<0.05$. Not differences were observed between H vs N (404.3 ± 42.17) or L vs N animals ($P>0.05$). Moreover, age at first collection (days ± SD) was lower in H animals (424.7 ± 47.6) compared to L animals (451.5 ± 37.43), $P<0.05$. Not differences were observed between H vs N (438.5 ± 42.21) or L vs N animals ($P>0.05$). The average number of viable embryos collected during first flush was not different between L (5.4 ± 4.8), N (6.9 ± 4.8) or H (5.3 ± 4.0) animals ($P>0.05$).

In conclusion this study confirms the importance of the nutrition of heifers before entering a MOET breeding program. Animals with a weight higher than 370 kg at 12 months will start in vivo embryo production earlier than animals weighting less than 320 kg. Reduced time of heifers lodging represents an economic and genetic gain.

Effects of melatonin on bovine embryonic developmental competence and kinetics *in vitro*

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Keywords: IVF, bovine, melatonin

Oxidative stress has been identified as a major factor affecting embryo development *in vitro*. Melatonin is a well-known potent free radical scavenger and broad-spectrum antioxidant and could thus protect early embryos from oxidative damage. The goal of the current study was to evaluate the effects of melatonin on developmental competence and kinetics of bovine embryos derived from *in vitro* fertilization (IVF). A total of 1131 oocytes were collected by slicing of ovaries obtained from a local abattoir and were cultured in the presence or not of melatonin (MT) to two different concentrations [MT-10^{-9} (0.0002328 mg/mL) and MT-10^{-11} (0.000002328 mg/mL)] during maturation, fertilization, and *in vitro* culture). As melatonin is a lipophilic hormone, it has to be dissolved in ethanol, thus an ethanol (ETH) as a "sham" group and a standard control group (without any supplements) were included in the experimental setting for a total of four experimental groups (Control: N=260, ETH: N=304, MT-10^{-9}: N=277, and MT-10^{-11}: N=290). Final concentration of ethanol in ETH and MT groups was 0.01%. Variables evaluated included cleavage rate (CR) 72 hours post-insemination (72 hpi), blastocyst rate (BR) (186 hpi), and hatching rates (HR) (210 hpi). Additionally, the embryonic developmental kinetics were analyzed. Data were statistically analyzed using the SAS/STAT® software (SAS. version 9.3) with the logistic procedure (PROC LOGISTIC). Significant differences were defined as $P<0.05$. A statistical tendency was considered at $P=0.08$. There were no differences ($P>0.05$) for CR in the control group when compared with the two melatonin concentrations. Ethanol supplementation reduced significantly ($P<0.05$) CR in comparison with all other groups. The blastocyst rates for control, ETH, MT-10^{-9} and M-10^{-11} were 20.8 %, 23.4%, 27.1%, and 25.5%, respectively. The addition of melatonin at the 10^{-9} concentration revealed a statistical tendency ($P=0.08$) towards improved BR compared with the control group. Furthermore, the groups supplemented with ethanol and melatonin showed higher ($P=0.002$) hatching rates than the control group. The hatching rates at 210 hpi (Day 9) for control, ETH, MT-10^{-9} and M-10^{-11} were 20.4 %, 52.1%, 53.3%, and 50.0%, respectively. No differences ($P>0.05$) were observed for HR in the ethanol and different melatonin concentrations. Supplementation with ethanol and/or melatonin accelerated embryo development kinetics. The percentage of blastocysts reaching the hatching stage at 186 hpi (Day 8) was lower ($P<0.05$) in the control Group (14.8%), compared with ETH, MT-10^{-9} and MT-10^{-11} (36.6%, 32.0%, and 33.8%), respectively. Likewise, the proportion of blastocysts which reached the advanced blastocyst stage (expanded and hatching) at 186 hpi was higher in MT-10^{-11} compared with the control group (68.98% vs 51.85%, respectively). In conclusion, the presence of melatonin and ethanol (0.01% v/v) during early embryo development *in vitro* affects the kinetics of embryo development and increased hatching of bovine oocytes fertilized *in vitro*.

Detection of cell-free DNA in embryo culture medium: its potential application as a noninvasive method for sex determination in cattle

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Keywords: cell-free DNA, culture media, blastocyst

The application of noninvasive sex determination methods of embryos is believed to be crucial in assisted reproduction procedures. The aim of this study was to detect cell-free genomic DNA (gDNA) in the embryo surrounding spent media for sex determination of individually cultured bovine embryos. For this, bovine ovaries were collected from slaughter house. Immature oocytes aspirated from follicles of 3-5 mm in diameter, were matured and fertilized in vitro. Embryos were cultured individually starting from eight-cell stage until day 7 blastocyst in 10 µl drops of culture media under oil (SOFaa-medium). To verify the effect of different media on the release of cell-free DNA, individual embryos were cultured either in SOFaa supplemented with 5% exosome-free serum (SOF-EXO) media (n=15) or SOFaa supplemented with 0.1% hyaluronic acid (SOF+HA) media (n=9). Parthenogenetically activated oocytes and the corresponding embryos were cultured in SOF-EXO medium (n=6) to be used as controls. Individual blastocysts and the corresponding spent media were collected at day 7. Cell-free DNA was isolated from each spent media using QIAamp®Circulating Nucleic Acid kit followed by whole genome amplification using REPLI-g single cell kit (Qiagen, Hilden, Germany). In parallel, extraction of DNA from individual blastocyst was performed using blastocyst Lysis buffer. Multiplex PCR amplification was done to detect sex related fragments in DNA samples from both individual blastocysts and amplified cell-free DNA recovered from corresponding spent media. For this, two different primers; bovine Y-chromosome specific primer and bovine autosomal centromere-specific were used for sex specific PCR amplification. DNA of female and male animal tissues was used as control. The results of the present study revealed that, cell-free DNA was detected in 53.3% of embryos cultured in SOF-EXO spent media. Of these, the sex of 87.5% of individually cultured blastocysts was accurately determined using the cell free DNA isolated from the corresponding spent media. Similarly, cell-free DNA was detected in 55.6 % when embryos were cultured individually in SOF+HA spent media. Among these, 60% of the sex determinations were in accordance between spent media analysis and determination using the blastocyst itself. Moreover, cell-free DNA was detected in 66.7% of culture media drops when harbouring individual parthenogenetic embryos. Noteworthy, sex determination using cell-free DNA in parthenogenetic embryos was achieved with an accuracy of 100%. Lack of any Y-chromosome specific DNA in these samples could therefore demonstrate absence of genetic pollution. Inaccurate sex determination in some samples could be due to lower amount of cell-free DNA. In conclusion, cell-free DNA released from embryos to their surrounding culture medium can potentially be used as a noninvasive sex determination method prior to embryo transfer in cattle. Further studies should be conducted to improve efficiencies with respect to DNA isolation and amplification in spent media.

The influence of flunixin meglumine (FM), hCG or a combination of hCG and FM on the conception results in embryo recipient heifers including the passage time through the cervix and the presence of a large follicle

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Keywords: flunixin meglumine, hCG, recipients

The application of hCG before embryo transfer (ET) causes luteinization of a dominant follicle (Rizos, 2012). Non-steroid anti-inflammatory drugs (NSAIDs) lower the pro-inflammatory action of prostaglandins during cervical manipulation (Scenna, 2005). Some authors, however, do not confirm this effect (Nogueira, 2004, Torres, 2013). It is possible that the administration of hCG increases the likelihood of pregnancy in embryo recipients. It should be higher in cows with both CL and the large ovarian follicle. The probability of pregnancy should also be higher in recipients receiving flunixin meglumine (FM) - especially with the prolonged passage time through the cervix (PT). Consequently the highest pregnancy rate should occur in recipients treated with a combination of FM and hCG. The aim of the study was to assess the probability of pregnancy in cows treated with FM, hCG or a combination of FM and hCG in relation to the PT and the presence of a large ovarian follicle.

The conception results of 952 recipients of embryos collected by in vivo delivery (IVD) were included in the analysis. Recipients (CL>15 mm) were selected on day 7 after heat on the base of the ultrasound examination. Each follicle with a diameter of > 5 mm was considered as a large. Follicle of this size is palpable during rectal examination. All embryos were transferred by one experienced veterinarian. The FM was administered intramuscularly (IM) in an amount of 500 mg (Flunimeg 50 ml Fluniksyna –Scanvet Poland Sp. z o.o.), while hCG - IM in an amount of 1500 IU. hCG (Chorulon, Intervet International) was administered 15-2 min. before ET. In order to evaluate the factors determining cows' fertilization the logit model was estimated with the use of maximum likelihood estimation method and STATA software.

The average time of passage through the cervix to the embryo deposition site was 70.1s and it had no significant impact on the probability of conception ($p<0.09$). The average conception rate was 60.7%. In particular groups the conception rate was 61.3%, 63.4%, 58.5% and 58.3%, respectively, in FM, hCG, FM/hCG and control groups. There was no significant effect of FM administration ($p<0.35$), hCG ($p<0.32$) or the combination of both FM/hCG on the conception results in embryo recipients. The presence of a large follicle did not affect the conception rate ($p<0.23$). The different PT did not impact the results of the hormonal application (odds ratio $p<0.08$). The presence of a large follicle did not significantly change the treatment effects of used hormonal solutions ($p<0.45$). Administration of hCG, FM and a combination of both FM and hCG did not affect the probability of pregnancy of embryo recipients. Likewise, the results of conception were not improved by administering hCG to the recipients with a large ovarian follicle. FM application in females with prolonged passage time through the cervix does not affect the probability of pregnancy.

The influence of dominant follicles and corpora lutea location on the conception rate in embryo recipient heifers

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Keywords: Dominant follicle, embryo transfer, dairy cattle

Development of the first follicular wave dominant follicle on the ovary ipsilateral to the corpus luteum is associated with a decreased conception rate in dairy artificially inseminated cows (Miura, 2015). In embryo recipients that phenomenon has not been studied yet. Meanwhile, the position of the dominant follicle (DF) could be an additional criterion for the pre-transfer selection of embryo recipients. The aim of the study was to determine the effect of the DF placed ipsi- or contralateral to the ovary with CL on the conception rate. 967 recipients were examined in the study. Heats were synchronized with the use of prostaglandin (2xPG14). On the day of the transfer, the ultrasound examination of the ovaries was performed. Recipients with CL > 15 mm were qualified for the transfer. Additionally, the DF position and diameter were defined. Each follicle with a diameter > 5 mm was described as dominant. 682 fresh and 287 frozen embryos were used in the study. Pregnancy was examined palpatively 2 months after embryo transfer. Statistical analysis was done with STAPA.

A dominant follicle was observed in 928 (95.8%) recipients. The diameter of DFs was between 5 to 22 mm, with the average 10.1 mm. In 443 (47.7%) recipients, the DF was contralateral to the ovary with the CL, in 485 (52.3%) the DF was ipsilateral to the CL. In 39 (4.1%) recipients there was no dominant follicle. The percentage of pregnant recipients, in which embryos were introduced into the uteri horn on the same side as both CL and DF was 60,7% and it was lower ($p>0.05$) than in recipients with embryos introduced into the uteri horn on the side of the ovary with the CL, and contralateral to the ovary with the DF (61.04%). If the CL was observed on the left ovary and the DF on the right one, 61.6% of recipients were pregnant, while if the CL was observed on the right ovary and the DF contralateral to it, the percentage of pregnant recipients was 59.5. If there was no DF on the ovaries, the conception rate was 55.5% ($p>0.05$). Introducing embryos to the left horn (with the CL on the left ovary, no DF) resulted in 42.8% of pregnant recipients, while introducing them to the right horn (with the CL on the right ovary, no DF) ended with 59.1% of pregnancies. To conclude, the presence of the dominant follicle on the ovary ipsi- or contralateral to the ovary with the CL had no significant effect on the pregnancy probability. It seems that the DF location on the ovary in relation to the CL is an insufficient additional criterion for the selection of embryo recipients.

Reproductive fluids added to embryo culture vs. standard culture in cow: first results on pregnancy rates

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Keywords: Reproductive fluids, IVP, ET

Reproductive fluids (RF), though being part of the natural environment of embryo development, are not yet included in current IVC media. It has been shown in bovine that inclusion of RF in embryo IVC produces blastocysts with higher quality (Hamdi, Rep Fert Dev. RD17286, 2017). In porcine, embryos produced with RF had gene expression and DNA methylation patterns closer to in vivo grown embryos (Cánovas, eLife. 6:e23670, 2017). However, it is still unknown if the transfer of these embryos to recipients can give rise to pregnancies, thus justifying the conduction of this experiment. IVM, IVF and IVC details were already described elsewhere (Hamdi, Rep Fert Dev. RD17286, 2017). In IVC two different culture groups were created, according to the supplementation: RF group (SOF-RF) - 1,25%(v/v) NaturARTs BOF-EL (Embryocloud, Spain) from day 1 to 4 and 1,25%(v/v) BUF-ML from day 4 to 8 - and BSA group (SOF-BSA) - 3 mg mL-1 BSA from day 1 to 8. Vitrification and warming were performed using commercial media (Kitazato-Dibimed, Spain) with an open-system Cryotop, following manufacturer's instructions. Vitrification took place on IVC day 7/8 with embryos on stage 6-7 of development. Warming of embryos was performed less than 4h before transfers, loaded in straws and kept at 38,5ºC. Embryo transfers (ET) were made non-surgically to Holstein multiparous recipients either on day 6 or 7 after oestrus detection. Synchronization was made using Double-Ovsynch protocol. A total of 64 ET (n=36, SOF-RF; and n=28, SOF-BSA) were made in a 6 months period, from November 2017 until March 2018. Pregnancies were detected by ultrasound 30 days post-ET. Data were analysed by t-test independent samples with $p<0,05$ resulting in significant differences (data are means ± SEM). Pregnancy rate (P%) per group did not have a significant difference when comparing recipients of the same day. However, when comparing P% by recipient day there were significant differences: recipients on day 6 had 10,00±5,57 P% (n=30), while recipients on day 7 had 35,29±8,32 P% (n=34). Specific values for SOF-RF were 11,08±10,1 and 36,8±9,6; and for SOF-BSA were 7,77±11,6 and 33,3±10,8, respectively. ET's are routinely made to recipients on day 6 to 8 post-oestrus detection. Our data are consistent with other reports for P% of IVP embryos for day 7 recipients, but not for day 6. Additionally, the source of the oocytes is not OPU but rather slaughtered cows, which could decrease our blastocyst development and viability, despite it was maintained for day 7 recipients. In conclusion, the presence of RF in embryo IVC gave rise to pregnancies at a similar level than a control group. Day 6 recipients showed an adverse effect on pregnancy rates, regardless of the group. Further data (calves phenotypes mainly) are necessary to evaluate if the improvements reported at the blastocyst stage by including RF in bovine IVP are also evident after birth.

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Transcervical embryo recovery in Lacaune ewes superovulated with different doses of FSH

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Keywords: Cervix, dairy sheep, uterine flushing

This study assessed the effect of different FSH dosages for superovulation and the feasibility of transcervical embryo recovery in Lacaune ewes. Ewes (n=25) received 60 mg medroxiprogesterone acetate sponge (Progespon®, Syntex, Buenos Aires, Argentina) for nine days, 37.5 µg d-cloprostenol i.m. (Prolise®, Tecnopec, São Paulo, Brazil) 24 h before sponge removal and 50 µg gonadorelin (GnRH analogue, Gestran®, Tecnopec, São Paulo, Brazil) 24 h after sponge removal. Superovulatory treatments consisted of 100 mg (G100, n=13) or 200 mg (G200, n=12) of porcine FSH (Folltropin®-V; Bioniche Animal Health, Belleville, Canada), given i.m. (twice daily) for three consecutive days, in decreasing doses (25, 25, 15, 15, 10 and 10%), starting at 60 h before sponge removal. Ewes were checked for estrus twice daily and were naturally mated by fertile rams (4:1 ratio) while in estrus. Transrectal ovarian ultrasonography was performed at the 5th day after estrus, to count the number of corpora lutea (CL) with Doppler mode ultrasound (Mindray M5VET®, Shenzen, China – 8.0 MHz). All ewes received 37.5 µg d-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil) and 1 mg estradiol benzoate (Sincrodiol®, OuroFino, Cravinhos, Brazil) i.m. 16 h before uterine flushing and 50 IU oxytocin (Ocitocina forte UCB®, São Paulo, Brazil) i.v. 20 min before uterine flushing. Embryo collection was performed at days 5 or 6 after estrus by transcervical technique (Fonseca et al., Theriogenology, 86:144-151, 2016) in all ewes that showed estrus and had more than 2 CL (n=17). Qualitative data were analyzed by Fisher exact test. Quantitative data were analyzed by generalized linear models, using SAS® software (v 9.3, SAS Institute, Cary, USA). The percentage of ewes that showed estrus and the percentage of responding donors (> 2 CL) did not differ (P>0.05) between treatments: 77% (10/13) and 62% (8/13) for G100 and 100% (12/12) and 83% (10/12) G200, respectively. The number of CL was higher (P<0.05) for G200 (10.5±1.5) than G100 (4.2±1.5). Overall, cervical transposition and uterine flushing was possible in 100% (17/17) of ewes. The total time procedure was 32.3±0.1 min for G100 and 27.7±0.1 min for G200 (P>0.05).The number of recovered structures and viable embryos per ewe collected was higher (P<0.05) for G200 (7.5 ±0.1 and 6.2 ± 0.1) than G100 (0.4 ± 0.6 and 0.4 ± 0.6), respectively.The dose of 200 mg of FSH promoted greater superovulatory response and recovery of viable embryos by transcervical technique. Probably, the poor ovulatory response with 100 mg of FSH was also affected by the formation of luteinized unovulated follicles. The protocol for cervical relaxation was efficient to allow the transcervical embryo recovery of Lacaune ewes.

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Porcine follicular fluid as chemoattractant improves sperm attraction and *in vitro* fertilization

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Keywords: Porcine spermatozoa, follicular fluid, chemotaxis

Under physiologic conditions, different biofluids (follicular fluid (FF), oviductal fluid (OF), and secretion of cumulus-oocyte complex (COCs)) take part in the spermatozoa attraction previous to fertilization. Despite the progesterone (P4) being part of the composition of these biofluids, it's also considered as the main chemoattractant (Blengini et al., Asian Journal of Andrology, 13, 769-773, 2011). However, there are other components not defined in these media that could attract spermatozoa more efficiently. Thus, the aim of this study was to study the ability of biofluids for sperm attraction and the effect on *in vitro* fertilization (IVF) parameters. Periovulatory OF, FF and conditioned medium (CM) were collected previously described by Soriano et al., 2017. In the present study, a chemotaxis system was designed using a Petri cell culture dishes (35 x 10 mm) with four wells separated by 3 mm (GN627170, Sigma). Six wells were filled with fresh spermatozoa ($20x10^6$/mL) from fertile boars (N=6) that were capacitated for 45 min in 180 µL of the capacitation media (TALP), previously equilibrated for 3h at 38.5ºC and 5% CO_2. The opposite wells (six) were filled with TALP (control group) and TALP supplemented with the different chemoattractants: FF, OF, CM, P4, and mixture of all chemoattractans (experimental groups). Afterwards, 3-4 mm long capillaries bridges were placed between the wells containing capacitated spermatozoa and the opposite ones for 20 min. After that, the capillaries were removed and 22 (per replicate) denuded *in vitro* maturated oocytes were deposited with spermatozoa previously adjusted to $22x10^3$ in each group (chemoattractans and control groups). The experimental groups were: 1) TALP (control), 2) FF (1%), 3) OF (1%), 4) CM (2%) 5) P4 (28.3 pM), and 6) FF, OF, CM, and P4: Σ. After 18 h the oocytes were fixed and IVF parameters were evaluated in each experimental groups. All the data were analysed by ANOVA followed by Tukey post hoc to compare means and standard error ($p<0.05$). A total of six replicates were carried out. Follicular fluid alone (FF) showed the highest values for ZP binding, penetration rate, spermatozoa/oocyte and pronuclear formation ($p<0.05$). This preliminary study suggests that FF is the most important chemoattractant for porcine spermatozoa in *in-vitro* conditions. However, further experiment will be performed on the embryo development and quality.

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Retrospective survey on equine embryo transfer activities in France in 2014 and 2015

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Keywords:

The objectives of embryo transfer in mares are (1) produce several foals from the same mare during a single breeding season, eventually bred by different stallions; (2) allow donor mares to pursue their sport career while recipient mares carry their foals; and (3) obtain foals from mares unable to carry a pregnancy to term due to genital lesions or diseases.
The aim of our study was to analyse data from equine embryo transfer centres in France during the 2014 and 2015 breeding seasons. Our goal was to have an overview of Equine Embryo Transfer activity in France and to compare methods used by centres and their results to evaluate which parameters influence success rates of embryo recovery and transfer.
A survey was sent to 62 French centres and included questions about general activities and methods, and specific information of the 2014 and 2015 breeding seasons including donor mares, embryo recoveries performed and every embryo recovered and transferred. Only 18% (11/62) of centres responded to our study. Data from 208 donor mares (19%) of the 1108 donor mares recorded in France during those years, 669 embryo collections and 305 embryos were analysed.
This survey shows that centres used similar methods, but some specific points were different in the collection procedure (flush medium, number of flushes/collection (4 to 6), use of oxytocin), transfer procedure (sedation, NSAID), and in the tools to synchronize ovulations between recipient and donor mares. On average, 3.3 ± 3.8 embryo collections were performed per year from each donor mare (range 1 to 10), with a recovery rate of 41% (277/669) but significantly higher in 2014 (46.9%) than in 2015 (36.3%). This embryo recovery rate was significantly higher in mares under 14 years old, and in cycles with ovulation induction. Embryo collections were performed at 7 (5.5%), 8 (81.5%) or 9 (13%) days after ovulation, without any statistical difference in the recovery rate.
Pregnancy rate after embryo transfer was 75% (229/305). Age of embryo, delay of ovulations between recipient and donor (+1 to -4 days), and physiological status of recipient (maiden or not) had no significant effect on the pregnancy rate.
As 19% of French donor mares have followed by the same proportion of French transfer centres, one could make a projection on the total embryo transfer activity in France. It could be estimated that about 1800 embryo collections are performed per year, producing between 900 and 950 embryos. This projection would indicate that the annual report of equine embryo transfer presented by AETE is largely under evaluating the current embryo transfer activity. For example, 606 embryo collections and 365 transfers were reported in 2014 in France by the AETE, while in our study, 322 embryo collections were performed and 169 embryos transferred from only 19% of registered French donor mares. Only donor mares and successful embryo transfers (pregnancy at Day 14) are officially recorded in France; by reporting this number of recorded pregnancies to our estimated number of transfers, the estimated success rate (70-80%) is comparable to that really observed (75%) in the survey.

Folliculogenesis, Oogenesis, and Superovulation

Retrospective analysis of superstimulation with Folltropin®-V in Wagyu versus other beef breeds

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Key words: Wagyu, Folltropin®-V, superstimulation

The demand for Wagyu cattle, which originate from the Kobe region of Japan, is growing in many countries. Nevertheless, Wagyu remains an exotic breed in many places and questions persist in relation to the most efficacious superstimulation protocols. The data in this retrospective study were collected from 2012-2018 at the OvaGenix® (Texas) facilities. A total of 792 donors were collected while under direct care of OvaGenix® and were of the following *Bos taurus* and *Bos indicus* breeds: Angus (AN; n=120), Beefmaster (BM; n=139), Brangus (BN; n=207), Charolais (CH; n=76), Hereford (HH; n=45), Wagyu (KB; n=99), and Red Brangus (RB; n=106). The superstimulation protocol was the same for all donors and began with insertion of an Eazi-Breed™ CIDR® (Zoetis, Parsippany, USA) on Day 0. An injection (i.m., 100 mcg) of GnRH (Cystorelin®, Merial, Duluth, USA) was administered on Day 2, followed by 7 AM/PM injections (i.m.) of decreasing doses of pFSH (Folltropin®-V, Vetoquinol, Fort Worth, USA) on Days 4-7 and prostaglandin $F_2\alpha$ (i.m., 500 mcg, Estrumate, Merck Animal Health, Madison, USA) in the AM of Days 6-7. After CIDR® retrieval on Day 7, GnRH was injected in the AM of Day 8 and donors were artificially inseminated with frozen/thawed semen the PM of Day 8 and AM of Day 9. Embryos were collected on Day 14 (Day 6 from GnRH). Data from the embryo collections was analyzed with an ANOVA and Tukey's test to compare breed effect with $p<0.05$ considered significant. The data indicate that KB donors produce numbers of total ova (number of ova produced by superstimulation) and viable embryos that are comparable to other breeds using the above defined superstimulation protocol. Indeed, KB donors produced more total ova than AN (16.6±1.1 versus 11.4±0.7) and more viable embryos per collection than AN and CH (9.1±0.7, 4.1±0.4, 5.6±0.6, respectively). The BM, BN, RB, and HH donors produced similar numbers of total ova and viable embryos when compared to KB donors. Wagyu donors were also similar to other breeds for the percentage of donors with no ovarian response (5.0%) and donors with no viable embryos (9.0%). It is acknowledged that virtually every other beef and dairy breed responds well to Folltropin®-V and these data indicate that KB donors can also be effectively superstimulated with Folltropin®-V using a similar protocol to other beef breeds. Moreover, these data are similar to mean viable embryo numbers that are audited and published yearly by various national and international embryo transfer associations.

Physiology of Reproduction in Male and Semen Technology

ADAM protein expression in avian sperm and female genital epithelial cells: relation to sperm storage

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Keywords: ADAM protein, Sperm storage tubes, chicken

The cell-cell interaction can be mediated by the interface of a protein-protein. "ADAM is a transmembrane protein that contains a disintegrin and metalloprotease domain and, therefore, potentially has cell adhesion and protease activities" (Primakoff & Myles, Trends Genet., 2000, 16 (2): 83 -7). Avian sperm storage tubes (SST) are uterine-vaginal junction (UVJ) epithelial structures that can store spermatozoa (spz) through an interaction mechanism that is not yet clearly understood. This study was devoted to investigating the presence or absence of ADAM proteins in chicken SST as a model of sperm-epithelial cell adhesion and interaction. For *in vitro* tests, 3 hens per experiment (2 triplicate experiments, n = 18 hens) were euthanized and the internal epithelium UVJ was collected, digested (1 µg / ml collagenase) and the SST fragments were isolated by Percoll gradient column. SST (100/well), sperm (25.10^4 sperm/well) cultures and co-culture SST + spz (25.10^4 sperm / 100 SST) were grown in DMEM 199 medium supplemented with 10% BFS and gentamycin at 37 ° C, 5% CO2 atmosphere at 3 times: 0, 2 and 24h. For *in vivo* tests, 3 other hens were inseminated with 200 x 10^6 spz (pool of 6 roosters) and euthanized 24 hours later, while 3 other non-inseminated were used as control. In all experiments, antibody anti-ADAM-1-CT rabbit peptide sequence was used: CSSPGSGGSVDSGP (C-terminal part, close to the transmembrane metalloprotease domain), as described by Fàbrega et al. (Reproductive Biology and Endocrinology, 2011, 9:96). UVJ were isolated and prepared for immunohistochemistry (IHC). The slides were incubated overnight at 4°C with the primary antibody and incubated for 30 min with the secondary antibody ImmPRESS™ HRP Anti-Rabbit/Mouse IgG (Vector) and revealed with peroxidase. Protein concentration was determined individually in the samples, and the pools were prepared with equal protein concentration of each sample. Ten µg of protein in Laemmli buffer (5v:1v) were loaded on SDS-10% PAGE, and then transferred to nitrocellulose filters. The membranes were incubated with anti-ADAM-1 antibody overnight in blocking solution (5% non-fat dry milk in TBST [10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4]). The ADAM peptide exhibited sequence homology with *Gallus gallus* ADAM-12 and ADAM-33 (www.clustal.org/ multiple alignment program). ADAM was detected as a single 90 kDa band in SST samples and 3 bands (90, 45 and 40 KDa) in spz samples. Signals decreased in SST + spz co-culture in the cells and the respective supernatant, but a 90 kDa band was observed in the supernatant after 24h co-culture. Anti-ADAM IHC UVJ slides showed positive staining for spermatozoa located in the SST lumen and in small vesicles on apical SST cells. We can conclude that ADAM proteins are present in SST epithelial cells and can be released in the presence of spermatozoa, probably modulated by cell-cell interaction.

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Analysis of zona pellucida binding properties of boar sperm subpopulations separated by carbohydrate affinity

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Keywords: carbohydrate affinity, flow cytometry sorting, ZP binding assay

In mammals, sperm-sugar interaction is involved in relevant biological processes such as the sperm binding to the zona pellucida (ZP) and the formation of the sperm oviductal reservoir. The aims of this study are the identification of sperm subpopulations based on their affinity for carbohydrates, the isolation of these sperm subpopulations and the characterization of their ZP binding properties. Ovalbumin (OVA) which has high-mannose type glycans and the Lewis^a epitope (Le^a), consisting of Galß1-3[Fucα1-4]GlcNAcß, were tested. Heterospermic sperm samples from boars of proven fertility were coincubated with OVA-Alexa594 or Le^a-FITC for 30 minutes in PBS with 1mM sodium pyruvate. Subpopulations separation was done in a Sony SH800Z flow cytometer with excitation lasers at 488 nm and 561 nm, sorting 3500-4000 cells/second. Sperm were collected in tubes containing 100µl of TEST- 2% egg yolk buffer to minimize oxidative damage. Sperm resuspended in capacitating TALP medium at a concentration of 10^5 sperm/ml were incubated with 50 intact isolated ZP (n = 3). Isolated ZP were obtained form porcine ovaries collected at a local slaughterhouse. Cumulus-oocyte complexes were taken from antral follicles and treated with hyaluronidase. ZP were isolated with very thin pipettes in PBS containing protease inhibitors. After 2 h of incubation at 37ºC and 5% CO_2, samples were washed twice to detach weakly bound spermatozoa, fixed in PBS with glutaraldehyde 0.5% for 30 min and stained with Hoechst 33342. Spermatozoa bound to each ZP were evaluated with an Olympus IX70 fluorescence microscope. A Student's test was used to compare between each pair of samples and differences were considered significant when $p<0.01$. Percentages of spermatozoa from each subpopulation; Le^a-, Le^a+, OVA- and OVA+ were 77%, 18%, 82% and 14% respectively. A 4-5% of spermatozoa between each positive and negative subpopulation were discarded. The number of sperm bound to the ZP of the Le^a-, Le^a+, OVA- and OVA+ sperm subpopulations were 51.8±22.5, 18.9±8.8, 58.1±32.3, 17.5±6.8. Two different controls were perfomed. A total of 113.1±32.4 spz/ZP and 38.2±14.1 spz/ were observed for untreated ejaculated spermatozoa and sorted sperm not labelled. In both Le^a and OVA, ZP binding was significantly higher in the negative sperm subpopulations compared to the positive ones. Le^a+ subpopulation showed a high percentage of non viable spermatozoa (40±10.6%, n = 4), as assessed with propidium iodide, which correlates to less sperm bound to the ZP, while Le^a- viability was over 90%. Our results showed the existence of different sperm subpopulations with a different affinity for carbohydrates. These sperm subpopulations had dissimilar affinity for the ZP and could have a different fertilization ability. The biological significance of these sperm subpopulations will be analyzed in *in vitro* fertilization assays and binding to the oviductal epithelial cells.

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Sperm motility in thawed bull semen is increased by a short incubation before analysis

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Keywords: incubation time, cryopreservation of semen, sperm motility

Cryopreservation of semen is a widely used technique, mainly to provide samples with certified origin and quality for use in artificial insemination (AI) in dairy cattle. After thawing, spermatozoa may require a period to recover their proper motility, which is a factor directly related with the semen quality. Our aim was to determine the effects of a short incubation on total and progressive motility after thawing bull semen. The samples were collected from 12 bulls at a commercial bull station (Viking Genetics, Skara, Sweden) and were extended in OptiXcell® (IMV Technologies, L´Aigle, France) to provide a sperm concentration of 69×10^6 spermatozoa/mL. All samples were frozen in 0.25 mL plastic straws and stored in liquid nitrogen. The straws were thawed at 37°C for 12 s and the semen content was split into four tubes and incubated at 38°C for 0, 5, 10 or 15 min. Sperm motility assessment was performed by computer assisted sperm analysis (CASA – SpermVision, Minitub GmbH, Tiefenbach, Germany), connected to a microscope (Olympus, Tokyo, Japan) with a heated stage (38°C). Aliquots of 5µL from the thawed sperm samples were placed on a warm glass slide with a coverslip. At least 1000 spermatozoa were analyzed in a total of eight fields using the software program (SpermVision) with settings adjusted for bull spermatozoa. Statistical analysis was performed with SAS (version 9.3), using the proc mixed procedure for linear mixed models. Scheffe's adjustment was used for multiple-post ANOVA comparisons. Results are presented as LSMeans ± standard error of means (SEM). An increased incubation time was associated with increased total motility (0 min 44.7±4.45%; 5 min 47.0±4.45%; 10 min 49.0±4.45; 15 min 56.9±4.45) and progressive motility (0 min 41.5±4.34%; 5 min 43.8±4.34%; 10 min 45.9±4.34; 15 min 53.5±4.34). Motilities were significantly greater after 15 min incubation in OptiXcell® than after 0 min ($p<0.05$). In conclusion, total and progressive motilities may be increased by incubation before analysis, depending on the extender used. This effect should be remembered when comparing sperm handling treatments, or to avoid introducing bias when multiple samples are to be analysed.

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Effect of cholescytokinin (CCK) protein on the motility of porcine sperm.

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Keywords: spermatozoa, cholecystokinin, motion parameters

The expression of cholecystokinin (CCK) gene has been demonstrated in the testis of several different species and CCK-like peptides have been found in spermatocytes and spermatids of the mouse, rat, and monkey (Persson, PNAS; 86: 6166, 1989). In a recent study, Zhou et al suggested its possible involvement in the regulation of protein tyrosine phosphorylation by modulation the uptake of HCO3- and demonstrated the presence of CCK receptors on sperm surface (Zhou, Reproduction; 150:257, 2015). In addition, our group has detected CCK in the porcine oviduct using microarray technology, real-time RT-PCR and immunohistochemical analyses (Acuña, Reprod Fertil Dev; 29: 2387, 2017). Therefore, the aim of this work was to analyse the effect of CCK on sperm motility. Spermatozoa from fertile boars (15x106/ml) were exposed to different CCK (Bachem, Bubendorf, Switzerland) concentrations according to the bibliography (25 µM and 50 µM) in a capacitating medium (TALP) for 1h at 38.5ºC. TALP medium had a bicarbonate concentration of 5 mM and the incubator was adjusted to 1% CO2 according to the Henderson-Hasselbalch equation. Moreover, a control group was incubated under the same conditions without CCK protein. Motion parameters (total motility (%) and progressive motility (%)) were determined using a CASA system (ISAS®, Proiser, Valencia, Spain) after of incubation period. For statistical analysis the mean measurements of CASA for each male (n=5) were entered into the ANOVA model and compared by post hoc test (Tukey). Differences were considered statistically significant at $p<0.05$. The results showed that incubation with CCK increased the percentage of total sperm motility (Control: 53.8±2.8%, 25 µM: 59.4±2.1% and 50 µM: 65.4±4.5%) with statistically significant differences between the three experimental groups. Moreover, an increase in the percentage of progressive sperm motility was observed (Control: 34.4±3.9%, 25µM: 38.4±4.8% and 50 µM: 43.2±5.0%) showing significant differences between the control and 50 µM CCK. In both cases the effect of the protein is dose-dependent. No statistical differences were found between the study groups for the rest of motion parameters. In conclusion, these data could suggest a possible implication of CCK protein in the improvement of the sperm motility in the oviduct before fertilization. More analyses are necessary for clarify the molecular mechanism of this protein.

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Porcine periovulatory oviductal fluid decreases the occurrence of Protein Kinase A (PKA) substrates trough sAC/cAMP/PKA during mouse sperm capacitation

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Keywords: Oviductal fluid, spermatozoa, phosphorylation

Several studies have identified important factors involved in the regulation of sperm capacitation, a physiological process necessary to achieve fertilization competence. These events are regulated through activation of the cyclic AMP (cAMP)/protein kinase A (PKA) pathway. On the other hand, porcine oviductal fluids (POF) decreases PKA activity of porcine sperm during capacitation; however, the molecular mechanism of this regulation remains unknown.
The aim of this study was to evaluate if porcine periovulatory oviductal fluid (POF) regulates PKA activity during *in vitro* sperm capacitation in mice.
POF was obtained from porcine oviducts (n=40) close to ovulation and it was frozen at -80ºC before use. Sperm samples were obtained from mice cauda epididymides and incubated for 1 hour in: i) non-capacitation media, ii) capacitation media, iii) capacitation media supplemented with 1% POF, iv) capacitation media supplemented with 1% POF and 100 µM of 3-isobutyl-1-methylxanthine (IBMX), which inhibit adenosine 3',5'-cyclic monophosphate phosphodiesterase (cAMP PDE), v) capacitation media supplemented with 1% POF, and 1 mM dibutyryl-cAMP (db-cAMP), that mimics the action of endogenous c-AMP and vi) capacitation media supplemented with 1% POF, 100 µM of IBMX and 1 mM db-cAMP.
The protein phosphorylation pattern on PKA substrates was evaluated by Western blotting (WB). Proteins were separated by electrophoresis on 8% SDS-polyacrylamide gels and electrotransferred to PVDF membranes. The latter were treated with the following antibodies: rabbit monoclonal antibody anti-phosphorylated protein kinase A substrates (1:10000) followed by goat anti-rabbit IgG-HRP (1:10000). After developing, the relative amount of signal was quantified by optical densitometry using ImageJ software. Data were analyzed by two-way ANOVA and Tukey post-hoc test ($p < 0.05$).
Our results indicate that in the presence of POF, spermatozoa showed a lower PKA substrates phosphorylation pattern than those incubated in capacitation media alone ($p < 0.05$). The effect of the POF was reversed by the presence of db-cAMP and/or IBMX in the media. Besides, our results indicate that POF decreases the sAC/cAMP/PKA pathway during sperm capacitation and that this is not a specie-specific phenomenon. These effects were not observed under non-capacitating conditions.
We identified one of the mechanisms by which POF regulates sperm capacitation. This finding would help to understand the oviductal physiology during gamete interaction and could explain some cases of idiopathic infertility.
Further studies are required to evaluate whether FOP regulates PKA-related molecular pathways during sperm capacitation in other species such humans.

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Effect of swim-up on the activation of apoptosis in frozen bovine semen: A flow cytometric study

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Keywords: Bovine sperm, swim-up, flow cytometry

Techniques universally used to prepare bovine spermatozoa for in vitro fertilization (IVF) include the swim-up (SU) method and differential density gradient centrifugation, among others. Although it is clear that not all of them have comparable efficiency in selecting high quality sperm, the use of these techniques can be harmful for sperm quality. The aim of the present study was to detect the sub lethal changes in bull spermatozoa immediately post-thawing (PT) and after SU, namely the levels of apoptotic sperm cells, to assess if the sperm treatment before IVF induces apoptosis in bull spermatozoa. For such purpose, three semen straws per bull from six bulls were thawed by placing them in a water bath (37 °C) for 30 sec. Then, sperm apoptosis level was analyzed immediately PT and after the SU procedure using a flow cytometric method by detecting the phosphatidylserine translocation across the plasma membrane using a fluorescein-labeled Annexin-V and propidium iodide (PI). By using these two dyes, four different subpopulations of sperm were observed: a population of apoptotic sperm, a population of early necrotic sperma, a population of necrotic sperm and a population of fully viable sperm cells.
The most consistent effect observed was a significant increase ($P < 0.05$) in the fraction of apoptotic sperm (Annexin-V $^{+}$, PI^{-}) after the SU treatment among the six bulls tested. The highest increase of apoptotic spermatozoa after SU was observed on bull four with an increase from 7.0 % ± 0.9 to 59.2 % ± 0.2, respectively after thawing and after SU. On average, including all bulls, apoptotic values raised from 11.8 % ±3.3 to 16.9 % ± 6.5, respectively before and after SU, proving that the bulls presented different values in the activation of apoptosis during the SU process. In addition, the proportion of necrotic sperm (Annexin V^{-}, PI^{+}) was also significantly different among bulls. In particular, bull four, which had the lowest proportion of necrotic sperm, approximately 2% and 4% for PT and SU, respectively. The percentage of viable sperm (Annexin V^{-}, PI^{-}) was significantly different ($P < 0.05$) among bulls. Bull 5 had the highest ($P < 0.05$) proportion of viable sperm in the sperm samples immediately PT. Overall, PT treatment had no significant effect in the number of viable spermatozoa. The present study indicates that the SU technique can have an adverse effect on the spermatozoa membrane stability leading to different degrees of apoptosis in sperm during SU. Apoptotic markers found in ejaculated spermatozoa may represent an important tool for the study of male infertility, and combine with IVF, may be a valuable laboratory routine technique.

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Progesterone induces sperm release from bovine oviductal epithelial cells by modification of the sperm protein and lipid compositions

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Keywords: Progesterone, BOEC, sperm

After mating or insemination, spermatozoa reach the oviduct where they bind to oviductal epithelial cells (OEC) for hours to days in the so called "functional sperm reservoir" before moving towards the fertilization site. During this storage, the interactions between spermatozoa and OEC are believed to play an important role in sperm selection and capacitation. Recently, after measuring progesterone (P4) concentrations in the post-ovulatory bovine tubal fluid (Lamy et al. Theriogenology 86:1409-1420, 2016) our group evidenced that P4(100 ng/mL) was able to trigger sperm release from bovine OEC (BOEC) *in vitro*, similar to what occurs *in vivo*, selecting a population of spermatozoa with a higher fertilizing competence (Lamy et al. Reproduction 154:497-508,2017). The aim of this study was to elucidate the underlying mechanisms of action. Frozen-thawed bovine spermatozoa ($4x10^6$/mL), after Percoll density gradient (45/90%), were incubated *in vitro* with confluent BOEC for 30 min (humidified atmosphere, 5% CO_2, 38.8 °C). After collecting unbound spermatozoa (UnS), P4 was added to the sperm-BOEC culture for 1h. Then, released spermatozoa (P4-ReS) were collected. A group of spermatozoa was similarly manipulated without BOEC nor P4 (CTRL) and another was treated with P4 without BOEC (P4-CTRL).
Proteomic and lipidomic profiles were assessed on Intact Cells by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (ICM-MS) for each group. The presence of Binder of Sperm Proteins (BSP)-1, -3 and -5, the most important proteins involved in sperm-BOEC binding, was studied by Western-Blotting. Fluorescence Recovery After Photobleaching (FRAP) analysis coupled with confocal microscopy were performed on P4-ReS and CTRL groups to evaluate the changes in membrane fluidity, event directly related to capacitation ($n \geq 3$). In total, 139 m/z (mass/charge) peaks were found as differential m/z on spermatozoa proteomic profiles by ICM-MS. The number of differential peaks was highest between P4-ReS and CTRL (97), followed by P4-CTRL vs. CTRL (61), and UnS vs. CTRL (33). By contrast, only 37 peaks were found as differential m/z on lipidomic profiles, all of them from P4-ReS vs. CTRL (33) and UnS vs. CTRL (32) comparisons. A 3 to 4-fold decrease of BSP-1, -3 and -5 was seen on P4-ReS compared to CTRL ($p<0.05$), but not in UnS and P4-CTRL groups. Lastly, FRAP analysis showed a higher membrane fluidity on P4-ReS compared to CTRL (KW, $p=0.014$). In conclusion, these results show that binding to BOEC and then P4-induced release from BOEC triggered major changes in sperm protein and lipid composition whereas P4 by itself had a moderate effect. The UnS displayed an intermediate level of changes in proteins and lipids, suggesting a "BOEC effect" that may be due to a short time binding-release process. A loss of BSPs at the surface and an increase in membrane fluidity were evidenced on P4-ReS, suggesting a membrane destabilization probably involved in the increase of fertilizing competence of this sub-population.

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Motility and atomic force microscopy observation of avian spermatozoa incubated in uterine fluid

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Keywords: Spermatozoa, Atomic Force microscopy, Avian species

In hens, spermatozoa are stored in the genital tract for long periods, and fertilized eggs are produced for up to 3 weeks after insemination. Storage mechanisms and oviducal environment impact on sperm are largely unknown. The aim of this study was to evaluate the effect of uterine fluid (UF) on semen using two different lines of hens that display a long (21 days, F+ line) or a short (10 days, F- line) period of sperm storage. UF from 3 hens of each line was collected 10h after oviposition and pooled. Fresh ejaculates (pool from 6 roosters) were used in both experiments. For the first one, spermatozoa were incubated *in vitro* in either PBS alone, PBS containing 25% or 50% UF, or in pure UF from both lines for 5 min, 1, 2.5, 5 and 24h at 4°C (3 replicates). For the second experiment, UF from both lines were depleted from proteins >3kDa (d-UF) using Vivaspin 500. Spermatozoa were incubated *in vitro* in PBS, d-UF, and pure UF for 5 min, 1 and 2.5h at 4°C (3 replicates). Sperm motility was assessed using computerized method at 41°C (HTM-IVOS II). Metabolites contained in d-UF from both lines were quantified by NMR and sperm morphology was analyzed using Atomic Force Microscopy (AFM). Wilcoxon test was used to compare sperm motility at the different time point. ANOVA analysis was performed to compare sperm motility between the two chicken lines. Welch t-test was used to compare differential metabolites between the two chicken lines. We observed that pure UF and d-UF improve sperm motility compared to PBS ($p<0.05$). Nevertheless the effect of d-UF was lower than pure UF. In both experiments, sperm motility was higher after incubation in UF or d-UF from F-line than F+ line ($p<0.05$). NMR analysis reveals that the concentrations of 5 metabolites were higher in d-UF from F+ than F- line ($p<0.05$), including alanine, succinate, dimethylamide and N-acetyl groups. Moreover, AFM analysis clearly showed an alteration of head morphology of spermatozoa incubated with d-UF from the F- line. This study clearly demonstrates the major role of UF proteins >3kDa on the sperm motility. Nevertheless, the UF fraction (<3kDa) which mainly contains peptides and metabolites improves sperm motility and leads to ultrastructural modification of spermatozoa. Our findings demonstrate that the microenvironment and complexity is a key element during sperm storage.

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Sperm selection by density-gradient centrifugation of Merino ram semen cold-stored up to 48 h improves viability and membrane integrity

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Keywords: sperm, cold-storage, selection

Liquid ram semen stored at 5°C would be more competent than frozen/thawed for sheep crossbreeding programs. The aim was to evaluate the kinetics and membrane integrity of Merino ram semen cold-stored up to 48h at 5ºC before and after density-gradient centrifugation (DGC) selection. Pools of 3 normospermic Merino ram (2-7 years) ejaculates were collected by artificial vagina in fifteen sessions (45 ejaculates), diluted to 200x10^6 spermatozoa/ml with skim milk-based extender contained 6% egg yolk and cold-stored up to 48h at 5ºC. Motile spermatozoa were separated by BoviPure® DGC (Galarza et al., 2018, Anim Reprod Sci 192: 261-270) using 250μl of fresh (n=30) and cold-stored semen (24h: n=10 and 48h: n=10). The final pellet of 300μl was used to assess semen quality. The kinetic parameters were evaluated by computer-assisted sperm analysis (CASA) while plasma, acrosomal and mitochondrial membrane status was analyzed by PI/FITC PNA/Mitotracker fluorescence. The effects of storage time (fresh, 24 & 48h) and sperm selection process were analysed by univariant ANOVA and Bonferroni´s test ($p<0.05$). In terms of sperm storage time, CASA analysis of non-selected semen samples showed a significant decrease after storage for 24 and 48h compared to fresh samples with regard to progressive motility [SPM (%): 52.30±4.1 and 36.9±5.5 vs 71.3±1.6], straight line velocity [VSL (μm/sec): VSL 132.2±6.1 and 109.7±6.3 vs 176.7±4.3], linearity [LIN (%): 69.2±3.5 and 59.0±5.0 vs 82.0±1.2], and straightness [STR (%): 75.7±3.3 and 66.0±4.3 vs 86.9±0.9], respectively. However, analysis of DGC-selected semen showed a decrease only at storage for 48h when compared to 24h or fresh samples with regards to SPM (35.6±3.9 vs 56.1±6.91 and 59.3±2.6), VSL (83.5±4.4 vs 105.3±6.5 and 110±2.0) and LIN (63.9±3.4 vs 75.0±3.7 and 80.7±2.4), respectively. A comparison between DGC-selected and non-selected samples showed a significant lower total motility [TM (%): 94.4±0.8 vs 85.4±1.90], VSL (176.7±4.2 vs 110.0±2.0) and wobble [WOB (%): 94.2±0.6 vs 88.5±1.5] only for fresh semen. Fluorescence analysis evidenced a decrease only in 24h cold-stored non-selected compared with fresh semen with regard to plasma membrane integrity [PMI (%): 64.8±2.9 vs 80.1±1.7], high mitochondrial function [HMF (%): 88.2±1.6 vs 93.9±1.0] and total intact plasma/intact acrosome/high mitochondrial function [IPIAHM (%): 61.8±3.1vs 78.7±2.0]. In contrast, no differences were observed between fresh and cold-stored DGC-selected semen. A comparison between selected and non-selected semen showed a significant increase of PMI (64.8±3.14 to 89.4±2.32), HMF (88.2±1.26 to 96.0±1.26) and IPIAHM (61.8±3.14 to 87.6±2.04) only for 24h. These results suggest that kinetic activity of cold-stored and DGC-selected ram spermatozoa is maintained and the selection process results in improved viability and membrane integrity. Therefore, liquid storage combined with DGC-selection might become a good alternative to fresh or frozen non-selected semen to be used for artificial insemination in sheep crossbreeding programs.

Embryology, Developmental Biology, and Physiology of Reproduction

Oviduct extracellular vesicles: a new strategy to optimize porcine *in vitro* embryo production

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Keywords: Oviduct extracellular vesicles, Embryo development, Porcine IVP

Oviduct extracellular vesicles (EVs) have been identified as important components of oviductal fluid (OF) and as key modulators of embryo-maternal communication. In an attempt to optimize porcine *in vitro* embryo production (IVP) outcomes by mimicking the maternal environment, we evaluated the effect of porcine oviduct EVs supplementation during *in vitro* culture (IVC) on porcine embryo development rate and quality. The effect of OF supplementation (10%) during IVC was also tested. Thus, five different treatments were used: Control (without supplementation); OF 0-2 (during the first 2 days of IVC); OF 0-7 (during 7 days of IVC); EVs 0-2 and EVs 0-7. Porcine oviducts were collected at local abattoir and flushed with PBS (Sigma, Saint-Louis, USA). EVs were isolated by ultracentrifugation and stored at -80°C until use (3 EVs/OF pools of 4 oviducts pairs each, from gilts at late follicular stage of estrous cycle). EVs protein concentration were measured (21.8-28.3 mg/mL) and diluted in IVC medium (NCSU-23; 0.2 mg/mL). *In vitro* matured and fertilized presumptive zygotes were cultured under mineral oil, 5% CO_2 and 38,8ºC, as detailed above (6 replicates) (Almiñana C., Theriogenology, 64, 1783–1796, 2005). Cleavage rates were evaluated at day 2 and blastocyst rates at days 5, 6 and 7 after fertilization. Embryo quality was assessed by hatching rates and number of cells/blastocyst at day 7. Data were analyzed by one-way ANOVA or Kruskall-Wallis test and expressed as mean ± SEM. EVs treatments (0-2 and 0-7) had a positive effect on cleavage rates being significantly higher than control ($P<0.05$). Furthermore, EVs 0-2 treatment increased significantly cleavage rates compared to both OF treatments (0-2 and 0-7) ($P<0.05$) (Cleavage rates: 44.4±0.8; 46.4±1.5; 45.7±1.5; 52.1±2.5; 51.1±2.4; for Control, OF 0-2, OF 0-7, EVs 0-2 and EVs 0-7). Similarly, EVs 0-2 improved blastocyst rates over time, being significantly different to both OF treatments at day 7 ($P<0.05$). Moreover, EVs 0-2 showed a tendency to increase blastocyst rates on day 5, 6 and 7 of IVC compared to EVs 0-7 ($P=0.052$-0.08) (Blastocyst rates on day 5: 8.9±0.8; 11.7±2.6; 3.8±0.4; 13.4±2.7; 7.6±2.2; on day 6: 16±1.5; 15±1.9; 9±1.3; 23±4.1; 16±3.6 and on day 7: 21±1.3; 19±1.7; 10±1.3; 29±2.2; 20±2.2; for Control, OF 0-2, OF 0-7, EVs 0-2 and EVs 0-7). However, the use of OF 0-7 decreased dramatically the blastocyst rates on day 5, 6 and 7 of embryo development ($P< 0.05$). There were no differences on hatching rates for any of the treatments tested. However, EVs 0-2 and OF 0-2 treatments increased significantly the number of cells/blastocyst when compared to the same treatments during longer IVC times (38.8±3.8; 44.3±4.2; 32.8±2.2; 43.2±3.1; 32.8±1.4; for Control, OF 0-2, OF 0-7; EVs 0-2 and EVs 0-7). In conclusion, these results show that oviduct EVs supplementation during the first two days of development improves blastocyst yield and quality, suggesting that EVs could be a new strategy to improve porcine IVP.

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Physiological concentrations of steroid hormones during in vitro culture changed lipid composition and improved cryosurvival of bovine embryos

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Keywords: Embryos, Steroid hormones, Lipids

Steroid hormones are highly concentrated in the bovine oviductal fluid in the post-ovulatory period, during fertilization and early embryo development. The objective of this study was to evaluate the effect of progesterone (P4), estradiol (E2) and cortisol (CO) at physiological concentrations on bovine embryo development rates and quality in vitro. Bovine oocytes were collected in a local slaughterhouse, matured and fertilized in vitro. Presumptive zygotes were cultured during 8 days in 500 µl of synthetic oviductal fluid supplemented with 55 ng/mL P4, 120 pg/mL E2, 40 ng/ml CO or their combination (P4/E2/CO) at the same concentrations, i.e. mean concentrations previously measured by mass spectrometry (MS) in post-ovulatory oviductal fluids ipsilateral to the corpus luteum. Control embryos were cultured with vehicle (0.1% ethanol). Cleavage and blastocyst rates were recorded at Day 2 and Days 6-7-8 post-insemination, respectively. At Days 7-8, blastocysts were evaluated for cell number after staining with Hoechst. Day-7 blastocysts were vitrified and evaluated for post-thawing survival for 72h (Live-Dead staining, confocal microscopy). Subgroups of fresh Day-7 blastocysts were individually evaluated for their lipid content by Intact Cell MALDI-TOF MS (ICM-MS) in positive ion and reflectron modes. The effect of hormonal treatments on embryo rates and cell number/MS data was evaluated by ANOVA and Kruskall Wallis tests, respectively. The effect on cryosurvival rates was evaluated by exact Fisher tests.
Exposure to steroids did not affect the proportions of embryos that cleaved on Day 2 (78-81%) and developed to the blastocyst stage on Days 6, 7 and 8 (25-31%, 31-36% and 30-37%, respectively; 6 replicates; n=474 oocytes/group). The mean number of cells per blastocyst did not change between groups (97.8-118.2 cells, n=10-24 embryos/group). However, P4 improved the rate of embryo survival at 24 h post-thawing compared with controls (95% vs. 65%, p=0.04) while E2 improved embryo survival at 72 h post-thawing (55 vs. 20%, p=0.02; n=14-22 embryos/group). By ICM-MS, a total of 323 m/z within the 400-1000 mass range, corresponding mostly to phosphocholines and sphingomyelins, were detected. Among them, 119 masses were differentially abundant between groups ($p<0.01$; fold-change >1.5 or <0.67; n=11 embryos from 4 replicates/group). Exposure to P4/E2/CO induced the highest changes in embryo lipid composition, with 51 up-regulated and 30 down-regulated lipid species, followed by P4 (10 up- and 24 down-regulated), CO (6 up- and 27 down-regulated) and E2 (6 up- and 7 down-regulated lipid species). In conclusion, the exposure of bovine embryos to physiological concentrations of steroid hormones did not affect in vitro developmental rates but improved blastocyst quality through increased cryotolerance and altered lipid composition, in particular cell membrane phospholipids. Further studies are required to identify the lipids potentially linked to a better embryo cryosurvival and underlying mechanisms.

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Lipidomic profiling of the bovine oviductal fluid across the estrous cycle

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Keywords: Bovine Oviductal Fluid, Tubal, Lipids

Sperm capacitation, fertilization and early embryo development take place within the oviduct during the periovulatory period. Phospholipids (PL) are known to be taken up by the spermatozoa, influencing sperm capacitation. PL are also crucial for the embryo membrane fluidity, permeability and post-cryoconservation survival. However, data on the nature of PL present in the oviductal fluid (OF) and their regulation across the estrous cycle are scarce. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to monitor the abundance of phospholipids in the bovine OF according to the stage of the estrous cycle and the side relative to ovulation. Bovine oviducts were collected at a local slaughterhouse as previously described (*Lamy et al, Theriogenology 86:1409, 2016*) and classified into 4 stages according to the ovarian and corpus luteum morphologies (n=19 cows/stage): post-ovulatory (Post-ov), mid luteal (Mid-lut), late luteal (Late-lut) and pre-ovulatory (Pre-ov) stages of the estrous cycle. Follicular fluid was also collected from the Pre-ov follicles: animals with intra-follicular progesterone >160 ng/ml (cystic follicles) were excluded. Oviductal fluids were collected from contra- and ipsilateral oviducts by squeezing and stored in liquid N before analysis. Lipid spectral profiles of individual OF (0.5 µL, 5 technical replicates) were acquired in the m/z range of 400-1100 in positive reflector mode using an UltraFleXtreme MALDI-TOF MS (Bruker). Differential analysis between stages and sides were performed by Kruskal-Wallis (followed by Bonferroni post-test for pairwise comparisons) and Wilcoxon tests, respectively, on normalized intensities using the R software. Peaks were considered to be differentially abundant between 2 stages or sides when the p-value was <0.05[MSD1] and the ratio of normalized intensities >1.5 or <0.67. Principal component analyses (PCA) and hierarchical clustering (HC) were performed on most differential peaks (p<0.01) using R. Lipids profiles were obtained for all OF samples: a total of 209 molecular species were characterized, including phosphatidylcholines, phosphatidylethanolamines and sphingomyelines. No significant differences were identified between ipsi- and contralateral OF at a given stage. However, in ipsilateral OF, 57 differential peaks were identified between stages, of which 52, 37 and 22 for Pre-ov vs. Mid-lut, Pre-ov vs. Late-lut and Pre-ov vs Post-ov comparisons, respectively. The PCA and HC clearly discriminated the Pre-ov stage from the three others. In conclusion, a global lipidomic approach by MALDI-TOF profiling was applied to the OF for the first time. By this way, we demonstrated that PL in the bovine OF are highly regulated across the estrous cycle, in particular between Pre-ov and the lutal phase. The identification of the different PL species is ongoing. This work will provide new candidates potentially able to regulate sperm physiology and early embryo development.

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Short- and long-term effects of progesterone and prolactin during the second phase of IVM on metaphase-II chromosomes in bovine oocytes

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Keywords: progesterone, prolactin, M-II chromosomes

Progesterone (P4) production by cumulus cells rises with oocyte maturation. We have previously shown that exogenous P4 and prolactin (PRL) exert similar inhibitory effects on abnormal changes of M-II chromosomes in bovine oocytes during the second step of two-step IVM (Lebedeva et al., Reprod Domest Anim, 52(S3):53, 2017). The goal of this work was to examine the role of the endogenous P4-related pathway on destructive modifications of M-II chromosomes and implementation of the PRL effect. Bovine cumulus-enclosed oocytes (CEOs) were matured for 12 h in control medium (CM; TCM + 10% fetal calf serum) containing 10 μg/ml FSH and 10 μg/ml LH at 38.5°C and 5% CO_2. The CEOs were further matured for 12 h in one of three IVM media: (1) CM, (2) CM containing 10 μM trilostane (TS, inhibitor of 3β-hydroxysteroid dehydrogenase), and (3) CM containing 1 μM mifepristone (MFP, antagonist of the genomic P4 receptor). The following additives to these three media were applied: no additives (Control), 50 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia), or 50 ng/ml P4. A part of CEOs matured for 12 h in all three media was cultured for additional 24 h in CM to test long-term effects during aging. At the end of culture, the state of oocyte chromosomes was evaluated by the Tarkowski's method. The content of P4 in culture media was determined by ELISA. Percentage data (n=4-5, 81-106 oocytes per treatment) were arcsine transformed and analyzed by ANOVA. Except for PRL, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 24 h maturation, the rate of M-II oocytes was similar in all groups (80.1-92.6%). In the medium 1, both P4 and PRL reduced ($P<0.05$) the frequency of M-II chromosome abnormalities (decondensation, adherence, clumping) from 28.4±2.0% (Control) to 16.8±2.7 and 15.2±1.7 %, respectively. In the media 2 and 3, the rates of M-II oocytes with abnormal chromosomes increased in all groups (at least $P<0.05$); however, these rates were 1.5 times lower in the PRL-treated groups than in the respective control groups. After aging of control groups, a higher incidence of chromosome abnormalities ($P<0.01$) was observed in CEOs exposed to TS (79.0±2.0%) than MFP (67.3±1.0%) or in untreated CEOs (66.6±0.9%). The addition of P4 to IVM media 1 and 2 led to a 1.2-fold reduction ($P<0.01$) in the rate of aged oocytes with abnormal chromosomes, while PRL exerted a similar long-term action only in the medium 2. Meanwhile, TS decreased ($P<0.001$) the content of P4 in IVM media both in the absence of exogenous P4 (27 to 28 times) and in its presence (4 times), whereas PRL and MFP had no effect. This content remained low in aging media for CEOs matured with TS, although it rose 5 to 11 times in other groups. Thus, during the second phase of IVM, endogenous P4 can exert short- and long-term inhibitory effects on abnormal changes of M-II chromosomes, acting via genomic receptors, but it is not involved in the effect of PRL.

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The effect of short-term cytoskeletal inhibitor treatment on embryo metabolism and viability

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Keywords: metabolism, cytoskeleton, nuclear transfer technologies

The short-term use of chemical inhibitors of the cytoskeleton in oocytes is necessary for a range of micromanipulation events, including mitochondrial replacement therapy (MRT) and somatic cell nuclear transfer (SCNT). These advanced methods have been shown to be effective; however the full safety of such techniques has yet to be demonstrated comprehensively. To begin to address this knowledge gap, we have measured the impact of short-term cytoskeletal inhibitor treatment on embryo development and metabolism.
Abattoir-derived bovine oocytes were matured and fertilized under standard conditions. At the early pronuclear (ePN) stage, after 9 hours co-incubation with motile sperm, presumptive zygotes were exposed to one of cytochalasin B, latrunculin A, or nocodazole for 15 minutes. Mitochondrial response to treatment was assessed in real-time, and metabolic activity was measured in resultant embryos at cleavage and blastocyst stage. At these stages, treated embryos were transferred into 4µl individual culture droplets for 24 hours. Spent media was analysed using enzyme-linked fluorometric assays to measure glucose, lactate and pyruvate depletion/appearance and HPLC to measure the turnover of 18 key amino acids. In total, 36 embryos were assessed per group (three treatment groups and one control group) across 3 independent replicates at each developmental stage (acute, cleavage and blastocyst). Significance was tested to the level of $p<0.05$ using one-way ANOVA with Tukey's post-hoc.
The presence of cytoskeletal inhibitors at the ePN stage did not have an immediate impact on mitochondrial activity using a real-time assessment of response. Moreover, embryo development rate to cleavage (78.6±6.4, 72.3±4.8 and 70.1±6.9 compared to 73.9±6.9 in Cyt B, Lat A, Nocod and control respectively) and blastocyst stages (37.7±3.7, 30.5±2.8 and 29.8±2.0 compared to 33.3±3.0) was unchanged. Oxygen, glucose and pyruvate consumption were not significantly altered at either cleavage or blastocyst stage, however showed higher variance in embryos derived from zygotes exposed to cytoskeletal inhibitors. Altered turnover of arginine, glutamine, lysine, threonine and tyrosine was observed in at least one treatment group at later stages of development, and changes in trend in overall amino acid turnover were noted.
These data indicate that short-term cytoskeletal treatment does not induce an immediate metabolic response and does not cause gross changes to mitochondrial function in pre-implantation stage embryos. A 15 minute exposure at the ePN stage does, however, induce subtle alterations in amino acid metabolism at both cleavage and blastocyst stages, indicating a legacy-effect on treated embryos. These data are critical to consider as MRT moves into the clinic and is also of relevance to SCNT. Overall, these findings support the need for further research to understand the impact of using cytoskeletal inhibitors during early embryonic development.

The effect of chilling on the viability of in vitro produced bovine embyos

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Keywords: cattle, blastocyst, elongation

Conventional freezing of in vitro produced bovine embryos has usually significant negative impact on subsequent embryo viability. Vitrification, on the other hand, does not allow direct embryo transfers. Since long distance shipment of fresh in vitro produced embryos is also not recommended without incubator-like shipment conditions, the aim of the present study was to evaluate the possibility to transport in vitro produced bovine embryos overnight as chilled aiming at direct embryo transfers on farms.
Unless stated otherwise, all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Slaughterhouse-derived oocytes were matured in TCM199 with glutamax-I (Gibco™; Invitrogen Corporation, Paisley, UK) supplemented with 0.25 mM Na-pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 ng/ml FSH (Puregon, Organon, Oss, Netherlands), 1 µg/ml ß-estradiol (E-2257) and 10% heat inactivated FBS (Gibco™, New Zealand) at 38.5°C in maximal humidity in 5% CO_2 in air. Zygotes were cultured in G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with FAFBSA (4 mg/ml) and L-carnitine (1.5 mM) at 38.5°C in maximal humidity in 5% O_2, 5% CO_2 and 90% N_2. Transferable day-7 embryos were packed in straws in Hepes-buffered TCM199 supplemented with FAFBSA (1 mg/ml). After 24-h storage at 5°C embryos were either cultured for 72 h in vitro (until hatching) or transferred (10 embryos/recipient) into recipient heifers (until elongation).
Following chilling, the in vitro hatching rates were lower for the morulae 18.2% (n=22) than for the blastocyst stage embryos 68.5% (n=89) ($p<0.05$, Fisher exact test). Four day-7 recipient heifers were subjected to three separate embryo transfers each (a total of 120 blastocysts). Following the 12 non-surgical embryo collections on day 14 intact elongated embryos were recovered in seven collections (58.3%) yielding on average 4 embryos per collection. The average length of the elongated conceptuses was 9.95 mm (range 0.5-60 mm). In conclusion, using the presented conditions, in vitro produced bovine blastocysts are able to start elongation phase in recipient heifers after 24-h chilling period.

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Interferon tau exerts concentration dependent actions on bovine neutrophil gene dynamics favoring implantation

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Keywords: Interferon tau, Neutrophils, Implantation

It is a proven fact that Interferon tau (IFNT) signals pregnancy recognition in ruminants. Apart from local actions on endometrium, it can reach systemic circulation and exert effects on various immune cells including neutrophils, thereby leading to immunomodulation via specific expression of interferon-stimulated genes (Meyerholz et al., Reprod Domest Anim 51(1):175-7, 2016). Though some studies have been performed to analyze the in vivo dynamics of neutrophils during bovine peri-implantation period (Shirasuna et al., Reproduction 150:217-225, 2015), there is no data available on exclusive effects of IFNT on neutrophils which is the basis of our study. The genes selected for the study were interferon-stimulated genes like Interferon-stimulated gene 15 (ISG15), 2'-5'-oligoadenylate synthetase 1 (OAS1), Interferon-induced GTP-binding protein MX1 and MX2, interferon-gamma-inducible protein 16 (IFI16), Interferon Induced Protein 44 (IFI44) along with other genes like Platelet-endothelial-cell adhesion molecule-1 (PECAM-1), L-selectin, Integrin alpha M and Progesterone-inducible molecule (PIBF) that determine many of the effector functions of neutrophils like motility, trafficking, phagocytosis etc. Healthy Sahiwal heifers (n=10) aged 1.5-2 years were used for blood collection by jugular venipuncture [10 ml]. Neutrophils were isolated by established protocol [Manjari et al., Vet Immunol Immunopathol 173:44–49, 2016]. Cells were incubated with recombinant bovine Interferon-tau (MyBioSource Inc., San Diego, California, USA) in three different concentrations [1, 5 or 10 ng/ml] for 3 hrs at 37°C. At the end of the treatment, cells were retrieved from the wells for RNA isolation and gene expression study using real time PCR using specific primers for selected genes [Manjari et al., Vet Immunol Immunopathol 173:44–49, 2016; Biol Rhythms Res 49(2),329-333, 2018] and of CD31. The relative expression ratio of the target gene was calculated as per existing method (Livak and Thomas, Methods 25:402–408, 2001). All the data were analyzed by repeated measures one-way ANOVA for within group analysis followed by Fischer's multiple comparison test using SAS software, version 9.1 (SAS Institute inc., CARY, NC, USA). It was observed in the study that at lower concentrations of IFNT, ISG15, IFI16, PIBF and L-selectin were significantly up regulated, whereas at higher concentrations the same were significantly down regulated. Irrespective of concentration, MX genes, IFI44, and OAS1 were significantly up regulated and CD31, CD11b were significantly down regulated. The results show that at lower concentrations of IFNT, the neutrophil activity with respect to chemoattraction is stimulated whereas at higher concentrations the same is reduced. Hence, it can be concluded that IFNT exerts concentration dependent actions on neutrophil gene expression dynamics finely modulating its activity enabling the cells to exert temporal variation in their destined functions ultimately leading to successful implantation.

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Analyzing the effects of bovine Interferon tau and female sex steroids on neutrophil pro and anti inflammatory triggers to understand implantation

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Keywords: Implantation, Inflammation, Neutrophils

Bovine Interferon tau, a hormone of maternal recognition of pregnancy exerts both paracrine and endocrine effects on reproductive organs and also proven to have an effect on neutrophils (Shirasuna et al., Reproduction 150:217-225, 2015) which is partly understood till date. Though neutrophils are detrimental to the foreign invading antigen, they can also play a role in favoring implantation. Two gens that encode proteins important in the balance of inflammatory responses are IL-8 receptor (CXCR1) and Glucocorticoid receptor alpha (GRα) transcription factor. IL-8 binds CXCR1 and activates NF-κB associated proinflammatory pathways implantation whereas GRα can repress the activity of NF-κB thereby reducing inflammation. The differential roles of Interferon-tau [IFNT], Progesterone [P4] and Estradiol in triggering pro- and anti-inflammatory responses during peri-implantation period were assessed in cows. An ex vivo study was done by isolating neutrophils from the animals post artificial insemination (AI) at defined intervals (day 0, 4, 8, 12, 14, 16, 18, 21, 24, 30, 40) and analyzing the expression changes of GRα and CXCR1 genes. Isolation of neutrophils from the blood was performed as per the established protocol [Manjari et al., Vet Immunol Immunopathol 173:44–49, 2016]. An in vitro study was also done isolating neutrophils from prepubertal heifers and subjecting them to supplementation of IFNT (MyBioSource Inc., USA) [1, 5, 10 ng/ml], Progesterone and 17β-estradiol (Sigma Chemical Co., USA) at [10, 50, 100, 500, 1000 ng/ml], and [10, 50, 100, 500, 1000 ng/ml] respectively. Post supplementation, RNA was isolated from cells using Trizol method and was reverse transcribed to cDNA which was used for qPCR via LightCycler® 480 Instrument [Roche, Switzerland] using the SYBR Green kit [Thermo Scientific, USA] and specific primers for GRα, CXCR1, GAPDH, beta actin. Average values of the two housekeeping genes were used as reference for normalization of target gene for relative quantification. The relative expression ratio of the target gene was calculated as per established method (Livak and Thomas, Methods 25:402–408, 2001). Repeated measures one-way ANOVA was used for analysis of Data for within group analysis followed by Fischer's multiple comparison test using SAS software 9.1 (SAS Institute inc., USA).
We observed that from day 14 post AI, there is a significant up-regulation of neutrophil GRα and CXCR1 in pregnant and non-pregnant cows respectively. The in vitro study showed that IFNT significantly up-regulates GRα whereas P4 significantly up-regulates CXCR1. Whereas estradiol significantly downregulates GRα but did not show any significant effects of CXCR1. The total leukocyte count and neutrophil count were also significantly higher in non-pregnant cows. We conclude that IFNT efficiently mediates neutrophil immunosuppression during peri-implantation period in cows by interacting with Interleukin 8 receptor and can also exert ligand independent actions on GRα.

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Role of RNA isoform expression in sex determination in mice

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Keywords: sex determination, splicing

The majority of eukaryotic genes produce multiple transcriptional isoforms from the same locus. Thus, mRNA isoforms are molecules of different exon composition and length, which may code for different forms of the corresponding protein. They may be produced from different transcriptional starting sites, terminated at different polyadenylation sites, or as a consequence of alternative splicing (AS). As isoform changes may be masked by gene-level measurements, estimation of isoform expression provides a better resolution than gene expression to evaluate dynamic developmental processes. The genes that determine gonadal sex determination (GSD)in mice are known, but knowledge of the molecular pathways specifying GSD is still incomplete, in part because of these AS mechanisms. To identify differentially expressed isoforms (DEI), differentially expressed genes (DEG) and AS changes during GSD in mice, we performed a transcriptional analysis of RNA isoforms, genes and AS of XX and XY gonads during GSD at embryonic day 11 (E11) and early sex differentiation at day 12 (E12) using RNAseq. Two pairs of genital ridges (dissected from 2 different fetuses) were pooled per sample. Three samples were collected per sex. RNAseq libraries were prepared from male and female samples (n=3 per sex) at E11 and E12. DEG was evaluated with DESeq and edgeR packages, and genes were considered differentially expressed when both tests returned a statistically significant result (cutoff: $P<0.01$). Quantification of transcript abundance was performed with Salmon, and DEI analysis was carried out with edgeR package (cutoff: $P<0.01$). As events were evaluated with vast-tools software. Gene analysis identified 729 and 1691 DEGs between males and females at E11 and E12, respectively. Hundreds of these genes are related with GSD and early sex differentiation and could be good candidate genes for sex reversal; also, many of them appeared to be grouped in clusters on several chromosomes. Interestingly, increased expression at E11 in males was significantly enriched in RNA splicing and mRNA processing gene ontology (GO) terms. Isoforms analysis identified 705 and 1348 DEIs between males and females at E11 and E12, respectively. We found 14 genes at E11 and 19 genes at E12 with different isoforms expressed in males and females. Many DEI did not shown differences in the DEG analysis. In addition to the isoforms, 1167 differentially AS events were observed between females and males at E11. At E11 there was an enrichment in intron retention (IR) in females, and at E12 there was enrichment in IR and exon skipping in females. Eighty-five genes exhibited expression of different AS events in both males and females at E11, and 184 at E12. Some of these AS genes are transcription factors that could play an important role in GSD, like *Jarid2*a Jumonji family member essential for AS sex determination in reptiles. Our data indicate that RNA isoforms expression and splicing regulatory mechanisms constitute a common feature among sex determination in distant phyla, including mammals.

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The effect of vitamin c on the developmental competences and quality of pig blastocysts obtained after in vitro fertilization

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Keywords: pig, embryo, vitamin c

Vitamin C is one of the antioxidants used in in vitro culture media for oocytes and embryos of animal species. It has been shown, that vitamin C has antioxidant properties and reduces the level of reactive oxygen species (ROS), which cause damage to structures in oocytes and embryos. The aim of the study was to determine the effect of vitamin C on developmental competences and the quality of pig embryos obtained after IVF and IVC. IVF embryos were obtained from in vitro-matured and in vitro fertilized oocytes. The putative zygotes were cultured in the NCSU-23 medium with the addition of 20 µg/mL of vitamin C (exp. group) or without it (control), at 39oC and 5% CO2, 5% O2 and 90% N2 in the air up to the blastocyst stage. The IVF derived blastocysts from the exp. (n=11) and control (n=7) groups 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 filters: 520 nm (TUNEL) and 358-461 nm (DAPI). Statistical analysis was performed using the t-test and chi-square test. It was observed that the rate of embryos cleavage was slightly higher in the control group than in the exp. group (22.0% and 17.5%, respectively, no statistical differences). Simultaneously, the percentage of blastocysts was significantly higher in the exp. group than in the control group (51.3% and 16.7%, respectively $P<0.01$). The largest percentage of blastocysts was obtained on day 6 of IVC supplementing with vitamin C (54.5%), while in the control group on day 7 (57.1%). It was observed also that the mean number of cell nuclei was slightly higher (41.4±7.7) and the mean number of apoptotic nuclei was slightly lower (1.3±1.2) in embryos cultured in the presence of vitamin C compared to embryos from the control group (37.0±6.7 and 1.9±1.8, respectively), but no differences were observed. The TUNEL index was 3.1% for the exp. group and 5.0% for the control group. The study showed that in vitro culture in the presence of vitamin C may improve production of pig blastocysts and has possibly no significant effect on their quality. It should be noted, these are preliminary results and it is planned to continue research on a larger number of pig embryos.

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Sperm storage in hen's reproductive tract: metabolic composition of the uterine fluid after artificial insemination

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Keywords: sperm storage, uterine fluid metabolite, ^{1}H-NMR

Avian uterine fluid (UF) has been demonstrated to prolong sperm survival, maintain the fertility potential of the fowl sperm, and impact the filling rate of sperm storage tubules from utero-vaginal junction (UVJ) (Ahammad et al., Journal of Poultry Science, 50, 74-82, 2013). Two lines of hens previously selected on hatched chicks were used, one exhibiting a long (F+, 14-16 days) and the other one a short (F-, 4-6 days) duration of fertile period (Beaumont et al., British Poultry Science, 33, 649-661, 1992), as a major consequence of a good or a poor sperm storage ability, respectively (Brillard et al., Journal of Reproduction and Fertility, 114, 111-117, 1998). Therefore, UF analysis from F+ and F- hens represents a good approach to study molecules involved in UVJ sperm storage process. In this study, the comparison of UF metabolites from F+ (n=5-7) and F- (n=7-9) hens was investigated and quantified by high resolution proton nuclear magnetic resonance (^{1}H-NMR). Moreover, the effect of sperm storage on UF metabolic composition was investigated 24 hours, 1 week, 2 weeks or 3 weeks after artificial insemination (n=5-9/ times). ^{1}H-NMR analysis was done on a Bruker DRX-500 spectrometer (Bruker SADIS, Wissembourg, France). Topspin 2.1 software and AMIX software package were used to process ^{1}H-NMR spectra prior to assign them using our in-house database and online databases, including HMDB (http://www.hmdb.ca) and the Chenomx NMR Suite 8.1 evualuation edition (Chenomx Inc, Edmonton, Canada). To identify discriminant metabolite between the two chicken lines and between the time-condition after insemination, Orthogonal Projections Latent Structures Discriminant Analysis (OPLS-DA) were performed using the SIMCA 13 software. Equality of means was tested using Welch's t-test for each discriminant metabolite ($p<0.05$). To identify the most significantly affected metabolic pathways, the discriminant metabolite signature of each chicken line was analyzed by metabolite set enrichment analysis, implemented in Metaboanalyst 3.0 (htpp://www.metaboanalyst.ca). Eleven discriminant metabolites between the two lines were identified by OPLS-DA in the UF. There was an over-representation of inositol and galactose metabolism pathways in the UF of F- line ($p<0.01$). On the other hand, metabolisms of steroid ($p<0.001$), tryptophan ($p<0.01$), arginine and proline ($p<0.05$), as well as mitochondrial electon transport chain ($p<0.05$), were over-represented in the UF of F+ line. While no metabolites were discriminant in F+ UF between before and after insemination, OPLS-DA revealed that ten metabolites were discriminant in F- UF. Among them, fumarate ($p<0.05$) and myo-inositol ($p<0.05$) metabolites were more concentrated after insemination than before, whatever the duration after insemination. In contrast, dimethylamine was less concentrated after insemination than before ($p<0.05$). Our results indicate that metabolic composition of uterine fluid is associated with avian sperm storage duration in female genital tract and is related to sperm storage capacity.

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Local embryo effect on the transcriptomic response of the oviductal epithelial cells results from *in vivo* and *in vitro* approaches

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Keywords: Bovine, oviduct-embryo interaction

Based on previous data, the presence of a single 8-cell embryo does not alter the transcriptome of the cells of the oviduct, although this apparent lack of response might be due to a local effect at the precise position of the embryo which is missed if the whole oviduct is studied. Thus, we aimed to study the local embryo effect on the transcriptomic response of the epithelial cells of the oviduct *in vivo* and *in vitro*. For the *in vivo* experiment, 15 cross-bred beef heifers were synchronized, artificially inseminated and slaughtered on Day 2.5 after estrus. The oviducts from each animal were isolated, trimmed free of tissue and divided between ampulla and isthmus. The ipsilateral isthmus was then divided into smaller sections (2 cm). Each section was sequentially flushed until the embryo was located (n=4; three at 2-cell stage and one at 8-cell stage), opened and scraped longitudinally to obtain the epithelial cells. Cells were snap-frozen in liquid nitrogen for gene expression analysis. The *in vitro* approach consisted of the co-culture of fifty *in vitro* produced embryos (2- to 4-cells) on a bovine oviductal epithelial cells (BOEC) monolayer. BOEC from the ampulla and isthmus of ipsilateral oviducts collected during the early luteal phase were mechanically harvested and separately cultured with TCM-199+10% FCS in 5% CO_2 in air at 38.5°C for 7 days until confluence. *In vitro* 2- to 4-cell embryos were produced in parallel. A day before co-culture, BOEC medium was replaced with SOF+5% FCS. The groups were: Ampullary BOEC co-cultured with (A+) and without (A -) embryos; isthmic BOEC with (I+) and without (I -) embryos. After 24h of co-culture, BOEC were recovered from each group and snap frozen for gene expression analysis (5 replicates). Ten transcripts previously reported to be differentially expressed between the isthmus of pregnant and cyclic heifers (*Maillo et al. Biol Reprod. 2015. 92: 144*) were analysed in BOEC recovered from both experiments: *STK32A*, *SLC26A3*, *KERA*, *QRFPR*, *MCTP1*, SOD3, *PRELP*, *VAT1L*, *SOCS3*, *CCL20*. Data were analysed using one-way ANOVA and t-test. The results from *in vivo* samples revealed that 6 out of 10 transcripts (*STK32A, SLC26A3, QRFPR, MCTP1, SOCS3* and *CCL20*) were different between the segment where the embryo was collected and other locations within the ipsilateral oviduct which suggested the presence of an embryo site-specific signal. Comparison between the ipsilateral embryo site with the contralateral site revealed only one transcript different (*VAT1L*). Regarding the *in vitro* BOEC co-culture, 3 out of 10 genes (*SLC26A3, KERA* and *QRFPR*) were not expressed. For the remainder of the genes analysed, no differences were detected. In conclusion, under our experimental conditions, *in vivo* the embryo elicits site-specific signals in the oviduct, while *in vitro* evidence for these signals were not observed neither by the presence of the embryo, nor by the spatial differences of the bovine oviduct.

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

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Keywords: oviduct, hyperglycaemia, oviduct fluid

The oviduct, or Fallopian tube, provides the environment for gamete activation, fertilisation and the early stages of embryo development. However, little is known of the composition of the oviduct fluid in health and disease. The aim of this study was to investigate the impact of a hyperglycaemic challenge on the physiology of oviduct epithelial cells and the composition of fluid that they create *in vitro*. We have used the bovine material as a model for determining the impact of metabolic diseases on human fertility. Primary bovine oviduct epithelial cells were cultured in DMEM-F12, at 39°C, 5%CO_2 for 6 days. Cell identity was confirmed using confocal, optical and TEM microscopy. The cells were grown to confluence on a semi-permeable membrane. Barrier integrity was confirmed by measuring TransEpithelial Electrical Resistance (TEER) and fluorescein transport assays. Once confluence was achieved, the apical medium was discarded and cells were cultured in an air:liquid interface. Once confluence was confirmed, physiological (7.3mM) and hyperglycaemic (mild:8.5mM, severe:11mM) concentrations of glucose were added together with or without 20ng/ml of insulin to the basal compartment for either 24h (Experiment 1; *Acute*) or 7 days (Experiment 2; *Chronic*). The nutrient composition of apical secretions was analysed by enzyme linked assays and high-performance liquid chromatography. RNA was extracted from the cells for quantitative real-time PCR analysis of key genes related to oviduct physiology (*OVGP1*, *ERa*) and glucose transport (*INSR, SLC2A1, SLC2A3*). Insulin-only and glucose-free controls were included. Data were analysed using Kruskal-Wallis test with Dunn's post-hoc or two-way ANOVA where appropriate. Experiment 1 showed that an acute hyperglycaemic challenge in the basolateral compartment did not change the luminal concentrations of glucose, pyruvate or lactate, or the amino acid content of the cell secretions. By contrast, in experiment 2, 7-day basolateral exposure to hyperglycaemia in the absence of insulin reduced the volumes of oviduct-derived fluid (8.5mM+insulin and 11mM+insulin vs all treatments without insulin, $p<0.05$), increased luminal concentrations of glucose (7.3mM+insulin and 8.5mM+insulin vs 11mM-insulin, $p<0.05$) and modified the secretion of alanine, glycine, glutamine and arginine. Gene expression was not significantly modified in any of the genes tested, associated with oviduct epithelial cell function or glucose transport. Using an *in vitro* oviduct model, we have shown that exposure to hyperglycaemia in the presence or absence of insulin enriched oviduct secretions for glucose and modified the amino acid composition. Furthermore, insulin reduced fluid flow across the oviduct monolayer. These data suggest that the periconceptual environment can be modified in hyperglycaemia; such modifications may affect gamete and embryo physiology.

Effects of cumulus cells and prolactin on histone acetylation during the prolonged culture of matured bovine oocytes

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Keywords: oocyte aging, histone acetylation

Aging of mammalian mature oocytes attained the metaphase-II stage heavily reduces their quality and developmental capacity. Therefore, the knowledge of physiological factors modulating the speed of oocyte aging is of great importance for successful assisted reproductive technologies. The goal of the present research was to study effects of cumulus cells (CCs) and pituitary hormone, prolactin (PRL), on the dynamics of age-associated epigenetic changes during the prolonged culture of bovine oocytes in vitro. Bovine cumulus-enclosed oocytes (CEOs) were cultured for 20 h in the following maturation medium: TCM 199 containing 10% fetal calf serum, 10 μg mL–1 porcine FSH, and 5 μg mL–1 ovine LH. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After IVM, CEOs were transferred to the aging medium consisting of TCM-199 supplemented with 10% fetal calf serum and cultured for 0, 12 or 24 h, in the absence (Control) or presence of 50 ng mL–1 bovine PRL (Research Center for Endocrinology, Moscow, Russia). A portion of in vitro-matured oocytes were denuded of their CCs and cultured for 12 or 24 h in the control aging medium. At the end of maturation or prolonged culture, levels of acetylation of histone H4 at lysine 12 (acH4K12) and histone H3 at lysine 14 (acH3K14) in M-II oocytes were determined by immunostaining with specific antibodies (polyclonal rabbit anti-histone H4 lysine 12 and monoclonal rabbit anti-histone H3 lysine 14, both from Abcam, Cambridge, MA, USA). The fluorescence signal was evaluated using ZEN 2 Pro software (Carl Zeiss, Oberkochen, Gemany) and assigned to one of four grades (intense, moderate, weak and absent). Also, before analysis oocytes were counterstained with DAPI to visualize chromosomes. The data from 4 replicates (71-88 oocytes per treatment) were analyzed by ANOVA. In the control group of CEOs, a rise in the rate of oocytes with the intense fluorescence signal of acH4K12 occurred by 12 h of aging (64.1 ± 2.5% (12 h) v.52.7 ± 1.6% (0 h); $P < 0.01$) and persisted up to 24 h (86.5 ± 2.5%; $P < 0.001$). At the same time level of acH3K14 in aged oocytes increased only between 0 and 12 h of the prolonged culture (from 73.5 ± 1.8 to 92.0 ± 1; $P < 0.001$) and then this elevation ceased (93.5 ± 1.0 (24 h)). The addition of PRL to the aging medium or removal of CCs decreased levels of acH4K12 and acH3K14 in matured oocytes. In the PRL-treated groups, these effects were found after 24 h-aging, but in the groups of oocytes cultured in the absence of CCs, the rate of oocytes with the intense signal of acH4K12 and acH3K14 was lower than in the control group of CEOs throughout all culture periods (at least $P < 0.05$). Thus, elevated levels of H4K12 and H3K14 acetylation in matured oocytes during their prolonged culture suggest that these epigenetic changes may be caused by aging of ova. Furthermore, in bovine CEOs, CCs accelerate acH4K12 and acH3K14, whereas PRL decelerate these actions of CCs.

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Apoptosis resistance of bovine cumulus-oocyte complexes is modulated via progesterone-dependent pathways at the terminal step of in vitro maturation

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Keywords: progesterone, prolactin, oocyte apoptosis

Cumulus-derived progesterone (P4) is an important pro-survival factor, with its production significantly increasing during bovine oocyte transition from M-I to M-II stage. The aim of the present research was to compare effects of endogenous and exogenous P4 during the terminal step of in vitro maturation on apoptosis resistance of bovine oocytes and cumulus cells. Bovine cumulus-oocyte complexes (COCs) were matured 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 and matured for 12 h in the following systems: (1) TCM 199 containing 10% FCS, (2) TCM 199 containing 10% FCS and 10 μM trilostane (TS, inhibitor of 3β-hydroxysteroid dehydrogenase), and (3) TCM 199 containing 10% FCS and 1 μM mifepristone (MFP, antagonist of the genomic P4 receptor). All these systems were supplemented with either 0 (Control) or 50 ng/ml of P4. After 24 h IVM, a portion of COCs was cultured for 24 h in TCM 199 containing 10% FCS to induce aging. Following culture, oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI. Cumulus expression of pro-apoptotic marker Bax was assessed by immunocytochemical staining using Bax antibodies (Bio-Rad, Hercules, CA, USA). All data (n=5, 83-100 oocytes per treatment) were arcsine transformed and analyzed by ANOVA. After 24 h IVM in System 1, the rate of M-II oocytes with apoptotic signs was lower in the P4-treated group than in Control (9.4±1.1 vs. 17.1±2.5%, $P<0.05$, respectively). However, P4 did not affect this rate in the presence of TS or MFP. In the control group in Systems 1, the addition of MFP to the IVM medium resulted in a 3.2-fold decrease ($P<0.05$) in the original apoptosis rate. Both TS and MFP enhanced 1.2 to 1.4 times ($P < 0.05$) Bax expression in cumulus cells surrounding matured oocytes, whereas P4 did not change it. In System 1 following 24 h aging, maturation of CEOs in the presence of P4 led to a reduction from 52.0 ± 5.1% (Control) to 28.3 ± 2.7% (P4) ($P < 0.05$) in the rate of apoptotic oocytes. In its turn, MFP diminished 3.2 times ($P < 0.001$) the apoptosis rate in the control group and did not affect this rate in the P4-treated group. The expression of Bax in aged cumulus cells increased 1.2 to 1.6 times ($P<0.05$), but was not related to the presence of TS, MFP, or P4 in IVM media. Our findings indicate that, during the terminal step of IVM, exogenous P4 inhibits apoptotic processes in oocytes through non-genomic receptors only with the availability of cumulus-derived P4, whereas blocking of the genomic receptors, in itself, also leads to a decrease in oocyte apoptosis. At the same time the expression of pro-apoptotic Bax in cumulus cells is enhanced at inhibition of genomic P4 receptors and does not depend on exogenous P4.

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Rescue potential of supportive embryo culture conditions on bovine embryos derived from metabolically-compromised oocytes

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Keywords:

Metabolic disorders like obesity are linked to subfertility. The upregulated lipolysis leads to elevated saturated (stearic; SA, palmitic; PA) and unsaturated (oleic; OA) fatty acids (FAs), both in serum and in follicular fluid. While their ratio determines the severity of lipotoxicity, exposure to these FAs has a detrimental impact on oocyte quality. Insulin-transferrin-selenium (ITS, as a mitogenic and antioxidative support) is used in *in vitro* culture medium to maximize blastocyst yields. We hypothesize that supportive culture media (containing e.g. ITS) can minimize cellular stress levels and rescue development and quality of embryos derived from metabolically-compromised oocytes. In this study, bovine oocytes were exposed to different ratios of PA, SA and OA; 1) pathophysiological concentrations: 150, 75 and 200 µM respectively (HI COMBI), 2) only high PA: 150, 28 and 21 µM respectively (HI PA), compared to 3) physiological basal concentrations: 23, 28 and 21 µM respectively as a control (BASAL). Presumptive zygotes were cultured in SOF medium with or without ITS. Cleavage rates were recorded 48h post insemination (p.i.) and blastocyst rates at day 7 (D7) and 8 (D8) p.i. (n=905 oocytes, 3 repeats). D8 blastocysts were evaluated for apoptotic cell indexes (n=227) by caspase-3 immunostaining or snap frozen for mRNA expression of genes involved in ER unfolded protein responses (UPR) (*Atf4, Atf6*), oxidative stress (*SOD2, GPx, CAT*) and mitochondrial UPR (*HSPE1, HSPD1*) (n=356). Categorical and numerical data were analysed using binary logistic regression and ANOVA, respectively, and were Bonferroni-corrected for multiple testing. In the absence of ITS during culture, HI PA exposure during maturation significantly reduced cleavage (64.2% *vs.* 78.3%) and D7 blastocyst rates (12.4% *vs.* 24.3%) and tended to reduce D8 blastocyst rates (22.0% *vs.* 32.5% P=0.098) compared to BASAL. Maturation in HI COMBI had no effect on development. However, surviving blastocysts derived from HI PA- and HI COMBI-treated oocytes showed a significant increase in apoptosis. In the presence of ITS during culture, maturation in HI PA or HI COMBI had no significant effect on developmental competence whereas apoptosis was not alleviated by ITS. Within the HI PA group, ITS supplementation rescued embryo cleavage rate (by 15.1%, P<0.05), proportion of ≥4-cell embryos (by 13.9%, P<0.05) and tended to increase D7 blastocyst rate (by 8.2%, P=0.076) compared to the HI PA-treated group cultured without ITS. In the absence of ITS during culture, *HSPD1* expression of D8 blastocysts from PA-treated oocytes was significantly increased compared to BASAL-treated oocytes (P<0.05), an effect that was normalised by ITS. Within the HI PA-treated group, ITS tended to decrease *HSPD1* expression (P=0.069). Other genes were not affected. We conclude that ITS supplementation during embryo culture enhances development and alleviates mitochondrial stress (at mRNA level) of embryos derived from metabolically compromised oocytes (HI PA). However, produced embryos still showed higher apoptosis, indicating inferior quality.

Oviductal cells express Stearoyl-CoA desaturase that can protect the embryo against saturated fatty acids

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Keywords: NEFA, oviduct, embryo

Metabolic stress conditions, characterized by elevated free fatty acid (NEFA) levels are associated with reduced fertility in mammals. Particulary saturated NEFAs have a negative impact on the developmental competence of the oocyte (Leroy et al., Reproduction; 130: 485-495, 2005), while mono-unsaturated NEFAs, present at relatively high levels in follicular fluid (Aardema et al., Biol Reprod; 88: 164, 2013), are able to protect against lipotoxic events induced by saturated NEFA (Aardema et al., Biol Reprod; 85: 62-69, 2011). Cumulus cells protect the oocyte against lipotoxicity by Stearoyl-CoA desaturase I (SCD-I) activity that converts saturated stearic acid (SA) into mono-unsaturated oleic acid (OA) (Aardema et al., Biol Reprod; 96:982-992, 2017). The oocyte appears to mature in a 'protected environment', which may be in contrast to the metabolic condition in the oviduct. The current study investigates the impact of physiologic NEFA levels on embryos and whether oviduct epithelial cells (OECs) express SCD-I. Cumulus-oocyte-complexes (COCs) originating from 2-8 mm follicles of bovine slaughterhouse ovaries were collected, *in vitro* matured (day -1) and fertilized (day 0) according to our standard protocol (Aardema et al., Biol Reprod; 85: 62-69, 2011). From day 1 embryos were exposed to CONTROL, or to BASAL NEFA (100 µM OA + 80 µM palmitic acid (PA) + 70 µM SA; the 3 dominating NEFAs in follicular fluid) or HIGH NEFA levels (200 µM OA + 150 µM PA + 100 µM SA) during the complete embryo culture period. Fatty acids (10 mM; NEFA) were complexed to BSA (FA:BSA ratio of 5:1). At day 8 of culture, blastocyst rate was scored. OECs were collected from infundibulum, ampulla and isthmus, at the pre-and post-ovulatory, early-and late-luteal phase of the reproductive cycle for quantitative RT-PCR analysis. RNA was extracted and reverse transcription was performed on total RNA, *SCD-I* mRNA expression levels were normalized by the geometric mean of reference genes *GAPDH* and *ACTB*. Statistical analysis was performed with SPSS 24.0, by general linear model. Exposure to NEFA dring embryo culture resulted in a significantly reduced blastocyst rate versus the control condition at day 8 of culture (19.7±2.7%), for the BASAL NEFA (7.2±4.5%, p=0.021) and HIGH NEFA condition (3.7±3.6%, p=0.020; n=~340 COCs, 3 runs per group). The *SCD-I* expression in the isthmus region was higher during the pre- versus the post-ovulatory and early-luteal phase ($p<0.05$). Interestingly, *SCD-I* mRNA was expressed in each region of the oviduct during all phases of the reproductive cycle. These data indicate that embryos are very prone to NEFA and are threatened by metabolic stress. In particular since total NEFA levels in oviductal fluid resemble the levels in blood (Jordaens et al., Reprod Biol; 17:281-284, 2017). At this point, the NEFA composition in the oviduct is largely unknown. A major question, which is currently investigated, is whether the OECs that express SCD-I are capable of influencing the NEFA composition in the oviduct in the benefit of the embryo.

Enhancement of the developmental capacity of metabolically compromised bovine oocytes and embryos by water soluble vitamin E (TROLOX) depends on the timing of the treatment

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Keywords:

Maternal metabolic disorders are associated with elevated concentrations of free fatty acids (FFA) in serum, follicular- and oviductal fluid. Previous studies have shown that pathophysiological FFA concentrations, and in particular the saturated palmitic acid (PA), jeopardize bovine oocyte and embryo developmental competence *in vitro*. Moreover, gene expression and proteomic analysis of FFA exposed bovine oocytes point towards oxidative stress related pathways. As such, antioxidants may be a key factor in improving oocyte and embryo developmental competence. We investigated if the use of TROLOX, a water soluble vitamin E analogue and antioxidant, during IVM or IVC could enhance developmental competence of PA-exposed oocytes and embryos *in vitro*. Hereto, 1279 bovine oocytes were routinely matured, fertilized and cultured until day 8 in 2 different experiments (6 repeats each). In Exp.1, oocytes were exposed to pathophysiological follicular PA concentrations (150µM), after which the zygotes were cultured under solvent control (ethanol, PA-SC) or TROLOX (100µM, PA-TROLOX) conditions. In Exp.2, oocytes were matured under SC or TROLOX (100µM) conditions, then exposed to pathophysiological oviductal PA concentrations (230µM) during culture (SC-PA, TROLOX-PA). In each experiment a solvent control was included (SC-SC). Cleavage (48h post insemination, pi), blastocyst rates (D8 pi), the rates of D8 blastocysts/cleaved zygotes and the rates of D8 expanded and hatched blastocysts/total blastocysts were calculated. Developmental competence data were compared using a binary logistic regression model and Bonferroni post-hoc test (IBM SPSS Statistics 24). In Exp.1, cleavage of PA-SC (71%) was not significantly different from SC-SC (79%, P=0.133). D8 blastocyst rates of PA-SC (22%) tended to be lower compared with SC-SC (32%; P=0.064). Compared to PA-SC, we showed that TROLOX during IVC was not able to neutralize the PA insult during IVM (PA-TROLOX, 23%; P>0.100). The rates of total D8 blastocysts/cleaved zygotes and D8 expanded and hatched blastocysts/total blastocysts were not significantly different. In Exp.2, cleavage, D8 blastocyst rates and D8 blastocysts on total cleaved zygotes of SC-PA (59%, 9%, 14%, respectively) were significantly reduced compared with SC-SC (79%, 32%, 39%, respectively; P<0.0001). Cleavage and D8 blastocysts/cleaved zygotes of TROLOX-PA (68% and 24%, respectively) tended to be improved compared with SC-PA (P<0.1). Moreover, the addition of TROLOX during IVM could significantly increase D8 blastocyst rates (17%) of PA-exposed embryos (P=0.022), but not to control levels (32%). TROLOX during IVM significantly improved blastocyst development into expanded and hatched blastocysts when embryos were exposed to PA (SC-PA, 49% vs. TROLOX-PA, 68%; P=0.025) to levels similar to controls (SC-SC, 63%). In conclusion, the antioxidant TROLOX can protect oocytes from metabolic stress insults after fertilization, but metabolically compromised oocytes cannot be rescued by the addition of TROLOX during embryo culture.

Elevated non-esterified fatty acid concentrations during *in vitro* maturation affect the transcriptome profile of day 14 bovine embryos 7 days after transfer

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Keywords: NEFA, transcriptome, embryo transfer

We showed earlier that exposure to elevated non-esterified fatty acid (NEFA) concentrations during *in vitro* oocyte maturation (IVM) affects post-hatching development of day (D) 14 bovine embryos (Desmet *et al.*, Anim. Reprod. 14,p947,2017). Lipotoxic conditions during IVM influence DNA methylation in the D7 embryo and may thus affect its transcriptome during later development. Therefore the aim was to analyse the transcriptome profile of D14 embryos to investigate which mechanisms mediate carryover effects of adverse maturation condition on post-hatching development.
Bovine oocytes were matured for 24h under 2 conditions: 1) basal physiological NEFA conditions (BAS; 28µM stearic acid (SA), 21µM oleic acid (OA), 23µM palmitic acid (PA)); and 2) high PA concentration (most predominant in follicular fluid during negative energy balance) (HPA; 150µM PA, 28µM SA, 21µM OA). After fertilization, zygotes were cultured in SOF with serum. 8 blastocysts (normal and expanded, equally distributed per treatment and per replicate) were transferred to healthy non-lactating Holstein Friesian cows at D7 (n=8, 5 replicates). Each cow was used once for each treatment in a cross-over design. After transcervical recovery, D14 concepti (n=45) were dissected into embryonic disc (ED) and extra-embryonic tissue (EXT). ED (n=11BAS/7PA) and EXT (n=13BAS/8PA) were subjected to RNA sequencing (without RNA amplification). Differential expression was established in a DESeq2 model based on Negative Binomial distribution. Samples were divided by sample type for further analysis. A false discovery rate (FDR) of 10% was used as cut-off for differentially expressed genes (DEG) and *P*-values were Benjamini-Hochberg corrected. Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) were performed.
Recovery rate at D14 was not significantly different between treatments. Within ED and EXT datasets, only 14 and 0 DEGs were detected in HPA embryos compared to BAS embryos, respectively. However, when comparing concepti of similar morphological class (spherical/ovoid/tubular) and sex, higher numbers of DEGs could be detected (e.g. in ED dataset up to 6 times more DEGs). Overall, more DEGs were observed in ED compared to EXT at each morphological stage (except male tubular embryos). IPA and GSEA showed that affected pathways were related to cell growth and adhesion, metabolism, endoplasmic reticulum stress, mitochondrial respiratory chain complex and epigenetic mechanisms.
To conclude, elevated PA exposure during IVM has carryover effects on the transcriptome profile of D14 concepti although only good quality D7 embryos with the same morphology have been transferred. D14 transcriptome patterns were dependant on morphology (elongation stage) and cell type (ED versus EXT) but common pathways affected were related to cellular development, metabolism and epigenetics. This suggests that metabolic stress during oocyte maturation may have long-lasting effects on embryo development that may lead to reduced fertility in high-yielding dairy cows.

The effects of undernutrition and supplementation with cactus silage in Boujaâd ewes on offspring growth performances

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Keywords: Dietary restriction, Cactus silage, physiological stage

In Moroccan agriculture, sheep farming is extremely diversified but it is facing many constraints like the extensive mode of its management and the effects of climate change. The later makes food resources getting more scarced, thing that can affect the survival of the embryo and fetus development. In this context the cactus plant is currently gaining an interest in several countries to feed animals as it ensur a food supply in the case of a critical drought situation (Arba et al., *Agri.Mar,* 2009, 215-222). Thus, the aim of this work was to study the effects of nutrient restriction and total substitution of concentrate by cactus silage in Boujaâd ewes during late pregnancy and early lactation on neonatal offspring performance. The experiments were carried out at INRA-CRRA-Settat- MAROC. Thirty-three Boujaâd ewes at similar ages (3 months of gestation) and with an average weight of 50.83±1.49 kg were used. The experiment started at 3 month ewe's gestation until 1 month lactation. Ewes were randomly assigned to three groups, a control group CG (n=11), a dietary restricted group RG (n=11) (deficit ration in energy and proteins) and a group cactus-based diet CBG (n=11). The CG and CBG groups received the same energy amount. The food rations, adopted in this work were randomly selected in a way to simulate the under nutrition in small livestock producers during drought periods in Morocco. The body weight was measured monthly for all ewes. While, the lamb's body weight was measured at birth and one month later and lamb mortality and the rate of abortion were assessed. Statistical analyzes were performed using the JMP SAS 11.0.0 program and lamb mortality and the rate of abortion data were assessed by X2 analysis of contingency tables. This study showed significant effect ($P < 0.05$) on all parameters. The ewe body weight in late pregnancy and early lactation was higher in CG (53.36±1.25 kg and 49.21±2.09 kg respectively) compared to the RG (49.71±2.08 kg and 46.02±0.98 kg respectively) and CBG (49.18±2.24 kg and 45.92±0.98 kg respectively). The rate of abortion and mortality after birth were higher in RG (27.27% and 36.36% respectively) compared to the CG (7 % and 8.2% respectively) and CBG (4% and 5% respectively). The weight of lambs at birth was significantly affected by the diet adopted, the weight was higher in CBG (3.4±0.51 kg) and CG (3.12±0.34 kg) compared to RG (2.4±0.12 kg). Similarly, weight of lambs one month later was higher in CBG (18.1±0.91 kg) and lower in the RG (6.98±2.15 kg), while in CG the weight was intermediary (11.16±0.28 kg). It is concluded that ewe undernutrition in late gestation and early lactation affect negatively lamb growth performances and mortality rate. However, the use of cactus silage as a feed for ewes can be used as an alternative to concentrate especially in arid area with limited food resources.

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Piglets obtained by transfer of embryos received after *in vitro* fertilization of oocytes matured with thymosin: a preliminary study

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Keywords: pig, oocyte, thymosin

One of the most important issues of *in vitro* production of pig embryos is the quality of oocytes. Thymosin (TH) is a 28- peptide hormone, released by the Thymus. TH accelerates the healing of dermal burn wounds, injured cells or tissues as well as regenerates skin and stimulates hair regrowth (Tseng et al.,2002, Kim and Kwon, 2014). Until now the regeneration functions were examined only on somatic cells. In our recent study we showed that maturation of pig oocytes in a medium supplemented with thymosin increased the number of matured oocytes with lower morphological quality and improved the quality of *in vitro* obtained pig blastocysts (Gajda et al., 2015). The purpose of this preliminary study was to investigate the effect of maturation of oocytes with or without thymosin on *in vivo* survival of pig embryos obtained after *in vitro* fertilization. Cumulus-oocyte complexes (COCs) were obtained from ovaries collected from slaughtered gilts. COCs were selected based on cytoplasm morphology and cumulus cell layers appearance and cultured for 42–44 h in a modified TCM-199 medium supplemented with 1 mg/ml of synthetic TH (LipoPharm.pl) (experimental group) or without TH (control group). After maturation, oocytes from the experimental and control groups were assessed and fertilized *in vitro*. Presumptive zygotes were cultured in the NSCU-23 medium for 72 h at 39^{o} C, in an atmosphere of 5% CO_2 and 5% O_2 in humidified air. Embryos were transferred surgically, under general anesthesia, into a single oviduct of synchronized 6 month-old recipients gilts. Each of the two gilts from the experimental group and one from the control group received fifty embryos. On days 28-30 and 40, the diagnosis of pregnancy was performed by ultrasonography. The pregnant recipients were monitored until parturition, and the total number of piglets born within individual litters was determined. After transfer of embryos from the experimental group (50 to each gilt) into 2 recipients and 50 from the control group into 1 recipient, both gilts that received embryos obtained after in vitro fertilization of oocytes matured with TH became pregnant and delivered a total of 16 live piglets. Pregnancy was not achieved after transfer of embryos from the control group. In conclusion, in our preliminary study we suggest that the maturation of pig oocytes with thymosin supports the *in vivo* survival of IVF embryos. This study permitted us to set up the IVP-embryo transfer successfully in our lab allowing the birth of the first piglets obtained after IVP and ET in Poland. Further experiments are ongoing to validate the results of this study.

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PredOSEgenesis: A two-layer classifier for identifying oogenesis, spermatogenesis and embryogenesis-related proteins

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Keywords: SVM, protein, fertility

Successful spermatogenesis and oogenesis are the two genetically independent processes preceding embryo development. Therefore, further studies are required to discover more proteins associated with the development of germ cells and embryogenesis in order to shed more light on the processes. Here, we extended our previous study (Rahimi, M., Bakhtiarizadeh, M. R. & Mohammadi-Sangcheshmeh, A. OOgenesis_Pred: A sequence-based method for predicting oogenesis proteins by six different modes of Chou's pseudo amino acid composition.
J Theor Biol 414, 128-136, doi:10.1016/j.jtbi.2016.11.028 (2017)) and offered a new algorithm to predict not only the proteins are involved in oogenesis, but also those implementing spermatogenesis and embryogenesis processes. First we extracted 345, 641, and 831 proteins through searching the UniProtKB database with gene ontology terms for “oogenesis”, “spermatogenesis” and “embryogenesis”, respectively. Then we developed a new method based on the support vector machine (SVM) and informative protein physicochemical properties (1920 different features including amino acid composition, autocorrelation, Quasi sequence-order, two types of pseudo-amino acid compositions and etc.) for predicting new fertility-related proteins and their classes (oogenesis, spermatogenesis and embryogenesis). Moreover, we employed a feature selection approach by using 10 different feature weighting methods on general datasets (combination of oogenesis, spermatogenesis and embryogenesis) to identify the more important protein features for fertility/non-fertility-related proteins. Our model achieved 80.79%, 80.54% and 79.74% prediction accuracy by five-fold cross validation test and 82.03%, 80.15% and 77.16% prediction accuracy using the independent test for datasets with 50% identity for Oogenesis, spermatogenesis and embryogenesis respectively. Furthermore, our results of feature selection revealed that Isoleucine and Serine frequency, Dipeptide Composition, Quasi sequence-order, composition, distribution and conjoint triad are important features for fertility-related proteins prediction. Interestingly, we found that the role importance of serine and isoleucine in fertility-related proteins and related biological process are highlighted. Isoleucine is believed to be related to male fertility through its synthetic and metabolic activities. For instance, mutation of encoding gene of ubiquitin-specific protease 26 (responsible for a valine to isoleucine change) has been reported to cause male infertility and adversely affect the testicular function. Cytochrome P4501A1 participates in isoleucine–valine exchange; mutation of its heme-binding region is also associated with infertile men. The importance of isoleucine in sex-determining region Y (SRY) protein has been highlighted, specifically the orientation of isoleucine side chain in DNA minor groove. The key role of the glycine and serine-rich sequences in oogenesis and folliculogenesis processes is highlighted by explaining their relationship to bone morphogenetic protein (BMP) family. Finally, we developed a two-layer classifier software, named as “PrESOgenesis”.

Characterization of sperm-oviduct extracellular vesicles interactions at various stages of the bovine estrous cycle and their effects on sperm physiology

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Keywords: Extracellular vesicles, sperm-oviductal interactions

Extracellular vesicles (EV), recognized as an important mechanism of cell-to-cell communication, have been identified in the oviductal fluid (OF) and have been proposed as key modulators of sperm-oviduct interactions. Hence, the aims of this study were: 1) to characterize sperm-oviduct EV interactions across the estrous cycle and 2) to identify the effects of these interactions on sperm viability and motility. Oviducts with their attached ovaries were collected from bovine reproductive tracts at a local slaughterhouse and classified into 3 stages of the cycle: Pre-Ovulatory (PE), Post-Ovulatory (PO) and Luteal Phase (LP). After OF collection, EV were isolated by ultracentrifugation and resuspended in 50 µL PBS (pool 300 µl from 2-3 cows). A pool of frozen-thawed washed spermatozoa (spz) from 2 bulls (Holstein and Normand; 85% viability and 60% motility after Percoll) was used. To analyze sperm-EV interactions and survival, EV were labeled with a green fluorescent dye (PKH67) and co-incubated (at 3.3% (v/v)) with spz for 2, 8 or 16 h at 37°C, 5% CO_2in IVF media (Tyrode medium), then stained with Hoechst 33342 and Ethidium Homodimer (to visualize dead spz) and evaluated by confocal microscopy (~900 spz counted). Sperm motility was assessed after 2, 8 or 24 h of co-incubation with and without (Control) EV (at 1 or 10% (v/v)) by CASA. Each experiment was done in triplicate. The effects of the stage on interactions and of interactions on sperm viability were analyzed by chi-squared statistical test. The effect of interactions on sperm motility were analyzed by Kruskal-Wallis test. The proportion of spz interacting with EV (EV-spz) increased over time, ranging from 1-2% of EV-spz after 2h of co-incubation to 4-10% after 8h and 62-80% after 16 h. Overall, a significantly higher proportion of spz were bound to LP-EV compared to PE- and PO-EV (8h: 9% vs 5 and 4% and 16h: 76% vs 66 and 63%, respectively; $p<0.01$). Besides, five different patterns of sperm-EV interactions were observed with a marked effect of the stage of the cycle. After 16h of co-incubation, PE- and PO-EV were mainly detected over the sperm head in comparison to LP-EV (70 and 54% vs 29% of EV-spz, respectively; $p<0.01$) whereas LP-EV were distributed over the head and intermediate piece compared to PE- and PO-EV (58% vs 41 and 28% of EV-spz, respectively; $p<0.01$). LP-, PE and PO-EV had a detrimental effect on sperm viability compared to controls at 8h (48- 52% vs 61%) and 16h (27-28% vs 35%; $p<0.01$). Furthermore, PE-EV (10%) decreased significantly the % of motile & progressive spz after 2h incubation (10.3% vs 31,1% and 4.0% vs 19.3%; $p<0.05$), and PO-EV (10%) the % of progressive spz after 8h incubation (2.6% vs 5.4%; $p<0.05$). In conclusion, we showed that oviductal EV interacted with cryopreserved bovine spz in a stage-specific manner. Long co-incubation times with EV impaired sperm viability and motility. These results pave the way for further research on sperm-EV interactions in the maternal tract.

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Health status in the Latvian native breed of Latvian Brown dairy cows that are intended for multiple ovulation and embryo transfer

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Keywords: extinct cow breed, preservation

The Latvian Brown (LB) dairy cows are native cattle breed endemic in Latvia. It is possible to save LB cow breed by using multiple ovulation (MO) and embryo transfer (ET) due to the ERAF project No.1.1.1.1/16/A/025, *BioReproLV*. In Latvia MOET in cows has been restarted after 35 years of interruption. Many of the LB gene fond (GF) animals are at advanced age, they live in small farms without a calculated feeding ration, and many of these small private farm owners do not have appropriate education relevant to the cow physiology beside their own experience. The aim of the present study was to find out the health status in genetically valuable LB GF cows intended for MOET. Clinical examination, blood morphological (leucocytes, erythrocytes, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, platelets) and biochemical analysis (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyltransferase (GGT), alkaline phosphatase (AP), urea, creatinine (CREA), total protein (TP), albumin (Alb), sodium (Na), potassium (K), calcium (Ca), phosphorus (P), chloride (Cl), cholesterol (CHOL), triglycerides (TG), as well as glucose (Glc) and ß-hydroxybutyric acid (BHB) by using express test *FreeStyle Optimum Neo H*) were performed in 12 animals. Data are expressed as the mean ± SD and one sample t-test and independent samples t-test were performed for statistical analysis considering the significance level of P=0.05. The donor cows were 5.3±2.66 years old (max. 9). Body score condition =2.0 points was in 3 cows (25%) and =3.5 was in 4 cows (33%). No signs of illness were established by general clinical and reproductive tract examinations in 9 cows (75%), but 3 cows (25%) were rejected because of ovarian cysts, pyometra and subclinical ketosis. Blood morphological parameters were in the reference ranges in all cows. ALAT (40.3±6.60 u/L) was increased in 4 (33%), GGT (39.5±6.72 u/L) was increased in 7 (58.3%) and BHB (1.7±0.25 mmol/L) was increased in 3 cows (25%), (P=0.05). Urea (1.6±0.86 mmol/L) was decreased (P=0.05) in 4 cows (33%). These results indicate impaired liver health and functionality due to inaccuracies in nutrition. Other biological parameters as ASAT (84.4±17.86 u/L), AP (60.0±83.16 u/L), CREA (72.9±27.81 µmol/L), TP(73.6±6.39 g/L), Alb (32.5±5.27 g/L), Na (142.3±2.3 mmol/L), K (5.1±0.48 mmol/L), Ca (2.5±0.63 mmol/L), P (2.1±0.33 mmol/L), Cl (98.7±1.92 mmol/L), CHOL (4.5±1.69 mmol/L), TG (0.2±0.06 mmol/L), Glc (2.9±0.72) were in the reference ranges in all cows. MO using pregnant mare serum gonadotropin and double artificial insemination were carried out for 7 cows till now and only 2 embryos were obtained. In conclusion, because of the inapropriate LB GF health status, it is necessary to conduct blood morphological and biochemical analyses in all cows intended for MOET. The express-tests (Glc, BHB) are the first signals of inaccuracies regarding cow metabolism, and blood biochemical analyses have proved it.

Cloning, Transgenesis, and Stem Cells

Adipose mesenchymal stem cells: a new tool to restore interesting genotypes by cloning in the rabbit

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Keywords: nuclear transfer, multipotent, adipose

Our objective was to investigate somatic cell nuclear transfer (SCNT) as a tool for restoration of particular genotypes (genome edited mainly) in the New-Zealand rabbit. SCNT efficiency is founded on the capacity of donor cells to be reprogrammed to a totipotent state. Consequently, the less differentiated donor cells are, the more easily they could be reprogrammed by a recipient ooplasm. In rabbit, the lack of functional embryonic stem cells is thus a problem. In Ali/Bas rabbit, V. Zakhartchenko *et al*. (Biol Reprod.84p229. 2011) opened interesting perspectives with the use of bone marrow multipotent cells as donor cells for SCNT. Thus, multipotent mesenchymal stem cells (MSC) could be attractive for our purpose. From this prerequisite but looking for multipotent cells accessible in the least invasive way for the donor rabbit, we tested the ability of adipose-derived mesenchymal stem cells (ASC) to give birth to cloned animals. ASC were easily recovered from abdominal fat under anaesthesia. For this preliminary study, we used 2 different batches of commercial ASC (RBXMD-01001/Cyagen Biosciences, Neu-Isenburg, Germany) chosen for their multipotent state and strong capacity to expand maintaining this state. We used cumulus cells (CC) as "control" of development potential since they have been used widely for SCNT and most rabbit live clones were produced from freshly prepared CC. Nuclear transfer and embryo transfer were performed as described by N. Daniel *et al*. (Methods Mol Biol.1222 p15. 2015 and Cold Spring Harb Protoc. 2010). The pregnancies were followed by ultrasound monitoring as described by P. Chavatte-Palmer *et al*. (Theriogenology.69 p859. 2008). *In vitro* and *in vivo* embryo developments were compared by Chi-2 or non-parametric Fisher's exact test and differences were considered significant at $P<0.05$. We first compared 2 ASC lines to make sure that the individual characteristics of each do not influence the developmental competence of SCNT embryos. No significant differences were observed for cleavage, blastocyst, implantation and pregnancy rates, nor for development to term. We then compared ASC versus (vs) CC. ASC showed higher *in vitro* development rates: 88% (492/559) vs 73.5% (180/245) and 46.1% (65/141) vs 32.2% (79/245) for cleavage and blastocyst rates respectively. At mid-gestation, pregnancy rates were not significantly different: 40.1% (9/22) vs 50% (4/8). Term pregnancies were obtained for 1 and 3 recipient females respectively. One clone was born from ASC and 5 from CC. Embryo competence to develop to term was thus significantly lower for ASC 0.4% (1/247) vs 3.6% (5/138). Large Offspring Syndrome was observed for 1 ASC and 2CC clones. Further studies are thus necessary to decrease LOS incidence in rabbit cloning, but our study showed that ASC, which are easily available for multiple cloning sessions, are compatible with full term pregnancy after SCNT.

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Detection of adult stem cell marker leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) transcripts in bovine oviduct epithelial cells

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Keywords: mRNA abundance, oviduct, stem cell

Oviduct epithelial cells (OEC) are cultured *in vitro* to study their function with the aim of improving conditions for *in vitro* embryo production. However, primary culture of OEC is hampered by their limited lifespan and low passage capacity. This study aimed to determine whether the OEC population includes adult stem cells of the type identified in other tissues. Stem cells have the capacity to both self-renew and differentiate into cell types of ecto-, endo or mesodermal lineages. The leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is considered an adult stem cell marker in the epithelia of many tissues. In a first step to identify adult stem cells in oviduct epithelium, we investigated the presence of *LGR5* in OEC. Oviducts from cows obtained from a commercial slaughterhouse were dissected post-mortem and classified into four estrous cycle stages based on ovarian morphology: immediate post-ovulatory, mid-luteal, late-luteal, and pre-ovulatory (n = 3/stage). The OEC from each oviductal segment (infundibulum, ampulla, and isthmus) from ipsilateral and contralateral (relative to the CL or pre-ovulatory follicle) oviducts were collected separately using a mechanical technique for OEC collection. Briefly, the OEC from infundibulum and ampulla were scraped with a thin blade, and the sections of isthmus were gently squeezed with forceps. Relative *LGR5* level was analyzed by RT-qPCR, and normalized using the geometric mean of the two best reference genes (GAPDH encoding glyceraldehyde dehydrogenase and RLP15 encoding ribosomal protein L15). Student's t-test was used to evaluate differences in *LGR5* levels between ipsilateral and contralateral oviducts and ANOVA with post-hoc Tukey-Kramer test to assess differences in OEC *LGR5* levels between the three oviductal segments and four estrous cycle stages (JMP software; SAS Institute Cary, NC). No differences in *LGR5* levels were observed between ipsi- and contralateral oviducts ($P>0.05$). However, when compared to ampulla, *LGR5* was detected 156.4 ± 121.3 times more in the isthmus, while 0.51 ± 0.22 lower in the infundibulum ($P<0.05$). *LGR5* levels were estrous cycle stage independent ($P>0.05$) with the exception of the isthmus in which 2.75 ± 1.15 and 1.73 ± 0.85 times higher *LGR5* levels were detected during the pre-ovulatory stage when compared to the mid- and late-luteal stages, respectively ($P<0.05$). In short, *LGR5* mRNA was detected in all oviductal segments throughout the estrous cycle with highest levels in pre-ovulatory isthmus. The detection of *LGR5* transcripts suggests the presence of adult stem cells among bovine OEC. Experiments are ongoing to confirm the presence and location of LGR5 protein and to isolate LGR5 positive cells for future improvement of OEC cultures.

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Early microinjection of bovine zygotes reduces mosaicism rates following CRISPR-mediated genome edition

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Keywords: CRISPR, Mosaicism, IVF

Direct gene ablation (knock-out, KO) can be achieved by CRISPR microinjection at the zygote stage. KO generation mediated by CRISPR is based on the generation of insertion-deletions (indels) at the coding region of a gene that can disrupt its open reading frame leading to a truncated protein. Gene disruption occurs only when the indel is not multiple of three, therefore not all randomly generated indels produce a KO. Direct KO generation requires all alleles (indels) harbored by an individual to be frame-disrupting. In this sense, a reduction in the number of indels generated in a given individual increases the chances of direct KO generation. Ideally, CRISPR should generate 2 indels, one on each parental chromosome, however, if the edition occurs after DNA replication, mosaicism (i.e. the presence of more than two indels on the same embryo) arises. The objective of this study has been to develop a protocol based on early microinjection of bovine oocytes to reduce mosaicism rate following CRISPR edition. A preliminary study was conducted to evaluate the shortest gamete co-incubation time compatible with normal developmental rates. Fertilization rates were dramatically reduced at 8 or 9 h co-incubation times, but 10 h yielded similar cleavage rates than the conventional 20 h *in vitro* fertilization (83.8±5.0 vs. 90.9±5.5 for 10 or 20 h groups, respectively, ANOVA ($p>0.05$)). Next, we evaluated DNA replication timing by 5-Ethynyl-2'-deoxyuridine (EdU) incorporation from 10 to 20 hours post-insemination (hpi), observing that most zygotes began their S-phase well before 20 hpi (zygotes in S-phase (%): 39.0±5.61; 78.0 ±7.54; 92.5±4.13 77.5±4.48; 56.1±6.41; 37.5±5.09, at 10, 12, 14, 16, 18 and 10 hpi respectively; mean±s.e.m., 3 replicates). Finally, we performed CRISPR microinjection (300 ng/µl Cas9 mRNA and 100 ng/µl sgRNA) at 10 or 20 hpi, targeting an intronic region where indels do not exert any detrimental effect on development. Genome edition rates of blastocysts derived from microinjected zygotes was similar in both microinjected groups (85.7 % -6/7- for 20 hpi vs. 92.3% -12/13- for 10 hpi). However, clonal sequencing of the edited embryos (10 clones sequenced per embryo) revealed a significantly lower incidence of mosaicism in blastocysts from 10 hpi group (100 % -6/6- for 20 hpi vs. 33.3% -4/12- for 10 hpi. Fisher's exact test ($p<0.05$)). In conclusion, an earlier deliver of CRISPR components reduces mosaicism rates and achieves similar developmental and genome edition rates.

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The use of a novel microfluidic culture device and predictive metabolic profiling as a means to improve murine embryo developmental competence *in vitro*

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Keywords: Microfluidics, metabolism, embryo quality

Successful embryo development in vitro is directly dependent on the provision of an optimal culture environment that supports coordinated embryonic cell division, metabolism, and genetic and epigenetic development. A number of attempts have been made over recent years to use microfluidic devices in IVF as a means to control the culture environment and so improve embryo developmental competence (quality) *in vitro*. In this study we have designed, engineered and tested a novel microfluidic device for the *in vitro* production of murine embryos from the 1 cell zygote stage to the blastocyst stage. Soft lithography was used to prepare microfluidic devices in polydimethylsiloxane (PDMS). The microfluidic device consists of a 400 nL circular chamber (radius 750 mm) where 10 embryos can be loaded, kept in static culture for the full period of culture and visualized by optical and fluorescent microscopy. A series of 2 experiments were conducted to evaluate the efficacy of our microfluidic device for mouse embryo culture. Cryopreserved, IVF-derived, mouse embryos of strain C57BL/6N, provided by MRC Harwell, UK were cultured in KSOM media. Microfluidic culture was used in conjunction with non-invasive analysis of glucose (G), pyruvate (P) and lactate (L) metabolism in spent zygote culture media as a means to improve embryo quality. In both experimental series, data from microfluidic cultures were compared to equivalent end point analyses of control embryos grown in conventional microdrop cultures under oil.Experiment 1: 2 cell embryos were thawed and cultured in groups of 8-10 in microfluidic devices (n=46) or 10µl control (n=32) drops for 3 days at 37^{o}C under 5%CO_2/5%O_2/N_2 balance. Embryos were removed to individual culture drops for 24h for analysis of energy substrate turnover using the method of Guerif et al. (PLOS ONE, 2013) followed by transfer to fibronectin-coated dishes for assessment of attachment and outgrowth according to the method of Hannan et al. (Endocrinology, 152 (12), p4948-4956, 2011). Blastocyst grade, hatching, attachment, outgrowth rates, and pyruvate and glucose consumption were assessed and were similar between device and control groups (P>0.05). However, lactate output was significantly reduced following device culture vs controls (4.1±0.8 vs 1.4±0.3 pmol/embryo/hr, P=<0.0001). GPL metabolism did not predict embryo attachment or outgrowth in either culture environment.
Experiment 2: 1 cell zygotes were cultured individually overnight for analysis of GPL metabolism and assigned to culture groups based on pyruvate consumption, with 1 device and 1 microdrop group per tertile per culture with 10 embryos per group (total n=60 device and n=60 control). Following group culture, individual blastocyst pyruvate consumption was reduced (5.4±2.2 vs 12±1.5 pmol/embryo/hr, P=<0.0001). Pyruvate consumption tertile was unaffected by device culture. Device culture was non-toxic and did not affect embryo development. However, blastocyst pyruvate consumption and lactate output were reduced compared to controls. This may suggest microfluidic culture can be utilised to achieve a controlled, moderate metabolic phenotype, reducing variation between embryo metabolism.

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

The use of different progestin devices in ovarian stimulation protocol affects gene expression in sheep cumulus-oocyte complexes (COC)

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Keywords: oocyte, progestogen

Progesterone (P_4) and its analogues (progestins) are commonly used in estrus synchronization protocols and to hold the LH surge during ovarian stimulation to allow oocyte recovery in live donor ewes. However, recent evidence suggests that some progestins may have a deleterious effect on embryo quality after long-term use. Thus, the present study aimed to evaluate the effect of two progestin devices used during ovarian stimulation on the COC quality in donor ewes. A total of 30 pluriparous ewes had their estrus and follicular wave synchronized by a short-term protocol (Bragança et al., Reprod., Fertil. Dev., published online, 2018). At 80 h after sponge removal, all ewes received 80 mg of pFSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) in three applications (50%, 30% and 20%) every 12 h. For stimulation, the ewes were allocated into three groups (n=10 each): MAP, in which ewes received intravaginal sponges containing 60 mg of medroxiprogesterone acetate (Progespon, Zoetis, São Paulo, Brazil); P_4, in which a silicone device impregnated with 0.33 mg of natural P_4 (CIDR, Eazi-Breed, Zoetis) was applied; and Control, in which the ewes did not receive any device (only luteal P_4). COCs were recovered by laparoscopy and morphologically graded as viable (GI/II, homogeneous ooplasm and at least a complete cumulus cells layer; and GIII, homogeneous ooplasm and/or partially denuded) or poor quality (GIV, heterogeneous ooplasm or degenerated). To infer development competence, viable COCs were stained with brilliant cresyl blue (BCB) and classified as BCB^+ (competent) and BCB^- (non-competent). Pools of 10 BCB^+ COCs/group were used for gene expression analysis by real-time PCR of oocyte competence markers (ZAR1, zygote arrest 1; MATER, maternal antigen that embryo requires; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15; RELN, reelin; Bcl-2, B-cell lymphoma 2; and BAX, Bcl-2 associated X protein) and steroidogenic pathway-related genes (ERα, estrogen receptor α; LHr, LH receptor; FSHr, FSH receptor; and StAR, steroidogenic acute regulatory protein). An ANOVA was then conducted to compare the variables followed by a Tukey test. No significant difference ($P>0.05$) was observed for the number of viable COCs per ewe (MAP: 5.7 ± 1.0, P_4: 7.7 ± 0.7 and Control: 5.7 ± 1.1) or the percentage of BCB^+ (MAP: 61%, P_4: 58% and Control: 65%). However, the gene expression profile was affected by the type of progestin used. FSHr, LHr and RELN genes were up-regulated ($P<0.05$) in the P_4 as compared with the MAP group, while LHr and RELN genes were down-regulated in the MAP as compared with the Control ($P<0.05$). Finally, FSHr, LHr, Erα, as well as the Bcl-2, ZAR1 and GDF9 were up-regulated in the P_4 as compared with the Control group ($P<0.05$). In conclusion, the progestin device alters the expression of genes related to quality and the steroidogenesis pathway in fully-grown COCs, and the use of a natural P_4 device may improve the development competence of COCs. Further studies including IVP are necessary to confirm our findings.

Analysis of correlation between zona pellucida birefringence using polarized light microscopy and resistance to trypsin digestion in different mammals

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Keywords: Zona pellucida, Hardening, Birefringence

The Zona Pellucida (ZP) is an extracellular matrix of mammalian oocyte and early embryo. It plays physiologically important roles in fertilization including binding of spermatozoa and prevention of polyspermic penetration. Sensitiveness of ZP to protease digestion and resistance to enzymatic solubility is species-specific. Polarized light microscopy allows monitoring the multilaminar ZP structure and identifying three differentiated ZP layers. This study investigated whether retardance and thickness of the inner, middle and outer layers of different species determines resistance to trypsin digestion. Immature oocytes from bitch (n=6), cow (n=10), mouse (n=5), pig (n=16) and rat (n=9) were collected from ovaries and analysed by polarized light microscope (polscope). The retardance and width of each layer of the ZP was analysed and measured by Oosight Meta®software (CRI, Woburn, MA, USA). Oocytes were incubated with trypsin solution (5mg/mL diluted in PBS) at 37ºC (Merck KGaA, Darmstadt, Germany). The time of ZP digestion was recorded and correlated with the birefringence and thickness outcomes. Analyses between groups were performed through a general lineal model, followed by Bonferroni's post-hoc test (p<0.05). Pearson's correlation between species and parameters was analysed.
The ZP of dog, mouse and rat showed lower ZP resistance to trypsin digestion (35.32±0.80 min, 71.38±2.16 min, 38.00±1.49 min, respectively) compared to cow (111.09±3.90 min) and pig ZPs (403.14±23.84 min).
As hypothesized, the inner, middle and outer layer were observed in bitch, cow, mouse, pig and rat oocytes. In all species the inner layer showed the highest birefringence. Furthermore, birefringence and thickness values differed between different species. Similar to ZP resistance, the relative thickness of inner ZP layer of pig (56.7%) and cow (56.8%) were higher than inner ZP layer relative width of bitch (47.6%), mouse (30.3%) and rat (47.7%). Finally, pig, cow and bitch showed higher birefringence, measured as retardance value, (5.98±0.24nm, 8.83±0.21nm and 6.75±0.30nm, respectively) than mouse (0.97±0.07nm) and rat (1.24±0.10nm) ZPs.
No significant correlations between ZP resistance to trypsin digestion and ZP birefringence or thickness were found in any analysed species.
In conclusion, differences were observed among the ZP of different species related with the ZP protein organization using the polscope microscopy. No correlations were observed between trypsin digestion and polscope analysis.
However, an increased number of oocytes should be analysed to confirm these preliminary results.

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Comparison of survival rates of two thawing methods for vitrified biopsied bovine *in vitro*-produced blastocysts

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Keywords: biopsied embryos, vitrification, survival rate

When embryos are genotyped, pregnancy rates after transfer are lower due to the need for biopsy and cryopreservation. The main objective of this study was to assess *in vitro* survival rates of biopsied vitrified *in vitro*-produced blastocysts using two different vitrification devices and a one- vs. three-step warming system.
Embryos were produced by a standard *in vitro* protocol and placed in the culture medium (synthetic oviductal fluid (SOF) supplemented with 5% oestrus cow serum (OCS), 40µl/ml basal medium Eagle (BME) and 10 µl/ml non-essential amino acid solution (MEM), at 38.5ºC in 5% CO_2 and 5% O_2). A total number of 215 of grade 1 blastocysts (IETS codes 6 and 7) were randomly divided into a biopsy group (BG, n=76) and a control group (CG, n=139). Blastocysts were biopsied using a microblade mounted on a micromanipulator. A small portion of the trophoblast, approximately 15%, was cut off. Both groups were then vitrified using the Cryotop ® (Kitazato, Tokio, Japan) or the new VitTrans device (Morató and Mogas, Cryobiology, volume 68, issue 2, pages 288–293, 2014). For vitrification, all blastocysts were exposed to an equilibration medium with 7.5% EG + 7.5% DMSO in holding medium (HM), consisting of TCM-199 with 20% fetal calf serum, moved into a drop with 16.5% EG + 16.5% DMSO + 0.5M sucrose in HM and then placed in a microdroplet on the Cryotop ® (Biopsy, n=39; Control, n=94) or VitTrans device (Biopsy, n=37; Control, n=45).
Warming of embryos using the VitTrans device for vitrification occurred in a one-step procedure by incubating them at 38.5ºC in 0.5M sucrose solution for 5 minutes. Embryos vitrified with the Cryotop system were warmed using the conventional three-step procedure by incubating them at 38.5ºC in 1M sucrose solution for 1 min, 0.5M sucrose solution for 3 min and 0M sucrose (HM) for 5 min. After warming, all embryos were placed in the culture medium.
Morphology and re-expansion were evaluated 24h post-warming. The embryo survival rate was defined as the ratio of blastocysts that were able to re-expand with regards to the total number of warmed blastocysts.
A 67% survival rate was observed in all biopsied embryos compared to controls (71%) (SAS, PROC GLM; p-value=0.5319). The ratio of re-expanded embryos of the 3-step warming system was 73% (biopsy group (69%) and control group (75%), Cryotop, n=133), in comparison with 63% using the 1-step warming system (biopsy (65%) and control group (62%), VitTrans, n=82); (SAS, PROC GLM; p-value=0.1123).
In conclusion, the biopsied embryos showed good *in vitro* development after vitrification and warming. However, based on our results, additional research is required to increase the re-expansion rate of embryos using the one-step warming procedure. The viability of the embryos needs to be confirmed by successful establishment of pregnancies.

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Design of pJuno-beads to study the molecular mechanisms of sperm-oocyte interaction

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Keywords: Porcine Juno, Conjugated beads, Gamete interaction

Juno, an essential protein in mammalian fertilization, was identified as the receptor for Izumo1 on mouse eggs and proven that the interaction between both proteins is conserved within mammals (Bianchi, Nature 508(7497): 483–487, 2014). Moreover, we have developed an *in vitro* 3D model mimicking oocyte's properties to study the molecular mechanisms involved in gamete interaction in pigs (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). Thus, the objectives of this study were i) to construct expression plasmids encoding the sequence of porcine Juno (F1STK4), ii) to express this recombinant protein in mammalian cells and, iii) to create a new 3D model consisting of magnetic beads (His Mag Sepharose™ Excel) conjugated with porcine pJuno. cDNA encoding nucleotide sequence (672 bp) of Juno protein in *Sus scrofa* was kindly donated by Dr. Enrica Bianchi (Cell Surface Signalling Laboratory, Wellcome Sanger Institute, UK). pJuno was PCR-amplified with reverse primer contained a sequence encoding a 6 histidine-tag and cloned into a pcDNA3.1 (+). pJuno-Cherry (pcDNA3.1 (+)) was designed including mCherry fluorescent protein in-frame with 3' region of pJuno and tagged with 6-histidines before stop codon (GeneArt, Life Technology). Expression plasmids were verified by DNA sequencing and purified. Both proteins were *in vitro* expressed in Chinese Hamster Ovary cells (CHO) and, once secreted, identified by SDS-PAGE and western blot. Juno-Cherry expression was monitored by fluorescence microscopy. Then, the secreted recombinant proteins were conjugated with the magnetic beads. Groups of 60-65 beads conjugated to Juno were co-incubated for 1 and 2h with a heterospermic dose of boar spermatozoa in TALP medium at a final concentration of 200.000 spermatozoa/mL. After co-incubation, the beads were washed twice in PBS, fixed and stained with Hoechst 33342. The percentage of beads with at least one sperm bound (BSB) and the mean number of sperm bound per bead (S/B) were scored by fluorescence microscopy. Secreted proteins and their adhesion to the beads was confirmed by western blot with anti-His antibody. Recombinant pJuno showed a molecular weight of ≈30kDa and pJuno-Cherry ≈50kDa. The BSB rate was similar at both incubation times, being 96.1 ± 1.7% at 1h (n= 128) and 92.8 ± 2.5% at 2h (n=111). The S/B was 15.0 ± 1.2 and 16.0 ± 1.4, respectively after 1 and 2h co-incubation. In conclusion, in this study, recombinant pJuno and pJuno-Cherry were *in vitro* expressed and identified for the first time. Moreover, the 6 histidine-tag of the recombinant protein allowed its successful conjugation to the magnetic beads generating a new and promising 3D model. Our preliminary results show that boar spermatozoa binds to Juno-beads but further studies are necessary to characterize this sperm-egg interaction and to gain more knowledge on the molecular basis of this interaction in pigs.

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Effect of vitrification on the functional activity of mitochondria in porcine oocytes during in vitro maturation

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Keywords: oocyte, mitochondria, vitrification

Problems in development an effective technology for vitrification of female gametes are determined by the features of oocytes structure, the sensitivity of cellular compartments to the action of ultralow temperatures, the toxicity of cryoprotectants. The aim of the present study was to evaluate the effect of pre-treatment of cumulus-oocyte complexes (COCs) with the fluid from the follicles (FF) d ≤3mm before vitrification on the mitochondrial activity (MA) in native and devitrified oocytes. MA was evaluated in: native oocytes; native oocytes pre-treated with FF (120 min, 37°C); devitrified oocytes; devitrified oocytes pre-treated with FF. Vitrification was performed by equilibration of oocytes in: CPA1:0.7 M dimethylsulphoxide (Me2SO) +0.9 M ethylene glycol (EG), 30 sec; CPA2:1.4 M Me2SO + 1.8 M EG, 30 sec; CPA3:2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose, 20 sec and loading into straws. After thawing COCs washed in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199. COCs were cultured in maturation medium (NCSU 23) supplemented with 10% FF (d≤3 - 6 mm), 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. 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. MA was measured by fluorescence probe MitoTracker Orange CMTM Ros, intensity of fluorescence expressed in μA/oocyte, chromatin status was evaluated with Hoechst 33342 (H.Torner et al., Reprod Dom Anim 42, 176–183, 2007). Oocytes were examined using confocal laser scanning system Leica TCS SP5. Chemicals were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test. Chromatin status and MA of 467 oocytes were evaluated (in 4 replicates, 27-30 oocytes/group). Before cultivation level of MA in native oocytes was significantly higher than in devitrified oocytes (331 ± 18,9 μA and 321 ± 17,7 μA vs. 105 ± 10, 3 μA and 150 ± 18,9 μA, $P<0.01$). Pre-treatment oocytes with FF increased level of MA in diplotene, metaphase-I and II devitrified oocytes (105 ± 10,3 μA vs. 150 ± 18,9 μA, 149 ± 12,1 μA vs. 209 ± 9,9 μA and 117 ± 17,1μA vs. 155 ± 11,9 μA, $P<0.05$). There were no differences in the level of MA at metaphase II stage of native and devitrified oocytes that have pre-treated with FF (158 ± 12,8 μA vs. 155 ± 11,9 μA). Features of the functional activity of mitochondria in native and devitrified oocytes during IVM have been identified. Treatment of porcine oocyte with FF (d≤3 mm) prior to vitrification had a positive effect on the mitochondrial function in devitrified oocytes. The obtained results supplement data concerning the functioning of mitochondria in porcine oocytes at the influence of ultralow temperatures.

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Cryodamage of oocytes frozen in antral follicles of bovine ovarian tissue fragments

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Keywords: cryopreservation, oocytes, antral follicles

Cryopreservation of ovarian tissues is largely developing method. Successful preservation of primordial follicles was documented in many animal species. There are reports that also oocytes in the antral follicles of ovarian fragments can be successfully cryopreserved, despite the fact that the cavity with follicular fluid is a barrier for freezing. The aim of our study was to assess histological and ultrastructural status of oocytes from small antral follicles cryopreserved in the ovarian fragments in order to judge about their developmental competence. Ovarian fragments (n=451; approximate size of 4x4 mm), containing antral follicles (2-4 mm), were isolated from undefined cows at a local abattoir, and frozen by two methods previously used for freezing of ovarian primordial follicles. For solid surface vitrification (SSV), ovarian fragments were exposed to 4% ethylene glycol (EG) in DPBS+10% FBS for 15 min and then rinsed in a vitrification solution composed of 35% EG and 0.4 M trehalose in DPBS+10% FBS. After 5 min equilibration in an ice bath, fragments were placed in a minimum volume of vitrification solution onto the surface of a metal plate pre-cooled by an immersion into a liquid nitrogen (LN). For liquid vitrification (LV), ovarian fragments were equilibrated in a vitrification medium containing 40% EG, 30% Ficoll 70, 1M sucrose and 4 mg/ml of BSA at room temperature for 5 min. Then the tissues in 1.8 ml cryovials were placed into LN. After thawing the fragments were processed for histology and ultrastructure analyses; part of oocytes were tested for the ability to mature in vitro (IVM). After 24 h of IVM in maturation medium (TCM 199 (Gibco), sodium pyruvate (0.25 $mmol.l^{-1}$), gentamycine (0.05 $mg.ml^{-1}$), fetal bovine serum 10% and FSH/LH (1/1 I.U., Pluset) at 38.5 °C and 5 % CO_2, the oocytes were fixed in formalin, stained with a DAPI dye and fluorescently evaluated. None of the oocytes from the SSV group were matured in vitro and only 8.4% from the LV group showed the signs of nuclear maturation in contrast to 60.6% in the control (fresh) group. In all frozen ovarian fragments, regardless of vitrification technique, serious damages of oocytes at the light or electron microscopy levels were detected like extensive vacuolization and disintegration of the ooplasm and organelle dislocation. Visible zona pellucida cracks and deformities of oocytes were caused likely by the mechanical action of ice crystals formed in the follicle cavity. The granulosa cell nuclei were largely pyknotic. Germinal vesicles showed disintegrated nuclear envelope. Microvilli of cytoplasmic membrane were disrupted. The damaged *zona pellucida* acquired layer-like structure, and cells of *corona radiata* showed extensive damages. In conclusion, our experiments did not confirm that the oocytes, frozen in small antral follicles from ovarian tissue fragments using SSV or LV, are able to mature *in vitro,* due to extensive cellular damages revealed by histological and ultrastructural analyses.

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MitoQ rescues early embryo development of metabolically-compromised bovine oocytes *in vitro*

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Keywords: MitoQ, embryos

Maternal metabolic disorders e.g. obesity have been linked with reduced oocyte quality and subfertility. In these cases, upregulated lipolysis increases free fatty acid concentrations, predominantly palmitic acid (PA), in blood and ovarian follicular fluid, causing direct detrimental effects on oocyte quality and IVF results. Using proteomic analyses, we found that high cellular oxidative stress, mitochondrial dysfunction and unfolded protein responses (UPR) are key mechanisms explaining PA-linked lipotoxicity in bovine oocytes. Carryover of cellular stress to subsequent early embryonic stages increases the risk of embryo cell death and explains the reduced blastocyst rates observed *in vitro*. Mitochondria-targeted therapeutics are increasingly used to treat metabolic diseases, however, their efficiency in rescuing development of metabolically-compromised oocytes has not been examined. In the present study, oocytes matured in media containing high pathophysiological PA concentration (150µM) or solvent (Control) were cultured in the presence or absence of mitoquinone (MitoQ; a mitochondria-targeted antioxidant, 1µM) (from day 1 post-fertilization (D1) until D8, in serum-free FA-free SOF media). Embryo cleavage and fragmentation (at D2) and blastocyst rates (D8) were recorded. Gene expression patterns of markers of cellular stress were examined in the resultant D8 blastocysts. Numerical data were analyzed by ANOVA, and categorical data by binary logistic regression, followed by Bonferroni correction. At D2, PA had no effect (P>0.1) on embryo cleavage (63.6 vs. 70.2%) and fragmentation (14.2 vs. 12.9%) rates compared to control. However, PA significantly reduced blastocyst rate at D8 (16.9 vs. 25.7%, P<0.05). MitoQ supplementation during culture to PA-derived embryos significantly reduced embryo fragmentation (7.3 vs. 14.2%) and rescued embryo development to the blastocyst stage (25.0 vs. 16.9%) compared with PA-group cultured in the absence of MitoQ. Day 8 blastocysts derived from the PA group had significantly (*P*<0.05) higher mRNA expression of genes related to oxidative stress (*CAT*, 5.5±3.1 folds; *SOD2*, 3.4±0.4; but not *GPx*, 1.99±1.03); mitochondrial UPR (*HSPE1*, 4.2±1.6 folds, and *HSPD1*, 10.6±5.8 folds); and ER stress (*ATF4*, 2.2±0.29 folds; *ATF6*, 25.99±12.5 folds; and *HSPA5*, 10.7±4.7 folds). In contrast, MitoQ supplementation to PA-embryos during culture significantly reduced the expression of the affected genes to control levels (P>0.05). For oocytes matured in control conditions, MitoQ supplementation during culture had no significant effects on development and gene expression patterns (P>0.1). In conclusion, these results provide further evidence that mitochondria play a central role in the pathogenesis of reduced oocyte developmental capacity under metabolic stress. Mitochondrial-targeted therapy (MitoQ) during early development of embryos derived from metabolically-compromised oocytes may be an efficient tool to reduce cellular stress level and maximize embryo development to the blastocyst stage. Further investigations are in progress to examine the quality of the rescued embryos.

mtDNA content of bovine cumulus cells derived from oocytes with different developmental competence following individual culture

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Keywords: Mitochondria, mtDNA, oocyte competence

Mitochondria are the most abundant organelles in the mammalian oocyte and early embryo, where they serve relevant metabolic and signal transduction functions. Oocyte´s mitocondrial DNA (mtDNA) content increases during folliculogenesis and it has been positively linked with fertilization outcomes following IVF in humans, bovine and pigs. Cumulus cells are closely associated with the oocyte they nourish and may constitute a valuable biological material to perform non-invasive assays of oocyte´s developmental competence. The objective of this study has been to determine whether oocytes exhibiting a diverse developmental competence following individual *in vitro* maturation (iIVM), fertilization (iIVF) and culture (iIVC) exhibit differences in the mtDNA content of their cumulus cells. Cumulus-oocyte complexes (COCs) were obtained from slaughterhouse ovaries aspirating 2 to 8 mm follicles.
Following conventional morphological COCs selection, iIVM was performed in 10 µl drops of maturation medium (TCM199 supplemented with 10 ng/ml EGF, 10 % serum and gentamicin) covered by mineral oil. Following maturation, cumulus cells were removed by pipetting in a 0.1 % hyaluronidase solution in PBS. Cumulus cells were pelleted by centrifugation at 1500 g for 5 min and pellet was snap frozen and stored at -80 ºC until analysis. Denuded oocytes were individually fertilized and cultured in 40 and 10 µl drops, respectively, covered under mineral oil. For mtDNA analysis cumulus cells samples were sorted in three groups based on the developmental competence of their corresponding oocyte following iIVF and iVC: 1) uncleaved (n=15), 2) cleaved but not reaching the blastocyst stage (n=15), and 3) developed to blastocyst (n=11). Cumulus cells DNA was extracted by Picopure and mtDNA was quantified by qPCR using specific primers for the mitocondrial gene *ND1* and the autosomal gene *RN18S1*, used to normalize mtDNA copy number to the number of cells present in the sample. Relative mtDNA abundance did not differ between the different groups analyzed (1.08±0.17 vs. 1±0.14 vs. 1.12±1.7 for oocytes resulting in not cleavage, cleavage arrest or blastocysts, respectively: mean ± s.e.m., ANOVA $p>0.05$), suggesting that cumulus cells mtDNA is not predicitive of oocyte developmental ability under these conditions.

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Good pregnancy rate of bovine biopsied and vitrified IVP embryos

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Keywords: IVP, biopsy, pregnancy

Genomic selection is now routinely used to select the best animals for breeding. Basically the same method can also be used to select the best embryo. Since the process of genomic selection (i.e. genotyping and breeding value estimation) takes a few days, the embryo need to be frozen. Good pregnancy results are already obtained using biopsied flushed embryos after slow freezing with Ethylene Glycol (Ponsart Reprod Fertil Dev., 26, 12-21, 2013). However, when using IVP embryos, pregnancy rates are too low for commercial use (30% compared to 50%).
Here we tested if vitrification of IVP embryos after biopsy give pregnancy results that are good enough to use the method in a commercial setting.
IVP embryos are made from OPU derived COCs using standard production method of our company CRV (Merton, Reprod Domest Anim. 47, 1037-42, 2012). At day 7 quality 1 and 2 embryos are biopsied using the blade biopsy method. The DNA in the biopsy is pre-amplified and used for genotyping. The remaining embryo is vitrified using a mixture of Ethylene Glycol, DMSO and Ficoll. The CVM Vitrification Block and CVM Fibreplugs™ with sleeves were used as vitrification method. The embryos are placed for 2 minutes in a 7,5% EG/DMSO solution and 40 sec in a 15% EG/DMSO/Ficoll solution. Subsequently, the embryo is transferred on the nylon hook of the fibreplug in a total volume of 1,5µl and plunged into the LN and stored. Before transfer the embryos are thawed in a 3 step washing method using base medium containing Fetal Calf Serum and a decreasing percentage of sucrose. Embryos (53 quality 1 and 57 quality 2) were transported at a temperature of 25ºC and subsequently transferred to recipient animals on 5 different farms. Pregnancy rates are scored at 3 months after transfer of the embryo by ultrasound scanning.
The results indicate that good pregnancy results are obtained from both quality 1 (55%) and quality 2 (44%) embryos. These pregnancy data are not different from those obtained with non-biopsied embryos (results not shown). As expected quality 1 embryos seem to give a higher pregnancy rate compared to quality 1, but numbers are yet too low to obtain a statistical significant difference.
We conclude that using vitrification as freezing method for biopsied IVP embryos, good pregnancy rates can be obtained (especially for quality 1) and can be used in a commercial setting.

Peri-natal basic blood biochemistry and health of calves born from frozen and vitrified IVP cattle embryos

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Keywords:

Techniques to cryopreserve bovine IVP embryos include vitrification/warming (V/W) and freezing/thawing (F/T). V and F differ in cryoprotectant concentration, cooling rates, and both may differently affect embryo physiology and survival. In this study we analyze whether calves born from V/W and F/T differ in metabolism and health status. Embryos were produced in vitro from slaughterhouse oocytes, fertilized with N=3 bulls (2 Holstein and 1 Asturiana de los Valles –AV-) and cultured in SOFaaci as described (Gómez et al, Reprod Fertil Dev, 29:1932-43; 2017). Subsequently, expanded blastocysts were subjected to V/W (Gómez et al, 2017), or to F/T with an ethylene-glycol based method modified from Sanches et al, 2016 (Theriogenology, 85:1147-51). In experimental herd, N=34 embryos were singly transferred to recipient heifers (Holstein, AV and their crosses), within replicates of N=5-7 embryos in order to obtain age- and sire-matched calves. Gestation was allowed to end without calving induction. At birth, calves suckled colostrum from mothers ad-libitum. Clinical examinations and blood samples were taken 1-4 h after feeding (Day 0), and subsequently at fixed times (10 a.m. on days 15 and 30 of life). Blood was collected in vacuum tubes (lithium heparin) from jugular vein and directly analyzed in a Vetscan i-STAT One analyzer (Scil Animal Care, Madrid, Spain; CG4+ and Chem-8+ modules). Calves analyzed were N=4 females and N=4 males (V/W); and N=5 males and N=3 females (F/T; with 1 male dead on Day 17). Data were analyzed by ANOVA, and Bonferroni and REGWQ tests for FDR. The following parameters were affected ($P<0.05$) by treatment (F/T vs V/W): Heart rate (149±5 vs. 136±5 beats/min); pCO_2 (39.4±2.1 vs 44.6±2.1 mmHg); packed cell volume (PCV; 22.6±1.2 vs. 26.0±1.2 %PCV) and calculated hemoglobin (7.7±0.4 vs. 8.8±0.4). V/W tended to show higher values than F/T within HCO_3, sCO_2 and glucose ($P<0.10$). Day x Treatment interactions ($P<0.03$) were detected in T, Heart rate, respiratory rate, pCO_2, pO_2, base excess, HCO_3, sCO_2, sO_2, Na^+, glucose and creatinine. Day effects were identified between Day 0 vs. Day 15 and Day 30, whereby base excess, HCO_3, sCO_2, Na^+, creatinine, PCV and hemoglobin decreased; conversely, calf body weight, PO_2, sO_2 and Ca^{2+} increased within both F/T and V/W (P values between <0.02 to <.0001). Calf sex affected Cl^-, PCV, hemoglobin and urea ($P<0.05$), while appearance of conjunctival membrane, nasal secretions, K^+, lactate and anion gap were not affected. Interestingly, all values were comprised between normalcy intervals described in previous calf studies. However, subtle epigenetic changes may underlie the differences observed between calves born from F/T and V/W embryos. Comprehensive metabolomics and genomics studies are in course.

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Identification and mathematical prediction of different morphokinetic profiles of *in vitro* developed bovine embryos

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Keywords: embryo, morphokinetics, prediction

The current method of embryo classification (IETS, 2013) is based on a static observation of *in vivo* derived embryos at day 7 post insemination (7 dpi). *In vitro* produced embryos (PIV) features impair their classification with this method. Morphokinetics is a powerful source of information to improve the comprehension of PIV embryo developmental behaviour. The objective of this study is to develop a methodology to read and predict different *in vitro* developmental potential of bovine PIV embryos by combinating morphokinetical parameters. Holstein embryos produced from oocytes recovered from slaughterhouse ovaries, *in vitro* fertilized with the semen of 4 different bulls and cultured for 8 days post insemination (8 dpi). Time lapse pictures were taken every 15 minutes throughout the culture period (672 pictures/embryo; Primovision™). The work was performed in 4 tasks (T): T1) identification of the profiles of *in vitro* development; T2) identification and standardisation of a reading method for bovine embryos morphokinetical parameters; T3) mathematical selection of a parsimonious subset of non-correlated parameters and construction of a predictor through the application of a supervised learning approach combining regression and classification (Random Forest) and creation of a mathematical predictor of the embryo development profiles. A total of 172 embryos were observed. T1: 6 morphokinetical profiles were retained: Arrested Embryos (AE: embryos without mitotic activity, showing signs of life); Dead Embryos (DE: embryos with all cells dead); Anarchic Embryos (ANE: embryos with abnormal morphological and/or kinetical development: some of these embryos can result in a blastocyst); Not Hatched Blastocysts (NHB: blastocysts not hatching by 8 dpi); Hatching Blastocysts (HB - blastocysts hatching *in vitro* from 7.3 dpi to 8 dpi) and Early Hatching Blastocysts (EHB - blastocysts hatching from 6 to 7.2 dpi). T2: a guideline was built to standardise reading of 116 parameters (i.e.: type, timing and duration of cell divisions and embryo cycles, LAG phase, cell degeneration, cytoplasmic particles, fragments, vacuoles,...); T3: a subset of parameters was selected and the mathematical predictor was built. The standardisation of the reading methodology is important to promote scientific exchange and study comparisons on the subject (to our knowledge this work resulted into the first morphokinetics reading guideline for the bovine PIV embryos). In addition, this initial work highlighted a new concept for the *in vitro* bovine embryo assessment and further valorisation: it takes into account the very early embryo's dynamic behaviour to predict its further potential of development. The robustness of the algorythm is satisfactory. The specificity, sensitivity, PPV, NPV of the predictive algorithm range respectively in the intervals [0,944; 0,977], [0,640; 0,962], [0,724; 0,892], [0,931; 0,992] for the different profiles. This predictive method can be useful in the field to select embryos for transfer and for research (groups of embryos sharing potential and morphokinetic similarities).

Enriched n-3 polyunsaturated fatty acid diet modified oocyte lipid composition and may influence oocyte quality in Prim Holstein dairy cows

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Keywords: n-3 PUFA, oocyte lipids, dairy cows

Administration of long chain n-3 polyunsaturated fatty acid (n-3 PUFA) diet to dairy cows may impact oocyte quality (Elis et al, Animal Reproduction Science 164:121, 2016). Addition of docosahexaenoic acid (C22:6 n-3) during IVM led to higher blastocyst rate after IVF (Oseikria et al, Theriogenology 85(9):1625, 2016) and significantly changed oocyte lipid content (Elis et al, J Ovarian Res 10(1):74, 2017).
The present objective was to compare lipid content of the oocytes from the dairy cows supplemented with n-3 or n-6 PUFA-enriched diet.
Oocyte-cumulus complexes were aspirated by OPU after hormonal ovarian stimulation, from 18 primiparous Holstein dairy cows after 3 or 9 weeks of supplementation with 1% dry matter of either n-3 PUFAs (n=9, micro encapsulated fish oil, OMG750®) or n-6 PUFA (n=9, micro encapsulated soy oil, OMG Soy®) (Kemin). N-3 PUFA level in plasma and follicular fluid was measured after 2, 5 and 7 weeks of supplementation. Immature oocytes from n-3 and n-6 diet groups (60 and 61 oocytes, respectively) were denuded from CC and analyzed individually using an UltrafleXtreme MALDI-TOF/TOF instrument in positive reflector mode, with DHAP matrix. Lipid spectral profiles (3000 shots per spectra) were acquired for each oocyte. M/z peaks were detected in the range of 160 to 1000 m/z. Values of the normalized peak heights (NPH) were quantified and compared between the two groups by t-test with Benjamini-Hochberg correction. Multivariate Principal Component Analysis (PCA) was performed using differential NPHs. Lipids were identified by high-resolution mass spectrometry LC-MS or by direct infusion combined to top-down MS/MS analyses, and annotated according to Lipid maps database.
Concentration of eicosapentaenoic acid (C20:5 n-3) and total n-3 PUFA significantly increased in n-3 group, after 2 weeks of diet in plasma, and after 5 weeks in follicular fluid, as compared to n-6 group. Body weight and milk production did not differ. Lipid profiles of the oocytes showed significant difference between n-3 and n-6 diets (97 up-regulated and 91 down-regulated peaks, $p<0.05$, fold change >2). PCA allowed clear discrimination of n-3 and n-6 groups. 40 differential peaks were identified (496-827 m/z); among them 12 phosphatidylcholines (PC), 3 phosphatidylethanolamines (PE, C36), 2 sphingomyelins (SM, C35) and lyso-phosphatidylcholine LPC 22:4 were more abundant in n-3 oocytes, whereas 14 PC, PE 30:0, SM 34:1, two LPC (16:0 and 18:0) and two triglycerides (46:1, 47:1) were more abundant in n-6 group. These variations indicated profound changes in composition of several lipid classes from oocyte membrane and intracellular pool, occurring after only few weeks of n-3 or n-6 PUFA dietary supplement. These cellular lipid changes may influence oocyte capacity to develop better blastocysts after IVF in n-3 supplemented cows (see Elis et al, AETE 2018), and highlight the importance of identifying beneficial oocyte lipid profile to improve embryo biotechnologies issues.

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Effect of seminal plasma proteins from stallions of proven fertility on frozen epididymal sperm

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Keywords: Seminal plasma, stallion, epididymal sperm

The incubation of epididymal sperm with stallion seminal plasma could help to identify the components of the seminal plasma responsible for a beneficial effect or not on cryopreservation process of sperm. Our previous study showed that the preincubation of epididymal sperm with seminal plasma obtained from three different stallions (A, F, O) affected post-thaw sperm parameters differently. While two of them (F, O) improved post-thaw sperm viability and acrosome status of epididymal sperm, the other one (A) reduced these parameters compared to control. The aim was to determine the components of seminal plasma that could be involved in the improvement of the sperm parameters. Seminal samples from these stallions were evaluated using 2D-DIGE and liquid chromatography-mass spectrometry (LC-MS/MS) to discover the proteins involved in protecting spermatozoa during freezing. To our knowledge, this is the first report in the literature that compare the seminal plasma protein composition of several stallions. Seminal plasma was labeled using the kit AmershamCyDye DIGE Fluors (General Electric Healthcare España S. A., Madrid, Spain, ref. 25-8010-65) for two dimensional-fluorescence difference gel electrophoresis (2D DIGE). In each 2D gel, two samples were compared. Cy3 and Cy5 fluors were used for labeling each sample (A, F and O) separately, whereas Cy2 fluor labeled a mixture of both (A, F and O) and was used as normalization reference. Data were analyzed by one-way ANOVA followed by Dunnett´s multiple comparison tests ($P< 0.05$). Eight differentially expressed proteins were identified in seminal plasma samples. Four of them showed a higher expression in F and O samples which improved the frozen-thawed epididymal sperm parameters: 1) Cysteine-rich secreted protein 3; 2) Seminal plasma protein A3-like that belongs to the same family of Cysteine-rich secreted protein 3; 3) Thrombospondin type-1 domain-containing protein 7B; and 4) Seminal plasma protein 1 ($P< 0.05$). On the other hand, the other four proteins showed a higher expression in sample A that reduced the seminal parameters: 5) Polymeric immunoglobulin receptor, 6) Protein serum albumin precursor, 7) Kallikrein-1E2 precursor and 8) Matrix-remodeling-associated protein 5 ($P< 0.05$). Our results showed that some proteins from F and O seminal plasma were previously related to freezability (Cysteine-rich secreted protein 3 and seminal plasma protein A3-like), whilst A seminal plasma proteins (Polymeric immunoglobulin receptor, Matrix-remodeling-associated protein 5 and Kallikrein-1E2 precursor) suggest some alteration in the male genital tract. However, further in-depth research is needed to know what proportion of proteins, related to freezing, should be present in seminal plasma to be considered as a good freezability.

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Workshop I: Sanitary and regulations on embryo transfer

IETS and HASAC: the genesis of the World Organization for Animal Health (OIE) recommendations for safe trade of embryos

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Keywords:

The International Society for Embryo Technologies (IETS) was created in 1974. To meet the needs for specific regulation in terms of health safety, IETS created an Import & Export committee, chaired by R Mapletoft, in the early 80's. A formal relationship between IETS and the World Organization for Animal Health (OIE) was then set up in 1987. With the development of new embryo technologies and the concern about the safety of animals produced with such technologies entering the food chain, the scope of the committee was expanded in 2000 under the initiative of M Thibier so as to include those issues, resulting in the current IETS Health and Safety Advisory Committee (HASAC, https://www.iets.org/comm_hasac.asp). HASAC functions to advise the IETS Board members, IETS members and OIE. HASAC is composed of IETS members from academic, regulatory and industry sectors, under the direction of a chair nominated by the IETS Board of Governors (BoG) (current chair: Julie Gard, succeeding to F Fieni, P Chavatte-Palmer and M Thibier).
Its aims are:
- to review regularly and extensively the literature in order to evaluate potential risks, based on scientific evidence, with regard to international trade of embryos and human consumption of animal or animal products derived from embryo transfer and related technologies.
- to provide guidelines such as codes of practice, recommendations and any information pertinent to the safe movement of embryos and safe introduction into the food chain of animal or animal products derived from reproductive biotechnologies without unduly restricting technological advances and commerce.
- to communicate to IETS members all its achievements and upon acceptance by IETS BoG, to all the relevant International Agencies.
Three HASAC subcommittees meet annually but work electronically throughout the year:
1. The Research subcommittee maintains and revises literature relevant to the animal health implications of current and emerging technologies. A large database, including an abstract, HASAC interpretation and conclusions on all publications examined, is updated each year. Based on these yearly evaluations, science-based recommendations are then handed for evaluation to the Regulatory subcommittee.
2. The Regulatory subcommittee examines new evidence provided by the scientific subcommittee and discusses whether current recommendations for the management of the risks potentially associated with embryos in terms of pathogen-embryo interactions should be modified. Any proposed modifications to alter text with regards to regulations and/or OIE recommendations are put forward to the BoG and, if approved, subsequently formally proposed to the OIE for incorporation into the Terrestrial Animal health Code. Commission members of OIE most often support the proposed amendments from IETS/HASAC before finally being voted on, and mostly accepted, by the OIE General Assembly.
3. The Manual, Forms and Certificates subcommittee develops, elaborates and publishes a code of practice providing guidelines to practitioners for managing their operations so that pertinent quality assurance standards will be met. Additionally, this committee creates and maintains recording and identification systems of embryos to ensure traceability.

Viral emergences and consequences for the risk of disease transmission via *in vivo* derived embryos

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Keywords:

Viruses can emerge unexpectedly in different regions of the world. These can induce negative consequences on reproductive performances. This paper presents an update on emerging diseases that may be of primary concern to ruminants and equids and whose impact should therefore be monitored in the context of the European embryo trade. Development of artificial insemination (AI) together with embryo cryopreservation has led to international trade of cattle germplasm for more than 60 years. Although experimental data show that many animal pathogens can be associated with semen and embryos, risk of disease transmission can be substantially reduced or eliminated by applying sanitary protocols recommended by the International Embryo Transfer Society (IETS) and the World Organization of Animal Health (OIE). The basic principle to ensure such a high level of biosecurity for semen relies on the concept of pathogen-free semen collection center. In the case of embryos, practical guidelines have been published in the manual of IETS in order to provide risk management procedures ensuring the safety of herds using embryo transfer, and embryo washing procedures which are the most effective means of reducing the number of microorganisms associated with germplasm. Although transfer of bovine embryos is much less likely to result in disease transmission than transport of live animal, the sanitary risk associated with bovine embryo transfer remains the subject of scientific investigations and adaptations of national and international legislations (OIE). Concerning transmission risk via ET, the IETS HASAC Committee reviews scientific publications on an annual basis and updates a complete set of more than 400 references, which can be consulted on their website (www.iets.org). All diseases and pathogenic agents have been placed into one of four categories (category 1 to 4) based on the amount of research indicating the likelihood of disease control through the use of embryo transfer. For category 1 diseases, risk of transmission of a given disease from donor to recipient via an embryo is negligible, providing biosecurity measures described for handling embryos, material disinfection, and animal health requirements (semen, donor, and recipients). For category 4, no conclusions are yet possible with regard to the level of transmission risk, or the risk of transmission via embryo transfer might not be negligible even if the embryos are properly handled according to the IETS Manual between collection and transfer. This paper will present the epidemiological situation in Europe of (re)emerging viral diseases affecting ruminants and belonging to the different categories: Bluetongue (cat 1, 2, 4), Schmallenberg (cat 4), Lumpy Skin Disease (cat 4) and Foot-and-Mouth Disease (cat 1, 3) but also on some diseases affecting the equids: West Nile and Equine infectious anemia.

The constrains for a registered Embryo Transfer Team concerning bovine embryo importation and exportation

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Keywords:

The goal of international embryo trading is to allow safe exchanges of genes. That's why they are only performed between registered Embryo Transfer Teams. Their responsibility is to guarantee health authorities and breeders that the embryos don't carry contaminant pathogens and that the filiation of the calves born will be certified. This part of the workshop presents the practical, economic and administrative constrains in a French context as an example of the European one. All these constrains increase the cost of the exchanges, above all with no European Union countries. Consequently they limit their development. Practical constrains are not very difficult as they consist by following IETS recommendations. Administrative constrains are inevitable since they are verified during annual controls for the team's approval. Some other ones could be minimize as, for example, health requirement for export outside European Union. Indeed each country has its own sanitary specifications even for the same disease. Thus, a huge progress will be noticed if all countries could follow the same OIE recommendations! Meanwhile a specific production for each country must be done. It is then more important to select good donors to maximise the chances and having sufficient number of embryos to limit health tests expenses.

Consequences of sanitary issues (diseases outbreaks) on bovine semen exportations from Europe to third countries: history and current situation

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Keywords:

Today, most of European Members States (MS) involved in bovine genetic or genomic selection are also trading germinal products (GP) all over the world. Exchanging semen and embryos is easier and safer than dealing with live animals and can provide incomes allowing to keep the cost of selection schemes more reasonable and to manage semen stocks. It allows customers to get access to a wider range of sires and provides sanitary guarantees since GP are produced according to high sanitary regulation standards. However, commercial competition with traditional exporting countries such as USA, Canada is very tough. Main criteria taken into account for buying are genetic merit of the sires, biological quality of the products (fertility) and their sanitary safety brought through the respect of EU Directives (88/407, 43/2003), of OIE recommandations and of IETS procedures.
Despite the full respect of the regulation, MS had to face various and unexpected emergent diseases during the last 20 years such as Foot and Mouth Disease (FMD) during 2001, Blue Tongue Virus (BTV 1 and BTV8) in 2006/2007 and then again in 2015, Schmallenberg disease (SBV) in 2012 and for France Blue Tongue again (BTV4) in 2017. These different outbreaks were linked to drops in volumes of semen sold from MS to third countries. (Eurostat: http://epp.eurostat.ec.europa.eu/newxtweb/). Surprisingly, this negative impact was rather moderate and short in time even if some third countries took benefit of the situation to stop importations from EU MS. This is still the case of USA, Canada, China and Mercosur who deny the reliability of the RT-PCR test to detect the presence of viral RNA in semen of seropositive bulls. This could be considered as a technical barrier to trade.
The positive impact of such outbreaks was to prompt MS to find common solutions to carry on exporting GP: health certificates were renegotiated with most of third countries and adapted, SBV additional declarations were proposed and new diagnostic tests were developped. Cooperation between MS was reinforced and the role of the EU Commission and of OIE was strenghtened.
Regarding Blue Tongue, the experience of 2006 was useful to manage and cope with the new outbreaks of 2015 and 2017 in France.
Today, new sanitary threats are surrounding EU 28 MS such as Lumpy skin disease in eastern Europe and Peste des Petits Ruminants in Bulgaria. This means that we have to remain vigilant all the time and to anticipate such negative situations. To do so, MS have to take benefit of their previous experiences and to carry on working in a joint and constructive way.

Workshop II: Preservation of *in vitro* produced embryos

Cryopreservation of IVP bovine embryos

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Keywords: cryopreservation, *in vitro*-produced embryos, bovine

The number of bovine in vitro produced embryos (IVP) has increased year by year. In 2016, a total of 666,215 IVP embryos were produced, exceeding for the first time, the volume of embryos generated in vivo (Perry G. Embryo Technology Newsletter, 35:1-46, 2017). Also, 121,490 frozen embryos and 326,623 fresh embryos were transferred. In the same year, 60,723 frozen embryos were transferred from the OPU-IVP bovine embryo in Brazil, while in the USA this number was 48,533. In this context, the total embryo production is sometimes higher than the number of embryos transferred, so the cryopreservation methods are a good alternative. However, differences have been reported regarding the freezability of Bos taurus vs Bos indicus IVP embryos. Studies have reported quantitatively higher lipid content rates in the *indicus* embryos rather than taurine counterparts. Despite the IVP advantages, cryopreservation represents a challenge for commercial laboratories and adaptations are necessary for each practice. The cryopreservation technique predominantly used for IVP embryos is vitrification. This ultra-rapid freezing process reduces cell damage caused by the formation of ice crystals but requires a high concentration of cryoprotectants. Moreover, it is necessary to be trained to perform a morphological evaluation of embryo quality before the loading process. Thereby, the process of thawing and the direct transfer (DT) of embryos to cows make the slow freezing protocol - method previously described for in vivo embryos - more efficient for IVP commercial use. The low concentrations of cryoprotectants are the main advantage of this technique since high concentrations are toxic to embryos. Also, the DT strategy has recently been performed by commercial laboratories, providing good embryo viability after thawing. A study conducted in Brazil with female Girolando donors (1/2 Gir and 1/2 Holstein) (Sanches, B.V. Theriogenology, 85: 1147-1151, 2016), compared pregnancy rates for fresh, vitrified or frozen IVP embryos. The conception rates obtained were 51.35 ± 1.87% (133/259) for the fresh embryos, 35.89 ± 3.87% (84/234) for the vitrified embryos and 40.19 ± 4.65% (125/311) for the frozen directly transferred embryos. These data showed that IVP embryos with sexed semen could be directly transferred with similar conception rates to vitrified embryos. Thus, by facilitating logistics, this technique may become a more practical approach for the transfer of cryopreserved embryos in the field. Due to the promising results, the DT strategy has been applied in large-scale operations, mostly in the US and Brazil. Finally, shortly, while other companies will start using DT, it is likely to reach the point where most IVP embryos will be frozen, as is the case of semen industry.

The challenge of vitrifying in vitro-produced porcine embryos

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Keywords: vitrification, porcine embryo, in vitro-produced

Significant progress in pig embryo cryopreservation has been achieved since the development of vitrification as an alternative to slow-freezing, which has resulted ineffective in this species. Currently, high in vitro survival rates and promising reproductive performance after ET can be reached with vitrified in vivo-derived porcine morulae and blastocysts. Although piglets have been obtained from vitrified in vitro-produced (IVP) embryos, their vitrification ability is still far behind that of their in vivo-derived counterparts. The development of successful vitrification procedures for IVP porcine embryos would be relevant not only for the livestock industry, but also for banking genetically modified embryos from swine models of human diseases, which are of great importance for biomedical research. It is well known that some factors affect vitrification outcomes regardless the origin of the embryo (in vivo or in vitro) such as the embryonic stage, the concentration and type of cryoprotectants, the vitrification device or the equilibration temperatures. However, two main aspects make the IVP embryos particularly sensitive to vitrification: A higher lipid content and a much poorer quality compared to in vivo-derived ones. Several studies have focused on the importance of embryo lipid content and its relation to vitrification tolerance. In this regard, piglets have been obtained after transfer of parthenogenetic and IVM/IVF embryos subjected to delipidation by micromanipulation. However, protocols that involve zona pellucida disruption should be avoided for sanitary reasons. Thus, stimulation of lipolysis with chemicals agents such as forskolin, has been proposed as an interesting and efficient tool to decrease the lipid content that hinder vitrification, while maintaining the zona pellucida integrity. With respect to the quality of embryos, many supplements have been added during in vitro embryo production in order to improve embryo quality and therefore the embryo vitrificability. Among them, those compounds protecting IVP embryos against oxidative stress during in vitro culturing and vitrification-warming procedures, such as L-ascorbic acid, seem to be key to improving vitrification efficiency. Also, the length of the in vitro culture, which is related to the embryonic stage, influences the quality of IVP embryos and, consequently, the vitrification efficiency. Finally, the high incidence of polyspermic penetrations in porcine IVF, which is still an unsolved problem, is a factor that should be taken into account. In this respect, the selection of monospermic embryos would be advisable when vitrification is to be performed. In this workshop we will discuss all these aspects related to the vitrification of IVP porcine embryos based on our results and also on those from other researchers working in this field.

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Effects of (cryo)preservation on the quality of in vitro produced embryos

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Keywords:

Numerous studies have shown in vitro produced embryos to differ from their in vivo produced counterparts in terms of timing of development and morphology, metabolism, cell number, gene expression patterns and also their chilling sensitivity and their ability to survive cryopreservation.
Cryopreservation of gametes and embryos is a crucial step for the widespread application and conservation of animal genetic resources. Differences between cryopreservation methods can be seen for the first time after cryopreservation in re-expansion and hatching rates. Therefore, comparisons between slow cooling and vitrification methodologies have mainly been drawn at a morphological level. However, the effect of cryopreservation on the quality of in vitro produced embryos is also clearly reflected at the molecular level. Over the last two decades it was possible to increase survival rates after thawing/warming by a multiple number of improvements. However, results for post-preservation survival vary immensely. Efforts to improve survival rates were mainly restricted to altering the IVC media or to modifications of the cryopreservation method itself. Additionally, an effect of the age and developmental stage of the embryo has been determined. As vitrification alters fewer transcripts than conventional cryopreservation, it seems to be the more favourable method for cryopreservation of embryos produced in a SOF-based culture system. Comparing vitrification media supplemented with or without DMSO, it was shown that DMSO-containing media result in an increased number of surviving bovine IVP embryos compared to DMSO-free media. Nevertheless, these embryos show a reduced quality at the molecular level. Due to lower pregnancy rates after transfer of biopsied and cryopreserved embryos especially when they are generated via in vitro production (IVP), an alternative preservation method also needs to be developed. Liquid preservation employing FBS might fulfill the requirements as shown recently.
Despite all the refinements, fundamental research is still needed to improve the results mainly with in vitro produced embryos, especially after biopsy, and oocytes.

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