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Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

28ème COLLOQUE SCIENTIFIQUE

28th SCIENTIFIC MEETING

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Dr. Danielle MONNIAUX

Special Celebration

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Saint Malo, France, 7th and 8th September 2012

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A TRIBUTE TO DANIELLE

When I joined the research unit at Nouzilly in 1987, the Division head at that time, Michel Courot, spoke to me of Danielle Monniaux with whom I was to work: « You haven't landed with one of the worst ». I agree: Several months later I learned that my application was preferred over that of a woman because (this time I'm repeating what the division head said to me): "There are already too many women working here". It is important to remember that Danielle was of course the only woman working in the team at that time …

Danielle Monniaux was a graduate of ENS (Ecole Normale Supérieure), who began her career in the 1980s after having successfully succeeded the Biology "aggregation", a high level exam required for teaching and a short period during which she taught in Africa. The subject of Danielle's research was the variability of the response of cows to superovulation treatment. This variability then and still today hinders research and development programs that implicate massive production of embryos, with some poor donating females being "lost" for the scientist. Through very simple but rigorous experimentation, Danielle was able to show that the response to such a treatment was very dependent on the population of growing follicles, which itself is variable amongst individuals. This result, summarized very briefly here, was published in Theriogenology and was cited more than 250 times (source: Isiweb of knowledge, April 2012), that is approximately ten times the number of average citations for a scientific article! Therefore underlining the importance of the result! This work has been reproduced many times in other breeds and species since. Several years later, Danielle returned to the subject following the invalidation of the AMH gene in the mouse, a gene known to end the blockage of ova and their massive recruitment. Since then, it has been shown that in this species but also in cows and women, AMH is produced by growing follicles and plays a major role in the management of the ovarian reservoir by having a negative retro-action on very small follicles. Everyone now knows the importance of Danielle's recent work showing that the variability of the follicular population in the cow is correlated to the variability of AMH concentration in the blood. Measurement of bovine AMH, developed by Danielle and her team, are now predictive tests for the cow's and heifer's response to superovulation treatment. All these results and this measurement are major advances in the field of reproduction control of our livestock species. Danielle's career hasn't

ended yet – she still must figure out how the key actors manage ova stock and their recruitment.

During this whole period, Danielle Monniaux dedicated herself to the training of several doctoral students: Flora Rabahi, on the follicle/oocyte during the peri-ovulating period; myself on the IGF ovary system; Clotilde Huet and Frédérique Lebellego, on the role of the extracellular matrix; Alice Pierre on the role of the BMP elements in the ovary; Charlène Rico and Anthony Estienne on the role of AMH. In all, she has more than 60 publications referenced in PubMed and a patent that has been deposited along with many requests for talks at congresses.

In addition to her personal studies, Danielle Monniaux during her whole career has helped many scientists and students, in other fields of studies, some which are cited as doctoral students for which she was a co-advisor and others that I will not cite here. She has also been very active in mathematical modeling, which is almost her second field of specialization. In fact, she had a very rich and successful partnership with Frédérique Clément from INRIA. And of course we cannot forget the help that Danielle regularly provides in statistics, but also lines of commands that she has written for scientists and students in her team so that they can use software for image analysis.

In short, here is a summary of a little over 30 years of research and success.

During this period, Danielle has also completed many administrative and scientific tasks including being head of the "ovary" team then directing the research unit to which she belongs, the Joint Research Physiology of Reproduction and Behaviour Unit, including approximately 160 agents. One of INRA's largest research units … During this period which lasted five years, Danielle was extremely efficient while showing great "diplomatic" qualities despite work that was not always interesting and required confrontation with people not as conscientious as she. It is important to underline that not many researchers return to science after having spent so much time doing administrative tasks. Her return to the lab bench, with the success that we know concerned AMH, has been particularly stunning. Besides this very difficult administrative task, Danielle has continued participating in technical, scientific and personnel coordination of the PRC dosage laboratory and regularly accepts to evaluate research projects that are sent to the National Research Agency (ANR).

I cannot finish this without underlining her human qualities, known to all: her modesty, her kindness which accompanies her rigour and clarity. I have never seen Danielle be surprised or trapped by an error of logic whatsoever. And when Danielle is in a meeting, if she hears an approximate reasoning or a question that is poorly formulated, which would push open a door if a method or another is followed (which often happens in labs), I enjoy observing her and listening to her when she explains to someone that their intellectual pathway is not adequate, while being as careful as possible not to hurt the person. She has a great, well-balanced style, which allows criticizes, because research makes progress only like that, but without the latter being sources of frustration.

I would like to say that for the longest time, Danielle worked part-time (80%) since on Wednesdays, she took care of her three children. One of her students, Clotilde, recently told me that she was amazed at how Danielle led such a double life. She imagined Danielle leaving the office on Tuesday nights and spending her Wednesdays in the kitchen preparing meals, doing laundry and driving her kids all over town to their activities. On Thursdays, Danielle would hand her an article that had been read and revised along with the several lines of the program necessary to debugging a problem for image analysis. And always smiling and

being just as kind. For Clotilde, Danielle is a Wonder Woman! A Wonder Woman who now spends more and more of her time doing one of her favorite hobbies, music and piano that she has continuously practiced and her more recent role as a grandmother.

I will not close this tribute without forgetting to mention a woman who worked with Danielle for nearly 20 years and who cosigned almost half of her articles, our dearest Claudine Pisselet. This tribute is also a little bit hers; I know that Danielle will be honored to share it with her.

Philippe Monget
INRA AGENAE, France

SUPEROVULATORY RESPONSES AND EMBRYO PRODUCTION IN RUMINANTS: LESSONS FROM OVARY

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In memory of Charles Thibault (1919-2003) and Pierre Mauléon (1926-2004), two French scientists who were pioneers in the field of ovarian function

Introduction

In the seventies and the early eighties, the development of embryo production and transfer in cattle was highly limited by the impossibility to be sure that a potential donor could provide a suitable number of good embryos at a given time. The major component of this problem was the large variation between and within individuals in their ovulatory response to gonadotrophin treatments. As suggested (Gordon *et al.* 1962; Saumande *et al.* 1978; Guay and Bedoya 1981), individual variation in ovulatory responses could be attributed to different parameters, i.e. the choice of the superovulatory treatment applied to the animals, the method of estimation of the ovarian response and the different responsiveness of animals to a given treatment.

Concerning the superovulatory treatments, eCG and FSH/LH from different hormonal preparations were available at this time. The question of the differences in their efficiencies for ovarian stimulation was the subject of controversy (Eldsen *et al.* 1978; Schams *et al.* 1978; Critser *et al.* 1980). Moreover, the doses to administrate and the schedule of treatments with FSH/LH preparations remained to be optimized for improving the mean number of good embryos recovered.

The method of estimation of the ovarian response was an important limiting factor in those days when ultrasonography did not permit to study ovarian follicular growth. The number of luteal or follicular structures on the superovulated ovaries was generally estimated using rectal palpation, and less frequently endoscopy or direct observation of the ovaries. Comparisons of the three techniques showed large discrepancies in the results, especially those obtained by rectal palpation (Guay and Bedoya 1981; Heyman and Chesne 1983). Interestingly, the analysis by ovarian ultrasonography of the large follicles present on the ovaries at the beginning of oestrus was proposed as a new promising method of prediction of ovulation rate after eCG (Chupin and Procureur 1983) but the technique was still in its infancy. Alternatively, interesting attempts were made to use steroid concentrations measured in plasma or milk before and/or after ovulation for estimating and predicting the ovarian responses (Lemon and Saumande 1972; Testart *et al.* 1977; Saumande 1980; Schilling *et al.* 1981). Estimation of the ovulation rate by plasma concentrations of progesterone after

ovulation was proposed, but the relationship with the number of corpora lutea was not sufficiently strong for an accurate prediction (Lamond and Gaddy 1972; Lemon and Saumande 1972; Sreenan *et al.* 1978; Saumande 1980). Also, prediction of the ovulation rate by steroid concentrations in plasma at the time of treatment was not possible (Schilling *et al.* 1981) and only oestradiol concentrations measured at the time of the preovulatory LH discharge before ovulation could be predictive (Saumande 1980).

The importance of the responsiveness of animals to a superovulatory treatment was investigated in a first time by trying to modify the status of the follicular populations present in ovaries before treatment. Results showed that prestimulation with eCG or cauterization of the largest follicles before treatment affected the follicular and ovulatory responses of cows to superovulation (Mariana 1969; Chupin and Saumande 1979). The question of whether the variability of ovulatory responses of cows could be related to the variability of ovarian status at the time of treatment was then asked (Saumande *et al.* 1980). The answers to this question are the subject of this text. I will consider both between- and within-individual variability, discuss about its origin and underlying physiological mechanisms and report the technical and scientific advances of the last 30 years which can help to improve its control and/or prediction.

1. Relationship between the ovulatory response to superovulation and the status of the ovarian follicular populations at the time of treatment

1.1. First evidence of relationship between follicle numbers before treatment and ovarian responses to superovulation

As said above, at the end of the seventies ultrasonography and endocrine methods were not sufficiently reliable for studying follicular growth and the only relevant method which was available at this time for ovarian studies was quantitative histology. This method was used in our laboratory to study the relationships between follicular populations at the time of eCG injection and ovulatory responses to this treatment in Friesian heifers (Monniaux *et al.* 1983). The experimental design was chosen such that each animal was its own control. One ovary was removed immediately before the other was stimulated by injecting eCG (2000 UI) and then removed just after ovulation.

From our results, the number of follicles larger than 70 µm diameter (follicles with more than 2 layers of granulosa cells) per ovary was found to be quite variable between animals: it varied between 53 and 755 in the "control" ovaries (recovered before eCG injection) and between 73 and 881 in the "stimulated" ovaries (recovered after ovulation) of the studied heifers. However, within each heifer the number of follicles in the two ovaries agreed closely (Monniaux *et al.* 1983). Further comparisons between the control and the stimulated ovary showed that eCG can increase the proliferation of granulosa cells (estimated by their mitotic index) in preantral and early antral follicles, stimulate the growth of the antrum in large antral follicles and reduce the number of the largest atretic ones, suggesting some "rescue" of antral follicles from atresia (Monniaux *et al.* 1984a).

An interesting finding was the existence of a close relationship between the total number of follicles in the ovary before treatment and the number of young corpora lutea (recent ovulations) plus luteinized unruptured follicles in the stimulated ovary ($r_s = 0.88$, Spearman rank correlation ; $p < 0.001$, **Figure 1**). Similar correlations were also observed

with different types of follicles in the control ovary (**Table 1**), but the number of ovulations alone was not correlated significantly with any kind of follicular variable measured before treatment. The relationship between the number of large antral atretic follicles before treatment and the number of luteinized follicles in the stimulated ovary suggested that some early atretic follicles cannot ovulate but only luteinize after eCG.

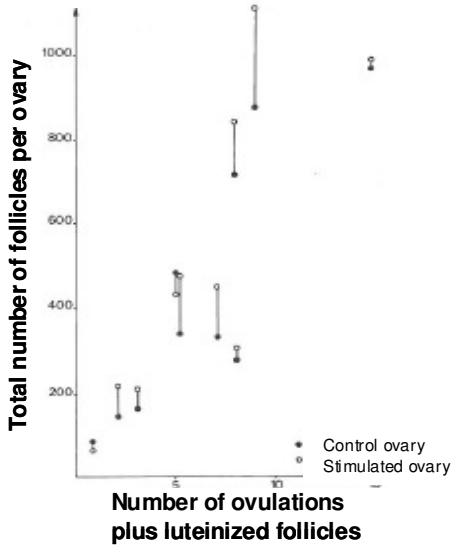

Figure 1: Relationship between total numbers of follicles per ovary and ovarian response to eCG (2000 UI) in heifers. For each heifer (n=10), the control ovary was recovered before eCG and the stimulated ovary after eCG treatment and ovulation. Each pair of black and open circles represents data from one animal. Adapted from Monniaux *et al.* 1983.

Ovarian response of the stimulated ovary	N° follicles on the control ovary					
	Preantral follicles		Antral follicles		Follicles > 0.5 mm	
	Normal	Atretic	Normal	Atretic	Normal	Atretic
N° ovulations	0.54	0.43	0.62	0.49	0.53	0.41
N° luteinized follicles	0.62	0.54	0.32	0.60	0.61	0.70*
N° ovulations and luteinized follicles	0.87***	0.81**	0.72*	0.87***	0.92***	0.83**

Table 1: Relationship between follicular populations before treatment and ovarian response to eCG in the heifer: Spearman rank correlation coefficients. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Adapted from Monniaux *et al.* 1983.

This study confirmed the existence of a large between-individual variability of follicular populations previously reported in cattle (Rajakoski 1960; Erickson 1966; Testart 1972; Mariana and Huy 1973; Scaramuzzi *et al.* 1980). Moreover, it showed for the first time that the variability of the ovarian status could explain a large part of the between-individual variability of the ovarian response to a superovulatory treatment. This result was later confirmed by other studies using histology or high-performance ovarian ultrasonography for the detection of antral follicles larger than 2mm diameter in cow (Kawamata 1994; Cushman *et al.* 1999; Taneja *et al.* 2000; Singh *et al.* 2004; Durocher *et al.* 2006; Ireland *et al.* 2007) and sheep ovaries (Mossa *et al.* 2007).

1.2. Between-individual variability in ovarian follicular populations and functional within-individual links

One of the main problems in embryo production is the existence of poorly responding animals, whatever the ovarian stimulatory treatment used. From our results described above, heifers with less than 200 healthy growing follicles at the time of stimulation are low-

responding animals and those with more than 500 of these follicles are high-responding animals.

The large numerical variability observed between ovaries recovered before treatment from animals of the same breed and age (18 months), with similar weights, raised in the same experimental station and studied on the same day of cycle (16th day) was intriguing. Further analyses on the ovarian populations of these heifers revealed the existence of strong intra-ovarian relationships (Monniaux *et al.* 1984b). Particularly, the total number of normal (or healthy) follicles per ovary was found to be tightly correlated to the total number of atretic follicles (rs = 0.95, **Figure 2**). This result indicates that the high differences in follicle numbers observed between heifers cannot be due to differences in atresia rates, but would be rather the consequence of different growth activation rates of primordial follicles. When different size classes of follicles were considered, significant positive correlations were found within and between classes for the numbers of normal follicles per class, the numbers of atretic ones, and the growth rate of the normal follicles, estimated by the mitotic index of their granulosa cells (**Figure 3**). Overall, these observations suggested that the heifers can differ importantly by their folliculogenesis activity. Schematically, animals with highly active ovaries are characterized by high numbers of normal and atretic follicles in all size classes and high-speed folliculogenesis. These highly active ovaries waste their ovarian reserve of primordial and growing follicles and interestingly they have a high capacity to superovulate in response to eCG. On the contrary, ovaries with a low folliculogenesis activity economize their ovarian reserve and respond poorly to eCG.

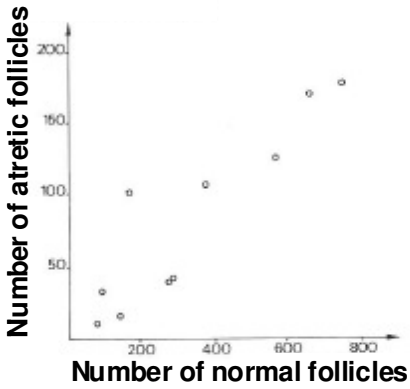

Figure 2: Relationship between the total number of normal and atretic follicles per ovary (control ovaries) in heifers (n=10) at Day 16 of the oestrus cycle. Each circle represents data from one animal. Adapted from Monniaux *et al.* 1984b.

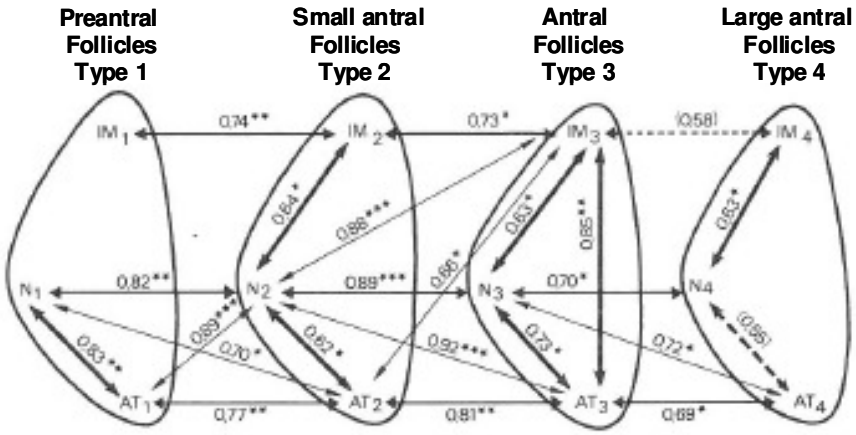

Figure 3: Rank coefficient correlations between the numbers of normal (N) and atretic follicles (AT) and the mitotic index (IM) of the granulosa cells of normal follicles, within and between 4 types of follicles: type 1 (preantral follicles 100-200 µm), type 2 (small antral follicles 200-300 µm), type 3 (antral follicles 500-1000 µm) and type 4 (large antral follicles > 1000 µm). Ovaries were recovered from heifers at Day 16 of the oestrous cycle. *: p < 0.05; **: p < 0.01; ***: p < 0.001. Adapted from Monniaux *et al.* 1984b.

These results were recently confirmed by comparing the total follicular populations of cattle selected for high differences in antral follicle count during follicular waves. Results show a high correlation between antral follicle count during follicular waves studied by ultrasonography and the numbers of healthy primordial, transitory, primary, secondary and small antral follicles studied by histology (Ireland *et al.* 2008). Overall, these findings and our previous results indicate that important differences in ovarian activity can exist between individuals, they concern the total population of growing follicles (numbers and growth rates) and they might be associated with numerical differences in the ovarian reserve of primordial follicles and their capacity to enter follicular growth. The mechanisms determining these differences between individuals remain unknown up to now and a genetic determinism is highly likely. It was recently proposed that maternal nutrition and disease during pregnancy have also a critical role in regulation of the high variation in the ovarian reserve of the offspring (Ireland *et al.* 2011).

Presently, the between-individual variability of ovarian folliculogenesis remains an important limit to control embryo production in ruminants. However, during the past 30 days, our progress in understanding the mechanisms regulating the follicular waves and the natural number of ovulations at each oestrous cycle (ovulation rate) helped to improve the ovarian responses to superovulatory treatments by reducing their within-individual variability.

2. Follicular waves, their regulatory mechanisms and their control for embryo production

2.1. General features of the follicular waves in ruminants

The existence of follicular waves throughout the oestrus cycle in cows was suspected as soon as 1960 by Rajakoski from histological studies (Rajakoski 1960). This hypothesis was then the subject of controversy (Choudary *et al.* 1968; Marion and Gier 1971; Swanson *et al.* 1972; Mariana and Huy 1973) until important methodological progress firmly established their existence. The progress concerned the parallel development of ovarian ultrasonography and sensitive hormonal assays for monitoring FSH, LH, steroid and inhibin variations with time. The combined use of these methods was the key to understand the endocrine dialogue between the follicular populations and the hypothalamo-pituitary complex.

It is now well established that antral follicles grow in cohorts, in two or three wave-like patterns during oestrous cycles in cows (Ireland and Roche 1982; Ireland and Roche 1983; Pierson and Ginther 1984; Savio *et al.* 1988; Sirois and Fortune 1988; Ginther *et al.* 1989b; Fortune 1994). During each wave, the synchronous growth of a cohort of 3- to 5-mm follicles is followed by the selection and development of a single dominant follicle and concomitant atresia of the remaining follicles in the original cohort. The dominant follicle which has reached an ovulatory size (15-20 mm) will either ovulate in a permissive hormonal environment or regress by atresia in the period of luteal progesterone secretion or during early pregnancy (Ginther *et al.* 1989a; Savio *et al.* 1990). The existence of follicular waves was also demonstrated in other species, particularly polycotous species in which more than one dominant follicle is observed, and extended to other reproductive periods (Mariana *et al.* 1991; Monniaux *et al.* 1997; Ireland *et al.* 2000; Evans 2003).

2.2. Selection of the ovulatory follicles: regulatory mechanisms

The finding that the emergence of the follicular waves is associated with small rises in FSH concentrations led to suggest that FSH could orchestrate the waves (Adams *et al.* 1992). Moreover, fluctuations in plasma oestradiol and inhibin concentrations were found to reflect the wave pattern and the dominant follicle was established to be directly responsible for these fluctuations (Martin *et al.* 1991; Guilbault *et al.* 1993).

Keys for understanding the mechanisms regulating the selection of the dominant, potentially ovulatory follicle(s), are the following ones: (1) There is a functional hierarchy among follicles of a wave with probably never two strictly identical follicles at the same time (2) The follicles of an emerging follicular wave are gonadotrophin-dependent and become atretic when FSH concentrations are below the threshold needed to sustain their development (3) FSH secretion is controlled by negative feedback at the pituitary level through a synergistic interaction between inhibin and oestradiol secreted by the largest follicle(s) of the wave (4) Vascularization develops strikingly during the last stages of follicular development, allowing an increased supply of hormones, particularly gonadotrophins, to the largest follicles (5) Acquisition of LH responsiveness by granulosa cells of the largest follicles will allow these follicles to become gradually less dependent on fluctuations of FSH. By these mechanisms, small functional pre-existing differences among follicles of a wave could be accentuated by the hormonal environment, resulting in selection of one or several dominant follicle(s) and atresia of the remaining follicles of the wave. The concept was born that only the follicles containing granulosa cells in the right state, according to the endocrine and paracrine environment (the "right" follicles in the "right" environment) are able to continue development until ovulation (Monniaux *et al.* 1997). These mechanisms were recently reviewed in ruminants (Scaramuzzi *et al.* 2011).

Our laboratory has participated to these scientific advances in three directions. The first one concerns the intra-follicular regulatory mechanisms that contribute to establish a functional hierarchy among follicles. The second one concerns some mechanisms regulating the number of ovulations in different ovine breeds and genotypes. The third one is the development of a mathematical model to study the selection of the ovulatory follicle(s).

2.2.a. Intra-follicular regulations: the example of the insulin-like growth factor (IGF) system

The concept that numerous intra-follicular factors, particularly growth factors, importantly modulate gonadotrophin actions on follicular cells has been widely accepted. Among growth factors, the IGF system appears to be particularly important for follicular development. In sheep, we showed that IGF-I can stimulate either the proliferation or the differentiation of granulosa cells *in vitro*, depending on the stage of development of the follicle (Monniaux and Pisselet 1992). *In vivo*, most IGF-I in follicular fluid is derived from blood, and intra-follicular IGF activity was shown to be regulated locally by IGF binding proteins (IGFBPs) (Monget *et al.* 1989; Monget *et al.* 1993). It is now established that follicular growth is accompanied by a decrease in free IGF-binding activity and concentrations of IGFBP-2, -4 and -5 in follicular fluid, whereas atresia is characterized by marked increases in these IGFBPs (Monget and Monniaux 1995; Monniaux *et al.* 1997). These variations result from both changes in expression of these IGFBPs by follicular cells and in local degradation by gonadotrophin-induced specific proteases (Besnard *et al.* 1996a;

Besnard *et al.* 1996b). The pregnancy-associated plasma protein-A (PAPP-A) was identified as the main protease responsible for IGFBP-2, -4 and -5 degradation in preovulatory follicles of different domestic species (Mazerbourg *et al.* 2001; Mazerbourg *et al.* 2003).

Interestingly, we showed that the IGFBPs present in follicular fluid can inhibit the proliferative effect of IGF-I on sheep granulosa cells by a mechanism involving specific trapping of IGF-I, preventing it from binding to its receptor (**Figure 4**) (Monget *et al.* 1993).

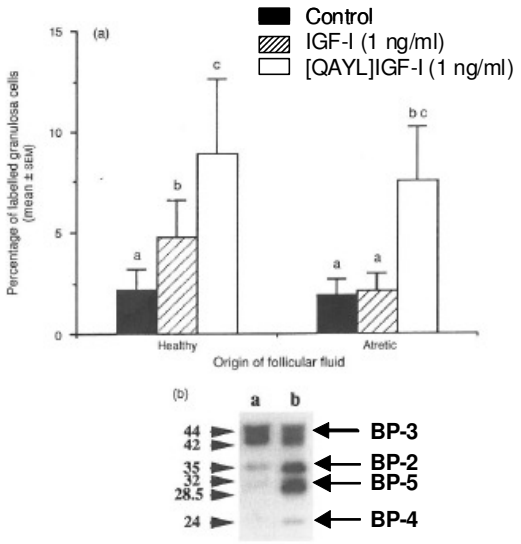

Figure 4: (a) Effect of IGF-I and an IGF-I analogue on ovine granulosa cells in the presence of follicular fluid from normal or atretic follicles. Granulosa cells were cultured with 1% follicular fluid from either normal or atretic follicles in the presence of IGF-I or an IGF-I analogue ([QAYL]IGF-I), which has a low affinity for IGFBPs. After culture for 24h, cells were incubated with [3H]thymidine for 2h, and then treated for autoradiography. Data represent the percentage of labelled cells. Columns with different letters are significantly different. (b) Patterns of IGFBPs in follicular fluid from normal (lane a) and atretic (lane b) follicles were visualized by western-ligand blotting. The bands corresponding to IGFBP-3 and to IGFBP-2, -4 and -5 (IGFBPs < 40 kDa) are indicated on the blot. Adapted from Monget and Monniaux 1995.

It was concluded that during the development of follicles in a wave, the decrease in intra-follicular concentrations of IGFBPs is responsible for an increase in IGF bioavailability, enhancing the efficiency of gonadotrophins in the largest follicles. When administrated at low doses for two days after follicular wave emergence, FSH can induce the selection two co-dominant follicles with high PAPP-A activity, low IGFBP-4 and oestradiol intra-follicular concentrations (Rivera and Fortune 2001; Rivera and Fortune 2003). In conclusion, changes in the IGF system are thought to be essential for follicular selection and development until the preovulatory stage.

2.2.b. Mono- and poly-ovulation: control of ovulation number by the Bone Morphogenetic Protein (BMP) system

In sheep, genetic studies have indicated that the natural ovulation rate can be regulated either by a set of different genes each having a small effect as in the Romanov breed (Ricordeau *et al.* 1990), or alternatively by the action of single genes with major effect, named fecundity (*Fec*) genes (Davis 2005). Different genetic models of poly-ovulation in sheep have shown that elements of the BMP system play a key role in regulation of the ovulation rate. The first mutations related to poly-ovulation were identified in Inverdale and Hanna New Zealander sheep in the *FecX* gene, carried by the X chromosome and coding for the BMP15 protein (Galloway *et al.* 2000). More recently, other mutations in this gene and the closely related *GDF9* gene have been described in different ovine breeds such as Belclare, Cambridge, Lacaune, Thoka, Santa Ines and Rasa Aragonesa (Fabre *et al.* 2006; Bodin *et al.*

2007; Nicol *et al.* 2009; Lahoz *et al.* 2011; Silva *et al.* 2011). These mutations are responsible for very similar phenotypes, i.e. increased fecundity in heterozygous ewes and infertility in homozygotes.

Our laboratory has been particularly involved in the discovery of the $FecB^B$ mutation responsible for the hyper-prolific phenotype of the Booroola ewes. Three independent groups published simultaneously this mutation, present in the coding sequence of the BMP receptor gene named *BMPR1B*, also known as Activin-like kinase receptor 6 (*Alk6*) (Mulsant *et al.* 2001; Souza *et al.* 2001; Wilson *et al.* 2001). The $FecB^B$ mutation was later reported in other prolific breeds such as Garole, Indian Javanese, Chinese Hu and Small-tailed Han (reviewed by Fabre *et al.* 2006). Homozygous and heterozygous $FecB^B$ carrier ewes are characterized by an increase in ovulation rate, associated with a precocious maturation of a large number of antral follicles which ovulate at a smaller size than non-carrier follicles (Henderson *et al.* 1985; McNatty *et al.* 1986a; McNatty *et al.* 1986b; McNatty and Henderson 1987).

The three fecundity genes are expressed in ovary and interestingly, oocytes are the major sources of GDF9 and BMP15 (Juengel *et al.* 2002a). These findings have led to a new paradigm in reproductive biology, namely that the oocyte can play a key role in regulating the ovulation rate (McNatty *et al.* 2005).

How mutations in these genes affect the ovulation rate remains not fully understood. Clearly, they impair the production of the biologically active ligands BMP15 or GDF9 (Galloway *et al.* 2000; Bodin *et al.* 2007) or the responsiveness of BMPR1B to BMPs (Mulsant *et al.* 2001; Fabre *et al.* 2003). It is suggested that mutations in *Fec* genes impair the proliferative action of BMPs in growing follicles and lower the BMP inhibiting action on the FSH pathway in granulosa cells, enabling a higher FSH sensitivity and an earlier expression of LH receptors (McNatty and Henderson 1987). The increased sensitivity to gonadotrophins of these follicles can promote their selection and maintenance when plasma concentration of FSH is decreasing in response to increasing concentrations of oestradiol and inhibin. The smaller mature follicles present in *Fec* gene carrier ewes each produce lower amounts of oestradiol and inhibin, but altogether they produce the same amounts than one larger wild-type follicle. As a consequence, the same endocrine dialog can establish between the ovaries and the hypothalamus-pituitary complex of both carriers and non-carriers of mutated *Fec* genes, leading to the selection and ovulation of numerous smaller follicles in mutated *Fec* gene carrier ewes (**Figure 5**).

There is no evidence for a direct link between the BMP and the IGF systems in regulation of the ovulation rate. From our data, the early decrease in intra-follicular IGFBP-2, -4 and -5 concentrations observed in $FecB^B$ carrier antral follicles accompanies their precocious maturation; it leads to an early increase in IGF bioavailability, which may at least partly account for the increased ovulation rate that characterizes the $FecB^B$ carrier ewes (Monniaux *et al.* 2000). However, to our knowledge, no mutation in the IGF system was found to be associated with an increase in ovulation rate, suggesting that the IGF system itself is not a key system for the regulation of ovulation rate.

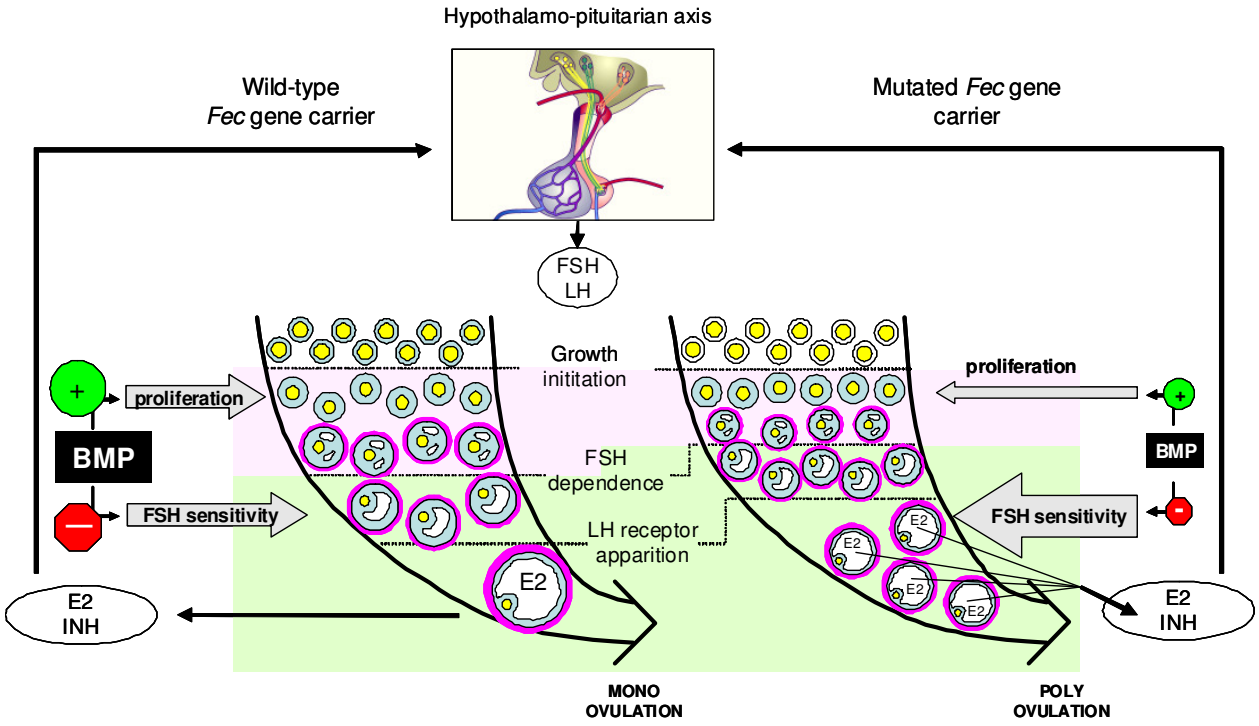

Figure 5: Schematic representation of the effects of a mutation in fecundity (Fec) gene on folliculogenesis and ovulation rate in sheep. See text above for specific comments. Adapted from Fabre *et al.* 2006 and Monniaux *et al.* 2009.

2.2.c. A mathematical model of follicular selection

In a collaborative work between our group and a team of INRIA French mathematicians, a multi-scale model of follicular selection using control theory concepts was developed (Echenim *et al.* 2005). In this model, for each follicle, the granulosa cell population dynamics is ruled by a first-order conservation law which describes the changes in the granulosa cell age and maturity. A control term representing FSH signal intervenes in the velocities, gain and loss term of the conservative law. The model accounts for the changes in cell number, cell growth fraction (percentage of proliferative cells) and maturity of both ovulatory and degenerating follicles for various intensities of the follicular selection rate. The selection rate depends on the follicular sensitivity to FSH, the pituitary sensitivity to the negative feedback of oestradiol and inhibin, and the hypothalamic sensitivity to the positive feedback of oestradiol. An example of the model results illustrating a competition between 3 follicles in a wave is illustrated in **Figure 6** (Monniaux *et al.* 2009).

This model is a useful tool to study the mechanisms regulating natural ovulation rate and anovulation in the case of ovarian pathologies. It can also be used to study the regulations of the multiple ovulations induced by FSH superovulatory treatments (**Figure 7**). Recent new developments of the model have been proposed (Michel 2010; Aymard *et al.* 2011) with the aim at the end to simplify it and to implement a user-friendly interface for biologists.

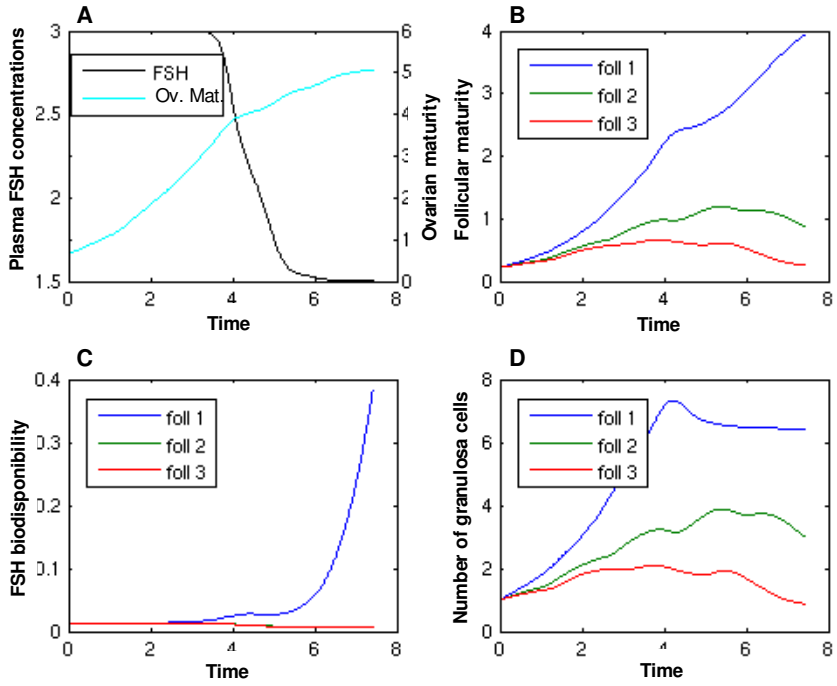

Figure 6: Modelisation of the follicular selection process: competition between 3 follicles during terminal follicular development. A: The increase in ovarian maturity (reflecting the increase in ovarian production of oestradiol and inhibin) triggers the decrease in plasma FSH concentrations. B: Changes in maturity (oestradiol and inhibin production) of the 3 follicles in competition. C: Intrafollicular FSH biodisponibility (related to vascularization and intra-follicular regulations) for each follicle. D: Changes in the number of granulosa cells of the 3 follicles in competition. At the initiation of the procedure, the 3 follicles had similar maturities and cell numbers, but slightly different FSH sensitivities. In this competition, Follicle 1 becomes dominant and reachs the ovulatory stage. Adapted from Monniaux *et al.* 2009.

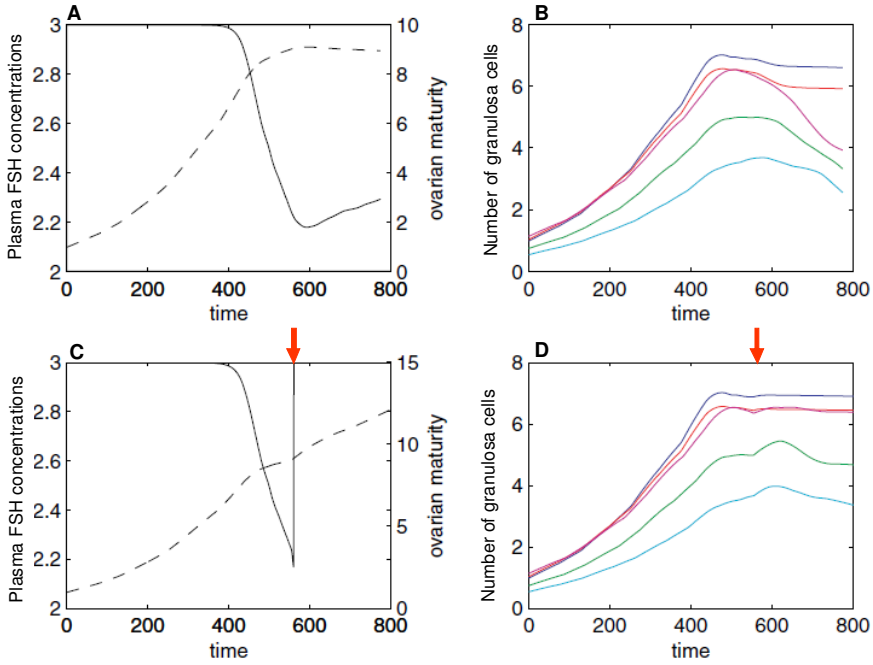

Figure 7: Selection process within a cohort of 5 follicles. A and C: Black lines correspond to ovarian maturity (dotted lines) and plasma FSH concentration (solid lines) outputs. B and D: Changes in the numbers of granulosa cells of the 5 follicles in competition. A and B: Natural selection process leading to 2 ovulatory follicles. C and D: Effects of an exogenous entry of FSH (red arrow). One follicle is rescued from atresia by exogenous FSH. Adapted from Echenim *et al.* 2005.

2.3. Control of the follicular waves to improve the results of embryo production

The concept of follicular waves laid the basis of improving superovulatory treatments. Attempts to optimize the ovarian responses and to decrease the within-individual variability followed two directions. First, the small growing follicles in the emerging cohorts appear to be the direct target of the gonadotrophin treatments and their number should be enhanced since it determines the ovulatory response to treatments. Second, atresia of the subordinate follicles of the wave can explain why the presence of a dominant follicle reduces the superovulatory responses to gonadotrophins in cattle (Pierson and Ginther 1988); overriding follicle selection or synchronizing the follicular wave emergence before treatment should improve the ovarian response.

It has been shown that the number of small growing follicles in the emerging follicular waves can be enhanced either directly or indirectly by nutritional changes and/or hormonal treatments. Clearly, the numbers of follicles lower than 4 mm in diameter and the superovulatory responses of heifers are increased by enhanced short term dietary intake (Nolan *et al.* 1998; Gong *et al.* 2002). Administration of recombinant somatotropin leads also to an increase in the number of small antral healthy follicles and enhances the superovulatory responses to FSH (Gong *et al.* 1996; Cushman *et al.* 2001). It is strongly suggested that both nutritional and somatotropin effects are mediated by enhanced plasma concentrations of IGF-I and insulin (Gong *et al.* 1991; Webb *et al.* 2004). In goats, the ovarian responses and embryo qualities are both improved by insulin administration at low doses (Selvaraju *et al.* 2003; Souza *et al.* 2008). However, detrimental effects of overfeeding have been observed on embryo quality in superovulated ruminants, and nutritional recommendations aiming to optimize follicular growth and the quality of oocytes evaluated by their developmental ability may be different (Boland *et al.* 2001; Lozano *et al.* 2003; Freret *et al.* 2006; Santos *et al.* 2008).

Alternatively, pre-treaments impacting on plasma FSH concentrations have been proposed to increase the number of small growing follicles able to respond to superovulatory treatments. Long term GnRH antagonist treatments have been shown to increase the number of small gonadotrophin-responsive follicles in ewes and goats (Cognie *et al.* 2003; Baril *et al.* 2004; Gonzalez-Bulnes *et al.* 2004b; Lopez-Alonso *et al.* 2005), but no clear effect was observed in heifers (D'Occhio *et al.* 1998). In cattle and small ruminants, active or passive immunization against inhibin is also associated with an increase in the number of small follicles at the time of wave emergence and a greater response to superovulatory treatments (Mizumachi *et al.* 1990; D'Alessandro *et al.* 1999; Medan *et al.* 2003; Medan *et al.* 2004; Takedomi *et al.* 2005; Sasaki *et al.* 2006; Mei *et al.* 2009) and similar results were found with immunization against follistatin (Singh *et al.* 1999). It is suggested that inhibin and follistatin antibodies may act primarily by an intraovarian paracrine action rather than by reducing the suppressive action of inhibin on pituitary FSH release (Li *et al.* 2011; Holtz *et al.* 2012).

Superovulation in cattle was shown to be optimal when the treatment is initiated at the time of follicular wave emergence (Adams *et al.* 1994). From this observation, protocols that control follicular wave emergence and ovulation have been the object of intense investigations (Bo *et al.* 2008; Bo *et al.* 2010; Mapletoft and Bo 2012). Oestradiol administration was shown to be highly effective for suppressing the growth of the dominant follicle (Bo *et al.* 1993) but it cannot be used because of the presence of oestrogenic substances in the food chain. Alternatively, transvaginal ultrasound-guided ablation of all follicles larger than 5 mm at random stages of the oestrous cycle followed by a single bolus of

gonadotrophin treatment 1 day later results in a superovulatory response that is comparable to the same treatment administered around the time of spontaneous wave emergence (Bergfelt *et al.* 1997), however follicle ablation is difficult to implement in farm. Later, other treatments of pre-synchronisation with progestogen, associated with administration of GnRH, have been shown useful in synchronising follicular wave emergence, and FSH diluted in a slow-release polymer (hyaluronan) can then be administered as a single intramuscular injection (Bo *et al.* 2010).

In conclusion, improvements in superovulatory treatments were permitted by increasing knowledge of the follicular waves and their regulations. They have resulted in optimization of the ovarian responses to superovulation by reducing the within-individual variability. However, despite this progress, the average number of embryos recovered from superovulated cows has not changed markedly in the past 20 years, and the use of embryo biotechnologies (presently MOET and OPU-IVP) is approaching a plateau (Hasler 2003). The major problem is that ovarian responsiveness to gonadotrophins remains highly variable between individuals in cows (Driancourt 2001; Mapletoft *et al.* 2002; Merton *et al.* 2003; De Roover *et al.* 2005; Pontes *et al.* 2011) and goats (Cognie 1999; Cognie *et al.* 2003; Baldassarre and Karatzas 2004; Cognie *et al.* 2004; Gibbons *et al.* 2007; Menchaca *et al.* 2010). Notably more than 20% of donors produce no usable embryos in cows. As said above (see part 1), it is suggested that intrinsic between-individual differences in follicular development result in marked animal-to-animal variations in the magnitude of the superovulatory response. In this context, a predictive test for selecting cows that can produce high numbers of transferable embryos or fertilisable oocytes would represent an important advance in embryo biotechnologies.

3. Anti-Müllerian Hormone (AMH), an endocrine marker of the gonadotrophin-responsive follicles and its use to select the best embryo donors

3.1. General features and ovarian origin of AMH in ruminant females

Anti-Müllerian hormone (AMH) also known as Müllerian inhibiting substance (MIS) is a glycoprotein of 140 KDa belonging to the transforming growth factor beta family (TGFβ) that is expressed only in the gonads (Cate *et al.* 1986). AMH has long been known for its developmental effect causing regression of the Müllerian ducts in male foetuses (Jost *et al.* 1973), but later studies have shown that AMH exerts inhibitory effects on the development and function of reproductive organs in both sexes (For review, see (Teixeira *et al.* 2001; Josso and Clemente 2003). In the ovary, AMH is of key importance: it inhibits the recruitment of primordial follicles into the pool of growing follicles and it decreases the responsiveness of growing follicles to FSH (Di Clemente *et al.* 1994; Durlinger *et al.* 1999; Durlinger *et al.* 2001; Durlinger *et al.* 2002). Interestingly, AMH expression is restricted to a single cell type, that is, granulosa cells of growing follicles, as shown in cow (Vigier *et al.* 1984; Takahashi *et al.* 1986; Monniaux *et al.* 2008), sheep (Bezard *et al.* 1987), rat (Ueno *et al.* 1989; Hirobe *et al.* 1992; Hirobe *et al.* 1994), human (Rajpert-De Meyts *et al.* 1999; Weenen *et al.* 2004), mouse (Dutertre *et al.* 2001; Salmon *et al.* 2004), possum (Juengel *et al.* 2002b) and hen (Johnson *et al.* 2008)). This pattern of expression makes AMH an ideal endocrine marker of the size of the ovarian pool of growing follicles.

We have shown recently that AMH is highly expressed in healthy 3-7 mm follicles in the cow and 1-5 mm follicles in the goat (Rico *et al.* 2009; Monniaux *et al.* 2011). Granulosa

cells of these follicles express higher *AMH* mRNA levels and their follicular fluids contain higher AMH concentrations, compared to larger antral follicles and to atretic ones (**Figure 8**).

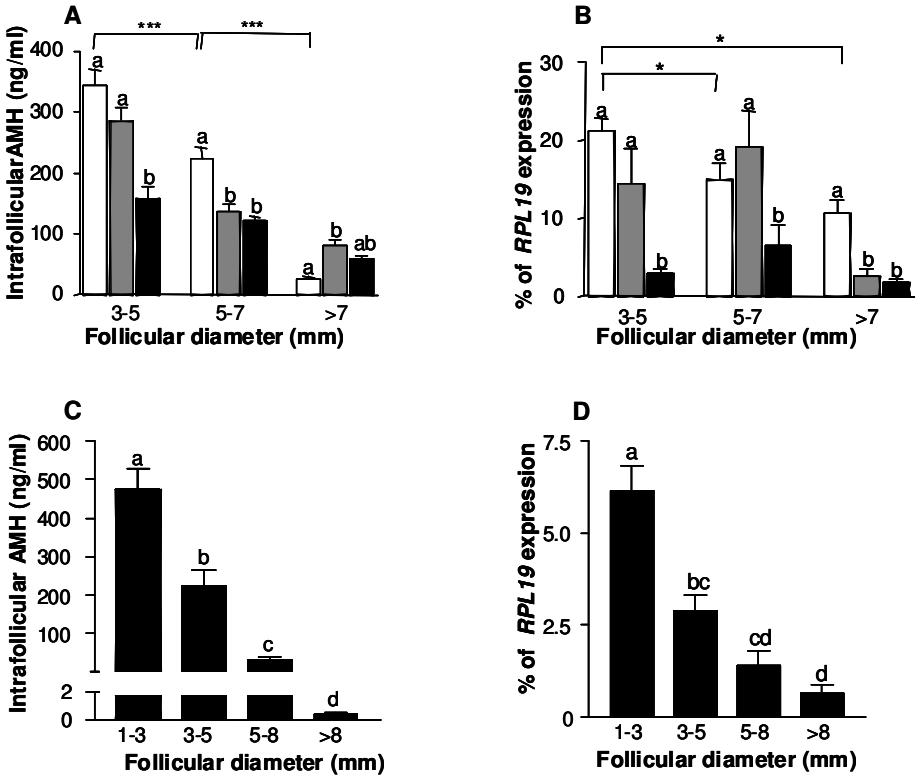

Figure 8: AMH concentrations in follicular fluid and *AMH* expression in granulosa cells from cow (A and B) and goat (C and D) antral follicles. A and C: intrafollicular concentrations of AMH in follicles of different size classes. B and D: *AMH* mRNA expression, expressed as percentage of the ribosomal gene *RPL19* expression, in follicles of different sizes. A and B: the bovine follicles were classified as normal (open bars), early atretic (grey bars) or atretic (black bars) follicles. Adapted from Rico *et al.* 2009 and Monniaux *et al.* 2011.

AMH expression can also be detected in the granulosa cells of small growing preantral and early antral follicles, but not primordial ones (Rico *et al.* 2011), in agreement with results of previous studies in different species (Bezard *et al.* 1987; Baarends *et al.* 1995; Juengel *et al.* 2002b; Visser *et al.* 2006). The cumulus cells and the outer layers of granulosa cells close to theca are preferred zones of high AMH expression in healthy antral follicles (**Figure 9**). Moreover, in atretic follicles AMH expression is strongly diminished, except in the cumulus cells surrounding the oocyte. Recently, we have shown that BMP6 and BMP4, known to be expressed in oocyte and theca cells, respectively (Elvin *et al.* 2000; Glister *et al.* 2004; Juengel *et al.* 2006), can enhance AMH expression in bovine granulosa cells *in vitro* (Rico *et al.* 2011). These results strongly suggest that BMP, of oocyte and theca origin, support AMH expression in growing follicles and participate in its regionalisation during follicular development.

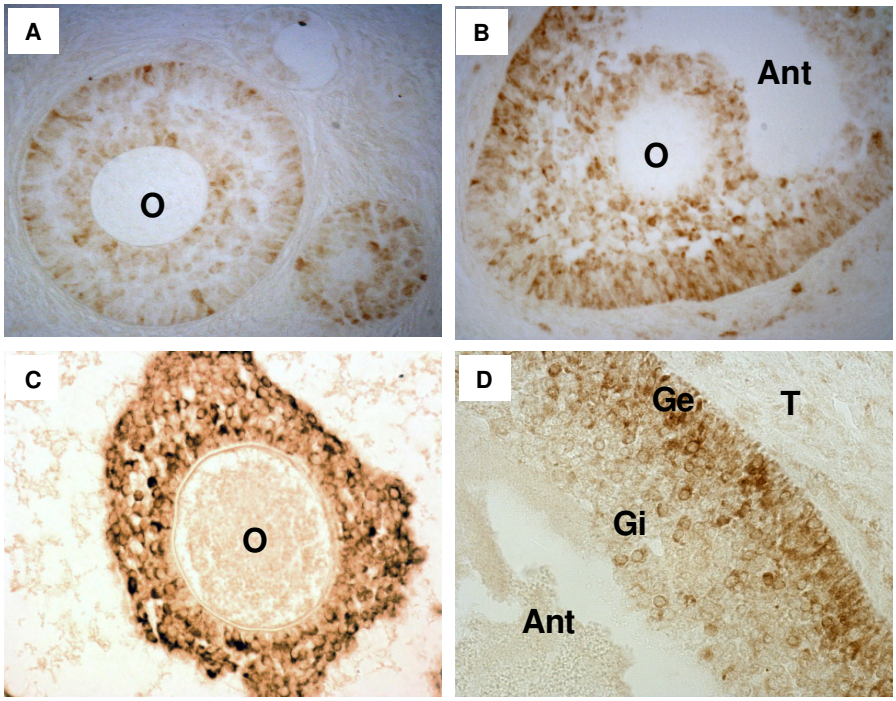

Figure 9: Immunohistochemical localization of AMH in bovine granulosa cells of follicles at different stages of development. A: preantal follicles. B: Early antral follicle. C: Cumulus of a large antral follicle. D: Wall of granulosa of a large antral follicle with a gradient of staining between the external (Ge) and internal (Gi) layers of granulosa. O: oocyte, Ant: antrum. Adapted from Monniaux *et al.* 2010b and Rico *et al.* 2011.

3.2. Follicular populations and endocrine variations of AMH

Plasma AMH concentrations were found to be highly variable between individuals in ruminants and AMH is now considered as a reliable endocrine marker of the population of small antral growing follicles in cows, goats and sheep. From our results, the numbers of 3-7 mm follicles in the cow and 1-5 mm follicles in the goat are strongly related to AMH concentrations in plasma, suggesting that these follicles contribute importantly to AMH endocrine levels (**Figure 10**, Rico *et al.* 2009, Monniaux *et al.* 2011). In agreement with these observations, cows selected for higher numbers of antral follicles during follicular waves are characterized by higher plasma AMH concentrations (Ireland *et al.* 2008). Altogether, these results support the existence of important differences in ovarian activity between individuals, as reported in cattle by pioneer studies using histology (Rajakoski 1960; Erickson 1966; Testart 1972; Mariana and Huy 1973; Scaramuzzi *et al.* 1980; Monniaux *et al.* 1983; Monniaux *et al.* 1984a; Monniaux *et al.* 1984b) and further studies using ultrasonography (Kawamata 1994; Cushman *et al.* 1999; Taneja *et al.* 2000; Singh *et al.* 2004; Durocher *et al.* 2006; Ireland *et al.* 2007).

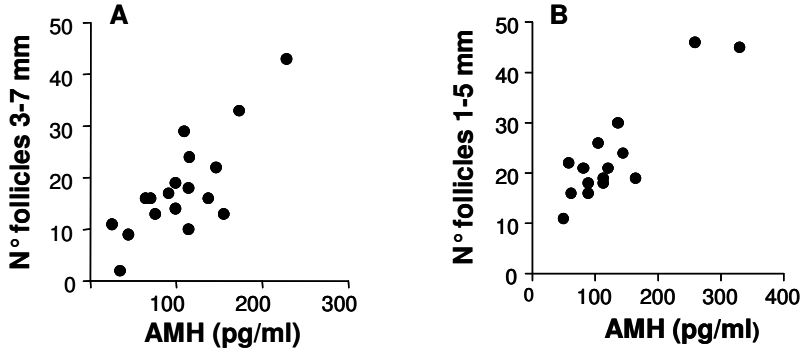

Figure 10: Relationships between the numbers of antral follicles and plasma AMH concentrations in cows (A) and goats (B). In cows, follicles were counted using ultrasonography. In goats, follicles were counted after dissection from ovaries recovered on slaughtered animals. AMH concentrations were measured in plasma samples recovered at the time of follicular counting. Each circle represents data from one animal. The correlation coefficients were 0.79 and 0.89 (both $p < 0.001$) in cows and goats, respectively. Adapted from Rico *et al.* 2009 and unpublished data.

In ruminant ovaries, the population of small antral growing follicles is considered to show little numerical change with time, unlike the gonadotrophin-dependent follicles which grow through a wave-like pattern (Scaramuzzi *et al.* 2011). As this follicular population produces the highest amounts of AMH, plasma AMH concentrations are expected to be rather steady with time for each animal. In agreement with this hypothesis, between-cow differences in AMH concentrations were found to be unchanged after a 3-month delay (Rico et al., 2009), and in cows undergoing repeated OPU protocols over 1 year, the AMH concentrations measured in plasma before each protocol are highly repeatable (Rico *et al.* 2012). Similarly, in the goat, a species that presents a seasonal breeding activity, plasma AMH concentrations show little change with season (Monniaux *et al.* 2011). These results reinforce the hypothesis that plasma AMH concentrations are characteristic of each animal on a long-term period.

In both high and low-responding cows, AMH follows a specific dynamic profile during the oestrous cycle, which occurs independently of the follicular waves of terminal development (Rico *et al.* 2011). Despite the existence of important differences between cows in AMH mean endocrine levels, oestrous cycle length and numbers of follicular waves per cycle, the profile of AMH changes during a natural oestrous cycle is quite similar between cows. It consists in a rapid decrease in AMH concentrations after oestrus, reaching minimal values between days 4 and 8 of the cycle, followed by a slow increase until the next oestrus (**Figure 11**).

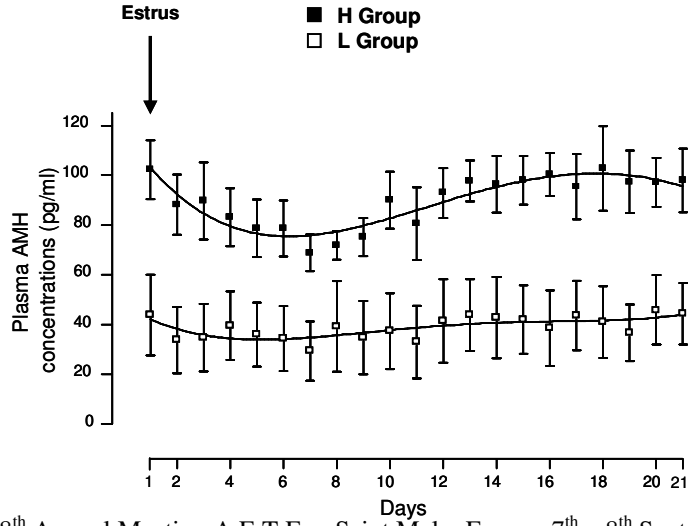

Figure 11: Daily changes in plasma AMH concentrations during the first 20 days of a natural oestrous cycle in the cow. Cows were allocated to 2 groups, according to their ovulatory response to a previous FSH stimulation test. Ovulations rates in response to the test were 21.5 ± 0.45 in cows of the high (H) group (n = 6) and 6.8 ± 0.97 in cows of the low (L) group (n = 5). Adapted from Rico *et al.* 2011.

These variations in AMH concentrations were not found to be associated with concomitant changes in the numbers of follicles detected by ovarian ultrasound scanning. Alternatively, FSH could be responsible for the sharp decrease in AMH concentrations after oestrus since it was shown that FSH can decrease AMH production by bovine granulosa cells *in vitro* (Rico *et al.* 2011). We propose that AMH endocrine levels decrease during the days following oestrus in response to the preovulatory and periovulatory FSH surges acting on the population of high-AMH secreting follicles of the basal follicular development stage. From these observations, a multiscale model of regulation of AMH production has been proposed (**Figure 12**).

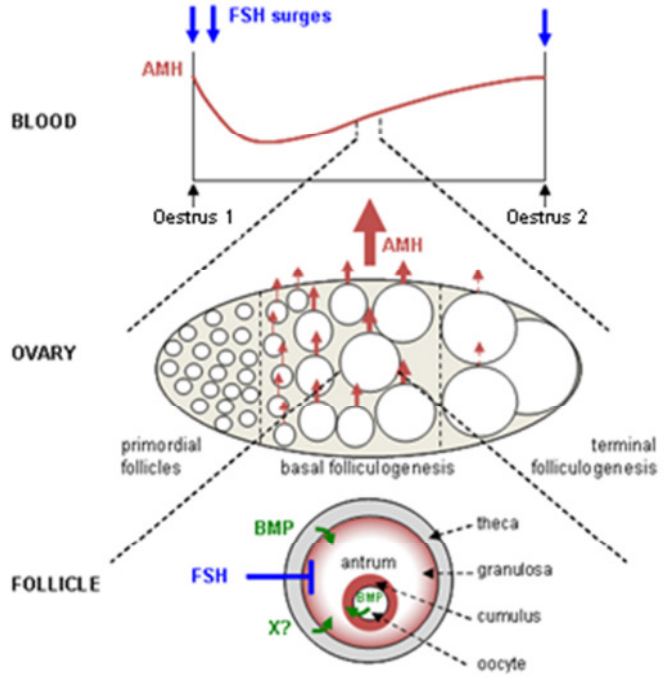

Figure 12: Multiscale model of the regulation of AMH production in the cow. In small antral follicles, BMP of thecal and oocyte origin can enhance AMH expression in granulosa cells, whereas FSH has an inhibiting effect. Plasma AMH concentrations decrease during the days following oestrus, likely in response to the preovulatory and periovulatory FSH surges acting upon the population of high-AMH secreting follicles of the basal folliculogenesis. Adapted from Rico *et al.* 2011.

3.3. Plasma AMH concentrations, ovarian responses to superovulation and capacity of embryo production

In the cow and the goat, we have shown that AMH concentrations in the plasma of individuals are indicative of their ability to respond to superovulatory treatments (Rico *et al.* 2009; Monniaux *et al.* 2011). AMH concentrations measured in plasma just before treatment were found to be highly correlated with the numbers of large follicles at oestrus and the numbers of ovulations (**Figure 13**). Moreover, they are predictive of the capacity of a donor female to produce high or low numbers of high-quality embryos (**Figure 14**). Recent results indicate that prediction could be made accurately from a single blood measurement of AMH performed several months before the start of embryo production campaigns in the cow (Monniaux *et al.* 2010a) and during either breeding or anoestrus seasons in the goat (Monniaux *et al.* 2011).

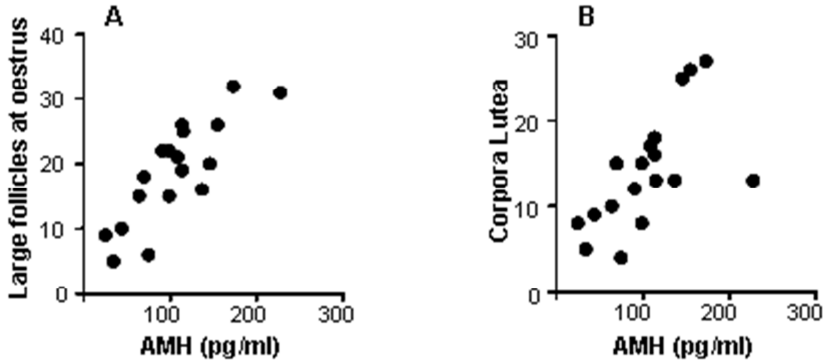

Figure 13: Relationships between plasma AMH concentrations before treatment and (A) the numbers of large follicles (> 7 mm) at oestrus, (B) the numbers of ovulations (corpora lutea), after administration of a superovulatory treatment in cows (n = 18). Follicles and corpora lutea were counted using ovarian ultrasonography. Each circle represents data from one animal. The correlation coefficients were 0.83 ($p < 0.001$) and 0.64 ($p < 0.01$) with the numbers of large follicles and corpora lutea, respectively. Adapted from (Rico *et al.* 2009).

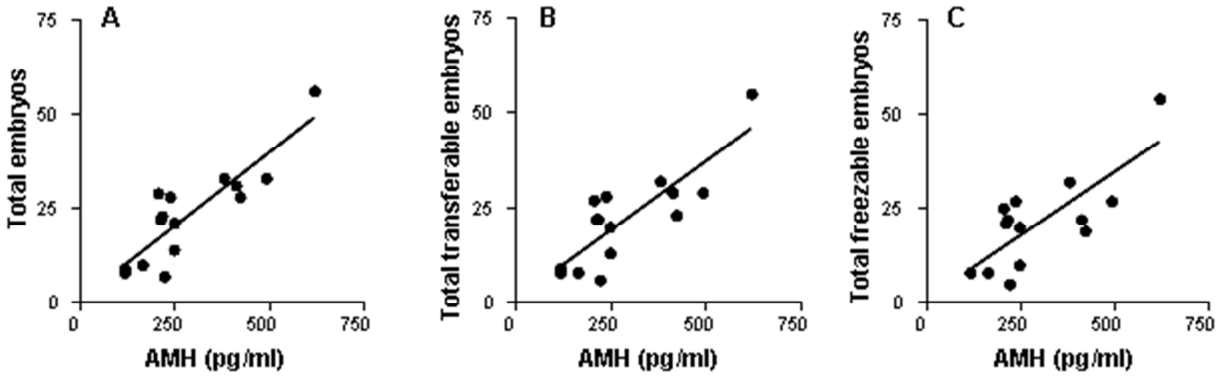

Figure 14: Relationships between AMH concentrations measured in plasma before the first superovulatory treatment and the total results of 3 sessions of embryo production, performed in January, April and October in goats (n = 15). A: total number of collected embryos. B: total number of transferable embryos. C: total number of freezable embryos. Each circle represents data from one animal. The correlation coefficients were 0.87, 0.83 and 0.78 (all $p < 0.001$) with the numbers of collected, transferable and freezable embryos, respectively. Adapted from (Monniaux *et al.* 2011).

3.4. Determination of AMH concentrations in blood as a tool to select donor ruminants for embryo production

The embryo production capacity of donor cows was shown to be a repeatable and heritable trait in MOET (Asada and Terawaki 2002; Benyei *et al.* 2004; Eriksson *et al.* 2007; Monniaux *et al.* 2010a) and OPU-IVP protocols (Machado *et al.* 2006; Merton *et al.* 2009). Similarly, in small ruminants, a high within-animal repeatability in embryo production by both methods was reported in sheep (Ptak *et al.* 2003) and goats (Monniaux *et al.* 2011). Thus, embryo production capacity is intrinsically dependent on the donor female and predicting the capacity of each animal to respond to a gonadotrophin treatment could improve the results of MOET and OPU-IVP protocols by identifying in advance and discarding the low-responding animals.

It was previously proposed that the response to superovulatory treatments in goats, in terms of the number of embryos, could be predicted from plasma inhibin A levels measured at the start of superovulatory treatment (Gonzalez-Bulnes *et al.* 2004a). In small ruminants, inhibin A is known to be secreted not only by small antral follicles but also mainly by the largest ones (Tsonis *et al.* 1983; Mann *et al.* 1993). As said above, large antral follicles produce low AMH amounts. From this difference in the pattern of expression, AMH is likely a better endocrine predictive marker of superovulatory responses than inhibin A in small ruminants. Presently, AMH is considered as the best known endocrine marker of the ovarian reserve of gonadotrophin-responsive follicles in ruminants, as also proposed in human (van Rooij *et al.* 2002; Gruijters *et al.* 2003; Visser and Themmen 2005; Visser *et al.* 2006) and mouse (Kevenaar *et al.* 2006).

The development of a prognostic method to determine the intrinsic capacity of a potential donor cow to produce an expected number of embryos might be based on the measurement of circulating AMH concentrations. However, several questions have to be answered before validating a prognostic test.

The first question concerns technical problems related to AMH determination in blood. Presently, AMH concentrations can be measured accurately in the plasma of cows using the commercially available kit supplied by Beckman Coulter for the quantitative measurement of AMH in human serum. The use of this kit has been validated for AMH measurement in various female mammals such as mouse (Kevenaar *et al.* 2006), rat (Yeh *et al.* 2007), cow (Ireland *et al.* 2008; Monniaux *et al.* 2008), goat (Monniaux *et al.* 2011), horse (Almeida *et al.* 2011), dog and cat (Place *et al.* 2011). In the cow, plasma AMH concentrations are about 10-fold lower than in human, so high sensitivity and close repeatability of the assay are important for an accurate AMH measurement. Moreover, we reported recently that the technical conditions of blood sampling and storage before assay are of crucial importance for an accurate determination of AMH concentrations in bovine plasma or blood (Rico *et al.* 2012).

The second important question to answer for developing a diagnostic test is how to schedule the optimal time for carrying out the blood test on cows. From our results, the optimal period of the oestrous cycle at which to measure AMH concentrations with the aim of selecting the best cows for embryo production was found to be at oestrus and after Day 12 of the cycle (Rico *et al.* 2011). A better knowledge of the AMH variations throughout life and of the effects of environmental changes on AMH endocrine levels would help to propose optimal times and breeding conditions for a blood test.

The third important question concerns the possibility to define a threshold (cut-off) AMH concentration enabling to discard the low-responding cows, which could be used in cows bred in farm conditions. Recently, we proposed that gonadotrophin-stimulated cows producing fewer than 15 large follicles at oestrus and fewer than 10 embryos in MOET protocols could be discarded efficiently with plasma AMH concentrations below 87 and 74 pg/ml, respectively (Rico *et al.* 2012). Whether these cut-off values are of interest for different breeds, ages and breeding conditions remains to be defined.

Conclusions

In ruminants, both the within- and the between-animal variabilities of embryo production in response to superovulatory treatments are determined in a large part by the status of the populations of gonadotrophin-responsive follicles at the time of treatment. The within-animal variability is time dependent and presents some cyclic features in direct relationship with the cyclic nature of emergence of the ovarian waves of terminal development. During the past 30 years, our increasing

knowledge of the follicular waves and their regulatory mechanisms has led to significant improvements in treatments and helped to reduce the within-animal variability in embryo production. The problem of the large between-animal variability of embryo production has not been solved but it is clear now that each donor female owns its intrinsic capacity of embryo production, determined by its ovarian activity.

Anti-Müllerian hormone has been found to be a useful endocrine marker of the populations of gonadotrophin-responsive follicles and can help to determine the intrinsic capacity of a potential donor cow to produce an expected number of embryos. The development of a prognostic method based on AMH measurement in blood could permit to discard the poorly-responding animals and consequently to improve embryo production by reducing the between-animal variability and increasing the average numbers of embryos produced in MOET and OPU-IVP protocols. The assessment of the AMH concentration in plasma may represent a very promising tool for the selection of embryo donor heifers with equivalent genetic value, but the integration of the AMH level in the genomic evaluation requires further physiologic and genetic investigations. The possible consequences of excluding cows with low AMH concentrations from MOET and OPU schemes are not known. From our observations, there was no relationship between AMH concentrations and milk production or body weight (data not shown), suggesting that excluding cows with low AMH concentrations would not concomitantly select cows against high milk yield or heavy carcass. There is no reason to think that AMH is related to important agronomic traits, except for reproduction. Recently, AMH was proposed as a diagnostic marker for fertility (Ireland *et al.* 2011), but AMH concentrations have not been found to differ between two groups of dairy cows contrasted at one female fertility QTL (Coyral-Castel *et al.* 2011). The relationship between AMH concentrations and fertility should be further investigated and the consequence of the selection of cows on the basis their plasma AMH concentrations should also be assessed on other important traits linked to reproduction such as sexual precocity and reproductive lifespan.

We do not know why so important differences in ovarian activity exist between females of similar breed, age, reproductive status and raised in similar breeding conditions. Follicular growth is triggered by withdrawal of inhibitory mechanisms maintaining the quiescence of the primordial follicles and supported by a finely tuned molecular dialogue between the oocyte and its surrounding granulosa cells (Monniaux 2010). However, our knowledge of the mechanisms regulating the size of the ovarian reserve of the primordial follicles and its management throughout the life remains limited. Answers to these questions need to assess the genetic as well as the environmental determinants of these mechanisms. They are likely the keys for improving embryo production in the future.

"The many slight differences which appear in the offspring from the same parents, or which it may be presumed have thus arisen, from being observed in the individuals of the same species inhabiting the same confined locality, may be called individual differences. No one supposes that all the individuals of the same species are cast in the same actual mould. These individual differences are of the highest importance for us, for they are often inherited, as must be familiar to every one; and they thus afford materials for natural selection to act on and accumulate, in the same manner as man accumulates in any given direction individual differences in his domesticated productions. These individual differences generally affect what naturalists consider unimportant parts; but I could show by a long catalogue of facts, that parts which must be called important, whether viewed under a physiological or classificatory point of view, sometimes vary in the individuals of the same species."

(Charles Darwin, 1859. In "On the Origin of Species by Means of Natural Selection")

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National Statistical Data of Embryo Transfer Activity in Europe (2011)

by Hiemke Knijn, The Netherlands

Commercial Bovine Embryo Transfer Activity in Europe 2011

General

Country	Name Collector	N° of approved ET teams	N° of teams provide ET data
Austria	G Wetchy	-	-
Belgium	P Vercauteren	6	4
Bosnia & Herzegovina	T Markovic	1	1
Croatia	M Matkovic	1	1
Czech Republic	P Bucek	-	3
Denmark	H Callesen	12	7
Estonia	J Kurykin	1	1
Finland	M Mikkola	4	3
France	C Ponsart	18	15
Germany	H Cramer	39	18
Greece	S Foteini	2	2
Hungary	F Flink	5	5
Ireland	P Lonergan	5	1
Italy	G Lazzari	-	-
Luxembourg	J Westphal	2	2
The Netherlands	JG Derksen	-	-
Norway	E Kummen	2	2
Poland	J Jaskowski	7	7
Portugal	J Chagas e Silva	5	-
Romania	S Zamfirescu	3	3
Spain	J de la Fuente	18	11
Sweden	A Tidström	3	1
Switzerland	R Saner	7	4
Turkey	E Emsen	3	2
UK	A Meacock	54	41

Bovine In Vivo Embryo Production

Country	N° of flushed donors	N° of embryos collected	N° of transferable embryos	N° of transferable embryos/flush
Austria	37	-	162	4.4
Belgium	1022	7062	3213	3.1
Bosnia & Herzegovina	-	-	-	-
Croatia	11	135	87	7.9
Czech Republic	82	455	275	3.4
Denmark	447	3780	3025	6.8
Estonia	8	20	17	2.1
Finland	418	4317	2557	6.1
France	5665	50660	29966	5.3
Germany	2215	24055	14780	6.7
Greece	2	9	6	3.0
Hungary	83	1157	773	9.3
Ireland	420	4074	2077	5.0
Italy	2103	19680	-	6.2
Luxembourg	118	1410	855	7.2
The Netherlands	4045	44495	27609	6.8
Norway	6	49	41	6.8
Poland	84	644	476	5.7
Portugal	134	1566	810	6.0
Romania	23	174	124	5.4
Spain	626	6919	3135	5.0
Sweden	39	226	150	3.8
Switzerland	533	5733	3880	7.3
Turkey	173	1018	717	4.1
UK	5186	14780	13976	2.7
Total	23.480	192.418	108.711	4.6

Bovine In Vitro Production

Country	N° of OPU sessions	N° of oocytes collected	N° of transferable embryos	N° of embryos/OPU session
France	265	2208	524	2.0
Germany	1012	4564	3217	3.2
Italy	168	2434	423	2.5
The Netherlands	3530	28678	3814	1.1
Turkey	-	100	56	-
Total	4975	37984	8034	-

Embryo Technologies in Bovine

Country	N° of biopsied embryos		N° of sexed embryos		N° of genotyped embryos	
	In vivo	**In vitro**	**In vivo**	**In vitro**	**In vivo**	**In vitro**
France	160	-	160	-	-	-
The Netherlands	269	--	-	-	269	-
Total	469	-	160	-	269	-

Bovine Embryo Transfer

Country	N° of transferred embryos				
	In vivo fresh	In vivo frozen	In vitro fresh	In vitro frozen	*Total*
Austria	34	113	-	-	147
Belgium	1207	3912	-	59	5178
Bosnia & Herzegovina	-	-	-	-	-
Croatia	29	30	-	-	59
Czech Republic	119	63	-	-	182
Denmark	1045	459	-	-	1504
Estonia	13	19	-	-	32
Finland	884	3149	-	-	4033
France	14669	14563	356	159	29747
Germany	5168	9508	1516	103	16295
Greece	-	12	-	-	12
Hungary	227	298	-	-	525
Ireland	744	909	1653	-	3306
Italy	5426	7193	50	782	13451
Luxembourg	160	780			940
The Netherlands	4130	16876	2688	581	24275
Norway	21	85			106
Poland	209	241	-	-	450
Portugal	87	193	30	36	346
Romania	60	-	-	-	60
Spain	1040	1622	-	-	2662
Sweden	-	46	-	-	46
Switzerland	895	2063	-	24	2982
Turkey	717	403	56	-	1176
UK	5623	8353	-	-	13976
Total	42.507	70.890	6.349	1.744	121.490

Embryo Activities in other Species (I)

Country	Sheep		Horses	
	N° embryo produced	N° embryo transfers	N° embryo produced	N° embryo transfers
Bosnia & Herzegovina	2	2	-	-
Czech Republic	30	16	-	-
France	-	-	376	376
Hungary	-	68	5	5
Italy	-	-	172	168
Portugal	-	-	8	8
Turkey	216	216	-	-
UK	-	13	133	110
Total	248	315	694	667

Embryo Activities in other Species (II)

Country	Goat		Swine	
	N° embryo produced	N° embryo transfers	N° embryo produced	N° embryo transfers
Czech Republic	7	-	-	-
France	10	4	-	-
Total	17	4	-	-

INVITED LECTURES

RECENT ADVANCES IN THE CONTROL OF FOLLICULAR DEVELOPMENT AND SUPEROVULATION PROTOCOLS IN CATTLE

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Abstract. Although some embryo transfer practitioners still initiate superstimulatory treatments during mid-cycle in donor cows, the elective control follicular wave emergence and ovulation developed in the early 1990's have had a great impact on the application of on-farm embryo transfer, especially when large groups of donors need to be collected at a given time. However, the most common treatment for the synchronization of follicular wave emergence involves the use of estradiol which cannot be used in many parts of the world. Therefore, alternative treatments had to be developed for the superovulation of donor cows in countries were estradiol is not available. An approach that has shown promise is to initiate FSH treatments at the time of the emergence of the new follicular wave following GnRH-induced ovulation. Alternatively, it has been shown that it may be possible to ignore follicular wave status, and by extending the treatment protocol, induce subordinate follicles to superovulate. Finally, the short half-life of pituitary FSH necessitates twice daily treatments which are time-consuming, stressful and subject to error. Recent treatment protocols have permitted superstimulation with a single or alternatively, two FSH treatments 48 hours apart, reducing the need for animal handling during gonadotropin treatments.

Keywords: Superovulation, FSH, follicle wave, GnRH, estradiol.

Introduction

Since the advent of commercial embryo transfer, superovulatory treatment protocols have been constantly evolving with the objective of obtaining high numbers of viable embryos from every donor. Traditional superstimulation protocols consisted of a single administration of equine chorionic gonadotropin (eCG) or twice daily injections of pituitary extracts containing FSH over 4 or 5 days (Mapletoft et al. 2002). Equine chorionic gonadotropin is a complex glycoprotein which has a long half-life (over 40 hours) representing a practical advantage, since a single administration will induce ovarian superstimulation (Murphy and Martinuk 1991). However, this also results in increased numbers of large follicles at the time of ova/embryo collection which affects collection efficiency and embryo quality (González et al. 1994). In contrast, the half-life of FSH is very short (~5 hours) in the cow (Laster 1972), necessitating frequent applications to induce superstimulation (Monniaux et al. 1983). Twice daily treatments with FSH have resulted in greater superovulatory responses than once daily administration (Looney et al. 1981; Monniaux et al. 1983, Walsh et al. 1993).

Variability in superovulatory response and the time and effort required for treatments and estrus detection have been the primary limiting factors affecting the use of embryo transfer technology in genetic improvement programs. Although the recent development of protocols that control follicular wave emergence and ovulation have not eliminated the variability in superovulatory response, they have had a positive impact on the application of embryo transfer by permitting the initiation of treatments at a self-appointed time (Bó et al. 2002; 2006; Mapletoft et al. 2002). In addition, protocols that synchronize ovulation in superstimulated donors have facilitated

artificial insemination (AI) of donor cattle at a fixed-time, thereby avoiding the necessity of estrus detection (Baruselli et al. 2006; Bó et al. 2006). Thus, treatments are now more "user friendly" and easier to implement on farm, and are not dependent upon estrus detection efficiency.

As the most commonly used treatment for elective synchronization of follicular wave emergence for superstimulation (i.e., estradiol) cannot be used in many countries (Lane et al. 2008), alternatives are now being investigated. Similarly, the elective induction of superovulation with pituitary FSH without the need for twice daily treatments has also been recently investigated. The objective of this manuscript is to review the different superovulation treatment protocols used today and offer some alternatives that may help to the simplification of treatment protocols without compromising ova/embryo production.

Traditional superovulation protocols

For many years, practitioners have been initiating superstimulatory treatments during the mid-cycle (i.e., 8 to 12 days after estrus). Although this was initially based on anecdotal evidence, and some experimental data (Lindsell et al. 1986), it is now known that this encompasses the time of emergence of the second follicular wave in cattle exhibiting two- or three-wave cycles (Pierson and Ginther 1987; Ginther et al. 1989). Superovulatory response has been shown to be higher when gonadotropin treatments are initiated precisely at the time of follicular wave emergence rather than 1 or 2 days later (Nasser et al. 1993), making it necessary to synchronize the timing of follicular wave emergence in groups of animals. Many practitioners prefer decreasing FSH dose schedules and treat with PGF on the third day of the treatment protocol, while others prefer to treat with PGF on the fourth day, and many do not treat with FSH on the day after the administration of PGF. Regardless, most superstimulation protocols have successfully induced superovulation under most circumstances (reviewed in Mapletoft et al. 2002). Still others incorporate a progestin device into the protocol which ensures that donors do not come into estrus early. In all cases, inseminations are normally done 12 and 24 hours after the onset of estrus (Mapletoft et al. 2002). Although the initiation of superstimulatory treatments during the mid-cycle has served the embryo transfer industry well over the years, conventional treatment protocols require that personnel are dedicated to the detection of estrus, and that all donors show estrus more or less at the same time in order to begin the superstimulatory treatments at most appropriate time i.e., mid-cycle.

Synchronization of follicular wave emergence and ovulation using progestins and estradiol-based treatments

The ability to electively induce follicular wave emergence permits initiation of superstimulation without regard to the stage of the estrous cycle and eliminates the need for estrus detection or for waiting 8 to 12 days to initiate gonadotropin treatments (Mapletoft et al. 2002). In the 1990's, we reported on the use of progestins and estradiol to induce synchronous emergence of a new follicular wave (Bó et al. 1995); this approach to superovulation in the cow has been reviewed extensively (Bó et al. 2002; Mapletoft et al. 2002). It has been used by practitioners around the world and has recently been incorporated into protocols that permit fixed-time AI of donors (Bó et al. 2006; Baruselli et al. 2006).

The most common protocol to synchronize the emergence of a new follicular wave for superstimulation involves the administration of 5 or 2.5 mg estradiol-17β or 2.5 mg estradiol benzoate plus 100 or 50 mg progesterone by intramuscular injection at the time of insertion of an intravaginal progestin device (reviewed in Bó et al. 2002; 2006; Mapletoft et al. 2002). The estradiol treatment suppresses FSH release and induces follicle atresia. Once estradiol has been metabolized, FSH surges and a new follicular wave emerge, on average 4 days later (Bó et al. 1995). Gonadotropin treatments are initiated at that time i.e., 4 days after treatment.

Barros and colleagues have developed a superstimulatory protocol in *Bos indicus* cattle that they refer to as the P-36 protocol (Barros and Nogueira 2005). In this protocol, the progestin device is left in place for up to 36 hours after PGF administration (thus the protocol was called P-36) and ovulation is induced by the administration of pLH 12 hours after withdrawal of the progestin device (*i.e.*, 48 hours after PGF administration). Since ovulation occurs between 24 and 36 hours after pLH administration (Nogueira and Barros 2003), fixed-time AI is scheduled for 12 and 24 hours after pLH, avoiding the need for estrus detection in donor animals.

In a series of experiments in which the timings of ovulations were monitored ultrasonically in *Bos taurus* donors, Bó et al. (2006) showed how synchronization of follicular wave emergence with estradiol could be incorporated into a protocol for fixed-time AI without estrus detection and without compromising results. Basically, the time of progestin device removal was delayed to prevent early ovulations and allow late developing follicles to "catch-up", followed by induction of ovulation with GnRH or pLH. In this protocol, follicular wave emergence is synchronized with estradiol and a progestin device on Day 0 and FSH treatments are initiated on Day 4. On Day 6, PGF is injected in the AM and PM and in the AM of Day 7, the progestin device is removed. On Day 8 AM (24 hours later), GnRH or pLH is injected and inseminations are done 12 and 24 hours later. From a practical perspective, fixed-time AI of donors has been shown to be very useful for busy embryo transfer practitioners (Larkin et al. 2006).

Studies in high-producing dairy cows (*Bos taurus*) in Brazil have indicated that it is preferable to allow an additional 12 hours before removing the progestin device (i.e., Day 7 PM; P-36) followed by GnRH 24 hours later (i.e., Day 8 PM) with fixed-time AI 12 and 24 hours later (Bó et al. 2006). In *Bos indicus* breeds, it was found that it is preferable to remove the progestin device on Day 7 PM (P-36), followed by GnRH 12 hours later (i.e., Day 8 AM; Baruselli et al. 2006). Although donors are typically inseminated twice, 12 and 24 hours after administration of pLH or GnRH, it is possible to use a single insemination 16 hours after pLH (Baruselli et al. 2006).

Non estradiol-based treatments for follicle wave synchronization and superstimulation

Follicle ablation

One alternative to estradiol for the synchronization of follicular wave emergence is to eliminate the suppressive effect of the dominant follicle by ultrasound-guided follicle ablation (Bungartz and Niemann 1994; Bergfelt et al. 1997). Initial studies involved the ablation of all follicles $\geq$ 5 mm (Bergfelt et al. 1997), but we subsequently showed that it was necessary to ablate only the two largest follicles (Baracaldo et al. 2000) to ensure that the dominant follicle was removed. Superstimulatory treatments are then initiated 1 to 2 days later, at the time of emergence of a new follicular wave. Although follicle ablation has been shown to be highly effective (reviewed in Bó et al. 2006), ultrasound equipment and trained personnel are required making it most appropriate for embryo production centers, where the equipment is maintained and personnel and donors are maintained.

GnRH

It has been shown that following GnRH-induced ovulation (Macmillan and Thatcher 1991), a new follicular wave will emerge approximately 2 days later. However, follicular wave emergence occurs only when GnRH induces ovulation (Martinez et al. 1999), and ovulation rates after GnRH treatment at random stages of the estrous cycle in cows have been reported to range from 44.3 % (Colazo et al. 2009) to 85 % (Pursley et al. 1995). Therefore, the interval from GnRH treatment to wave emergence may be too inconsistent for superstimulation. Indeed, Deyo et al. (2001) reported unsatisfactory embryo production following synchronization of follicular wave emergence with GnRH. However, recent results from commercial embryo transfer practitioners (Hinshaw, pers. comm.; Steel and Hasler 2009) and a research report involving 411 dairy donors (Wock et al. 2008) have revealed more promising results. Basically, a progestin device is inserted at random stages of the estrous cycle and GnRH is administered 2 or 3 days later with superstimulation treatments beginning 1.5 to 2.5 days later.

Superovulation during the first follicular wave

Nasser et al. (1993) have shown that superstimulation can be initiated at the time of emergence of the first follicular wave, and they subsequently showed that follicles in the first wave were as responsive to gonadotropin treatments as second wave follicles (Adams et al. 1994). However, a progestin device must be in place during FSH treatments in the first wave to ensure embryo quality (Nasser et al. 2011).

An alternative approach is to combine the use of GnRH and a progestin device (reviewed by Bó et al. 2010). In this protocol, a persistent follicle is induced by the strategic use of PGF and a progestin device (Small et al. 2009), and GnRH or pLH is used to induce its ovulation, at which time gonadotropin treatments are initiated. The most user-friendly and efficacious protocol consists of insertion of a progestin device and the administration of PGF on random days of the estrous cycle (Day 0). Progestin devices remain in place until the end of the FSH treatment protocol. GnRH was given on Day 7 AM and 36 hours later (i.e., Day 8 PM) at the expected time of ovulation FSH treatments were initiated. On Day 12 AM, the progestin device is removed and PGF is administered, and by adding a second GnRH injection 24 hours later, it is possible to do fixed-time AI 12 and 24 hours later. This protocol resulted in 90% of cows ovulating to the first injection of GnRH, which ensures that follicle wave emergence is synchronized. It can be used at random stages of the estrous cycle, without estrus detection, and with no decrease in embryo production.

Subordinate follicle break-through

During a normal follicular wave, subordinate follicles regress because of decreasing concentrations of circulating FSH, caused by the secretions (estradiol and inhibin) of the cohort and especially of the dominant follicle (Adams et al. 1992; 1993). Small follicles require FSH to continue their growth, and more importantly, recent evidence indicates that follicles as small as 1 mm in diameter will commence growth under the influence of FSH (reviewed by Adams et al. 2008). Perhaps all that is required for superstimulation is to cause these follicles to grow to a diameter of 3 or 4 mm at which time the regular 4- or 5-day superstimulatory treatment protocol could be initiated. Assuming a growth rate of 1 to 2 mm per day, this should take 2 to 3 days i.e., add 2 to 3 days to the superstimulation treatment protocol. Thus, the presence of a dominant follicle may not adversely affect superovulatory response under these circumstances; the exogenous FSH replaces that being depressed by the secretory products of the dominant follicle. Indeed, we successfully superstimulated donors using this approach at random stages of the estrous cycle, without regard to the presence of a dominant follicle (Bó et al., 2008). Small doses of FSH were administered twice daily starting 2 days before initiating the regular FSH treatment protocol. Alternatively, the 2 days of FSH pretreatment could be replaced with an injection on 500 IU of eCG. Caccia et al. (2000) reported that the administration of 500 IU of eCG 2 days before initiating FSH treatments tended to increase the superovulatory response in beef donors, and pretreatment of

poor responding donors with 500 IU of eCG resulted in increased embryo production over that achieve previously without eCG (Bo et al. 2008). It was hypothesized that pretreatment with eCG resulted in additional follicles being recruited into the wave.

Other changes in the superstimulation treatment protocols

Long FSH treatment protocols

We recently performed a study to determine if lengthening the superstimulation protocol from 4 to 7 days would result in an increase in the superovulatory response with no adverse effects on oocyte/embryo competence in beef cows (Garcia Guerra et al., 2012). All cows (n=24) were subjected to ultrasound-guided follicular ablation, to synchronize the emergence of a follicle wave, and a progesterone-releasing intravaginal device was inserted at the same time. Superstimulation treatments were initiated 1.5 days later (Day 0) with one of two treatments: Control (4 days of FSH) or Long (7 days of FSH). A total dose of 400 mg NIH-FSH-P1 (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada) was distributed equally over 8 (Control) or 14 (Long) intramuscular injections at 12-hour intervals. Prostaglandin F2α was administered twice, 12 hours apart, on Day 2 (Control) or Day 5 (Long), and progestin devices were removed 12 hours after the second PGF. Both groups were given 25 mg pLH intramuscularly 24 hours after progestin removal and AI was done 12 and 24 hours later. Ova/embryos were collected 7 days after the pLH injection. The mean (±SEM) number of follicles ≥9 mm in diameter at the time of first the AI did not differ (P = 0.24) between groups, but more ovulations (30.9 ± 3.9 vs. 18.3 ± 2.9, P = 0.01) and CL (27.2 ± 2.1 vs. 20.8 ± 2.2, P = 0.04) occurred in the Long group. A higher proportion of the ≥9 mm follicles ovulated between 12 and 36 h after pLH in the Long group (93 vs. 69%; P = 0.001). Although numerically higher in the Long group, mean numbers of total ova/embryos, fertilized ova, transferable or freezable embryos did not differ (Table 1). We concluded that a lengthened superstimulatory treatment protocol sustained follicle growth and resulted in more follicles acquiring the capacity to ovulate with an increased number of ovulations, and without a decrease in oocyte/embryo competence. These very interesting findings warrant further investigation, especially in lactating high-producing Holstein cows, which are usually harder to superovulate than beef donors, or non-lactating Holstein cows or heifers.

Table 1. Superovulatory response (mean±SEM) of cows subjected to a conventional 4-day (Control) or a 7-day (Long) treatment protocol

Treatment	N	CL	Ova-embryos	Fertilized ova	Transferable embryos
Control	12	20.8 ± 2.2[a]	11.3±2.0	5.6±1.5	4.2±1.5
Long	12	27.2±2.1[b]	13.8±2.3	8.0±2.0	6.3±1.6

[ab] Means within columns with different superscripts differed (P<0.05).

Replacing FSH by eCG to stimulate the final stages of follicle growth

The need for FSH and LH at different times during superstimulation has been debated. Basic studies on follicular development have shown that FSH is required for follicle recruitment and growth (Adams et al. 1992; 1993), until the dominant follicle is selected i.e., reaches 8.5 mm in diameter in Bos taurus cattle (Ginther et al. 1996) and 6.2 mm in Bos indicus cattle (Gimenes et al. 2008, Sartorelli et al. 2005). Following selection, dominant follicles acquire LH receptors and

become LH-dependent (reviewed in Mihm and Evans 2008). Therefore, follicles of superstimulated cattle may benefit from the inclusion of LH near the end of the superstimulatory treatment protocol. Equine chorionic gonadotropin is a gonadotropin with both FSH and LH activity (Steward et al. 1976; Litch et al. 1979; Murphy and Martinuk, 1991) and could provide a constant stimulus to the LH receptors of the growing follicles near the end of a conventional FSH superstimulation treatment protocol.

Barros et al. (2008) conducted an experiment in which Nelore cows were superstimulated with FSH over 3 days; the last two FSH injections (on the fourth day) were replaced by two injections of 200 IU of eCG. This treatment significantly increased the number of ova/embryos and numerically increased the number of transferable embryos over controls. Although Sartori et al. (2009) found no beneficial effect of eCG in Nelore heifers, more transferable embryos were produced in Red Sindhi cows (Mattos et al. 2011).

To further investigate this alternative, an experiment was conducted in Brangus cattle (Reano et al. 2009). Thirty-eight cows and 25 heifers were randomly allocated to three treatment groups to be superstimulated with twice daily injections of FSH over 4 days or to receive a single injection of 400 IU of eCG after the first four injections of FSH (i.e., on Day 6, when PGF was given and no treatments on Day 7) or to receive two injections of 200 IU eCG on Day 7 (after the first 6 injections of FSH on Days 4, 5 and 6). There were no differences between the Control group and those receiving a single dose of 400 IU eCG on the third day (i.e., Day 6) which suggests that it may be possible to reduce the numbers of treatments required to induce superovulation. However, of even greater importance, treatment with two doses of 200 IU of eCG on Day 7 resulted in an increased number of transferable embryos.

Taking all these reports into consideration, it can be concluded that this approach can result in an improved superovulatory response and embryo production, especially when the potential response of the donor cow is not maximized. In a recent report published at the 2012 IETS meeting (Davis et al., 2012), eCG treatment did not result in improved embryo production in Angus donors; however, in this report, the mean number of transferable embryos in both groups (FSH: 9.4±0.7and FSH+eCG: 8.1±0.7) were higher than the usual mean number of transferable embryos reported (i.e., 6.2 to 6.5; Looney et al. 1986, Mapletoft et al. 2002, Stroud et al., 2011).

Superstimulation using a single or two injections of FSH

The need to inject FSH twice a day requires constant attention by farm-personnel and increases the possibility of failures due to mishandling and treatment errors. In addition, twice daily treatments can cause undue stress in donors with a decreased superovulatory response (Bó et al. 1994), and/or altered preovulatory LH profiles (Stoebel and Moberg 1982). Simplified protocols of superstimulation may be expected to reduce donor handling costs and improve response, particularly in less tractable animals.

A single subcutaneous injection of the total cumulative dose of FSH has been shown to result in a superovulatory response equivalent to that of a twice daily intramuscular treatment regime over 4 days (Bó et al. 1994). However, response seemed to depend on body condition; a more consistently high superovulatory response occurred when the subcutaneous injection was made behind the shoulder rather than the neck region, and in cows that had more subcutaneous adipose tissue. In fact, anything that increased the rate of absorption of FSH (e.g., intramuscular injection or injection in the neck region of lean cows) resulted in a reduced superovulatory response. A 0.5 inch need was also used to ensure that the injection was into subcutaneous tissue and not muscle. Results were inconsistent in Holstein cows because of a lesser amount of subcutaneous fat, but this was partially overcome by splitting the total dose of FSH so that 75% was given subcutaneously behind

the shoulder at the start of treatment and 25% was given at the time of PGF injection 48 hours later (Lovie et al. 1994). Regardless, it was important to ensure that the injection was made subcutaneously and into a fat pad. As a result, this approach was not widely used, especially in animals with little subcutaneous fat.

An alternative to induce a consistent superovulatory response with a single injection of FSH would be to combine the pituitary extract with agents that result in a slow and sustained release of the hormone for several days. These agents are commonly referred to as polymers, and are biodegradable and non-reactive in the tissue, facilitating use in animals (Sutherland 1991). Several reports of single administration of FSH in various polymers have appeared in the literature, but all have had limitations (reviewed by Bó et al. 2010).

We have recently conducted a series of experiments involving a single intramuscular injection of FSH diluted in a slow-release polymer (20 mg/mL hyaluronan). Over a total of 325 embryo collections, mean numbers of ova/embryos, fertilized ova and transferable embryos did not differ from cows treated with twice daily intramuscular injections of FSH in the regular diluent (Bó et al. 2010; Tribulo et al. 2011). However, hyaluronan at a concentration of 20 mg/mL was viscous and difficult to mix with FSH, especially in the field. A 50% reduction in the concentration of hyaluronan (10 mg/mL) was much less viscous and easier to mix, but a single intramuscular injection resulted in a lower superovulatory response, presumably because a lesser depot effect. Mean follicle sizes in the 50% hyaluronan group were identical to the original 100% hyaluronan group on Days 4 (day of FSH injection), 5 and 6, but visibly less on Days 7 and 8, and larger on Days 10 and 11, suggesting that several follicles did not reach an ovulatory size and continued to grow after estrus. We speculated that an additional injection of FSH in the 50% preparation 48 to 60 hours after initiating treatment would have caused these follicles to grow and ovulate.

In a subsequent study, beef cows (n=29) were randomly assigned to one of three treatment groups (Control, 5 mg/mL hyaluronan or 10 mg/mL hyaluronan) to be superstimulated three times in a cross-over design (Tribulo et al. 2012). Dilution of FSH lyophlized powder was done using 20 mL of saline for Control cows (Group 1) or 10 mL of hyaluronan (5 mg/mL, MAP-5 50 MG; 10 mg/mL, MAP-5; Bioniche Animal Health,) for cows in Groups 2 and 3, respectively. All cows received a total dose of 300 mg NIH-FSH-P1 (Folltropin-V) by intramuscular injections; in Groups 2 and 3, 200 mg was given on Day 4 and 100 mg was given on Day 6. Results are shown in Table 2. Although numbers of ova/embryos and fertilized ova favored the split-single injection groups (Groups 2 and 3), follicle development, superovulatory response and the numbers of transferable embryos did not differ among groups. Data confirmed that the split-single intramuscular administration of FSH diluted in either of the hyaluronan solutions (5 mg/mL or 10 mg/mL) results in a comparable number of transferable embryos to the traditional twice-daily intramuscular injection protocol.

Table 2. Superovulatory response (mean ± SEM) of beef cows treated with 300 mg Folltropin-V given by twice daily IM injections over 4 d (Control) or diluted in 10 mg/mL or 5 mg/ml hyaluronan and given two IM injections 48 hours apart.

Treatment	N	CL	Ova-embryos	Fertilized ova	Transferable embryos
Control	29	12.0 ± 1.3[a]	10.2 ± 1.8[a]	6.7 ± 1.3	4.0 ± 0.8
Split-single (10 mg/ml hyaluronan)	29	15.1 ± 1.0[b]	14.4 ± 2.0[b]	8.9 ± 1.4	5.0 ± 0.9
Split-single (5 mg/ml hyaluronan)	29	15.3 ± 1.1[b]	14.3 ± 2.1[b]	9.3 ± 1.9	6.1 ± 1.3

[ab] Means within columns with different superscripts differed (P<0.05).

Summary and conclusions

Protocols designed to control follicular wave emergence offer the convenience of being able to initiate superstimulatory treatments quickly and at a self-appointed time, without the necessity of estrus detection. However, estradiol, which has proven to be most useful for these purposes in the field, is being withdrawn from many veterinary markets, leaving only follicle ablation as a reliable method to synchronize follicular wave emergence for superstimulation. Unfortunately, follicle ablation is difficult to utilize in the field. Although the synchrony of follicular wave emergence following the administration of GnRH or pLH has been considered to be too variable for superstimulation, recent reports suggest that GnRH can be used along with a progestin device to synchronize follicle wave emergence for superstimulation. An alternative is to initiate FSH treatments at the time of emergence of the first follicular wave following GnRH-induced ovulation, with the inclusion of a progestin device. Results of preliminary studies suggest that another alternative for the superstimulation of donors may be to ignore follicle wave status and cause subordinate follicles to enter the wave by lengthening the gonadotropin treatment protocol. There is also evidence that superovulatory response and ova/embryo production can be increased by incorporating eCG into the last day of the superstimulation protocol. Finally, a single or two intramuscular injections 48 hours apart of FSH in hyaluronan has now been shown to reliably induce a satisfactory superovulatory response, simplifying the entire protocol and reducing the stress of treatments and animal handling.

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EFFECT OF MATERNAL METABOLISM ON EMBRYO AND FETAL DEVELOPMENT

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Summary

It is now well established that the maternal environment has profound effects on embryo and fetal development and that this persists into adulthood impacting health and welfare. Most studies in the literature have investigated effects on cardiovascular development and there are few studies on the effects on reproductive function. We have recently completed a study that found that restricting maternal nutrition (to 0.6 of maintenance energy requirements) during the first trimester of pregnancy (the period encompassing ovarian follicle formation in foetuses) resulted in their calves having 60% fewer ovarian follicles compared with calves born to mothers fed control diets. Most studies in the literature have investigated under-nutrition or chronic under- of over-nutrition we examined the effects of daily transient hyperglycaemia (100 ml oral propylene glycol twice per day) during the third trimester of pregnancy in sheep and found that it resulted in the birth of offspring that were both larger and had higher growth rates than lambs born to mothers that received the control (water) treatment. The challenge for human medicine and animal production systems is now to understand the mechanism by which maternal environment affects offspring development and, of greater urgency, is to recommend diets that have a positive impact on the health, development and welfare of offspring.

Introduction

The Thrifty Phenotype Hypothesis, (Hales and Barker, 2001; Barker, 1992) suggests that environmental influences early in fetal life are reflected in impaired growth, development and metabolism leading to increased risk for diseases in adulthood. The Hypothesis proposes that some diseases originate through fetal adaptations to malnutrition that permanently alter body function. This hypothesis is supported by animal models (McMillen and Robinson, 2005). Our research group has begun to investigate this phenomenon and here I summarise the findings of two studies in contrasting models of maternal metabolism. In the first study we investigated the consequences of maternal under nutrition during the first third of gestation on ovarian development in their offspring in cattle (Mossa et al., 2009a) and in the second study we investigated the effects of a high glycaemic diet (over nutrition) during the last trimester on offspring growth and development in sheep (Smith et al., 2009).

Effects of maternal nutrition on ovarian follicle numbers in their offspring in cattle

Variation in ovarian follicle numbers

We have conducted a number of studies that have systematically counted and catalogued the numbers of antral follicles on different days of the oestrous cycle in both beef and dairy heifers and in post-partum dairy cows and have established that the numbers of follicles in ovarian follicular waves of the oestrous cycle are highly variable among animals but very highly repeatable within individuals (Burns et al., 2005; Ireland et al., 2007; Ireland et al., 2008; Jimenez-Krassel et al., 2009; Mossa et al., 2010). This observation holds true when considering the peak and nadir numbers associated with follicle waves and the mean numbers across all days of the cycle. However, the count must include all the follicles $\geq$ 3 mm in diameter in both ovaries (the antral follicle count or AFC) and explains why many studies in the last 20 years that have focused on

follicles ≥ 5 or 6 mm in diameter have not noted the high variability of follicle numbers growing during follicular waves among animals nor the remarkably high repeatability of follicle numbers during waves in individuals. We have noted that the AFC in both ovaries during different follicular waves of an oestrous cycle may be consistently lower than 5 during follicular waves in some animals and greater than 50 in others (Burns et al., 2005; Ireland et al., 2007; Mossa et al., 2012) (Figure 1). Moreover, this high repeatability of follicle numbers during waves persists for at least one year (Burns et al., 2005).

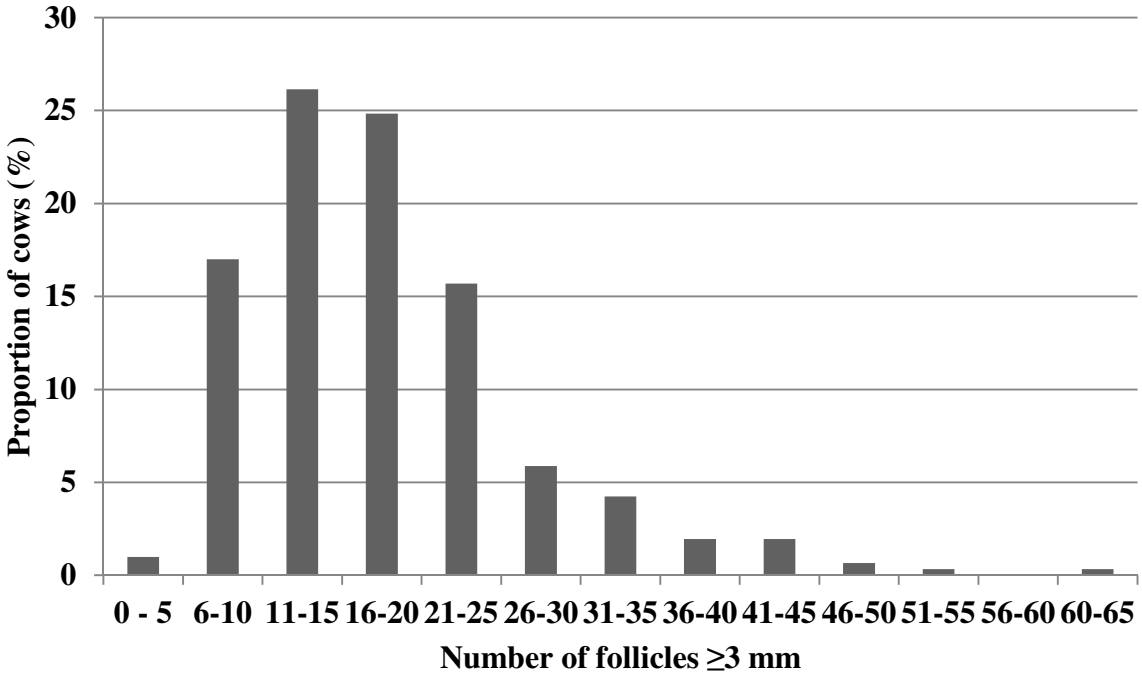

Figure 1 Frequency distribution of the total number of ovarian follicles ≥ 3mm in diameter detected using ovarian ultrasonography on two consecutive days during the first wave of follicular growth of the estrous cycle in 306 lactating dairy cows (Mossa et al., 2012).

Once this high variation in follicle numbers had been established we asked if there is an association with fertility. In cattle the number of follicles prior to superovulatory treatment is correlated with the numbers of follicles and corpora lutea after superovulation, and total ova and transferable embryos recovered (Kawamata, 1994; Cushman et al., 1999; Taneja et al., 2000; Singh et al., 2004). In addition, lower circulating concentrations of progesterone in cattle with low AFC are associated with a much lower endometrial thickness from Day 0 to 6 of the oestrous cycle (Jimenez-Krassel et al., 2009) and endometrial thickness is positively associated with implantation and pregnancy rates in humans subjected to in vitro fertilization programs (Raga et al., 1999). Taken together these findings indicate that AFC is positively associated with fertility in cattle. To test this hypothesis we performed ovarian ultrasonography on 306 dairy cows (aged 3.48±1.66 years) during the first wave of follicular development 1 to 4 months post-partum and recorded their reproductive performances during the breeding season. Cows with a high AFC had higher pregnancy rates, shorter calving to conception intervals and received fewer services during the breeding season compared with cows with a low AFC (Mossa et al., 2012). This is supported by a study in beef heifers showing higher pregnancy rates in heifers with high AFC versus low AFC (Cushman et al., 2009).

Maternal environment and offspring follicle numbers

Most studies that have examined the effects of maternal environment during gestation on offspring development have examined the link between malnutrition with cardiovascular disease, obesity and diabetes (McMillen and Robinson, 2005; Boo and Harding, 2006), but only a few studies have examined the impact of maternal environment on reproduction. Hence, it is reasonable to speculate that the maternal environment during gestation, at the time of ovarian development in their fetuses, may impact oogonia proliferation and thus follicle numbers postnatally.

We have recently tested this hypothesis by restricting nutrition of beef heifers to 0.6 of their maintenance energy requirements, from shortly before conception to the end of the first trimester of pregnancy (period encompassing the peak in oocyte numbers in foetuses (Erickson, 1966)). Results show that calves born to nutritionally restricted mothers had a 60% lower peak, minimum and mean AFC during follicular waves compared with calves born to mothers fed control diets (Mossa et al., 2009b) (Table 1). Similar studies in *Bos indicus* cross heifers support the notion that prenatal maternal nutrition affects ovarian measures in their offspring (Sullivan et al., 2009).

We conclude that there is large between animal variation in the numbers of ovarian follicles (AFC) in adult cattle and that this is at least partly explained by effects of maternal environment during the first trimester of pregnancy, the time of ovarian follicle formation during fetal development.

Table 1. Mean (± SEM) number of follicles ≥3 mm in diameter (Antral Follicle Count) during follicle waves in heifer calves born to mothers fed a control diet (Control) or who were nutritionally restricted (Restricted, 60% energy requirement) for the first 110 days of gestation. From (Mossa et al., 2009a).

Weeks of age	Control (n=13)	Nutrient Restricted (n=10)
7	23.8 ± 2.1	14.1 ± 0.9**
18	26.0 ± 2.8	16.2 ± 1.1**
35	23.9 ± 2.2	16.6 ± 1.2**
56	21.9 ± 2.0	17.5 ± 1.9
86	23.6 ± 1.9	15.8 ± 1.8*

*P<0.05 and **P<0.01 compared to the control animals at the same age

Effects of transient high glycaemic intake in the last trimester of pregnancy on offspring birth weight and postnatal growth rate in sheep

Most studies that investigate the effects of the maternal environment during gestation on embryo and fetal development examine under nutrition. However, over nutrition (in our well-fed society) during gestation is an issue in humans and has implications for animal production in agriculture.

Experimental evidence in humans has established that maternal weight and maternal weight gain during pregnancy significantly influences infant birth weight (Abrams and Laros, 1986; Frentzen et al., 1988; Johnson et al., 1992; Mahony et al., 2007). Fetal macrosomia (large for dates with birth weight >4.0 Kg) is associated with an increased risk of maternal perineal trauma (Sultan

et al., 1994) and an increased risk of trauma to the infant (Geary et al., 1995; Ecker et al., 1997). Additionally, studies have found that infants born at the highest end of the distribution for weight were at a higher risk of being obese in childhood, adolescence and adulthood when compared to normal sized infants (Eriksson et al., 2003; Baird et al., 2005). In humans, the source of maternal glucose originates either from the maternal liver or from the maternal diet. High maternal glucose concentrations are thought to increase maternal weight gain, result in feto-placental overgrowth as well as a higher risk of fetal macrosomia, while low maternal glycaemic diets result in normal maternal weight gain and produce infants with birth weights between the 25th and 50th percentile (Clapp, 2002). Also, there is a relationship between elevated maternal glucose concentrations during gestation (below those levels diagnostic of diabetes) and increased birth weight (Metzger et al., 2008). With the above evidence in mind, it is apparent that there is a need to control the level of glucose consumption during pregnancy to help reduce maternal and fetal trauma at parturition, as well as reducing the risk of obesity related adult diseases later in life.

Consumption of high glycaemic index diets increase postprandial glucose peaks as well as increasing fasting glucose levels compared to low glycaemic diets (Moses et al., 2006). In addition, lifestyle choices mean that pregnant women in the developed world often consume high glycaemic snacks between meals that temporarily but dramatically increase circulating glucose and insulin concentrations. The impact of this type of diet, causing transient elevations in glucose concentrations, on fetal development has not been studied.

On the basis of this we conducted a study to investigate the consequences of transient, intermittent high glycaemic intake during the last trimester of pregnancy on neonatal size and postnatal growth in a sheep model. To achieve this aim, we studied lambs born to pregnant ewes that received a twice daily high glycaemic oral dose of propylene glycol or water (control) in addition to their normal meals during the last trimester of pregnancy. We found that short duration, high glycaemic intake during late gestation, that causes transient elevations in glucose and insulin concentrations (analogous to snacking on high glycaemic foods) compared to the control ewes (Figure 2), had substantial effects to increase offspring birth weight and postnatal growth rates (Table 2).

We concluded that our animal model has relevance for the investigation of the effects of transient maternal hyperglycaemia on offspring development and health. In the context of human health, altering the source and pattern of intake of maternal dietary carbohydrate will prove valuable in the management of pregnancies at risk for fetal overgrowth, trauma at parturition and in the subsequent prevention of childhood obesity. However, in the context of animal production manipulating nutrition late in gestation is a useful tool to maximise the size of new-borns (calves and lambs in particular) as this has significant consequences for the age at puberty and/or the age that animals can go to slaughter.

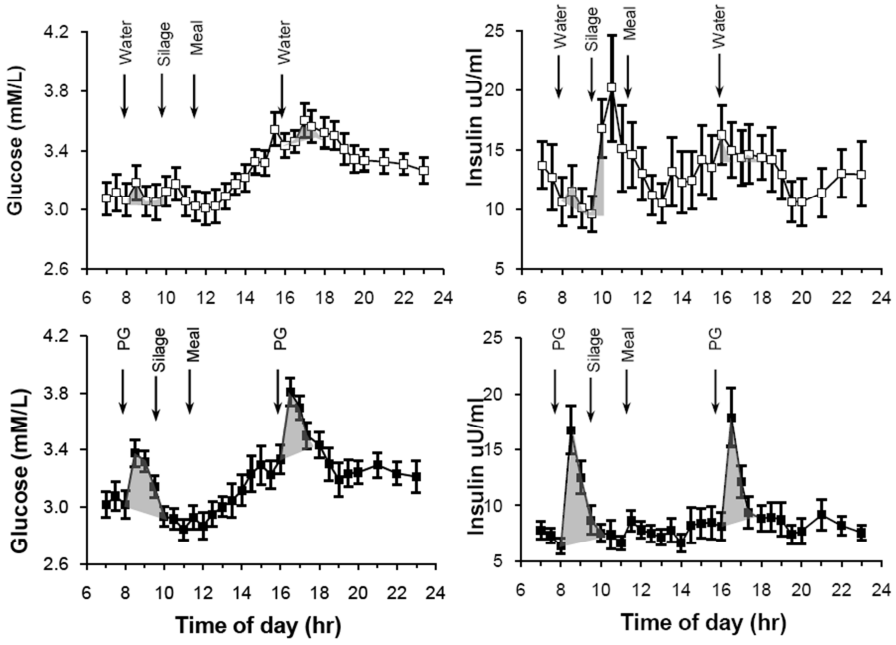

Figure 2. Glucose and insulin concentrations on Day 109 of gestation in ewes administered propylene glycol (n=12) or water (n=12) daily from Day 98 of gestation. Ewes received 100 mls of propylene glycol (PG) or water (control) at 08.00 and again at 16.00 hours. Grass silage was fed ad libitum and replenished as shown and a concentrate meal was fed as shown. The response to propylene glycol or water was calculated as AUC for the 2 hours post administration (shaded area). Glucose (8.00 hours, p=0.002; 16.00 hours, p=0.006) and insulin (08.00 hours, p=0.006; 16.00 hours, p=0.006). AUC response in ewes fed propylene glycol was significantly larger when compared to ewes administered water (Smith et al., 2009).

Table 2. Characteristics of lambs born to ewes fed 100 ml propylene glycol or 100 ml water (control) twice per day from Day 98 of gestation to term (about Day 147) (Smith et al., 2009).

	Propylene Glycol	Water	P value
Gestation length (days)	147.9 ± 0.2 (n=58)	147.6 ± 0.2 (n=63)	P=0.192
Birth Weight (kg)	5.27 ± 0.11 (n=70)	5.01 ± 0.01 (n=80)	P= 0.032
Birth Glucose (mM/L)	3.88 ± 0.29 (n=61)	2.87 ± 0.17 (n=76)	P= 0.001
Birth IGF-I (pg/ml)	629.8 ± 122.6 (n=70)	774.8 ± 172.4 (n=80)	P= 0.253
Growth Rate 0 to 6wks (kg/day)	0.36 ± 0.09 (n=55)	0.33 ± 0.01 (n=55)	P= 0.036
Growth Rate 0 to12 wks (kg/day)	0.31 ± 0.01 (n=51)	0.29 ± 0.01 (n=60)	P= 0.002
Age at slaughter (Days)	166.0 ± 6.75 (n=35)	183.4 ± 7.02 (n=36)	P= 0.039
Carcass Weight at slaughter (kg)	20.6 ± 0.28 (n=35)	20.0 ± 0.26 (n=36)	P= 0.148

Conclusion

It is well established that the maternal environment during gestation affects embryo and fetal development. Here I have summarised the findings of two studies in which offspring ovarian follicle numbers were impacted by reduced maternal nutrition during the first trimester in cattle and also a study where fetal and post natal growth were increased by twice daily transient elevations in glucose (and insulin) in sheep. The challenge for human medicine and animal production systems is now to understand the mechanism by which maternal environment affects offspring development and, of greater urgency, is to recommend diets that have a positive impact on the health, development and welfare of offspring.

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PERI-CONCEPTION ENVIRONMENT

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What is peri-conception environment, and why is it important?

The peri-conception environment is defined as the milieu of the female reproductive tract at the time of mating. It involves the presence of the gametes in the tract, the fertilization and the early embryonic development and implantation. The initiation of the peri-conception environment can be marked by the arrival of spermatozoa in the female reproductive tract or the release of oocyte in the oviduct after ovulation. The arrival of the gametes is then followed by fertilization. Fertilization is the process in which the sperm and the oocyte unite and begin a dynamic developmental program that produces all the cell types of the ultimate adult organism. In internal fertilisers the female reproductive tract is the venue of fertilization. The fertilization is followed by the early embryonic development and implantation which lead to the establishment of pregnancy. All these events take place in the female reproductive tract of mammals. Research has shown that the environment of the female reproductive tract has a profound effect on the successful accomplishment of pregnancy.

Today's perception of the maternal tract environment is different from that in the past. The effect of the periconception environment is not just restricted to events taking place during the early stages of conception and pregnancy but its influence continues well into adulthood. It seems that the peri-conception environment leaves its signature on the offspring by affecting its future development and health.

This view is based on many studies performed in several cohorts of patients and individuals around the world. Probably the most famous is the research carried out on Dutch adults, born to mothers pregnant during the final year of the Second World War known as "Winter Hunger" period. The adults, born by mothers who were exposed to famine at the early stages of pregnancy, showed a higher occurances of coronary artery disease (Painter *et al.* 2006). The male offsprings whose mothers had poor nutrition during the conception and the early stages of pregnancy showed an increased prevalence of intra-abdominal obesity (Ravelli *et al.* 1976). This was in stark contrast with those male offsprings whose mothers suffered from famine during the middle or the late stages of gestation.

Thanks to the work of a number of research groups around the world it is now clear that the *in utero* effects on the childrens are not just limited or uniqe to humans. The pregnancy and the peri-conception environment also affect the decendants of other animals (Fleming *et al.* 2011). This has two consequences for scientific research. Firstly, we may see a universal physiological phenomenon which is shared by many taxa. Secondly, it may allow us to use experimental models which eliminate the potential problems of research in humans. The use of animal models is crucial to accelerate research which aims to understand the causes and mechanisms of the effect of the maternal environment on the offsprings.

How this maternal influence is regulated is not known yet. We also do not understand which particular substances affect the development of the fetus *in vivo,* and how they result in changes in growth of adults, or why the offspring can become vunerable to certain diseases in later life. It is

clear, however that this *in utero* effect is inherited through the maternal (particularly the peri-conception) environment. This underlines the importance of investigating the characteristics and the regulation of the peri-conception environment.

What are the factors influencing the peri-conception environment?

The peri conception environment is a complex milieu. It is confined to the female reproductive tract, and can be influenced by all the factors which have effect on the mother. Some of these factors are general or systemic. For example, several studies have shown that the quality of the maternal nutrition during peri-conception period will have lasting effects on the offspring through fetal and post-natal development into adulthood (Fleming *et al.* 2011). Stress, diseases, toxins and pollutants are also general factors which affect the peri-conception environment. In the past few years many reports showed that the mother's environment has a profound effect on the health and development of the offspring (Feil and Fraga 2012). Finally, I can add another factor on the list of the general factors. These are sex hormones produced during reproductive/menstrual cycle and pregnancy. Hormones affect the maternal milieu and have a substantial impact on peri-conception environment. They influence the growth and differentiation of the cells in the female reproductive tract, and regulate the secretion of these cells (Seytanoglu *et al.* 2008).

Not all the factors affecting the periconception environment have a general and systemic nature. Some of these have a local impact on the gametes and the embryo, and may only influence their immediate vicinity in the maternal tract (Fazeli 2008; Fazeli 2011). The arrival of spermatozoa in the tract, for example, initiates signals which alter the composition of secretory cells in the oviduct (Holt and Fazeli 2010). As the spermatozoa progress in the oviduct and eventually meet the egg, fertilization itself sends unique signals which changes the response of the oviductal cells and the milieu of the oviduct (Georgiou *et al.* 2011; Alminana *et al.* 2012). These alterations continue during the further development of the embryo in the oviduct. We should always take into consideration this dynamic flow of events when we study the peri-conception environment. Investigation of the peri-conception environment in isolation is not a fruitful endeavour.

In summary, several factors can affect the periconception environment. As metioned above these can be general factors such as the hormonal and nutritional status of the mother, or local factors provided by the fertilization process through the gametes, and the embryo. All these prepare a complex milieu and a complex developmental response which eventually establish a dynamic and evolving environment (Fazeli and Pewsey 2008). Hence understanding the peri-conception environment cannot be separated from learning the factors which influence it. These factors may interact with each other, and as a result create a unique effect on the female reproductive tract. Research of these factors have been going on for many years, and a substantial knowledge base has been collected. Research on how these factors interact and dynamically change the unique environment of the female reproductive tract, however, is limited and requires further investigation.

Because of the dynamic nature of the peri-conception environment it is very difficult to predict what the exact composition of this environment at a specific point of the time is. Computational modelling is probably the only method which is able to predict the status of the peri-conception environment with a degree of certainity (Van Soom *et al.* 2010). In my lab we have developed a number of simple computational models (Burkitt *et al.* 2011a; Burkitt *et al.* 2011b; Burkitt *et al.* 2011d; Burkitt *et al.* 2011c; Burkitt *et al.* 2012) to forecast the composition of the periconception milieu at a given stage of development. These models are the first attempts, and there is still a long way ahead of us to use computational models reliably for the prediction of the composition of the peri-conception environment.

Can we influence the peri conception environment and if we do, what would we gain?

The prediction of the composition of the tract at a given time by computational modelling should be for a useful purpose. We can influence the peri-conception environment as the nature has been doing it for billions of years. There are numerous examples that famine, war, change of diet can result in a change in preconception envoirenment (Rosenfeld 2011). The question is how to influence the peri-conception environment in order to have a predictable outcome.

One theory is that changes in the peri-conception environment alter the epigenetic profile of the offspring. These modified epigenetic profiles will affect the development and health of the offspring (Feil and Fraga 2012). One of the best studied models is the agouti viable yellow (*Avy*) allele in the mouse. This locus comprises an upstream intracisternal A-particle (IAP) retrotransposon. When this retrotransposon is unmethylated, the agouti gene is aberrantly expressed, resulting in a yellow coat colour, obesity and diabetes (Rosenfeld 2010). Not all epigenetic changes, however can be reversed by providing methyl donors in the diet. Probably other mechanisms such as chromatin remodelling are at work too (Feil and Fraga 2012).

By influencing the peri-conception environment we can alter the characteristics of the offspring even without knowing the precise mechanisms. Hence this is a novel tool in our hand with translational impact on the quality of life of human beings and on the production of livestock. One can imagine its profound consequence in public health. Maternal diet which influences the peri-conception environment can prevent the occurrence of many degenerative diseases in adulthood. This could generate huge savings in the healthcare budgets and increase the efficiency of the workforce of a country. The impact on economical growth and prosperity is teremendous.

Artificial insemination (AI) and embryo transfer (ET) are well accepted technologies in animal breeding. They have been used for many years to help increase the efficiency of livestock production. However, these technologies have never been used as tools to influence the epigenetic traits of the offspring in the animal breeding practice. Both AI and ET provide excellent opportunities to intervene and modify the environment of the maternal tract. For example, the diluent of spermatozoa or embryo inserted in the maternal tract during AI or ET can be vehicles to carry particular drugs or substances which can localy affect the maternal environment and effect the epigenetic profile of embryo.

ET can be used to transfer embryos which have been treated *in vitro* in a particular environment to set their epigenetic signature for a particular form of development and plasticity. A recent study reported by Banrezes et. al.,(Banrezes *et al.* 2011) showed an increase in weight gain in adulthood of embryos which were incubated from one-cell stage to two-cell stage in a culture media that promoted cytosolic alkalization and increased NAD(P)+ reduction resulting in offspring of normal birth-weight but significantly and persistently larger than controls in adulthood. Such techniques combined with ET in livestock (although it is still not entirely clear how the extra weight gain characteristics are set in the memory of these cells), would be an easy and already accessible technology for the improvement of livestock production.

Future

The society is probably more receptive to interventions through peri-conception environment as a tool for animal breeding or for correcting human health compared to other methods which

involve radical genetic modifications. Still in many parts of the world the general public is very sceptical of food produced by genetic modifications (GM food) or of therapies based on genetic engineering. Such negative views would not be encountered if nutrition were used to intervene in the peri-conception environment resulting in a better quality livestock or a healthier human being.

We must realise that this is the way how nature has always done it. For many years nature has used alterations in peri-conception environment as a strategy to increase the adaptive ability of the offsprings to survive in their new environment even before they were born. Understanding how peri-conception environment affects the newborn will enable more reproductive success, not only in the number of offsprings but in their physiological well being and health.

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EMBRYO GENOTYPING: FROM DNA AMPLIFICATION TO FIELD IMPLEMENTATION

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Abstract

Genomic tools are now available for most livestock species and are used routinely for Genomic Selection (GS) in cattle. Recently, multiple markers detection has been achieved from biopsies of pre-implantation stage embryos, thus paving the way to develop new strategies based on pre-implantation diagnosis and genetic screening of embryos. Different strategies were tested and used to estimate genomic breeding values for traits of particular interest and/or the presence of genetic abnormalities. This review will focus on the practical aspects ensuring a successful embryo genotyping under field conditions. Used alone or in combination with embryo sexing, results are influenced in many different ways including skill of embryo micro-manipulation, strategies used to produce DNA, method of embryo genotyping. Different steps for commercial services and in genetic schemes were investigated to implement efficiently genetic screening of embryos.

Introduction

Advances in genetic analysis and mapping technologies have permitted the implementation of genomic selection (GS) in cattle. With this method, animals can be selected for breeding on the basis of their genetic merit predicted by markers tagging the entire genome. Since a few years, the genomic revolution enables i) to evaluate the "meiosis alea" (difference between the genetic value of the progeny and the mean parental value) without any progeny testing with a reliability increasing with development of new calculation algorithms ; ii) to evaluate more candidates than permitted with progeny testing iii) to select more efficiently animals for functional traits that have low heritability, thus paving the way for sustainable management of populations (Boichard et al., 2012). This is accompanied by an increase of the selection pressure for those traits. Subsequently, the way to produce numerous candidates becomes critical. In this context, artificial insemination (AI) alone may be inadequate to generate sufficient animals in a given period of time. Then, using "intensive" embryo-based reproductive techniques is a common strategy to increase the number of candidates by increasing the number of flushes in Multiple Ovulation Embryo Transfer (MOET) and Ovum Pick-up combined with in vitro Fertilization (OPU-FIV) schemes (Humblot et al., 2011).

One of the main bottlenecks experienced by breeding organisations in Europe is the limited availability of female recipients. Since 20 years, ET teams commonly used embryo biopsy technology combined with a PCR based sex diagnosis to limit the number of embryos to be transferred (Bondioli, 1992 ; Lopes et al., 2001). As soon as marker assisted selection based on a limited number of micro satellites could be used, advantages of embryo genotyping were found due to its potential value for screening the embryo for several traits (Peippo et al., 2007). Today the potential advantages of combining intensive embryo production and genotyping are even higher. However, one of the major challenges of using genotype information in GS is the detection of multiple single nucleotide polymorphisms (SNPs) from a small biopsy sample of a pre-implantation

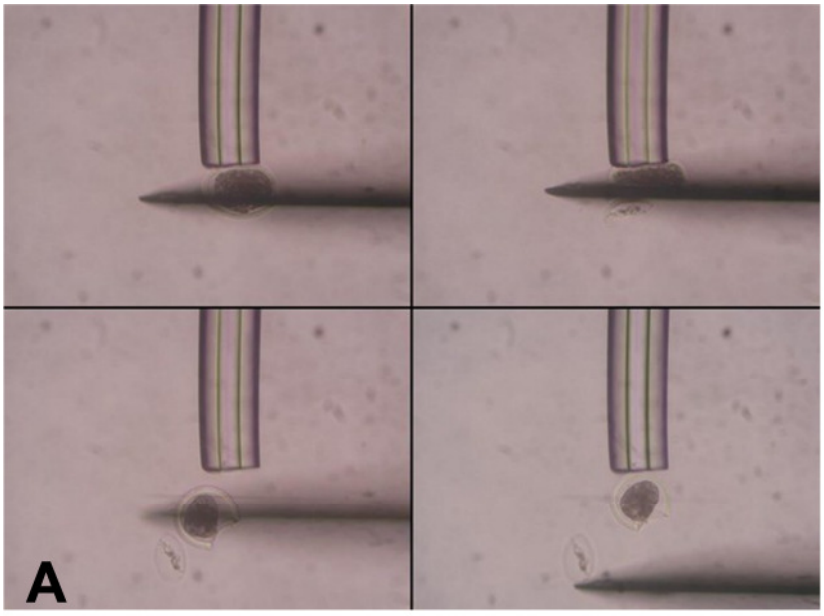
stage embryo. To obtain enough starting material for simultaneous analyses of several markers, different strategies have been attempted (Zhang et al., 1992 ; Lien et al., 1999 ; Peippo et al., 2007 ; Humblot et al., 2011).

Here, combining in vitro approaches and field data, we will present the different steps leading to evaluate the embryo breeding potential and the main practical aspects ensuring a successful embryo screening under field conditions.

Embryo biopsy : how to preserve viability ?

The bottleneck in biopsy procedure remains to manipulate the embryo without decreasing its viability. At the same time, the compromise to be reached consists of removing few numbers of blastomeres to preserve the viability potential together with sampling sufficient DNA quantity for further analysis. Embryo biopsy required highly skilled and trained operators and specific equipment such as an inverted microscope combined with micromanipulators. Depending on the embryo stage two methods are available :

-The microblade biopsy (Figure 1): a microblade is placed on the embryo end and moved down in order to separate 5 to 10 embryo cells. At the morula stage, cells can be cut whatever the embryo position. However, when embryo is biopsied at the blastocyst stage, trophectoderm cells have to be removed preferentially. To enhance the operative procedure, embryo can be maintained by a holding pipette, thus allowing carrying out more standardized biopsies (Figure 1). To prevent gDNA contamination between embryos, microblade and holding pipette have to be washed using ethanol-acetic acid and rinsed in a water bath between each biopsy.



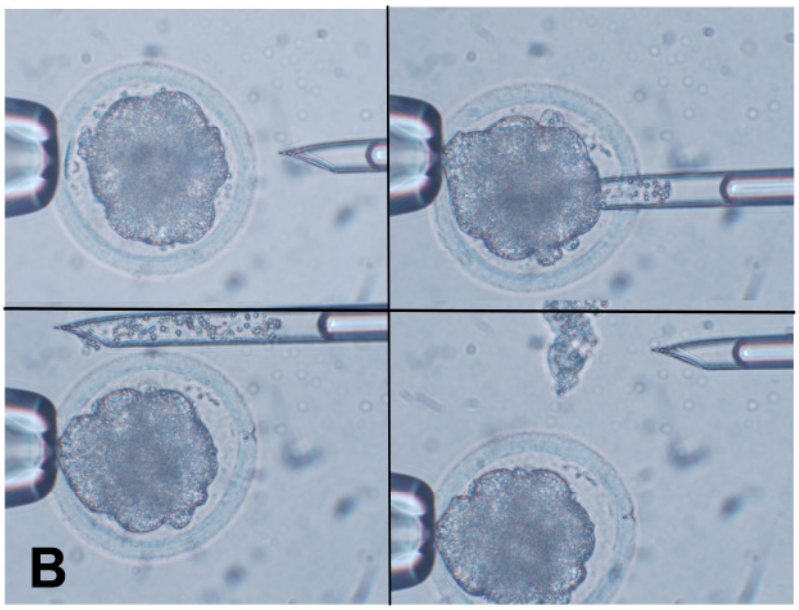


Figure 1: Steps describing embryo biopsy using a microblade (A) or an aspiration method (B ; source UNCEIA)

-The aspiration biopsy (Figure 1): the embryo is maintained by a holding pipette. With an aspiration believed pipette of 20 µm in diameter, the zona pellucida is perforated and cells are gentle aspirated through the pipette.

When carrying out a biopsy, 5 to 10 cells per embryo are removed using both methods, then aspirated from the holding medium and submitted to protocols of gDNA production prior to genotyping. Biopsied embryos are either *in vitro* cultured for further 24 to 48 hours or immediately transferred as fresh into synchronized recipients or even frozen (Table 1). Pregnancy rates ranged from 47.3 to 62.3 % after direct transfer of frozen biopsied embryos, without any significant effect of embryo stage or biopsy size (Tables 1 and 2). Only when embryos were transferred as fresh, pregnancy rates tended to be related to embryo stage and quality. These results indicate that the effects of the biopsy size on subsequent embryonic development are very limited whatever the system used to produce embryos. Freezing of IVP biopsied embryos remains the bottleneck of genotyping technics combined with IVP.

Using a microblade method, high survival rates were observed after a 48 h *in vitro* culture period when embryos were biopsied and frozen respectively at the blastocyst and morula stages (97.1% (100/103) and 88.4% (38/43), UNCEIA unpublished data). The post-thaw survival rate of *in vitro*-produced and biopsied blastocysts after termination of slow cooling at -25°C in 0.7 M glycerol alone (53.8%) tended to be lower than that at -25°C in 0.7 M glycerol plus 0.05 M sucrose (91.3 %) or -30°C in 0.7 M glycerol plus 0.05 M sucrose (91.3 % ; Tominaga et al., 2007). Positive effects of sucrose were also reported in biopsied *in vivo* derived embryos by Lacaze et al (2008), with pregnancy rates averaging 55.8 % when embryos were frozen in 1.5 M ethylene glycol plus 0.1 M sucrose, compared to 40.8 % when embryos were frozen in 1.5 M ethylene glycol plus 40% of fetal calf serum (FCS ; p=0.06).

Table 1: Pregnancy rates following transfer of biopsied embryos in cattle, under farm or experimental conditions

Author	Biopsied embryos	Biopsy procedure	Freezing	Pregnancy rate
El-Sayed et al., 2006	138 D7 *in vitro* produced blastocysts (experiment)	Beaver™ microblade fixed to a micromanipulator ; 2 hours *in vitro* culture to allow re-expansion (77.7 % of embryos)	No freezing	33 % on D 40 or D 50 of pregnancy, 3% of abortions
Gonzales et al., 2008	56 day 7 *in vivo* derived embryos (experiment)	Home-made microblade fixed to a micromanipulator + holding pipette	G1 (24), G2 (7) and G3 (23) embryos frozen in 1.5 M EG[2], direct transfer	On D 35 and D 90 respectively : G1 : 62.5 54.2 % G2 : 57.1 57.1 % G3 : 52.2 47.8 %
Lacaze et al., 2009	671 day 7 *in vivo* derived G1 embryos (farm)	Home-made microblade fixed to a micromanipulator + holding pipette	Fresh (284)	59 %
			1.5 M EG + 40 % FCS[1] (53) or 0.1 M sucrose (340)	50 %
Ghanem et al., 2011	49 D 7 *in vivo* derived G1 Blastocysts (experiment)	Beaver™ microblade fixed to a micromanipulator ; 2 hours *in vitro* culture to allow re-expansion (77.7 % of embryos)	No freezing	63.1 % on Day 28 (24/38 re-expanded blastocysts)

[1] *FCS : Fetal Calf Serum ;* [2] *EG : Ethylene Glycol*

Table 2: Effect of embryo stage and biopsy size on pregnancy rates following direct transfer of frozen biopsied embryos (Lacaze et al., 2008)

Variation factor	Class	Number	Pregnancy rate
Embryo stages	Morula	167	47.3
	Early blastocyst	39	48.7
	Blastocyst	16	62.5
Biopsy size (nb of cells)	<3	26	55.6
	3-7	65	47.8
	>7	37	49.5

Strategies to ensure sufficient dna production from biopsied cells for genotyping

Several studies reported that few cells collected per biopsy are sufficient for embryo pre-implantation diagnosis of one single trait using PCR (Thibier & Nibart 1995, Bredbacka et al., 1996 ; Le Bourhis et al., 1998). Nevertheless, few cells result in limited amount of genomic DNA, thus restraining the number of markers to be tested. Indeed, Lacaze et al. (2008) demonstrated that the efficiency of a PCR method for sex determination was inversely related to the number of cells : <3 cells: 85.5% (n=83); 4-6 cells: 97.4% (n=432) and >7 cells: 100% (n=255 ; $p<0.05$). Therefore, new strategies have been investigated and developed in order to increase the total amount of gDNA available for multi-genotyping.

The first strategy selected for DNA production was to use nuclear transfer (NT) in order to clone the biopsied cells, thus increasing number of cells before genotyping. After biopsy, cells were isolated and fused to an enucleated-activated recipient oocyte. Reconstituted embryos from each biopsy were then *in vitro* cultured for 8 days to the blastocyst stage (Table 3). This strategy can only be applied to embryos at the morula stage. Our results showed that the production of cloned blastocysts per donor embryo was correlated with the *in vitro* fertility of bulls. Using NT, the quantity of DNA from one single NT blastocyst enabled to perform multi-genotyping with an efficiency averaging 90 % using a set of 45 microsatellites markers (source UNCEA). Unfortunately, this strategy was time consuming and was not further developed when high density chips requiring few micrograms of gDNA emerged.

Table 3: Use of nuclear transfer combined with *in vitro* culture to produce blastocysts from biopsied cells (source UNCEIA)

Embryos	Bull	Number of embryos per group	In vitro survival rate	Number of cells	Number of cloned blastocysts
In vitro produced	1 (high fertility)	5	100%	9.8± 2.9	2.8±1.8
	2 (high fertility)	28	96.4%	7.4±2.4	1.7±1.2
	3 (low fertility)	11	81%	7.3±1.8	1.0±0.9
In vivo derived	A	7	100 %	6.7±1.6	0.6±0.8
	B	5	80 %	5.8±0.4	0.6±0.5
	C	5	0 %	7.4±1.9	0
	D	5	100 %	7.6±1.3	1.4±1.1
	E	6	100 %	8.8±2.3	1.3±0.8

A second strategy aimed to produce a large number of cells (high quantities of gDNA) for genotyping using *in vitro* culture of biopsied cells issued from bovine blastocysts (Gamarra et al., 2009). Individual biopsies were cultured *in vitro* in 4-well culture dishes coated with collagen type 1 under 3 medium conditions : i) DMEM/F12 + 10% FCS and 0.25% ITS (insulin, transferrin, selenium), ii) DMEM/F12 + 20% FCS supplemented with 1 mM sodium pyruvate, 1 µg mL^{-1} of heparin, and 1 µg mL^{-1} of FGF4 and iii) complex medium composed of 30% of [DMEM/F12 + 20% FCS] and 70% [DMEM/F12 + 20% FCS conditioned medium using mitomycined VERO cells] supplemented with 1 mM sodium pyruvate, 1.5 µg mL^{-1} of heparin, and 1.5 µg mL^{-1} of FGF4. For each culture conditions, none of the biopsied cells attached to the coated dishes and no colony were observed after culture. This strategy didn't provide sufficient amount of DNA for genotyping (Gamarra et al, 2008), in agreement with observations from Moros-Mora et al. (2011) indicating that only 50% of biopsies has proliferated after a 10 days period of *in vitro* culture.

Recently, Whole Genome Amplification (WGA) by multiple displacement amplification was tested. This WGA method has been developed for the pre-amplification of small amounts of gDNA, including gDNA from one single cell (Dean et al, 2002; Lovmar et al., 2006). In addition, WGA gDNA have been used in a variety of applications including high-throughput genotyping (Lovmar et al., 2006), Affymetrix^{TM} array (Paez et al., 2004) and Illumina Bovine SNP50^{TM} chip (Gunderson et al., 2005). Different commercially available WGA kits have been independently utilized in previous microarray studies with a high reliability and accuracy (Treff et al., 2011). Furthermore, WGA of genomic DNA from embryos of various species (pig, goat, bovine and human) have been used in many studies for pre-implantation diagnosis including specific traits such as sex, PRPN (PRioN Protein) genotype determination in caprine embryos or Duchenne muscular dystrophy (DMD) in human embryos (Akasaka et al., 2011; Guignot et al., 2011, Polisseni et al., 2010; Ren et al., 2009).

Practical aspects related to embryo genotyping implementation under field conditions

Different experiments were designed to refine the conditions ensuring a sufficient quality of gDNA following WGA before field implementation. First experiments aimed to detect a set of 58 SNPs markers depending on the number of cells, i.e. size of the biopsy before WGA. Samples including an increasing number of starting cells from 1 to 10 cells have been prepared from isolated blastomeres (Table 4). After WGA, samples with 5 to 10 cells presented significantly highest call rates (CR: proportion of detected markers) compared to samples with fewer number of cells (98% vs 75%, Le Bourhis et al, 2009). In a second set of experiments, WGA from biopsied *in vivo* derived embryos has been used to compare call rates using an Illumina BovineSNP50^{TM} beadchip according to the number of cells per biopsy. Mean CR were significantly higher ($P<0.05$) when the embryo biopsy included 8 to 10 cells compared to a biopsy including 5 to 7 cells (Table 5). In the same way, Ling et al. (2009) demonstrated that CR increased significantly when one, two, five and 10 cells were subjected to MDA.

Table 4: Efficiency of embryo genotyping according to the number of cells (Le Bourhis et al, 2009)

Number of cells per biopsy	Number of biopsied embryos	Rate of markers detection (%)
1	10	45[a]
2	10	75[b]
5	10	98[c]
10	10	98[c]
20	5	93[c]

[a,b,c]: $P<0.01$

Table 5: Call Rates following WGA and genotyping with a Illumina BovineSNP50^{TM} beadchip according to the number of cells (Le Bourhis et al., 2011)

Number of cells per biopsy	Number of biopsies	CR (mean± SEM)
5-7	12	85±8 [a]
8-10	14	90±4 [b]

[a,b,]: $P<0.05$

Under field conditions, DNA from bovine embryo biopsies are currently amplified using WGA- REPLI-g UltraFast Mini Kit (Qiagen, France) according to the manufacturer recommendations with slight modifications (Le Bourhis et al., 2012). After WGA, the process generated a DNA quantity ranging from 5 to 7 µg (PicoGreen analysis ; Table 6), which represents an increase of at least 40,000 times compared to the initial amount of gDNA products. When genotyping is performed in a central molecular laboratory including WGA and genotyping on chip,

embryo cells are transported in a small volume of medium (less than 2 µl) and sent per post. Then, transport conditions may be a crucial point to ensure success of genotyping and two transport conditions have been compared: room temperature or frozen. Different sets of experiments led to recommend a control of transport temperature, with biopsies sent as frozen (Tables 6 and 7). Except DNA content, no significant differences were observed between both conditions. However, call rates were higher and more constant when pre-amplification was realized without any transport.

Table 6: Amount of DNA after WGA and genotyping results (call rates, allele drop out) with a Illumina BovineSNP50^{TM} beadchip in cattle embryos following different transport conditions

Source	**Transport conditions**	**Number of biopsies**	**DNA following WGA (µg)**	**Call rate (%)**	**ADO (%)**	**Parentage error (%)**
UNCEIA (Le Bourhis et al., 2011)	**Frozen**	13	7.0±1.9[a]	87±7	18±16	0.26±0.33
	Room temperature	16	5.0±0.4[b]	90±6	17±10	0.32±0.47

Table 7: Call rates with a Illumina BovineSNP50^{TM} beadchip in cattle embryos following different transport conditions (source CRV)

Transport conditions	**# biopsies**	**Mean call rate**	**% samples with a call rate >0.85**
Fresh	45	0.64	32%
Frozen	46	0.48	17%

From genotyping to embryo breeding value

Assessment of genotyping efficiency

WGA gDNA from biopsied *in vivo* embryos has been used to compare the efficiency and accuracy of CR and ADO using an Illumina BovineSNP50^{TM} beadchip with blood DNA from corresponding calves. Mean CR from WGA of gDNA (embryo biopsy) was significantly lower than CR from calf blood, averaging 85 % in experiments from 2 breeding companies (Table 8). However, when comparing heterozygous SNP from each embryo/calf couple and considering that no ADO was present in blood samples, embryo ADO rates were correlated to the embryo CR. More precisely, embryo CR higher than 85 % showed ADO lower than 1 % (Le Bourhis et al., 2011).
As a consequence, CR higher than 85 % seemed to be a minimum CR threshold value to estimate breeding values for embryos. This was achieved for 74 % (275/371) of the biopsied embryos. Those results indicated that accurate genotyping chip results can be obtained using WGA gDNA from embryo biopsies.

Table 8: Call rates following DNA WGA from embryo biopsies (Le Bourhis et al., 2012)

ET team	**Nb. biopsies**	**CR (%)**	**% biopsies CR >85 %**
CRV	254	84	70
UNCEIA	117	87.7 ± 12.5	85.5

Breeding value estimation

Due to ADO, only markers that were still heterozygous in genotype of an embryo were selected for breeding value estimation. Furthermore, taking into account the SNP50 chip genotyping results of the parents, imputation was done using Beagle software and. breeding values (milk production and morphological traits) were calculated and compared with those of the corresponding calves (Table 9).

Table 9: Correlations between breeding values from 26 Holstein (HOL) et 11 Montbéliards (MON) from embryo cells and subsequent calves after birth (source UNCEIA)

Race	Genomic synthetic index (ISU)	Economic Index for dairy traits (INEL)	Morphology	Milk	Fertility (cow)	Cells
HOL	0.996	0.997	0.997	0.979	0.991	0.985
MON	0.994	0.998	0.997	0.998	0.989	0.937

From 37 couples including breeding values estimated from an embryo biopsy and the subsequent born calf, synthetic indexes (ISU) were compared and only few differences between breeding values were observed, with a maximum deviation of 5 points of index between calf and embryo observed in one single case. In 95 % of the cases (35 couples), the maximum deviation reached 2 points of index between calf and embryo (Figure 2).

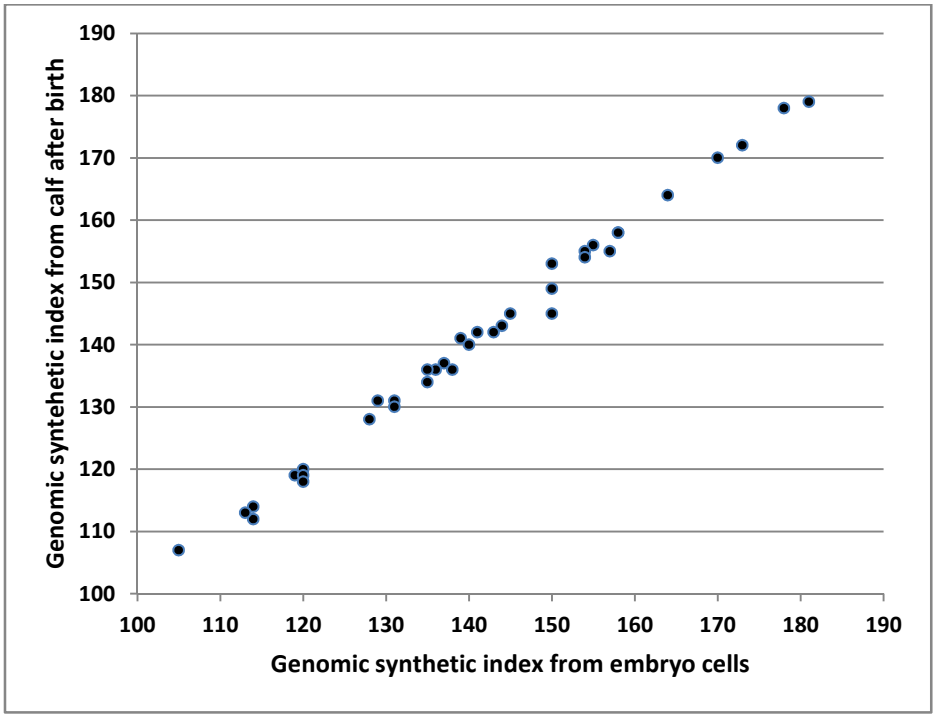


Figure 2: Comparison between genomic synthetic indexes estimated from 37 embryos and subsequent born calves (source UNCEIA)

Conclusion

Genomic Selection induced a revolution in cattle breeding schemes. In addition to new genomic tools, strategies to genotype embryos for multiple markers were developed and combined with embryo biospy at morula or blastocyst stages. This provides the opportunity to estimate breeding values for traits of particular interest and/or the presence of genetic abnormalities, thus allowing to select embryos before transfer. In addition, it enabled ET teams to produce valuable material from a very limited resource to perform clinical and functional studies. Finally it demonstrates the feasibility of a pre-implantation genetic diagnosis combined with freezing and transfer of embryos. Used alone or in combination with embryo sexing, development of embryo screening strategies should also depend on "breeder" demand for specific traits, regulatory changes and also changes related to the evolution of breeding strategies.

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SHORT COMMUNICATIONS

FOLLICLE SIZE IS NOT RELATED TO THE CONCENTRATIONS OF ESTRADIOL AND PROGESTERONE IN BOVINE FOLLICLES AFTER SUPERSTIMULATION

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Superstimulation (SO) protocols are widely used in dairy industry, but the response to the treatment varies and results in an unpredictable outcome for an individual cow. Previous studies related a higher success rate of SO to the presence of a high number of small-medium sized follicles at the initiation of the SO (Singh et al., 2004, Theriogenology **62**, 227-43) and a lower success rate to aberrant blood progesterone concentrations and timing of the LH peak during the SO treatment (Callesen et al., 1986, Theriogenology **25**, 71-86). Studies with non-stimulated cows, showed estradiol (E2) dominance in the follicular fluid around the LH peak shifting towards progesterone (P4) at the time of ovulation (Dieleman et al., 1983 J Endocrinol **97**, 31-42; Callesen et al., 1986, Theriogenology **25**, 71-86; De Loos et al., 1991, Theriogenology **35**, 537-546). Thus E2 and P4 concentrations are used to discriminate between "healthy" follicles and "aberrant" follicles after SO. As follicular hormone concentrations are not readily available at the moment of oocyte collection, we investigated whether follicular E2 and P4 concentrations are correlated to the size of the follicle and if follicle size can be used for selection of "healthy" oocytes.

Heifers (n=16) were synchronized with a CIDR® intravaginal device (Pfizer AH) during 7 days and a prostaglandin injection (PG; 5 ml Enzaprost® CEVA AH i.m.) one day before CIDR® removal. On day 8 of the synchronized cycle (estrus: day 0) the dominant follicle of the first follicular wave was removed and the standard SO protocol was started two days later (twice daily decreasing doses for four days, total 200 mg Folltropin-V®, Bioniche AH). Heifers received a CIDR® device during SO to suppress a spontaneous LH surge. To collect ovarian tissue at 22 h after the LH peak by ovariectomy, a controlled LH surge was induced by a GnRH injection (1 mg Fertagyl® Intervet SP AH) at the time of CIDR® removal (48h after PG injection; Vos et al., 1994 J Reprod Fert **100**, 387-393). Immediately after ovariectomy, all follicles > 8 mm were collected and follicle size was estimated by the volume of follicular fluid. To determine the quality of each individual follicle, follicular fluid was analyzed for E2 and P4 concentrations. Follicles at 22 h after the LH peak were defined as "healthy" when showing low E2 and high P4 > 0.5 μmol/L, (E2/P4<1; Dieleman et al., 1983 J Endocr **97**, 31-42). All procedures performed on the animals were in accordance with national regulations and established guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee.

In the category of follicles ≥ 13 mm (volume of 1 ml) a higher percentage of follicles showed the "healthy" hormone profile (50%; 30/60) compared to follicles < 13 mm (27%; 33/123). This study shows that larger follicles more often compose a healthy hormone concentration. However, follicles with diameters between 8-13 mm were still responsible for half of the total amount of healthy follicles. Therefore we conclude that follicular hormone concentrations and not follicle size should be used for obtaining competent oocytes from a pool of superstimulated follicles. Future research should focus on the development of a more rapid test to select the healthy follicles.

Notes

INFLUENCE OF THE LENGTH OF A PROGESTERONE TREATMENT ON THE GROWTH OF THE PREOVULATORY FOLLICLE INDUCED BY THE RAM EFFCT IN ANESTROUS EWES

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The objective of the present study was to investigate the effect of the length of a progesterone treatment (12 vs. 2 days) on the growth of the preovulatory follicle induced by ram exposure in anestrous Ile de France ewes.

The ram effect was performed with 9 sexually active males subjected to long daylength (16h light and 8h darkness) for 60 days followed by exposition to short daylength (8h light and 16h darkness) for 50 days in order to improve the stimulatory signal. The end of the photoperiodic treatment coincided with the day of ram introduction (D0).

Forty-one Ile de France ewes were selected from a group of healthy primiparous and multiparous ewes. The anovulatory status was checked by analysis of progesterone concentration in blood samples collected once a week for 3 weeks before the introduction of the ram. The anovulatory ewes were divided into three homogenous groups: **Control group** (n=20) received no hormone treatment, **CIDR-2** group (n=10) received a short-term progesterone treatment (intravaginal progesterone release device "CIDR" for 2 days) and **CIDR-12** group (n=10) received a long-term progesterone treatment "CIDR" for 12 days, before the introduction of the ram. CIDR was removed 24h before ram introduction (D0).

Estrous behavior was recorded twice daily from day 0 to day 4 by direct visual observation. The quality of the induced cycle (short or normal cycle) was determined based on plasmatic progesterone profiles over 25 days after ram effect (blood sampling once a day). The parameters of growing dynamic of preovulatory follicles were also analyzed (day of emergence, size at D0, day of ovulation, ovulatory size, the time for follicular growth, growth rate and follicle lifespan) by transrectal ultrasonography once a day from day -5 to day 7, and in days 8, 10 & 14.

Results show that the length of a progesterone treatment had no effect in the proportion of ewes ovulating. However, 95% of the treated ewes developed a normal cycle as expected. In contrast, most of the ewes from the control-group developed a short cycle (80%). Estrous was observed in all ewes from CIDR-12 group and only 2 ewes from the CIDR-2 group. Any ewes of control group exhibited estrous.

The time for follicular growth was greater ($p=0.003$) in ewes treated with progesterone for 12 days (4.4 ± 0.3days) than in ewes treated for 2 days (3.2 ± 0.3days) and untreated ewes (2.9 ± 0.2days). In the same way, the lifespan was longer (0.01) in CIDR-12 group (5.7 ± 0.3days) than in CIDR-2 group (5.1± 0.4days) and Control-group (4.3 ± 0.3days). These observations were related to the time of ovulation, which occurred later ($p=0.08$) in the CIDR-12 group (3.2 ± 0.7) than in the CIDR-2 group (2.4 ± 0.3) and Control group (2.6 ± 0.2days). However, the day of emergence, the size at D0, the ovulatory size and the growth rate, did not differ among groups. These results suggest that a long-term progesterone treatment prior to ram exposure leads to a better follicular maturation of pre-ovulatory follicles induced to ovulate.

Notes

EXPRESSION OF INTEGRIN BETA 2 (ITGB2) AND *ZONA PELLUCIDA* GLYCOPROTEINS (ZP3, ZP3A) IN DEVELOPMENTALLY COMPETENT AND INCOMPETENT PORCINE OOCYTES

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The mammalian oocyte's fertilization ability may be regulated at the molecular level by expression of species specific sperm-egg interaction molecules, which proper activity and/or cellular distribution determines gamete recognition and fusion. Although there are several published data indicating the expression of integrins (ITG's) and zona pellucida glycoproteins (ZP's) in developmentally full competent mammalian oocytes, the mRNA's level encoding these proteins in immature and developmentally incompetent porcine oocytes have to be elucidated. Therefore, the goal of this investigation was to determine the differences in ITGB2, ZP3 and ZP3α mRNA' s expression in porcine oocytes before IVM, oocytes stained with BCB test but colorless and BCB positive oocytes after IVM.

The porcine COC's were collected from 32 crossbred Landrace pubertal gilts and then separated into three groups; (i) oocytes analyzed immediately after collection-before IVM (n=50), (ii) oocytes stained with BCB test and colorless (n=50), and (iii) oocytes stained blue-BCB+, analyzed after IVM, (n=50). After collection and cultivation, all denuded oocytes (DO) were analyzed regarding ITGB2, ZP3 and ZP3α by using RQ-PCR assay.

We found an increased expression of ITGB2, ZP3 and ZP3α in oocytes before IVM and BCB+ oocytes, as compared to BCB- porcine female gametes ($P<0.001$, respectively). Regarding ITGB2 and ZP3 we did not observe differences in mRNAs level between oocytes analyzed before IVM and BCB+ oocytes. In addition, BCB- oocytes revealed decreased transcript expression in all three investigated genes.

It has been suggested that similar mRNAs levels of ITGB2 and ZP3 between oocytes before IVM and BCB+ oocytes may be associated (i) with toxic effect of BCB staining test on the oocytes and/or (ii) with degradation of accumulated maternal templates in immature porcine oocytes. Moreover, decreased ITGB2, ZP3 and ZP3α transcript levels pointed to the down-regulation of mRNA's synthesis of stored maternal transcripts in developmentally incompetent porcine oocytes.

Notes

SYNCHRONIZATION PROTOCOL FOR BOVINE OPU-IVP EMBRYO TRANSFERSOF MURCIANO-LEVANTINA BREED IN RECIPIENTS IN A DAIRY FARM: PRELIMINARY RESULTS

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Oocytes from three Murciano-Levantina donors included in a recuperation program of this bovine breed were recovered by ultrasound guided ovum pick up (OPU). The dominant follicle was retrieved by follicular ablation or exogenous GnRH 48h before i.m. administration of 500 IU FSH-LH (Pluset®, Calier, Spain). Follicles larger than 3 mm in diameter were aspirated. Cumulus oocyte complexes (COCs) from categories I, II and III were considered viable and suitable for IVP. Maturation, fertilization and *in vitro* culture were performed according to established procedures.

The mean cleavage rate obtained was 43.14% (22/51). The blastocyst rate was 21.57% (11/51) and the number of embryos per OPU session was 1.57. Depending on the recipients availability the *in vitro* produced embryos were vitrified (Morató et al. Reprod Fertil Dev. 2010) and rehydrated just before transfer or fresh transferred.

The synchronization protocol for the recipients was the following:
Heifers from a dairy farm were previously explored. Pubertal, healthy ones, >14 months old, with adequate body condition were selected. **Day 0**: progesterone-releasing intravaginal device (CIDR®, Pfizer, Paris AH, France) + 0.2 mg of GnRH i.m. (Dalmarelin, Fatro Iberica, Spain); **Day 10**: 0.15 mg of Dinoprost (Dinolytic®, Pfizer AH, Paris, France) i.m.; **Day 12**: CIDR-removal; **Day 14**: GnRH and oestrus recording; **Day 21**: Exploration of the recipients and ET. Only heifers with ≥1 CL with an area of >2 cm^2 (based on ultrasonographic examination) were used for embryo transfer. Flunixin meglumine (2.2 mg/kg i.m. Fynadine®, MSD, Boxmeer, Netherlands) was administered, at least 30 min before ET; **Day 28**: CIDR reintroduction; **Day 40**: CIDR removal and **>Day 45**: Pregnancy diagnosis. Non pregnant heifers with oestrus on days >42-44 got a doses of prostaglandin $F_{2\alpha}$ (Dinoprost) on day 48 after synchronization begin and were artificially inseminated.

The aims of this protocol are 1) to maximize pregnancy rates after ET, 2) to minimize the rejection rate of synchronized heifers on the day of ET and 3) fast oestrus synchronization after ET.

By now, a total of four vitrified-warmed embryos have been transferred to recipients, with one pregnancy as result, diagnosed with ultrasound at 60 days. Two additional embryos have been freshly transferred. At the moment no pregnancy diagnosis is available from these last transfers. The rate of heifers used for transfer per synchronization was 66.66% (6/9; rejection rate of 33.33%) and the mean CL-area in transferred heifers was 2.38±0.66 cm^2. Noticeably, the one pregnant heifer had the maximal CL area (3.3 cm^2 of luteal tissue).

The program is still going on and we expected to perform a total of 20 transfers until December 2012, with a pregnancy rate of 20 and 50% for vitrified and fresh embryos, respectively, of this endangered bovine breed.

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Notes

EMBRYO RECOVERY RESULTS AFTER TIMED ARTIFICIAL INSEMINATION IN NORMAL CYCLING AND IN SUPEROVULATED CATTLE WITH REDUCED DOSAGES OF UNSORTED SPERMATOZOA AND WITH SEXED SPERMATOZOA

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Different factors determine the effectiveness of the use of sires in artificial insemination. Most important factors are the number of inseminated spermatozoa, the quality of spermatozoa and the time of insemination. Especially in superovulated animals the insemination scheme plays in important role to cover the whole ovulation period. Using well-tried and approved schedules of timed artificial insemination in normal cycling cattle (Becker et al.2003) the influence of three different dosages of spermatozoa ($15x10^6$, $5x10^6$ and $1x10^6$) on fertilization rate was examined in experiment A. In experiment B one dosage of female and male spermatozoa of three different bulls were used for timed artificial insemination in 31 heifers. In total embryos and oocytes were flushed from the oviduct of 116 hemicastrated or slaughtered heifers on day 4 after insemination. The ovulation rate in heifers was 95.4%. In total 80% of the oocytes or embryos were recovered. There was no significant difference in the fertilization rate (93.3, 96.2 and 78.8%) and in the proportion of normally developed embryos (84.6, 80.7 and 75.8%) between groups. Significant differences were found in the mean number of accessory sperms/embryo and in the proportion of embryos with >10 accessory sperms/embryo or without accessory sperms; however the proportion of intact embryos was similar. Using sexed semen in experiment B similar results were obtained after flushing of the oviducts on day 4 after insemination of hemicastrated or slaughtered animals. In total an ovulation rate of 91.7%, a recovery rate of 70% and a fertilization rate of 86.8% were obtained. There were no differences between female and male sorted spermatozoa and between the control group.

In experiment C altogether 13 heifers were treated 8 times with FSH for 4 days starting between day 8 to 12 of estrous cycle. PGF2 was given 48 and 60 hours after the first FSH injection. Insemination with sexed semen (n=5 heifers) and with unsorted semen (n=8; $15x10^6$ and $1x10^6$) was done after 55 and 71 hours after induction of luteolysis. Flushing of the uterus was performed on day 7. Using the time oriented insemination after superovulation of animals fertilization rates varied between 65% and 85 %. There was no difference between groups regarding the number of transferable embryos (5.5; 4.9 and 4.8) Results demonstrate that the application of an approved insemination schedule may accomplish high fertilization rates after insemination with sexed or reduced dosages of spermatozoa in in normal cycling as well as in superovulated cattle.

Notes

USE OF DOUBLE-FLUSH TECHNIQUE TO IMPROVE EMBRYO RECOVERY RESULTS IN SUPEROVULATED HIGH PRODUCING DAIRY COWS

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The objectives were to evaluate potential improvements in recovery rate of superovulated dairy cows after a single (SF) or double-flushing (DF) technique. Holstein cows (n=95), were milked twice daily and housed and fed individually in tie-stalls. All cows were synchronized and superovulated using a modified 5d-Double Ovsynch protocol with 4 d of decreasing FSH (Pluset®) treatments. Cows were producing 32.4kg ± 5.0 and at 205DIM ± 23. Non-sexed frozen semen ($15x10^6$ sperm/straw) were produced from single ejaculates of five proven-fertility sires and cows were inseminated twice at 12h and 24h after final hCG treatment. To minimize variation, one experienced AI technician performed all breedings and four experienced technicians performed all flushings, which occurred 6d after synchronized ovulations. Cows were initially flushed with a silicone two-way catheter (Minitube of America, Verona, WI) in each horn individually, with a liter of flush media per horn (SF). Immediately following the initial uterine horn flush, the catheter was moved back to the cervix and flush media was placed in the uterus. After 30 minutes, cows were re-flushed in the whole uterus with a full liter of flush media (DF). In addition, a single technician searched and graded all embryos. Data was analyzed with the proc GLIMMIX (with cow used as a random variable) and proc CORR of SAS. Cows having no CL on the day of the flush (n=5) were not flushed and excluded from the recovery analysis. As expected, the double-flush technique increased (P<0.01) percent recovery from 46% to 56%, improving recovery rates in nearly ~22%. Overall, out of total average of structures collected (9.8±1.0), 80% were collected in the first flush and 20% were recovered in the second flush. In addition, most improvements in recovery seem to happen in cows with greater numbers of CL (1-12 CL: SF=43%^{a} vs DF=47%^{a}; 13-20 CL: SF=48%^{a} vs DF=62%^{b}; 21-40 CL: SF=47%^{a} vs DF=57%^{b}; a≠b at P<0.05). Proportion of total structures found in the second flush went from 11% in cows with less CL numbers to nearly 20 to 30% in cows with more CLs (1-12 CL: SF=3.4^a structures vs DF=3.6^a; 13-20 CL: SF=7.7^a structures vs DF=9.9^b; 21-40 CL: SF=13.1^a vs DF=15.8^b). In conclusion, cows presenting greater superovulatory response (greater CLs counts on the day of the flush) seemed to have improved recovery rates only after DF technique but not SF (P=0.04). The double-flush technique increased recovery rates in nearly 20% of the cows flushed and seems to be highly advantageous particularly in donor cows with greater CL numbers.

KEYWORDS
Superovulation, Dairy cow, flush technique

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Notes

EFFECTS OF DIFFERENT *INVITRO* MATURATION SYSTEMS ON BOVINE EMBRYO DEVELOPMENT

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The search for optimized media and culture conditions for assisted reproduction techniques in veterinary and human medicine has been a main research focus for decades. Recently, Albuz et al. (2010) reported a novel maturation system for cumulus oocyte-complexes (COCs), including supplementation with cyclic adenosine 3´, 5´-monophosphate (cAMP) modulators and an extended *in vitro* maturation (IVM) phase, which resulted in improved blastocyst quality and yields in mice and cattle. In the study presented here, we investigated the effects of cAMP modulators and an extended IVM phase on blastocyst yields and quality, and on mRNA expression profiles of developmentally important genes and epigenetic marks.

Oocytes were retrieved from slaughterhouse ovaries and subjected to three different IVM protocols. The TCM-based maturation protocol was employed that is routinely used in our laboratory (Wrenzycki et al. 2001), with either oocytes from sliced ovaries (TCM24slicing) or aspirated oocytes (TCM24aspiration). For the present study, a novel protocol was established, in which aspirated oocytes were treated with forskolin and IBMX in a 2h pre-IVM period followed by a 30 h extended IVM phase with cilostamide (cAMP30aspiration).

Maturation rates did not differ significantly between *in vitro* maturation treatments (TCM24slicing: 70.4±5.1%, TCM24aspiration: 79.3±2.6%, cAMP30aspiration 74.2±8.8%). Blastocyst rates were significantly lower ($p>0.003$) in the TCM24slicing (23±7%) and cAMP30aspiration (22±5%) groups compared to the TCM24aspiration (32±7%) group. Total cell numbers did not differ significantly between IVM treatment groups and *in vivo* derived blastocysts. The mRNA expression of selected genes indicative of embryo quality and developmental competence including *DNMT3a*, *SLC2A8*, *COX2*, *PCK2* and *EGR1* was determined by RT-qPCR and revealed that blastocysts derived *in vitro* did not differ between treatment groups in their expression profile. Only *EGR1* was significantly ($p>0.009$) up-regulated in *in vivo* derived blastocysts compared to their *in vitro* produced counterparts suggesting the potential to be used as a molecular marker for blastocyst quality. Investigation of the methylation status of two satellite sequences (Bovine testis satellite I/ BTSI) and Bos taurus α satellite I/ BTαS) revealed significant hypomethylation in bovine blastocysts produced with cAMP modulators and extended IVM (BTSI 7.9% /BTαS 35.44%) compared to blastocysts from the other *in vitro* groups (TCM24slicing 30.6%/48.4% and TCM24aspiration 31.7%/53.3%). *In vivo* blastocysts are currently investigated for their epigenetic profile.

Results so far indicate a significant influence of *in vitro* maturation protocols on blastocyst quality and epigenetic marks and the search for optimal culture conditions needs to be perpetuated.

Notes

GLOBAL TRANSCRIPTOME ANALYSIS OF ELONGATED EMBRYOS PRODUCED BY SOMATIC CELL NUCLEAR TRANSFER AND IN VITRO FERTILIZATION

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Among the different experimental approaches used in the past to learn how the cell fate can be determined through reprogramming, somatic cell nuclear transfer (SCNT) is the oldest. However, the efficiency of this technology is by far low. The boldly said reason for such failure is error in reprogramming of differentiated donor cell by oocyte's cytoplasm. In an effort to gain further insights into transcriptional reprogramming, we performed large scale gene expression profiling of day 16 bovine embryos derived from SCNT, IVP and AI and fibroblast donor cells obtained from ear biopsy of a bull using affymetrix microarray analysis. Following total RNA isolation from 3 replicates of each group, biotin labelled cRNA was hybridized on 12 bovine chips. Data was normalized by using Guanine Cytosine Robust Multi-Array Analysis (GCRMA) and analysis was performed using LIMMA written on R package which maintained the Bioconductor. The results revealed 477 transcripts that participated in 8 metabolic pathways including arginin and prolin, glycerolipid and fatty acid metabolism were differentially expressed between SCNT embryos and AI. Besides, 365 transcripts involved in TNRF-1 signalling, tight junction and other pathways were differentially expressed in IVP embryos compared to AI, while only 26 genes were found differentially expressed compared to SCNT. A subset of 280 transcripts including (TKDP2, CLDN1 and JAM2) were identified to be common in NT and IVP conceptuses and classified as genes affected by pre transfer culture condition. Interestingly, among 193 genes which were exclusively differentially expressed in SCNT embryos, 72 transcripts such as HSPB1, H2AFJ, and RCN2 were found to be not attaining transcriptional reprogramming. In addition, 92 and 30 transcripts were found to be incompletely and partially reprogrammed respectively. Trophoblast elongation size dependent QPCR expression profiling of selected genes in IVP (1mm-3mm, n=3) and SCNT (1.25mm-5mm, n=3) day 16 embryos revealed differential expression levels of FGF2R, HAND1, CCND2 and JAM2 with elongation size. The results of the present study evidenced deviation in expression of genes involved in various biological processes between elongated embryos derived from IVP and SCNT pregnancies and AI counterparts besides, error in transcriptional reprogramming between SCNT embryos and donor cell may contribute to abnormal maternal embryo cross-talk during this period.

Notes

DMSO INCREASES THE PROBABILITY OF SUCCESSFUL BOVINE IVP EMBRYO VITRIFICATION

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Vitrification of bovine in vitro derived embryos has been shown to be a successful and promising alternative to conventional cryopreservation. The success of a vitrification method is - amongst others - dependent on the employed cryoprotectants. DMSO is a very strong cryoprotectant but is also known to have cell-toxic effects. Therefore, it was the aim of the present study to compare the efficiency of two commercially available vitrification kits one with DMSO (V1) and one without DMSO (V2) on bovine embryos both at the morphological and the molecular level.

A total of n=503 day 7 expanded blastocysts were randomly allocated to one of four treatment groups: V1, n=142 blastocysts vitrified and thawed; V2, n=151 blastocysts vitrified and thawed; V1 CTL, n= 107 blastocysts, contact to vitrification and thawing media only; V2 CTL, n=103 blastocysts contact to vitrification and thawing media only. Reexpansion and hatching rates were assessed 24 h and 48 h after thawing or media contact, respectively. Reexpansion rates for embryos vitrified with the V2 media were significantly lower than for all other groups (V1: 89.16 ± 1.6^{a} vs. V2: 67.03 ± 4.48^{b} vs. V1 CTL 90.04 ± 3.09^{a} vs. V2 CTL 90.01 ± 5.45^{a}). Hatching rates of embryos vitrified with the V1 media (62.9 ± 3.68^{ac}) were significantly higher than those of embryos vitrified with the V2 media (29.36 ± 4.19^{b}). Neither hatching rates of V1 CTL (69.23 ± 4.54^{a}) nor of V2 CTL (47.88 ± 6.30^{c}) differed from those of embryos treated according to the V1 protocol.

Single hatched blastocysts of all groups were stained (V1 n= 25, V2 n= 20, V1 CTL n= 25, V2 CTL n= 25) in order to assess total cell numbers and live-dead-cell ratios. In addition to embryos derived from one of the four experimental groups, single hatched blastocysts (n=24) that had not been treated were stained. Total cell numbers did not vary among groups. The live dead ratio of cells was significantly lower in the V2 group compared to all other groups.

The relative abundance of SLC2A1, HSP1A1, IFNT2, TJP1, DSC2 and PTGS2 was analyzed via RT-qPCR in single hatched and snap-frozen embryos of all groups. Again hatched in vitro derived embryos that had not been treated were added as standard.

No differences regarding the expression of IFNT2 and PTGS2 could be detected among embryos of all 5 groups. The relative abundance of SLC2A1 was significantly lower in embryos derived from the V1 group compared to all other groups. A significantly decreased number of TJP1 transcripts was detected in embryos of the V1 and V2 CTL group in comparison to those belonging to the control group. A lower relative TJP1 abundance was measured in tendency in embryos of the V2 group in comparison to the control group. Embryos treated according to the V1 and V2 CTL protocol had a decreased amount of HSP1A1 and DSC2 abundance compared to those treated according to the V1 CTL or V2 protocol or those not treated prior to analysis.

The results show that vitrification of bovine in vitro produced embryos with DMSO-containing media can be regarded to be more successful in terms of survival and gross morphology. Nevertheless, in those embryos vitrified according to and with the V1 protocol three of the six analyzed genes differed from the control embryos, whereas only one gene differed after vitrification with the DMSO-free media.

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Notes

ALTERATION OF PROSTAGLANDIN SYNTHESIS REGULATION IN CUMULUS CELLS MIGHT AFFECT THE OOCYTE AND EMBRYO QUALITY IN DAIRY COWS WITH UNFAVORABLE HAPLOTYPE "FERTIL-" OF ONE FEMALE FERTILITY QUANTITATIVE TRAIT LOCUS LOCATED ON CHROMOSOME 3

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A decrease in fertility has been noted in high milk producing Prim'Holstein dairy cows, and a link with one Quantitative Trait Loci located on chromosome 3 (QTL-F-Fert-BTA3), enclosing several known genes, was recently evidenced. Prim'Holstein heifers presenting an unfavorable QTL-F-Fert-BTA3 haplotype "Fertil-" had a lower success rate after the first artificial insemination and a poorer blastocyst quality after *in vitro* maturation (IVM) and *in vitro* fertilization (IVF), in terms of embryo cell number, compared to favorable haplotype "Fertil+" counterparts. However, molecular mechanisms involved in these alterations are yet unknown.

In the present study, we showed that during the third lactation, "Fertil-" cows had lower oocyte quality than "Fertil+" as revealed by significant differences in oocyte maturation rate and kinetics both in vivo (after ovarian stimulation) and after IVM. Cumulus cells (CCs) enclose the oocyte and participate directly in the maturation process and in the acquisition of the latter to early developmental potential. Quantitative analysis of mRNA abundance by RT-qPCR in CCs surrounding individual fully mature metaphase-II oocytes from "Fertil+" and "Fertil-" cows after *in vivo* maturation and after IVM was performed. No significant difference was evidenced in expression of twelve QTL-Fert-F-BTA3 genes, adipokines (*RARRES1, RBP4, ADIPOR1/R2*) or presumptive marker of oocyte quality *HAS2*. However, relative expression of Prostaglandin G/H synthase (*PTGS2*) and microsomal prostaglandin E synthase-1 (*PTGES1*) genes was lower in CCs surrounding preovulatory metaphase-II oocytes of "Fertil-" cows compared to "Fertil+" suggesting a discrepancy in prostaglandin synthesis pathway in CCs between these haplotypes. *PTGS2* is known to increase in bovine CCs during maturation participating to cumulus expansion and meiosis progression. Inhibition of PTGS2 activity *in vitro* delayed oocyte maturation, inhibited MAP kinases 3/1 phosphorylation and led to lower embryo quality. In our study, addition of 10 and 50µM of NS-398, specific inhibitor of *PTGS2* activity, during IVM in optimized serum-free medium also resulted in oocyte maturation delay (Chi-2 test, $p<0.0001$) and abnormality of meiotic spindle in mature oocytes. Significant overexpression of *MOS* and *RAP1A* genes, which belong to the MAPK pathway, was found in 50µM NS398-treated oocytes. Moreover, after IVM, *MOS* was also overexpressed in metaphase-II oocytes of "Fertil-" cows, whereas no significant variation in expression of several QTL-F-Fert-BTA3 genes was found compared to "Fertil+". At day 3 after IVF, significant delay in kinetics of embryo development was observed for the group treated with 10µM of NS-398 during IVM ($p<0.001$), which resulted in lower embryo rate and in diminished cell number in morulae at day 5 (17 ± 1vs 23 ± 1, $p<0.0001$).

Altogether these data clearly indicate that alterations of prostaglandin pathway in CCs before fertilization could affect fertility in dairy cows and explain in part the early embryo loss in "Fertil-" females. Current work using *in vitro* PTGS2 inhibition in CCs promotes investigation of molecular mechanisms taking part in diminishing of oocyte and embryo quality in "Fertil-" cows and genes involved in these processes.

Notes

APPLICATION OF SINGLE FIXED-TIME LAPAROSCOPIC INTRAUTERINE INSEMINATION IN PIGS TO PRODUCE LOW-DIVERSE EMBRYOS

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Double fix-time insemination after ovulation induction is commonly used in pigs to produce embryos for ongoing biotechnological application. Variations in the time of ovulation and fertilization of ovulated oocytes by spermatozoa mainly of one of both inseminations can cause diversities in embryo development. However, sometimes there is a need to reduce such embryo diversity and to realize an 'equal outcome' of embryo stages. Single laparoscopic fix-time insemination can be used to minimize porcine embryo diversity. The potential of laparoscopic intrauterine insemination (LIUI) has been demonstrated in sperm mediated gene transfer (Fantinati *et al*., Theriogenology 2005) and evaluation of sperm migration (Brüssow *et al*., JRD 2006, 2011).

The aim of the present study was to analyze the development and possible diversity of embryos after LIUI. Altogether, 48 pubertal German Landrace gilts were included. Estrus was synchronized by 15-day long Regumate® feeding; follicle development was stimulated with 850 IU eCG 24 h after Regumate® and ovulation was induced by 500 IU hCG 80 h after eCG. LIUI was performed 31 h after hCG. Ketamine/azaperone anaesthetized gilts were fixed in a dorsal position, a pneumoperitoneum was produced and three trocar cannulas were inserted into the abdomen for optics and instruments. Laparoscopic handling was observed on a TV monitor. Each uterine horn was carefully fixed with atraumatic forceps 10 - 15 cm caudal from the utero-tubal junction and the uterine wall was punctured with a 2.5 mm diameter trocar. A 2.2 mm catheter connected to a syringe was inserted about 3 cm into the uterine lumen and 20 ml of extended, fresh boar semen (32.2 x 10^6 sperm cells/ml; 65% motility) were deposited in the lumen. Embryos were flushed from the genital tract on d2 and d3, respectively

Results of oocyte recovery and embryo development are presented in Table 1. The mean oocyte recovery rate was 68±17%; 45 of 48 gilts (93.8%) revealed fertilization and 76.1% of the recovered oocytes were embryos at the 2- and 4-cell stage.

Table 1. Ovarian response, oocyte recovery and embryo development in gilts after single fixed-time LIUI

Group (No. gilts)	No. of ovulations (Mean±SD)	No. of oocytes (Mean±SD)	No. of embryos (Mean±SD)	Gilts with 2-cell embryos (%)	Gilts with 2- / 4-cell embryos (%)	Gilts with 4-cell embryos (%)
d2 (n=25)	580 (23.2±9.2)	420 (16.8±8.2)	270 (10.8±8.8)	16 (72.2%)	5 (22.7%)	1 (4.6%)
d3 (n=23)	571 (24.8±9.1)	358 (15.6±5.9)	322 (14.0±7.2)	1 (4.3%)	0 (0%)	22 (95.7%)

Results demonstrate high rates of fertilization and of evenly ('non-divers') developed embryos after single fix-time laparoscopic intrauterine insemination in gilts. Furthermore, the number of sperm cell per insemination dose could be diminished by 75%.

Notes

EXPRESSION AND DISTRIBUTION OF *ZONA PELLUCIDA* PROTEINS 3 AND 4 IN MORPHOLOGICALLY ABNORMAL CANINE OOCYTES - A CONFOCAL MICROSCOPIC OBSERVATION BASED STUDY

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Morphology of mammalian oocytes significantly determines their ability to grow and develop. It was clearly demonstrated that COC's of abnormal quality have low developmental competence with particular attention given to their maturation and fertilization ability. However, few data are available about expression of proteins responsible for fertilization in morphologically abnormal oocytes, as a possible reason of failed fertilization. This study was aimed to analyze expression and distribution of ZP3 and ZP4 glycoproteins within canine oocytes.

The canine cumulus-oocyte-complexes (COCs) were morphologically evaluated following their recovery from anoestrus mongrel bitches after ovariohysterectomy. Only morphologically abnormal, denuded oocytes with a strongly heterogeneous and dark cytoplasm, were used in further steps of experiments.

In the results we found a decreased expression of ZP3 glycoprotein within the oocyte's cytoplasm, whereas the ZP4 manifested a significantly more pronounced expression. In almost all investigated oocytes ZP3 glycoprotein was distributed in oocyte's cytoplasm. However, a less pronounced expression of ZP4 was detected also within the *zona*. Using DAPI staining assay, we found an appropriate chromatin configuration only in approximately 15% of analysed canine oocytes. All confocal microscopic observations and analyses ware made using Imaris 7.2 (BitPlane, Zurich, Switzerland) software.

Our results suggested that a decreased expression of ZP3 glycoprotein, as the primary sperm receptor, as well as its pronounced cytoplasmic localization might explain failed fertilization in this group of canine oocytes. Moreover, specific staining of ZP4, distributed within the *zona*, might reflect a significant role of this glycoprotein in fertilization in canines or this partial distribution may represent remnants of the protein's strong expression during early steps of oogenesis and *zona* formation. Several irregularities found in the expression profiles of both ZP3 and ZP4 reflect differential distribution of these proteins in oocytes during oogenesis.

Notes

IN VITRO MATURATION CONDITIONS AFFECT mRNA AND PROTEIN EXPRESSION OF THE PROGESTERONE RECEPTOR ISOFORMS IN BOVINE OOCYTES AND CUMULUS CELLS

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In vitro maturation (IVM) of bovine COC has commonly been performed using medium covered by paraffin or mineral oil. However, it has recently been demonstrated that the concentrations of progesterone (P4) in the medium are reduced due to the high absorbing capacity of the oil. P4 plays a key role in female reproduction. Its effects are moderated by specific receptors. Changes during IVM are known to influence the oocyte and thus the quality of the later embryo.

The aim of the present study was to assess whether nuclear progesterone receptor (nPR) levels in oocytes (OO) and cumulus cells (CC) are altered due to different maturation conditions. Nuclear maturation to the MII stage was assessed after 24h of IVM. P4 concentrations were measured in maturation medium employing a RIA progesterone kit. The mRNA abundance of the nPR and the protein levels of the nPR (isoforms A, B and C) were analysed either via RT-qPCR or western blot. Groups (n=30, 7 replicates) of immature (IO) and IVM COC [5 or 20% O_2 with or without (+/-) oil overlay] were analysed. Separated CC and OO were allocated to the following groups: [1] CC from immature COC (n=120), [2] IO (n=245), [3] CC from mature COC (n=120, 20% O_2 +oil), [4] mature OO (n=240, 20% O_2 +oil), [5] CC from mature COC (n=121, 20% O_2 -oil), [6] mature OO (n=243, 20% O_2 -oil), [7] CC from mature COC (n=118, 5% O_2 +oil), [8] mature OO (n=239, 5% O_2 +oil), [9] CC from mature COC (n=122, 5% O_2 -oil), [10] mature OO (n=240, 5% O_2 -oil). Data were analysed employing an ANOVA.

Rates of nuclear maturation to the MII stage did not differ between oocytes from the different treatment groups (83.4-88.5%, n=70-75 oocytes per group). P4 concentration (calculated per COC) was significantly increased in medium from COC matured with 20% O_2 -oil compared to that from COC of all other groups (20% O_2 -oil: 304 pg/COC; 20% O_2 +oil: 11 pg/COC; 5% O_2 -oil: 133 pg/COC; 5% O_2 +oil: 6 pg/COC; respectively; 6-8 replicates). Maturation medium itself did not contain any detectable amount of P4 (detection limit <0.02 ng/mL). MessengerRNA amounts of the nPR was significantly increased in all matured oocytes compared to their immature counterparts. Only the nPR B protein was detected in oocytes being significantly higher in IO and those matured in 20% O_2 +oil compared to matured oocytes from the other groups (20% O_2 -oil, 5% O_2 +/- oil). The relative amount of PGR mRNA was significantly increased in all matured CC compared to CC from immature COC. Protein levels of nPR A were significantly higher in CC 20% O_2 +oil than in CC from immature COC, and those in CC 5% O_2 -oil were significantly higher than in immature CC, CC 20% O_2 -oil and CC 5% O_2 +oil. NuclearPR B had a significantly higher protein level in CC 20% O_2 +oil than in all other CC groups. Protein levels of nPR C were significantly higher in CC 20% O_2 +oil than in immature CC, CC 20% O_2 -oil and CC 5% O_2 +oil. Total nPR protein levels were significantly increased in CC derived from COC matured in 20% O_2 +oil and 5% O_2 -oil compared to CC from all other groups. The results suggest that nPRs are involved in maturation and that their expression depends on the maturation conditions.

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Notes

EFFECT OF POSTPARTUM BODY WEIGHT CHANGE AND CIRCULATING NEFAS ON EMBRYO PRODUCTION IN SUPEROVULATED HIGH PRODUCING DAIRY COWS

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This experiment was designed to evaluate the effect of body weight change (BWC) between calving to third and tenth week postpartum and NEFA concentration at third week postpartum on embryo production in superovulated cows with synchronized ovulations. Holstein cows (n=72), were milked twice daily and housed and fed individually in tie-stalls. All cows were synchronized and superovulated using a modified 5d-Double Ovsynch protocol with 4 d of decreasing FSH (Folltropin®, 400mg/cow) treatments. All animals were flushed between 65 to 75DIM, near peak production (39.6 kg/d). Non-sexed frozen semen ($15x10^6$ sperm/straw) were produced from single ejaculates of two high-fertility sires and cows were inseminated twice (12h and 24h after final GnRH treatment). To minimize variation, a single batch of FSH was used and two experienced AI technicians performed all breedings and flushings, which occurred 6d after synchronized ovulations. In addition, a single animal-blinded technician graded all embryos. The proc CORR of SAS was used to correlate embryo characteristics with NEFA at third week postpartum and percentage of BWC between calving to third and tenth week postpartum. Interestingly, circulating NEFA at third week postpartum was negatively correlated with % transferable embryos (r^2=-0.25, *P*=0.03) and positively correlated with % of degenerated embryos (r^2=0.23, *P*=0.05). Similarly, greater %BW loss between calving to third week postpartum was associated with greater % of degenerated embryos (*P*=0.04), lower % of transferable embryos (*P*<0.01) and % of fertilized structures (*P*=0.02). In addition, cows that lost more weight between calving to tenth week postpartum had more degenerated embryos (*P*=0.01), much lower % of transferable embryos (*P*<0.01) and % of fertilized structures (*P*=0.09). In conclusion, the degree of the negative energy balance measured by NEFA concentrations at third week postpartum and BW loss between calving to third or tenth week postpartum dramatically impaired embryo quality, even in synchronized donors in which oocytes were developed under a high progesterone environment. Measurements of NEFA and BW loss by third week post-partum can be used as a tool to select donors that will enhance the efficiency of embryo production from high producing dairy cows.

KEYWORDS
Superovulation, NEFA, Body weight loss, Dairy cow

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Notes

ADDITION OF L-ASCORBIC ACID DURING *IN VITRO* CULTURE OR/AND DURING CRYOPRESERVATION ENHANCES PORCINE EMBRYO SURVIVAL

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Cryopreservation causes significant oxidative stress on embryos. Thus, including an antioxidant, like L-ascorbic acid (AC), in *in vitro* culture systems or/and in cryopreservation solutions may help to reduce the effects of harmful oxygen radicals during vitrification/warming procedure. Against this background, the present study was conducted to evaluate the effects of adding L-ascorbic acid to the *in vitro* culture (NCSU23) and vitrification media. After culture, embryo development and quality, total number of cells and cryotolerance of *in vitro* produced (IVP) blastocysts were evaluated.

IVP blastocysts were derived from porcine fertilised oocytes cultured with 0.17mM pyruvate and 2.73mM lactate from Day 0 to 2 and then with 5.5mM glucose up to the blastocyst stage. The effects of L-ascorbic acid on cryotolerance were assessed by supplementing embryo culture and/or vitrification media. Thus, embryos were incubated in culture medium supplemented with 100 µM AC or non-supplemented (control) for a 144h period. After culture, embryo quality according to the IETS and development were assessed by stereomicroscopy. The total cell number was analysed by Hoechst-33342 staining. Then, grade I- and II-blastocysts were vitrified following the Cryotop method and using two different vitrification media, one was supplemented with 100 µM AC while the other was the control. Finally, embryos were warmed and incubated in NCSU23 for 24 h to examine survival rate. Data was evaluated following a two-way ANOVA procedure where culture (with/without AC) and vitrification (with/without AC) were the two intersubject factors.

Supplementing culture medium with 100 µM of AC did not affect the cleavage and the blastocyst rates, and the total number of cells. However, culturing embryos with 100 µM of AC resulted in a significant increase ($P<0.05$) of their cryotolerance as the blastocyst survival was significantly higher than in embryos cultured without AC (39.4% ± 3.5 *vs.* 17.8% ± 3.0). In contrast, there was no significant effect of supplementing vitrification media with AC, even though the addition of AC to vitrification media slightly increased the percentage of survival in those embryos cultured without AC (29.7% ± 3.3 *vs.* 17.8% ± 3.0).

In conclusion, supplementing culture medium with L-acid ascorbic does not enhance the blastocyst yield and the total number of cells, but increases their cryotolerance. However, adding AC to the vitrification medium only shows a slight but not significant increase in cryosurvival.

Notes

ASSESSMENT OF MITOCHONDRIAL ACTIVITY AND DISTRIBUTION DURING IVM IN LAMB OOCYTES

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Mitochondria are maternally inherited organelles that use oxidative phosphorylation to catalyse the formation of adenosine triphosphate (ATP) and give energy to the cell. Different patterns of distribution have been described, shape and activity of mitochondria at different stages of the IVM. Apparently high levels of mitochondrial activity are necessary for further maturation events that are dependent on ATP generation, such as maturation of the nucleus and cytoplasm, rearrangement of the cytoskeleton and accumulation of the mRNAs necessary for early development before the onset of embryonic transcription (Mol Cell Endocrinol 1998: (145) 27–37). Using prepubertal animals as oocyte donors could reduce the generation interval and also serve as a good model for studying low quality oocytes for human reproduction. Therefore the aim of this study was to analyse mitochondrial activity, shape and distribution of prepubertal sheep oocytes before (oh) and after (24h) in vitro maturation.

Lamb oocytes were stained with 200nM fluorescence probe MitoTracker Orange CTMTRos (Molecular Probes,Inc., Eugene, OR, USA) for 60 min. After staining, oocytes were fixed at 38ºC in 3% paraformaldehyde and then stained with 1 mg/mL Hoechst for 5 min. Stained oocytes were analysed under a confocal microscope. Results are shown in table 1.

Table 1: Mitochondrial fluorescence intensity before (0 h) and after IVM (24 h).

IVM	Nº oocytes	Mitochondrial Activity (Fluorescence intensity)	Shape		Distribution		
			D (%)	G (%)	P (%)	H (%)	Pol (%)
0 h	57	3176.42±223[a]	68.4	31.6	56.1[a]	43.9	0[a]
24 h	47	2467.15±315[b]	65.9	34.1	6.4[b]	53.2	40.4[b]

Different letters ([a,b]) are significantly different (Fisher test; $P<0.05$). D: diffuse; G: granulate, P: peripheral, H: homogeneous, Pol: polarize.

Our results showed a diminution of mitochondrial activity after IVM, probably due to the low quality oocytes used. In human, a correlation between reduction of mitochondrial activity and low embryo development has been shown (Hum. Reprod. 2001, 16(5):909-917). Mitochondrial shape did not show variation during IVM, while in bovine mitochondrial shape becomes diffuse after maturation (Reprod Domest Anim. 2006 Feb;41(1):5-11). The distribution was mostly peripheral and homogeneous in immature oocytes and polarized around the metaphase and homogeneous in matured oocytes. In bovine oocytes, Tarazona *et al.* (Reprod Domest Anim. 2006 Feb;41(1):5-11) showed a homogeneous distribution in the immature and homogeneous and peripheral distribution in the mature stage.

The analysis of these changes in mitochondrial organization may provide insights into the regulation of normal embryo development and might serve as predictors of oocyte developmental competence.

Notes

FERTILIZATION AND DEVELOPMENT OF EQUINE AND SWINE OOCYTES FOLLOWING ICSI WITH REFRIGERATED AND FROZEN SEMEN OF FERTILE AND INFERTILE STALLIONS

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The clinical use of Ovum Pick Up-ICSI in the horse is increasing not only for the treatment of female and male infertility, but also for obtaining offspring from performing sporting mares and for producing embryos outside the breeding season. The OPU-ICSI cycles requested for treating male infertility are cases of poor or no field fertility after artificial insemination and in some instances this request is associated to OPU-donor females also infertile or aged. In order to provide an estimate of success for these extreme cases it can be informative to perform an ICSI test on slaughterhouse oocytes, using in parallel semen of known fertility to demonstrate if the stallion infertility can be solved with OPU-ICSI cycles. However, the availability of equine oocytes is limited and decreasing due to the growing aversion to slaughter horses in many countries.

The scope of the present work was to develop a fertility test to assess fertilization ability of fresh or frozen stallion semen, using slaughterhouse horse oocytes but also pig oocytes that are much more easily available and in larger numbers.

The frozen semen of 5 stallions with good field fertility (HF) and the semen refrigerated (2) or frozen (3) of 5 stallions with no field fertility (LF) was used for ICSI with horse oocytes, while the semen of two stallions (one each category) was also used for ICSI with pig oocytes.

Horse oocytes were matured in vitro for 24-28 h, pig oocytes for 40-42 h, afterwards they were injected with Redigrad separated spermatozoa of HF or LF stallions, selected for motility and immobilized prior to injection. A third group of pig oocytes was sham-injected. The horse oocytes were allowed to cleave and develop to blastocyst. Half of the pig oocytes were fixed 20-24 h after ICSI to assess pronuclear formation (both male and female pronuclei developing synchronously) and the remaining were cultured for another 24 h to assess cleavage and then fixed to evaluate the presence of nuclei.

Seventy two percent of horse oocytes injected with HF stallions cleaved (254/351) and 21% developed to blastocyst while in the LF group 62% cleaved (193/312) and 9% developed to blastocyst. Using pig oocytes and semen of HF stallions fertilization was 85% (83/98) and cleavage rate was 75% (53/71) while with LF stallions fertilization was 25% fertilization and cleavage rate was 30%, indicating a very good correlation between fertilisation rate as measured by the formation of two pronuclei, and cleavage rate. No cleaved hybrid embryos developed to blastocyst. Sham injected oocytes gave a 12% fertilization and 14% cleavage rates.

In conclusion, this study indicates that stallion with low in vivo fertility also perform less efficiently in vitro and that in vitro matured pig oocytes could be used as a tool to evaluate the fertilising ability of horse semen to be used for ICSI, both at the pronuclear stage after fixation or by evaluating cleavage.

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Notes

REGULATION OF EARLY CLEAVAGE KINETICS AND EMBRYONIC GENOME ACTIVATION BY BOVINE OVIDUCTAL EPITHELIAL CELLS IN VITRO

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It has been demonstrated that the length of early cell cycles of bovine embryos could be related to their viability. Many studies suggest that cleavage pattern should be used as embryo developmental potential predictor. On the other hand, co-culture of early embryos with bovine oviduct epithelial cells (BOEC) has been widely used to mimic maternal environment in order to improve in vitro embryo production (IVP) and to study embryo-maternal interactions. The exact mechanisms of BOEC action have not been fully elucidated yet. Several studies suggest that this co-culture could improve embryo quality. We showed previously that the presence of cells during culture appears more critical during early stages of development. Therefore, the purpose of this study was to compare gene expression differences between early embryos co-cultured in presence of BOEC or not. Confluent monolayers of BOEC were derived from slaughterhouse oviducts. Immature cumulus oocyte complexes were aspirated from slaughterhouse ovaries. Zygotes were produced by *in vitro* maturation and fertilization, and cultured in SOF medium supplemented with 10 % FCS in the presence of BOEC or not. Two groups were compared: (C) control embryos, cultured in medium alone and (B) embryos co-cultured on BOEC during 4 days. Cell numbers were evaluated at 96, 115, 120 and 139 h post fertilization, using Hoechst 33342 fluorescent staining. At 115h (BOEC) and 120 h (C) post insemination (pi), embryos reaching the 20-cell stage were selected for RNA extraction, amplification, reverse transcription and hybridization on a home made cDNA array displaying 3,000 independent bovine ESTs obtained from a subtracted cDNA library (morula minus 4-Cell stage, Bui LC *et al*., 2005. BMC Genomics 6:155). Data were analysed using Limma R-package. The rate of embryos with 20 or more cells was significantly increased in group B as compared to C at 115 (C: 14.1%, B: 21.8%, $P<0.05$) and 139 hpi (C: 25.9%, B: 42.2%, $P<0.0001$). Embryos from group B developed beyond the 20 Cell stage (EGA stage) earlier than group C. Furthermore, it was observed that co-cultured embryos reached the 20-25 cell stage (maximum at 115 hours pi) earlier than the control group (maximum at 120 hours pi). Array hybridization evidenced 52 genes differentially expressed (raw p-value 1%) between group B and group C embryos. From these, 17 (32.69%) of them were upregulated in BOEC treatment whereas 35 (67.31%) were downregulated. Several genes involved in regulation of transcription, DNA replication, protein translation and export, ion transport and metabolic processes were found amongst these differentially regulated genes and will be confirmed now using qPCR. In conclusion, embryos co-cultured with BOEC reached the 20-cell stage earlier than controls. Co-culture could then be critical during early stages of development by accelerating the kinetics of the first cleavages and regulating embryonic genome expression, resulting in improved embryo quality.

Notes

ADRP LOCALIZATION WITH IMMUNOFLUORESCENCE IN BOVINE EMBRYOS

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Background: Adipocyte Differentiation-Related Protein (ADRP) also known as adipophilin is a protein found in bovine embryos which links lipids. Its expression is highly correlated with stored lipids during lipid droplet formation. It could be thus used as a marker of the lipid accumulation and of the embryos' quality.

Methods: The aim of this study was to localize ADRP in bovine embryos using immunofluorescence. Three groups of embryos were produced, 1) *in vivo*, 2) *in vitro* in basic medium (modified synthetic oviduct fluid, mSOF, supplemented with 5% foetal calf serum), and 3) *in vitro* on a bovine oviduct epithelial cell (BOEC) monolayer with basic medium. Embryos produced *in vivo* were recovered from superovulated beef heifers at Day 7.5 post insemination. All blastocysts were fixed with 4% paraformaldehyde, permeabilized with Triton 0.5% and blocked in 2% bovine serum. Then they were incubated with guinea pig anti-ADRP (Progen, #GP-40, 1:1000) overnight and with secondary donkey anti-guinea pig antibody conjugated to fluoroprobe Dylight 649. Lipid droplets were stained with the Nile Red dye and chromatin using Hoechst 33258. The embryos were then individually placed between glass coverslips before submission to laser excitations.

Results: As the commercial anti-ADRP was a polyclonal antibody, it was purified by Western blot and concentrated. The results observed with these 2 anti-ADRP (commercial form and purified form) were similar, thus the commercial form can be considered as "monoclonal". Adipophilin is strongly localized around lipid droplets in embryos produced *in vitro* (figures 1 and 2). The same result was observed in embryos produced *in vivo* (figure 3). ADRP is pointed up by the arrows below.

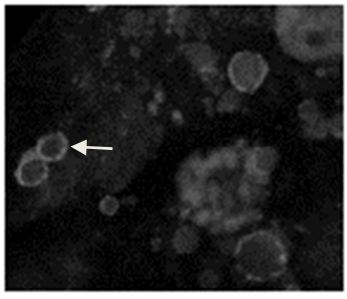

Figure 1: *in vitro* embryos in basic medium, x63

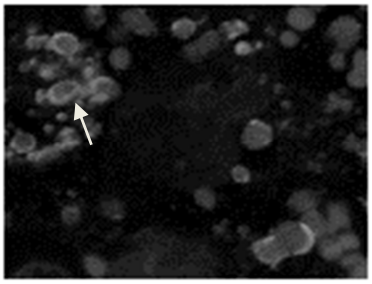

Figure 2: *in vitro* embryos with BOEC monolayer, x63

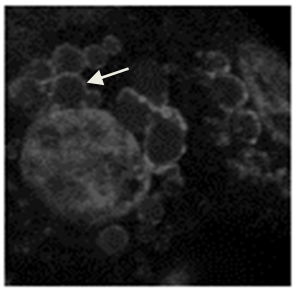

Figure 3: *in vivo* produced embryos, x63

Conclusion: In *in vitro* and *in vivo* bovine embryos, Adipophilin wraps lipid droplets. Its quantification can be a fine marker to evaluate lipid droplets in bovine embryos which are known to be related to embryo cryoresistance.

Notes

DETECTING LH PEAKS IN ORDER TO OPTIMIZE THE RATIO OF VIABLE EMBRYOS USING PREDI'BOV®, A NEW ON-FARM OVULATION TEST

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The LH peak initiating the ovulation 24 hours later, in cattle, is the more precise event for predicting ovulation and thus, insemination time. Previous studies demonstrated that success rate following artificial insemination (AI) and embryo production results were improved when AI was conducted 12 hours before ovulation ie 12 hours after the LH peak.

This study aimed to evaluate the benefit of LH monitoring with Predi'Bov® (ReproPharm, France) following superovulation in order to optimize numbers of viable embryos. Predi'Bov® is a rapid (40 minutes) and easy to use on-farm test allowing LH peak detection from a few drops of blood. The test was also used to estimate the variability of the onset of LH peak in relation to the onset of estrus.

This study was conducted by the embryo transfer teams of three French cooperatives, in collaboration with UNCEIA. Forty heifers in donor stations (Creavia, Midatest) and thirteen cows in farms (GEN'Iatest), all super-ovulated, were used in the experiment performed during the 2011-2012 embryo transfer campaign. Except for GEN'Iatest cows, each female was treated, in a latin square design during 2 consecutive collections, one in a reference protocol where AI was conducted 12 and 24 hours after the onset of heat, and the other in an experimental protocol where AI was conducted 12 and 24 hours following a positive Predi'Bov® test. Semen used was from different sires. The ovulation test was carried out on three blood samples collected every six hours from the last day of treatment (BS1,2,3) to detect the earliest LH peaks and 24 hours later for the latest ones (BS4). The use of the test showed that, in stations, 37.5% (15/40) of the LH peaks occurred during the last day of treatment at BS1 or BS2 and, on farm, 30.8% (4/13) at BS1. At Creavia station, LH monitoring showed that the LH peak was detected anytime during an interval of time starting 24 hours prior to the onset of heat to 9 hours after (n=24). In station, the viability rate of collected embryos was not significantly different between both protocols (63.1% and 61.8% in reference and experimental protocols respectively). But, the percentage of viable embryos collected from the 9 females with a LH peak detected at FSH7 (BS1) in station was 9% higher than in reference (respectively 50% and 41%, not significant). On farm, the percentage of viable embryos was higher but not significantly different in experimental protocol (65.7%, n=9) when compared with the reference (56.4%, n=4).

In station, when comparing results for heifers showing an early peak (BS1,2,3) according to collection rank, those in the reference protocol for the 2nd collection showed a reduced production of viable embryos (-15.1%, not significant) compared to heifers in the experimental protocol for the 2nd collection (+8.7%, not significant). Further research is needed to investigate the effect of collection rank, taking into account sire and female effects.

In conclusion, Predi'Bov® can be used as easily in donor stations as on farms. Its use allows the detection of animals which have early or late LH peaks, which in turn gives the opportunity of carrying out AI at the optimal time for such females.

Notes

EFFICACY OF BLUETONGUE VIRUS (BTV) DECONTAMINATION TECHNIQUES FOR CAPRINE EMBRYOS EXPERIMENTALLY INFECTED

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Previous experiments demonstrate that the washing procedure recommended by the IETS for bovine embryos failed to remove BTV from ZP intact caprine embryo [Ali Al Ahmad *et al.* , 2011]. The objective of this study was to investigate methods of decontaminating early goat embryos that had been infected *in vitro* with BVT8.

Embryos were isolated from 20 *in vivo*-fertilized BTV-free goats. One hundred and thirty one Zona Pellucida (ZP)-intact 8-16 cell embryos were randomly divided into two groups: nearly two thirds (n=95) were used in the "treated group" for one to three replicates conducted for each decontamination protocol (5 to 10 embryos per treatment), and one third (n=36) in the "positive control group" (infected but not decontaminated embryos; n=18) and "negative control group" (non-infected and non-decontaminated embryos; n=18). Embryos from the treated group and positive control were co-cultured for 36 h in an insert over a Vero cell monolayer infected with BTV8 at a multiplicity of infection of 1 (MOI=1).

The embryos were then treated with one of five different washing procedures. The treatment standard (TS) comprised PBS + 0.4% BSA (five times over for 10 s), Hank's + 0.25 % trypsin (twice for 45 s), and then PBS + 0.4% BSA again (five times for 10 s). The four other washing procedures all included the same first and last washing steps with PBS but without BSA (five times for 10 s) and with PBS + 0.4% BSA (five times for 10 s), respectively. The intermediate step varied for each washing procedure. Treatment 1 (T1): 0.25% trypsin (twice for 45 s). Treatment 2 (T2): 0.25% trypsin (twice for 60 s). Treatment 3 (T3): 0.5% trypsin (twice for 45 s). Treatment 4 (T4): 1% hyaluronidase (once for 5 min). After washing, the embryos were transferred and cocultured with BTV indicator Vero cell monolayers for 6 h, to detect any cytopathic effects (CPE). The effectiveness of the different washing techniques in removing the virus was evaluated by RT-qPCR analysis.

The TS, T1, T3, and T4 trypsin or hyaluronidase treatments did not eliminate BTV from infected treated and washed embryos, whereas they were eliminated by the T2 treatment with 0.25% trypsin twice over for 60s.

In conclusion, BTV shows a strong tendency to bind to caprine embryos after experimental exposure to the virus. The virus could not be removed by the trypsin washing procedure recommended by the IETS for bovine embryos. However, enzymatic treatment including PBS without BSA (five times), 0.25% trypsin (twice for 60s-60 s), and PBS + 0.4% BSA again (ten times) is an effective method for decontaminating caprine embryos infected with BTV8 *in vitro*. These results need to be confirmed using embryos that have been infected *in vivo*.

Ali Al Ahmad MZ, Pellerin JL, Larrat M, Chatagnon G, Cécile R, Sailleau C, Zientara S, Fieni F. Can bluetongue virus (BTV) be transmitted via caprine embryo transfer? Theriogenology 2011;76(1):126-132.

Notes

MOLECULAR MECHANISMS ASSOCIATED WITH EFFECT OF ENVIRONMENTAL FACTORS DURING BOVINE BLASTOCYST FORMATION

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Blastocyst formation is one of the critical events of pre-implantation development since it is the stage at which the embryo starts coordinated cross-talk with the mother. Identification of transcripts involved in blastocyst formation and influenced by culture conditions may help us to understand the mechanisms that control blastocyst formation. Therefore, we aimed to examine the effect of alternative in vivo and in vitro culture conditions during the time of blastocyst formation on the transcriptome profile of bovine blastocysts. Using the advent of non-surgical transvaginal endoscopic technology, two different blastocyst groups were produced. The first group (Vitro_morula) was matured, fertilized and cultured in vitro until morula stage then transferred to synchronized recipients and blastocysts were collected at day 7 by uterine flushing. The second group (Vivo_morula) was matured, fertilized and cultured in vivo until morula stage then flushed out and cultured in vitro until day 7. Complete in vitro (IVP) and in vivo blastocysts were produced and used as controls. Gene expression pattern between each blastocyst group and in vivo blastocyst control group were compared using EmbryoGENE's bovine microarray over six replicates of each group.

Interestingly, all blastocyst groups showed high number of differentially expressed genes (DEGs) compared to in vivo control group. Vivo_morula, Vitro_morula and IVP groups showed 773, 842 and 841 DEGs, respectively compared to control group ($FC \geq 2$, $FDR \leq 0.05$). Ontological classification of DEGs indicating that cell death was the most significant function in all groups with up-regulation for most of the DEGs involved in this function compared to in vivo control group. In addition, a clear significant pattern of lipid metabolism related genes was found in Vitro_morula and IVP groups, but not in Vivo_morula group, with down-regulation for most of lipid metabolism related genes compared to control group. Pathway analysis revealed that integrin signalling and NRF2-mediated oxidative stress response pathways were the dominant pathways in Vivo_morula group. However, TNRF1 signalling pathway was the dominant in Vitro_morula and IVP blastocyst groups. A total of 183 transcripts found to be commonly expressed in Vitro_morula and Vivo_morula groups and gave the same pattern of expression in both groups with highly abundance of cell death related genes. In conclusion, in vitro culture conditions critically determining embryo quality, measured in terms of gene expression patterns, during the time of blastocyst formation. Moreover, here we identified molecular mechanisms and pathways that influenced by in vitro conditions and this will enable to launch strategies to modify the in vitro culture conditions at this critical stage of development to enhance the development of competent blastocyst.

Notes

INCUBATION WITH LENTIVIRUS DID NOT AFFECT BOAR SPERM FUNCTIONALITY

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Transgenic pigs would be of high relevance for biomedical and agricultural research (Gadea & Garcia-Vazquez ITEA 2010). However up to now the efficiency and safety of the techniques is not good enough for a wide use of transgenic pigs. In one hand, lentiviruses have been used for the generation of transgenic farm animals mainly by the injection in the perivitelline space of early zygotes or oocytes (reviewed by Pfeifer Transgenic Research 2004). On the other hand Sperm Mediated Gene Transfer (SMGT) has been used with different performances (García-Vazquez et al. Reproduction 2010). An alternative methodology could be the use of lentivirus to infect spermatozoa that later could be applied by AI or other assisted reproductive techniques as recently reported (Zhang et al, PlosOne 2012). In this way the first objective is to establish the experimental conditions to optimize the infection of the spermatozoa with the lentivirus and minimize the possible deleterious effect on the sperm functionality.

In this study we incubated the boar spermatozoa in a non-capacitating media in presence or absence of lentivirus at 37ºC and evaluated the sperm functionality in terms of motility measured by CASA (ISAS, Proiser, Valencia, Spain) and simultaneously viability and acrosome status by flow cytometry. In brief, boar spermatozoa were diluted (10^7 spermatozoa/mL) in Belstville Thawing Solution (BTS, Pursel et al. 1975) in presence or absence of 10^6 viral particles/mL (MISSION TurboGFP Control Transduction Particles, Catalog Number SHC003V, Sigma) that are produced from the lentiviral backbone vector, pLKO.1-puro, containing a gene encoding TurboGFP, driven by the CMV promoter. Motility and motion parameters were evaluated at 0. 30, 60 and 90 min of incubation by CASA system. Viability and acrosome status were evaluated by flow cytometry after staining with Propidium Iodide and PNA-FITC lectin (Gadea et al., J Androl. 2005) at 0, 30, 60, 90 and 180 min.

No differences were found for the parameters evaluated among experimental groups (control vs. lentivirus). Viability, acrosome status, motility and motion parameters were affected by time of incubation The percentage of viable spermatozoa with intact acrosome ranged from near 90% in the beginning of the incubation to 80% after 3 hours of incubation ($p<0.01$). On the contrary, the percentage of spontaneous acrosome reacted cells increased from less than 5% to near 10% ($p<0.01$).

In summary, we can conclude that the title of lentivirus used in the present experiment has not effect on sperm functionality. So, these samples can be used to be applied in different reproductive processes (AI, IVF, ICSI) to attempt to produce transgenic embryos or animals.

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Notes

SET-UP OF OVUM PICK UP AND IN VITRO EMBRYO PRODUCTION IN MIDATEST: FIRST RESULTS

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MIDATEST opened in October 2009 a donor station in DENGUIN (South West of France) in collaboration with UNCEIA to produce in vitro embryos from high genetic merit females, combining ovum pick up and in vitro embryo production (OPU-IVP) with Multiple Ovulation Embryo Transfer (MOET) sessions.

After a 3 months adaptation period (October to December de 2009) including training from slaughterhouse ovaries and OPU sessions from two non pregnant Brown cows, a genetic program including in vitro embryo production started with Holstein and Brown Swiss donor heifers or cows.

Before each OPU session, animals were stimulated with decreasing doses of pFSH twice a day during two and half days (Stimufol®; total dose: 250 µg pFSH / heifer and 350 µg pFSH /cow). OPU was performed 12 hours after the last FSH injection using a 240 Parus Vet ultrasound scanner (Pie Medical) equipped with a 7.5 MHz annular-array probe. Oocytes were in vitro matured in M199 supplemented with FCS, FSH/LH, oestradiol and EGF for 22 hours at 38.5°C. They were fertilized with frozen – thawed semen in TALP medium using different bulls without any testing IVF program. Presumptive zygotes were cultured in SOF medium (Gamarra *et al.*; AETE 2010) up to day 8 at 38.5 °C in 5% CO2 , 5% O2 and 90% N2 atmosphere with maximum humidity.

Blastocysts and expanded blastocysts of excellent and good qualities Grade1 (G1) (according to IETS classification) were recorded. All G1 embryos were frozen for Direct Transfer. Embryo development was analysed by Chi-Square analysis.

Cleavage and G1 blastocysts rates were no significantly different between Holstein heifers and cows using conventional semen. Using the same Brown donors cleavage and G1 blastocysts rates per OPU session were lower when using sexed semen but these results were not significantly different. However, due to the reduced number of sessions using sex semen, these results need to be confirmed.

Table 1:
Number of collected oocytes and blastocysts produced and used in Brown and Holstein donor females

Semen	Breed/parity	N sessions	Collected oocytes	Inseminated oocytes /session	Cleavage (%)	Blastocysts G1/ insem. %	Blastocysts Q1 /session
Conventional	Holstein cows	16	164	10	141 (88) [a]	**61** [a]	**6.1** [a]
Conventional	Holstein heifers	39	429	10.2	328 (82) [a]	**43** [a]	**4.4** [a]
Conventional	Brown cows	10	201	19.6	140 (72) [a]	**29** [b]	**5.7** [a]
Sexed semen	Brown cows	3	48	15.6	19 (40) [b]	**17** [b]	**2.7** [b]

Different letters in the same column (a,b) differ significantly (Chi-Square Test, P<0.05).

In conclusion, oocyte collection and embryo production were not influenced by the status of donors (cows or heifers) and there were no significant differences in embryo production when using conventional semen. Our limited experience in using sexed semen showed a lesser efficiency of IVP due to significantly lower cleavage rates.

Notes

OPU OOCYTE YIELD AND EARLY EMBRYO DEVELOPMENT AFTER FOLLICULAR ABLATION OR EXOGENOUS GNRH (DALMARELIN) IN MURCIANO-LEVANTINA COWS

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OPU oocyte yield results in increased ovarian stimulation by FSH, with best results when it is administered early in a follicular wave. Control and synchronization of follicular dynamics has been achieved successfully with dominant follicle removal by follicle ablation using transvaginal ultrasound-guided aspiration or GnRH administration to induce synchronous early follicular wave 1.5 to 2 days later. These methods have been used interchangeably in different cattle breeds in MOET programs to induce and synchronize ovulation, but it is still unknown which treatment may be more effective on OPU oocyte yield and early embryo development.

The aim of this study was to evaluate OPU oocyte yield and subsequent early embryo development after two treatments, dominant follicle removal (DFR) by follicular ablation and exogenous GnRH, in Murciano-Levantina bovine breed. 3 cows were used, each animal received 4 sessions of OPU, 2 for each treatment applied alternately, with a frequency of weekly OPU: DFR using transvaginal ultrasound-guided aspiration and exogenous administration of synthetic GnRH (0.2 mg, i.m. Dalmarelin, Fatro Iberica, Spain), 48h before i.m. administration of 500 IU FSH-LH (Pluset®, Calier, Spain). Follicles larger than 3 mm in diameter were aspirated and follicular fluid was washed with PBS medium supplemented with FCS and sodium heparin. Cumulus oocyte complexes (COCs) from categories I, II and III were considered viable and suitable for IVP. Maturation, fertilization and in vitro culture were performed according to established procedures. Means of follicles aspirated were compared by Student T-test for independent samples and oocyte quality parameters, maturation and cleavage rates were analyzed by comparing proportions (Chi square test).

The number of follicles was higher in the GnRH-Group (19.33±5.94 follicles) compared to the follicles aspirated in the DFR-Group (13.00±5.62; $P<0.05$). However, this treatment delivered a greater percentage of type I COCs ($P<0.05$) than with GnRH (26.41 vs. 10.58). The treatment with GnRH resulted in a higher proportion ($P<0.01$) of type III (29.41 vs. 11.32) and mature (type V) COCs (31.76 vs. 7.54) compared to DFR. There were no significant differences in type II (8.23 vs. 9.43) and total COCs suitable for IVP rates (48.23 vs. 47.16) between GnRH and DFR, respectively. Percentage of denuded oocytes (type IV) was higher ($P<0.01$) in OPU sessions performed with DFR than GnRH treatment (45.28 vs. 20.0).

In both treatments, all COCs suitable for IVP completed their maturation. Cleavage rates obtained, 48h after IVF, were 43% for GnRH (18/41) and 36% (8/25) for DFR. These results do not differ significantly. There were no differences between treatments on OPU oocytes yield and early embryo development. In conclusion, follicular ablation can be replaced by exogenous GnRH to synchronize follicular waves in OPU and IVP procedures in Murciano-Levantina bovine breed.

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Notes

EFFECT OF SHORT TERM PROGESTERONE TREATMENT ON EMBRYO YIELD IN SHEEP

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Multiple ovulation and embryo transfer (MOET) technologies have made substantial contributions to the genetic improvement of sheep in several countries around the world. However, an important limiting factor still affecting the success of MOET programs is the variability of the ovarian response and embryo yield (Cognie et al., 2003). The most widely used protocols for superstimulation consist of 14 d of progesterone exposure, with FSH treatment. However, as indicated by Menchaca et al. (2009) progesterone treatment for 14 d does not have clear support, whereas the use of a shorter progesterone treatment should be sufficient which avoids maintenance of persistent follicles. Besides, it was also underlined that gonadotropin-releasing hormone (GnRH) after FSH treatment could be advantageous by improving synchrony of ovulations and producing more embryos (Walker et al., 1989). The objective of the current study was to evaluate the superstimulatory response in ewes by using short term progesterone treatment (8 d) along with one dose of GnRH given 24 h after progesterone treatment cessation compared with the Traditional Protocol.

A total 13 adult ewes were used out of season (May). Ewes (n=7) synchronized with the traditional method (TM) received progesterone treatment for 14 d. Superovulation was performed using FSH-p with 200 mg NIH-FSH-P1 (total of 20 ml) (Folltropin-V; Vetrepharm, Canada) applied in 8 decreasing doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, 1 ml i.m. at 12 h intervals starting 60 h before sponge withdrawal. Donors received 1ml estrumate and 100 I.U. eCG 36h prior sponge removal and an additional 200 I.U. eCG were injected at sponge removal. In the short term (ST) progesterone treatment group, donors (n=6) were treated with progesterone sponges for 8 days and 9 decreasing doses of FSH-p (2.5, 2, 2, 1.5, 1.5, 1, 1, 0.5, 0.5ml i.m.) at 12 h intervals starting 80 h before sponge withdrawal. At sponge removal ewes were injected with 200 I.U. eCG and additional one dose of GnRH was administrated 24 h after sponge removal. Donors underwent intrauterine insemination with fresh diluted semen (10 x 107) 40 h after sponge removal. Ewes were tested for estrus and were laparotomized and surgically flushed to recover embryos 6 d later.

The number of corpora lutea (CL) and the total number of embryos were recorded. Estrus and superovulatory response did not differ between donors in TM (100%; 100%) and ST (100%; 83%) groups. Ovulation rate was found significantly higher in ewes in TM (20.14± 1.99) compared to ST (8.00±2.35). However, the number of transferable embryos were similar in both groups (TM: 8.33±3.56; ST: 8.00±2.33). It was concluded that short term progesterone treatment along with GnRH administration is recommended due to the similar embryo yields.

Notes

EARLY PREGNANCIES AFTER TRANSFER OF BIOPSIED EQUINE EMBRYOS

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Embryo transfer is a powerful tool for genetic selection. Moreover, few embryonic cells are needed for preimplantation genetic diagnosis before transfer. The biopsy by section of Day 7 equine blastocysts, as done previously in our laboratory in small ruminants, is made difficult because of the presence of a capsule and greater diameter and volume (Guignot et al, MRD, 2009; Guignot et al, RDA, 2011). Recently, biopsy by cell aspiration has given acceptable results in horses (Choi et al, Reproduction, 2010).

The aim of the present study was 1/ to biopsy equine embryos at the blastocyst stage by cell aspiration, 2/ to diagnose the embryos sex on biopsied cells and 3/ to test the viability of biopsied embryos after transfer to recipients.

Embryos were recovered 7 days after ovulation from Welsh pony mares. Twenty five expanded blastocysts 155-655 µM in diameter were collected, 11 for *in vitro* survival (6 biopsied and 5 control) and 14 for *in vivo* survival (8 biopsied and 6 control). Biopsy was performed using a glass pipette attached to a Piezo drill. Approximately 2 to 4 trophectoderm cells were aspirated. For the *in vitro* survival study, biopsied and control embryos were individually cultured for 24 h in 30 µL of modified synthetic oviduct fluid (mSOF) with 10% foetal calf serum and 19 mM glucose under paraffin oil (38.5◦C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2). Morphological quality was estimated according to scale of notation (Squires EL, Collection and transfer of equine embryos, 1995) at t0 (collection) and t24 (after *in vitro* survival). For *in vivo* survival, biopsied and control embryos were transcervically transferred to recipient pony mares within 4 to 5 h after collection/biopsy (one blastocyst per recipient). During this period, embryos were held in enriched mSOF (38.5◦C, 5% CO_2, 5% O_2, 90% N_2). Pregnancies were ultrasonographically monitored until an embryonic heartbeat was detected (Day 25-30). Sex diagnosis was carried out by PCR amplification of ZFX/ZFY and SRY sequences.

After 24 h of *in vitro* survival, morphological notation was not significantly different for the two groups (1.1 ± 0.2 at t0 and 1.2 ±0.4 at t24, P=0.90, for biopsied embryos; 1.0 ± 0.0 at t0 and 1.2 ± 0.4 at t24, P=0.32, for control embryos). After embryo transfer, there were no differences in the number of embryonic heartbeats detected between biopsied embryos (50%) and control embryos (33%; P=0.53). Sex diagnosis was efficient for 82%; compared to control embryos, 100% of diagnosed sex were correct.

Equine embryo biopsy by cell aspiration is compatible with viability after transfer to recipients. The rate of pregnancy is very encouraging. The next step will be to try to transfer biopsied embryos after cryopreservation in our lab.

Grant from IFCE (Institut français du Cheval et de l'Equitation) was received to perform this experiment.

Notes

EFFECT OF ACTIVIN-A IN IVM MEDIUM OF PREPUBERTAL GOAT OOCYTES ON BLASTOCYST RATE AND QUALITY

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Activin-A is involved in regulation of oocyte maturation in several species. In bovine, Silva and Knight (1998) suggested that activin-A could modulate oocyte maturation and had dose-dependent beneficial effects on the proportion of oocytes that reach to blastocyst stage. The aim of this study was to determine the optimal concentration of recombinant human activin-A added during *in vitro* maturation (IVM) of prepubertal goat oocytes on the blastocyst yield and mean cell number. Cumulus Oocyte Complexes (COCs) were randomly assigned to one of the three IVM groups. (1) **CM** (control): COCs matured in TCM199 supplemented with 10% Donor Bovine Serum (DBS), 10 µg/mL FSH, 10 µg/mL LH, 1 µg/mL 17β-oestradiol and 100 µM cysteamine; (2) **CM+10:** COCs matured in CM+10ng/mL activin-A and; (3) **CM+100:** COCs matured in CM+100ng/mL activin-A. COCs were matured in groups of 25–30 COCs/ 100 µL drops for 24h. After IVM, oocytes were fertilized with fresh goat sperm at a final concentration of $4x10^6$ spz/mL and after 24h presumptive zygotes were cultured in synthetic oviduct fluid (SOF) during 8 days. The cleavage rate was evaluated at 48h post-insemination and blastocyst rate at the final of *in vitro* embryo culture. The mean cell number of those blastocysts produced was evaluated after hoechst 33342 staining. The results are shown in the table 1.

Table 1: Effect of activin-A in IVM medium on embryo development of prepubertal goats oocytes.

Culture treatment	**No. oocytes**	**No. Cleavage (%)**	**No. Blastocysts (%)**			**Blastocyst mean cell number ± (SEM)**
			Day 7	**Day 8**	**Day 9**	
CM	398	164 (41.20)	19 (04.77) [a]	27 (06.78)	35 (08.79)	176.72± 16.61
CM+10	406	188 (46.30)	32 (07.88) [b]	40 (09.85)	45 (11.08)	192.83± 20.86
CM+100	406	187 (46.06)	24 (05.91) [ab]	35 (08.62)	40 (09.85)	187.94 ± 17.38

Values in the same column with different letters [(a,b)] differ significantly ($P<0.05$). PROC GLM.

The current study shows that IVM of prepubertal goat oocytes in the presence of 10 ng/ml of activin-A significantly enhanced the percentage of blastocysts compared to the control treatment (CM) at day 7.

Notes

OPTIMAL USE OF EMBRYO RECOVERY, EMBRYO TRANSFER AND SEX-SELECTION IN REPRODUCTION OF A DAIRY HERD

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Present technologies offer many possibilities for animal breeding and thus challenges for the management of reproduction in a dairy herd. In this study, a linear programming model was developed to maximize the gross margin of milk production by determining the optimal use of different reproductive technologies in a dairy herd. The model has the potential to vary the use of conventional AI, AI with X-sorted sperm, and the use of unselected or sex-selected embryo recovery and transfer. Data from Finnish dairy herd recording systems were used to solve the results for a herd size of 60 dairy cows. Six different scenarios were estimated.

In 2010, 628 and 172 embryo recoveries with unsorted semen were registered for heifers and for cows, respectively. The average yields of transferable embryos in these recoveries were 7.2 for heifers and 12.0 for cows. Average embryo yields of 132 registered heifer and 110 cow recoveries with X-sorted sperm in 2010 were 6.8 for heifers and 4.5 for cows. In the model, 90% of these embryos were expected to be of desired sex. After AI with unsorted semen, recovered embryos can be diagnosed for sex by polymerase chain reaction (PCR) from an embryo biopsy. The method is efficient in sorting the sex but embryo viability may be compromised after the biopsy. Therefore, the yield of female embryos was set 10% lower than it would have been without sorting the embryos.

The average non-return rates of AIs in Finland in 2010 were used as inputs in the model. With unsorted semen they were 56.3% and 43.8% and with sex-sorted sperm 42.3% and 30.2% for heifers and for cows, respectively. The mean non-return rate in 2009, 66.2%, was used as an estimate for the success of embryo transfer.

In the basic scenario, the optimum economic combination for Finnish conditions was to inseminate 10 heifers and 22 cows with unsorted semen, 8 heifers with X-sorted sperm, and to use 20 cows as embryo donors which was the upper constraint for this technique. The embryo donors were inseminated with conventional semen for both embryo production and their subsequent pregnancy. Without restriction on embryo recovery, the optimum combination was to use all heifers as donors of sex-selected embryos and all cows as donors of unselected embryos. It was more profitable to produce female embryos with X-sorted sperm than by sorting embryos. Embryo recipients were not economically justified in any scenario.

In practice, the optimal strategy is herd-specific depending on the input costs, output values and the technical success of each reproductive technology in that herd. This single-year linear programming model adequately differentiates between breeding technologies within a herd, but further research is needed to develop dynamic models to consider genetic improvement and herd expansion.

Reference

Heikkilä, A.-M. & Peippo, J. 2012. Optimal utilization of modern reproductive technologies to maximize the gross margin of milk production. Animal Reproduction Science 132:129-138.

Notes

DEVELOPMENT OF BOVINE 2-CELL STAGE EMBRYOS CORRELATES WITH EXPRESSION OF GENES RELATED TO OXIDATIVE STRESS RESPONSE

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Transcriptome profiling has been used to identify genes related to developmental competence in bovine embryos and oocytes for several years. However, this technique is invasive, avoid subsequent embryonic development and therefore can only be related to developmental competence indirectly. Indeed, the introduction of blastocyst bisection enabled a direct correlation of a typical transcriptome profile and the potential to induce pregnancy and to develop to term of the same single embryo. This technique was applied to 2-cell stage embryos in the present work. Its well known from several species, that both sister-blastomeres are capable to develop to blastocyst. Furthermore, studies in mice revealed highly similar transcriptome profiles in both blastomeres of a single 2-cell stage embryo. The aim of the present approach was to define molecular fingerprints and to select candidate genes of bovine 2-cell stage embryos, which correlate directly with its developmental competence. To characterize molecular markers for preimplantation developmental competence, 2-cell stage embryos were bisected; one blastomere was cultured individually, its sister-blastomere was snap-frozen. According to the development of individual cultured blastomeres, the corresponding frozen samples were pooled into three groups for global gene expression analyses. We defined three groups: I. embryos which did not cleave after separation (2CB), II. embryos which stopped cleaving at 4- cell stage (8CB) and III. embryos reaching blastocyst stage (BL).

Global transcriptome analysis revealed 771 and 190 genes to be differentially regulated (fold change $\geq$ 1.5, P$\leq$ 0.05, FDR$\leq$ 0.1) between competent (BL group) and incompetent 2-cell stage embryos (2CB group and 8CB group). Functional annotation of differentially regulated genes evidenced that those genes to be involved in oxidative stress response (e.g. *NDUFS1, MAPK14, CAT, PRDX1* and *PRDX6*) including oxidoreductase-, peroxidise- and antioxidant- activity and as well in oxidative phosphorilation (OXPHOS). To confirm these findings, early and late cleaved 2-cell stage embryos, as a proven model for developmental competence, were investigated for relative transcript abundance of these candidate genes by real time PCR. Selected candidate genes are known to function as direct and indirect scavengers of reactive oxygen species (ROS). In accordance, higher accumulations of ROS were detected in late cleaved 2-cell stage embryos, implicating a lower abundance of ROS scavengers. Correspondingly, we found low ROS levels in early cleaved 2-cell stage with higher expression of ROS scavengers. The overall findings of the present study indicate that developmental competence of bovine embryos is largely determined in 2-cell stage and strongly correlated to enriched expression of transcripts regulating oxidative phosphorilation, oxidative stress response and ROS scavenging. These results were additionally supported by localization of CAT- and PRDX1 Protein performing Immunohistochemestry. Given the nature that embryonic major genome activation starts at 8-cell stage, we suggest that individual developmental competence of bovine 2-cell stage embryos is largely maternally inherited.

Notes

GROUP CULTURE INFLUENCES BOVINE BLASTOCYST QUALITY BUT NOT QUANTITY IN SERUM-FREE MEDIUM

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Individual culture of cattle embryos is commonly associated with low blastocyst rates. We have previously observed that, whereas serum containing medium does not support blastocyst development in individual culture, serum-free medium is able to produce comparable blastocyst rates in individual or in group culture. The aim of the present study was to determine if group culture has an influence on embryo quality, both in serum and serum-free containing medium, in terms of total cell number (TCN), inner cell mass (ICM) ratio and apoptotic cell ratio (ACR).

Bovine oocytes (n = 1130, 3 replicates) were matured either in the presence of serum (TCM199 + 20% FBS) or serum-free (TCM199 + 20 ng/ml EGF). Serum matured zygotes were cultured individually (20 µl drops) or grouped (25 embryos in 50 µl drops) in SOFaa + 5% FBS. Serum-free matured zygotes were cultured individually or grouped in SOFaa + 0.4% BSA + ITS (5 µg/ml Insulin – 5 µg/ml Transferrin – 5 ng/ml Selenium) obtaining four experimental groups: Ind FBS, Gr FBS, Ind BSA and Gr BSA. Blastocyst evaluation occurred 7 and 8 days post insemination (dpi). Hatching rate was measured as the proportion of hatching/hatched blastocysts out of total 8 dpi blastocysts. Blastocysts were collected at 8 dpi for differential apoptotic staining (Wydooghe *et al,.*2011). Developmental data were analyzed using a binary logistic regression model, while data concerning blastocyst quality were analyzed using a linear mixed model analysis. Differences at $p<0.05$ were considered significant.

Regarding blastocyst development, Ind FBS yielded significantly lower blastocyst rates than the rest of the groups both at 7 dpi (Ind FBS 7.8%, Gr FBS 29.7%, Gr BSA 29.6%, Ind BSA 24%) and at 8 dpi (Ind FBS 12.4%, Gr FBS 31.2%, Gr BSA 36.5%, Ind BSA 35.2%). Hatching rates were significantly higher in Gr FBS (34%) compared to all other groups (Ind FBS 2.6%, Gr BSA 15%, Ind BSA 12.5%). Concerning embryo quality, TCN was significantly lower in individual culture (Ind FBS 143.12 ± 7.25, Ind BSA 157.34 ± 6.48) than in group culture (Gr FBS 224.38 ± 7.31, Gr BSA 231.58 ± 5.76) for both media. ICM ratio was significantly lower in Gr FBS (0.34 ± 0.01) compared to Gr BSA (0.39 ± 0.01), otherwise no differences were observed (Ind FBS 0.36 ± 0.02, Ind BSA 0.37 ± 0.01). Embryos cultured individually had a significantly higher ACR (Ind FBS 0.05 ± 0.004, Ind BSA 0.05 ± 0.003) than group cultured embryos (Gr FBS 0.03 ± 0.002, Gr BSA 0.03 ± 0.002) for both media.

In conclusion, we confirmed the detrimental effect of serum for embryo development in individual culture. The higher hatching rate reached in Gr FBS showed that there is a component present in serum, which, in combination with autocrine secretions produced by the embryos, promotes hatching from the zona pellucida. Interestingly, although group and individual culture yielded equal blastocyst rates in serum-free medium, group culture (and exposure to autocrine secretions) showed to have a significant positive effect on blastocyst quality in both media conditions. Group cultured blastocysts presented higher TCN and less ACR than embryos cultured individually.

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Notes

MITOCHONDRIAL CLUSTER FORMATION AND ATP PRODUCTION IN BOVINE OOCYTES WITH DIFFERENT MEIOTIC COMPETENCE DURING THEIR MATURATION

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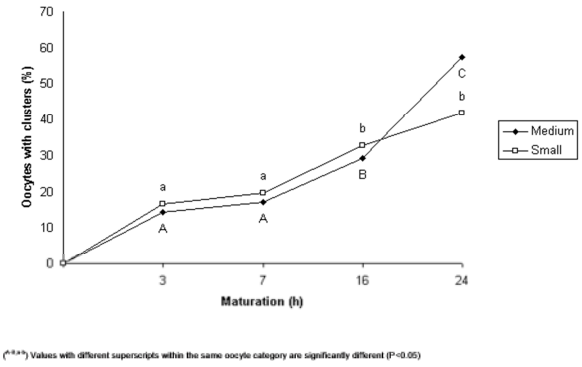
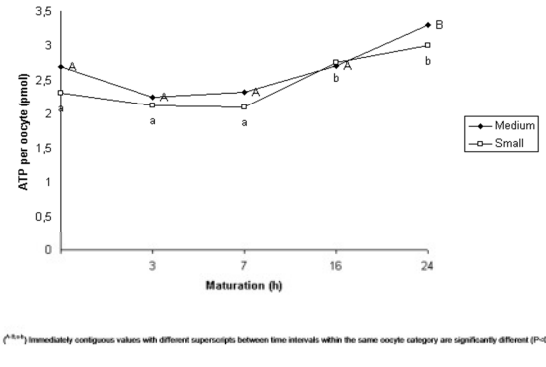
Meiotic and cytoplasmic competences of bovine oocytes closely correlate with the size of follicles from which the oocytes have been recovered. Important markers of cytoplasmic maturation of oocytes are mitochondrial status and energy production. During maturation, some changes in mitochondrial morphology and ATP production have been documented. The aim of this study was to characterize changes in mitochondrial cluster formation and ATP production in bovine oocytes with different meiotic competence during maturation. Cyclic cows, examined for ovarian status, were used as oocyte donors. Oocytes with greater or lesser meiotic competence were collected from medium (MF) and small follicles (SF) as two subpopulations. Only morphologically healthy oocytes were matured for 24 h using a standard protocol. Mitochondria were stained with MitoTracker Orange and ATP was determined using a bioluminescent cell assay kit. Proportion of oocytes with clusters and ATP content per oocyte were assessed at 0, 3, 7, 16 and 24 h intervals. The results were analyzed by the Chi-square test. Before maturation, the higher ATP content (2.7 vs. 2.3 pmol) was detected in MF-oocytes compared with SF-oocytes. The proportion of oocytes with mitochondrial clusters increased significantly from 7 h to 16 h and then again from 16 h to 24 h in MF-oocytes, but only from 7 h to 16 h in SF-oocytes. ATP content was increasing significantly between 7 h and 24 h in MF-oocytes, but only between 7 h and 16 h in SF-oocytes ($P<0.05$). After maturation, the significantly higher proportion of oocytes with clustered mitochondria (57.5% vs. 41.7%) and ATP content (3.3 vs. 3.0 pmol) were found in MF-oocytes compared to SF-oocytes ($P<0.01$).

Mitochondrial cluster formation in oocytes with different meiotic competence during maturation



(A,B,C) Values with different superscripts within the same oocyte category are significantly different (P<0.05)

ATP content in oocytes with different meiotic competence during maturation



(a,b,c) Immediately contiguous values with different superscripts between time intervals within the same oocyte category are significantly different (P<0.05)

It can be concluded that maturing bovine oocytes differing in their meiotic competence did not differ too much in kinetics of mitochondrial reorganization and intracellular ATP level. However, the meiotically more competent oocytes were more advanced in terms of cluster formation and ATP production in comparison with meiotically less competent oocytes after maturation.

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Notes

EFFECTS OF ELEVATED NON-ESTERIFIED FATTY ACID CONCENTRATIONS ON THE BOVINE OVIDUCTAL MICRO-ENVIRONMENT

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Metabolic disorders such as obesity and type II diabetes in women but also negative energy balance conditions in high yielding dairy cows, play an important role in the pathogenesis of subfertility. Previous research has indicated that elevated non-esterified fatty acid (NEFA) concentrations in the serum, arising from upregulated lipolysis, are detrimental for the follicular environment. However, the consequences on the oviductal micro-environment remains uncertain despite its importance on sperm capacitation, final oocyte maturation, fertilization and development of the pre-implantation embryo. For this study we hypothesized that elevated serum NEFA concentrations are reflected in bovine oviductal micro-environment.

Slaughterhouse oviducts of cows in the early luteal phase were selected. After mechanical isolation of Bovine Oviduct Epithelial Cells (BOECs) from the ampulla, the cells were cultivated in a flask and subsequently transferred in a PCC-system (polyester membrane, pore size 0.4µm, $4x10^6$ pores/cm^2.) until the Transepithelial Electric Resistance (TEER) reaching approximately 700Ωcm^2. The average increase of the TEER recordings was 27.14% per 24h (inter assay coefficient of variation = 5%). On day 7 the basal medium was supplemented with 360µM NEFA: 115µM oleic acid (OA), 140µM stearic acid (SA) and 105µM palmitic acid (PA). The medium of both the apical and basal compartment was sampled after 24h and 48h incubation (3 replicates with each 9 wells per time point). Samples were analyzed with photometric and gas chromatographic methods for total NEFA and specific fatty acid analyses respectively.

The results showed that on average the total NEFA concentration in the apical medium was 4 times lower than in the supplemented basal compartment. A small increase of total NEFA concentrations (Δ_{24h} = 4.76µM, $\Delta_{24h\text{-}48h}$ = 6.50µM) was detected in the apical compartment, more specifically an elevation of stearic (mean Δ_{SA} = 14.35µM) and oleic acid (mean Δ_{OA} = 7.37µM) ($P < 0,05$). A significant decrease in NEFA was observed in the basal compartment at 24h (Δ_{24h} = -7.1µM) exposure and is visible for the 3 NEFAs tested (mean Δ_{PA} = -29.09µM, Δ_{SA} = -14.70µM, Δ_{OA} = -30.90µM). However, the differences basally measured between 24h and 48h turned out to be statistically insignificant for both photometry and gas chromatography, possibly due to a relatively short time interval. BOEC monolayers without NEFA exposure did not induce significant changes in fatty acid concentrations in the apical medium. These *in vitro* results showed that NEFA-transport from the serum to the oviductal lumen can occur, but is tightly regulated. Many other factors such as hydrostatic pressure and a BSA concentration gradient need to be studied to further optimize this PCC system.

Notes

NEUTRAL RED (NR) AS A TOOL TO ASSESS PRE-ANTRAL FOLLICLE SURVIVAL IN BOVINE OVARIAN CORTICAL BIOPSIES CULTURED *IN VITRO*

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The development and optimization of pre-antral follicle culture methods are a crucial step in fertility preservation strategies for women undergoing anticancer therapy. When follicles are removed from their natural environment, they are subjected to *in vitro* culture conditions with an altered microenvironment. Pre-antral follicle stage transition and -growth is usually assessed by invasive histological evaluation, rendering it impossible to evaluate the same follicle subsequent times during culture. Therefore, there is a need for alternative, non-invasive follicle viability test methods. The aims of this study were to examine if NR can be used to: 1) visualize and quantify pre-antral follicles within ovarian cortical fragments and morphologically determine follicle stage; 2) evaluate follicle survival, -growth, and follicle stage transition; 3) assess the effects of *in vitro* follicle culture conditions, such as oxygen pressure (5% vs. 20% O_2), during culture.

Cortical slices (n = 132; 6 replicates) were cut from bovine ovaries and incubated for 3h at 37°C in Leibovitz medium with 50µg/ml NR. All NR stained follicles were evaluated '*in situ*' for follicle diameter and morphology. Next, cortical fragments were individually cultured McCoy's 5a medium for 6 days at 37°C, 5% CO_2 and 5% or 20% O_2. On Days 4 and 6 the fragments were re-stained by adding NR to the McCoy's medium and follicle survival, diameter and morphology was assessed.

Stained follicles could be visualized after 3h incubation on Day 0 and 1h incubation on Days 4 and 6 and follicle diameter and morphology could be assessed. In both treatment groups, within 6 days of culture a decrease of primordial follicles and increase of secondary follicles could be observed (Table 1). This suggests primordial follicle activation and follicle stage transition. No significant difference between follicle survival, stage transition and growth after 4 or 6 days of *in vitro* culture could be determined ($P > 0.05$) between both treatment groups (Day 4: 15.5 ± 3.9µm and 13.4 ± 2.4µm; Day 6: 28.8 ± 8.5µm and 23.9 ± 4.6µm; 20% and 5% O_2 respectively).

Table 1: Follicle survival and follicle stage on Day 0, 4 and 6 of the *in vitro* ovarian fragment cortex culture.

	N follicles		Follicle survival (%)		N primordial (%)		N primary (%)		N secondary (%)	
	20% O_2	5% O_2	20% O_2	5% O_2	20% O_2	5% O_2	20% O_2	5% O_2	20% O_2	5% O_2
DAY 0	427	413	427 (100)	413 (100)	297 (69.6)	279 (67.6)	118 (27.6)	127 (30.8)	12 (2.8)	7 (1.7)
DAY 4	427	413	357 (83.6)	337 (81.6)	72 (20.2)	54 (16.0)	255 (71.4)	255 (75.7)	30 (8.4)	28 (8.3)
DAY 6	270	289	183 (67.8)	195 (67.5)	6 (3.3)	12 (6.2)	148 (80.9)	145 (74.4)	29 (15.9)	38 (19.5)

In conclusion, the use of NR permits to determine the number of viable follicles in an ovarian cortical fragment and assess their survival, growth and stage transition in the same fragment throughout *in vitro* culture. This way, the effect of environmental conditions can be evaluated. However, 5 or 20% O2 oxygen tension had no effect on follicle survival, -growth and developmental stage transition.

Notes

INFLUENCE OF ENROFLOXACIN AND GENTAMICIN OVER SOME PARAMETERS OF BOAR SPERM

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Worldwide practice in semen preservation is adding of different antibiotics for protection of sperm from the different pathogens. The influence of these antibiotics on the sperm parameters like motility, enzyme activity and lipid peroxidation are important both for science and practice. The high activity of the enzyme LDH leads to better vitality of boar sperm. Increased levels of lipid peroxidation are associated with increased morphological and functional damage to germ cells. High GGT enzyme activity leads to increased levels of glutathione in semen, causing reduction of lipid peroxidation.

Aim of this study was to clarify the influence of enrofloxacin and gentamicin on the motility, enzyme activity and the rate of lipid peroxidation, of a sperm when stored for 72 h at 15 ºC.

Thirty three ejaculates, taken with artificial vagina from 8 clinically healthy boars, were studied. The ejaculates were diluted with semen extender "Sredets". Each ejaculate was divided into control and two experimental samples. Half of the experimental samples were treated with enrofloxacin (Baytrill, Bayer) and the other half with gentamicin (Vetprom), at concentrations of 20µg and 200 µg per ml of the semen extender, respectively. Sperm motility was determined on 24 th, 48 th and 72 th h after obtaining, by Sperm Class Analyzer (CASA, Microoptic s.l.). The enzyme activity of LDH and GGT was also measured at the same hours, using semi-auto clinical chemistry analyzer BA – 88(Mindray). The rate of lipid peroxidation was characterized by the amount of malondialdehyde (MDA), detected with Spekol 11(Carl Zeiss, Jena) at 532nm.

With regard to sperm motility at 24, 48 and 72 h, no significant differences have been found, both in the control and in the treated with enrofloxacin or gentamicin samples.The activity of LDH and GGT in the controls, decreased during storage from 24 to 72 h (0.751 UI/mg to 0.661 UI/mg, LDH; from 0.108 UI/mg to 0.022 UI/mg,GGT). In the samples treated with enrofloxacin and gentamicin, LDH activity increased both at 48th h (from 0.531 UI / mg to 0.707 UI / mg) and at 72 th h (from 0.531 UI / mg to 0.743 UI / mg) while for GGT activity was observed the same trend as in the control samples. When determining the lipid peroxidation at the 24 th h, the amount of MDA in the controls was higher compared with the experimental samples (1.15 nmol/20x10^6-control; 1.001 nmol/20x10^6- enrofloxacin, and 0.95 nmol/20x10^6 -gentamicin). At the 48 th and 72 th h the amount of MDA in the control samples decreased (0.9 nmol/20x10^6), while in the experimental, the values remained at the same level. In the enrofloxacin treated group even a slight increase (from 1.001 to 1.05 nmol/20x10^6) was observed.

Our results suggest that adding of gentamicin and enrofloxacin at the defined concentrations in boar semen stored at 15 ºC for 72 h, have a weak effect on sperm motility, and they both increased the activity of LDH, an enzyme which is responsible for sperm vitality. The activity of GGT in all treated samples decreased, which lead to the delay of lipid peroxidation reduction.

Notes

TIME OF CONCEPTION DURING LACTATION IN HOLSTEIN COWS INFLUENCES THE BASAL METABOLIC PARAMETERS AND PANCREATIC B-CELL FUNCTION OF THE NEWBORN CALVES

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Maternal undernutrition during early pregnancy alters organ function in the offspring. High-yielding dairy cows obviously suffer from negative energy balance (NEB) during early lactation and at least in some cows during early pregnancy. The latter may hamper embryonic development. The present study was undertaken to determine whether fetal undernutrition caused by maternal NEB alters the function of the endocrine pancreas in newborn calves (n=86) born out of multiparous Holstein cows fed a routine diet throughout pregnancy. The calves born out of dams that conceived at <80 days-in-milk (DIM) were considered undernourished (UN) during their early development, while the calves were considered normally nourished (NN) if their dam conceived later. The newborn calves were weighed before colostrum feeding and blood samples were taken on their 3rd day of life at least 4 h after a milk meal to evaluate basal glucose (G_0) and insulin (I_0) concentrations.

The mean body weight of the calves (46 female and 40 male) was 45.3±6.98 kg, and was not correlated (P=0.25) with G_0 nor I_0 concentration. The female calves had a higher G_0 (6.6 vs 6.1 mM/L, P=0.02) and I_0 (8.4 vs 6.6 mU/L, P=0.07) than the male calves. Although the G_0 (6.4 versus 6.4 mM/L) did not differ (P=0.81) between the UN and NN calves; the I_0 (6.6 vs 8.6 mU/L) and insulin sensitivity index (ISI, I_0/G_0, 1.0 vs 1.3) of the UN calves was significantly (P=0.05) lower compared to the NN calves. Pancreatic β-cell function as assessed by homeostatic model assessment ($HOMA_B$, $(I_0 \times 3.33)/(G_0-3.5)$) in the UN calves was also significantly (P=0.04) lower (8.0 vs 10.3) compared to the NN calves. These preliminary data suggest a significant effect of conception time during lactation on basal metabolic parameters and pancreatic β-cell function of newborn Holstein calves that warrant further investigation. These disturbances in pancreatic β-cell function programmed during early pregnancy by NEB may predispose calves to fertility and metabolic problems in later life.

Notes

EXPRESSION AND CELLULAR DISTRIBUTION OF CYCLIN-DEPENDENT KINASE 4 (CDK4) AND CONNEXIN 43 (CX43) IN PORCINE OOCYTES BEFORE AND AFTER *IN VITRO* MATURATION

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It is recognized that connexin 43 (Cx43) and cyclin-dependent kinase 4 (Cdk4) are involved in the cumulus cell-oocyte communication via gap junctions and the control of cell cycle progress. However, little is known about their mRNA expression pattern and encoded proteins distribution in porcine oocytes during *in vitro* maturation (IVM). Cumulus-oocyte-complexes (COCs) were collected from 31 pubertal crossbred Landrace gilts and analyzed for their Cdk4 and Cx43 mRNA expression using RQ-PCR and for the respective protein expression by confocal microscopic observations. We found an increased Cdk4 and Cx43 mRNA expression in oocytes after IVM ($P<0.001$, $P<0.05$, respectively). Confocal microscopic observations revealed a significant increase of Cdk4 protein expression in cytoplasm of oocytes during the maturation process. The localization of Cx43 changed from *zona pellucida* before to cytoplasmic one in oocytes after IVM. It is supposed that the increased expression of Cdk4 and Cx43 mRNA in oocytes after IVM is linked with accumulation of large amount of templates during the process of oocytes maturation. The translocation especially of Cx43 from the *zona pellucida* into the cytoplasm may be associated with a decrease in gap junction activity in fully grown porcine oocytes. Both Cdk4 and Cx43 can be used as "checkpoints" of oocyte maturation.

Notes

SHORT TERM CULTIVATION OF PORCINE CUMULUS-GRANULOSA CELLS IS RELATED TO CYCLIN-DEPENDENT KINASE 4 (CDK4) AND CONNEXIN 43 (CX43) PROTEIN EXPRESSION - THE REAL TIME CELL PROLIFERATION APPROACH

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The proper mammalian oocyte maturation requires a bi-directional communication between female gamete and surrounding cumulus-granulosa somatic cells (CGCs). Although genes involved in this process and expressed in oocytes are well recognized, still little is known about the CGCs specific genes as well as proliferation potential of these cells in separated culture system. Therefore, the goal of this study was to investigate the CGCs proliferation index as related to Cdk4 and Cx43 protein expression and distribution, the crucial factors for oocyte maturation.

The cumulus-oocyte-complexes (COCs) were recovered from pubertal crossbred Landrace gilts, treated with collagenase, and then separated CGCs were cultured in the standard TCM199 medium for 44h. At each step of *in vitro* cultivation (IVC) of CGCs, normalized proliferation index was assessed. Moreover, confocal microscopic observation was applied to determine Cdk4 and Cx43 protein expression and CGCs specific cellular distribution.

The normalized proliferation index differed significantly between the first 12h of CGCs IVC ($P<0.01$) and the period between 12h and 24h of cultivation ($P<0.001$). When later phases of proliferation were analyzed, no significant differences were observed. Between 24h-44h of IVC the CGCs proliferation rate was stable. However, an increased proliferation index was found after the first 12h of IVC. Using confocal microscopic observation we found an increased expression of both Cdk4 and Cx43 after 44h of IVC, as compared to expression of these proteins before IVC. Moreover, after IVC a substantial translocation of Cdk4 and Cx43 was noted from nucleus to CGCs cytoplasm.

In conclusion, it was demonstrated for the first time that CGCs may be cultured in a separate culture system without oocytes and that proliferation index was increased significantly in the first 12h of IVC, which may be related to cumulus cells expansion in CGCs models with an enclosed oocyte. Furthermore, expression of both Cdk4 and Cx43 in CGCs suggested that these proteins may be regarded to represent markers not only of proper oocyte maturation but also may be involved in the mechanism of CGCs differentiation. Translocation of these proteins into CGCs cytoplasm after 44h of IVC may be related to expansion process in CGCs models with an enclosed oocyte.

Notes

EXPRESSION OF CYCLIN-DEPENDENT KINASE INHIBITORS (CDKN1, CDKN5) IN DEVELOPMENTALLY COMPETENT AND INCOMPETENT PORCINE OOCYTES

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The oocytes developmental competition involves their ability for reaching maturation of MII stage, fertilization and achieving of blastocyst stage. Although several reports have been published till now, regarding the cyclin-dependent kinases (Cdk's) in developmentally competent mammalian oocytes, still little is known about their expression pattern in developmentally incompetent female gametes. The Cdk's are the main cell cycle division regulators, described also as "checkpoints" in the MI into MII transition. However, the role of cyclin-dependent kinase inhibitors (Cdkn's) remains not entirely known. Therefore, the aim of this study was to investigate the differential mRNA's expression of genes encoding Cdkn1 and Cdkn5 in developmentally competent and incompetent porcine oocytes.

The porcine COC's were collected from 26 crossbred Landrace pubertal gilts, following BCB staining test and in vitro maturation (IVM). After processing, COC's were divided into three groups; (i) oocytes analyzed soon after collection-before BCB staining test and IVM, (ii) oocytes after BCB test and colorless (BCB-), as well as (iii) COC's stained with blue (BCB+) and cultured in standard porcine medium (TCM199) for 44h. After grouping, the oocytes were analyzed by using RQ-PCR regarding Cdkn1 and Cdkn5 mRNA's expression.

After using RQ-PCR analysis, we found the highest Cdkn1 mRNA expression in the oocytes before IVM (group I), as compared to BCB+ and BCB- oocytes, ($P<0.01$, $P<0.001$, respectively). Regarding Cdkn5, an increased mRNA level was observed in BCB+ oocytes (group III) compared to oocytes before IVM and BCB- oocytes ($P<0.001$, for both comparisons, respectively). Both genes revealed higher mRNA's expression in BCB+ oocytes as compared to BCB- oocytes.

It has been suggested that Cdkn1 and Cdkn5 are differentially expressed in developmentally distinct groups of porcine oocytes, which occurs in a maturation stage-dependent manner. Moreover, Cdkn1 may be involved as the molecule regulating cell cycle arrest and specific "block of maturation" following MI to MII transition. It is also hypothesized that Cdkn1 may be associated with inhibition of RINGO/Speedy protein during cell cycle transition.

Notes

EFFECT OF SPIRULINA PLATENSIS ON THE REPRODUCTION PERFORMANCES IN GILTS

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The strong requests to the food quality and safety for human health necessitate searching for new approaches to the enhancement of reproductive potential in farm animals. Discovering the metabolic hormones (leptin, grelin) related directly to the reproduction provokes the interest of studying the relationship between metabolic state and the reproductive performances. Now it is known that the changes in the diet content of female animals lead to the change of glucose content in the follicular fluid reflected in the number of follicles and ovulations.

The aim of our investigation was to study the effect of microalgae Spirulina platensis, added to the gilts diet, on ovarian state. This microalgae possesses biological active properties due to high content of protein, vitamins, macro and micro elements.

21 gilts of Danube white breed from animal base of the Agricultural Institute-Shumen were divided in three groups: control (7 animals) and two experimental (7+7). All the animals received standard forage and water *ad libitum*. Dry biomass of microalgae Spirulina platensis was added (2g/per head -I experimental group and 3g/ per head -II experimental) to the forage of the two experimental groups. The duration of the experiment was 3 months (from the 4^{th} to 7^{th} month of age). After slaughter the morphometric and histological analysis of ovaries was performed. Developmental competence of collected oocytes was tested by brilliant cresyl blue. Data were analysed by computer software STATISTICA (Ver.6.0 of the Stat Soft Inc.).

The morphometric and histological estimation of gilts ovaries showed that all groups had reached the sexual maturation at the end of the experiment. The number of follicles having diameters between 2-6 mm was equal in the control and I^{st} experimental group (20 ± 2.2 and 21 ± 3.1 , respectively), but in II^{nd} experimental group there were only half as many of follicles (10 ± 1.6). Despite this observation in the II^{nd} group there was a higher number of follicles having a size beyond 6 mm (8 ±1.1 vs 4 ±0,8 in control) and yellow bodies(7 ± 2.1 vs 3 ± 0.4 in control). Histological analysis confirmed these findings: the number of preovulatory follicles and yellow bodies were also significantly higher in the II^{nd} experimental group. No differences were found between the groups related to the developmental competence of oocytes collected from the follicles with sizes between 2-6 mm. Approximately equal percentages of CB^+ and CB^- oocytes were observed in each group.

The obtained results allow to conclude that the addition of Spirulina platensis in a dose of 3g/per head, probably provokes the earlier development of the first follicular wave and ovulation in the ovaries of gilts.

Notes

EFFECT OF LEPTIN DURING MATURATION OF OPU-DERIVED BOVINE OOCYTES ON EMBRYO DEVELOPMENT AND PREGNANCY RATE

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Several studies suggest that Leptin plays an important role in female reproduction and can influence the outcome of in vitro culture of embryos. In an earlier study, we showed a significant increase of blastocyst rate after addition of 1000 ng/ml human Leptin during maturation of slaughterhouse-derived oocytes. No effect on embryo quality according to IETS standards and freezability, according to in vitro survival after thawing was found (abstract in proceedings AETE 2010 page 182). In order to investigate if addition of Leptin could also improve the efficiency of our OPU-IVP system, without negative effects on pregnancy rates, a field trial was performed.

During September and October 2010, immature Cumulus-Oocyte-Complexes (COCs) were obtained twice weekly by ultrasonic guided transvaginal oocyte collection. The OPU-derived oocytes were matured one week with Leptin (+ Leptin group) and the next week without Leptin (- Leptin group). Maturation was done in M199 with 10% FCS, FSH/LH and 1000ng/ml Leptin (Sigma, Missouri, USA) or without Leptin. Fertilization and culture was performed in SOFaaBSA as described earlier (van Wagtendonk et al. 2000, Theriogenology 53: 575-597). At Day 8 the blastocysts rate and quality according to IETS standards were recorded. The embryos were transferred fresh into recipients. The pregnancy was checked 4 months upon transfer. Results were analysed by Chi-square analyses.

No significant difference was observed in percentage embryos at Day 8 and in pregnancy rate after transfer (Table 1).

Table 1: Effect of Leptin on embryo production and pregnancy rate

	No. of OPU sessions	No. of oocytes	embryos D8 (%)	No. of embryos transferred fresh	Pregnancy rate (%)
- Leptin	142	954	27	69	45
+ Leptin	116	602	27	73	42

Although addition of Leptin during maturation improved the developmental competence of slaughterhouse-derived oocytes, it did not improve the efficiency of our OPU-IVP system. This suggests that the origin of the oocytes influences the effect of Leptin. OPU-derived oocytes are collected from follicles 5-8 mm in size and therefore prematuration has occurred in all collected oocytes. Oocytes derived from slaughterhouse ovaries however, are collected from follicles 2-8 mm in size. It may well be that Leptin only affects the developmental competence of oocytes which are in the prematuration phase (follicles 2-4 mm in size).

Notes

INFLUENCE OF QUALITY AND STAGE OF FROZEN – THAWED DIRECT – TRANSFER OVUM PICK UP IN VITRO EMBRYONS ON PREGNANCY RATES: PRELIMINARY RESULTS

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Cryopreservation is essential for a large-scale diffusion of bovine embryos. In vitro produced (IVP) bovine embryos have been shown to be more sensitive to cryopreservation than their in vivo counterparts, this could be explained by morphological and ultrastructural differences (Wrenzycki, *et al*., 2007 Theriogenology 68;77-83). Following cryopreservation, IVP embryos often yield lower pregnancy rates after transfer (Hasler, *et al*., 1995 Theriogenology 43, 141-53). These low rates highlight the need to combine several embryo quality criteria to evaluate IVP embryos before freezing. Since October 2009, MIDATEST's lab is producing embryos in vitro from high genetic merit donor Holstein females using Ovum Pick Up and In vitro production (OPU-IVP). The aim of this study was evaluate the pregnancy rates observed after direct transfer of IVP frozen Holstein embryos of different developmental stages and qualities.

Donors (heifers and cows) were stimulated with decreasing doses pFSH twice a day during two and half days (Stimufol® a total dose: 250 µg pFSH / heifers and 350 µg pFSH /cows). OPU was performed 12 hours after the last FSH injection using a 240 Parus Vet ultrasound scanner (Pie Medical) equipped with 7.5 MHz annular-array probe.

Oocytes were in vitro matured in M199 supplemented with FCS, FSH/LH, estradiol and EGF for 22 hours at 38.5°C. They were fertilized with frozen – thawed semen in TALP medium using different bulls without any previous testing in IVF program. Presumptive zygotes were cultured in SOF medium (Gamarra et al.; AETE 2010) up to day 8 at 38,5 °C in 5% CO2 , 5% O2 and 90% N2 atmosphere with maximum humidity. 38 Blastocysts (B) and 79 expanded blastocysts (EB) of excellent and good qualities (Grade 1 according to IETS classification) were recorded.

All embryos were slow frozen and directly transferred in heifer recipients under different environmental and field conditions. Pregnancy was determined by rectal palpation or ultrasonography between 60-90 days after embryo transfer. Pregnancy rates were analyzed by Chi-Square analysis.

The results are presented in Table 1. The overall pregnancy rate observed after direct transfer of IVP embryos in different field conditions was 45.2% (n=117). A higher pregnancy rate was achieved with EB whatever the quality of embryos at freezing was (but significantly not different P>0.05). Impact of embryo quality was evidenced after transfer of blastocyst stage for which higher pregnancy rates were observed after transfer of excellent (41.4% vs 22.2% for good quality embryos). However, due to the reduced number of transfers of Good B, these results need to be confirmed.

Table 1: Pregnancy rate following direct transfer of frozen OPU- IVF embryos

		Percent pregnant (%)		
Embryo Stage	N° Transferred	Excellent	Good	All
Blastocysts (B)	38	41.4 (12/29)	22,2 (2/9)	36.8 (14/38)
Expanded Blastocysts(EB)	79	48.5 (33/68)	54,5 (6/11)	49.4 (39/79)
Total	117	46.4 (45/97)	40 (8/20)	45.2 (53/117)

These results suggest that the optimal development stage of an embryo for freezing and direct transfer is the expanded blastocyst; but excellent blastocysts can also be successfully frozen. Further research is required to confirm the results obtained.

Notes

EFFECT OF LIGHT EXPOSURE ON DEVELOPMENT OF PORCINE PARTHENOGENETICALLY ACTIVATED EMBRYOS

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Oocytes and embryos are sensitive to different types of stress during in-vitro handling, e.g. low or high temperatures and certain substances in the media. Visible light is another type of stress that is considered to be harmful, as it has been shown in cool and warm light on zygotes of hamster and mouse (Takenaka et al. PNAS 2007, 104(36):14289-14293). However, similar experiments have not been made on porcine embryos, so our aim was to test effects of different types of normal light on the development of parthenogenetically activated porcine embryos.

Cumulus–oocyte complexes (COCs) were aspirated from 2 to 6 mm follicles in slaughterhouse-derived sow ovaries and matured at 38.5°C in 5% CO2 with maximum humidity for 42 h. Parthenogenetic activation (Day 0) was made first by an electric pulse (1.26 kV/cm, 80 μs) and then by incubation with 5 μg/ml cytochalasin B and 10 μg/ml cycloheximide in PZM-3 medium for 4 h. The activated oocytes were exposed to different light (daylight: near to window, but no direct sunlight; lab-light: approx. 40 cm from warm white lamps (12V, 40W)) in PZM-3 and on the heating plate (38.5°C) with the culture dish covered in crystal plastic foil filled with the appropriate gas (5% O_2,5% CO_2) for different intervals (0h, 0.5h, 1h, 4h or 24h), and then cultured in PZM-3 in incubator. On Day 6 total and good blastocysts were counted. A good blastocyst was defined as a blastocyst having expanded to 1.5 times the size of oocytes, having cells of uniform colour and distribution,and having formed a regular blastocoel cavity.

The results are summarized in the table. An adverse effect occurred earlier with daylight on good blastocysts, i.e. after 1 h exposure. For both light types, there was a clear adverse effect on all blastocysts after 24 h exposure, most severely after daylight.

It can be concluded that a limited exposure to light has no immediate adverse effect on parthenogenetic porcine embryos, and that indirect daylight is more harmful that lab-light.

Light type	Exposure time (h)	Total activated oocytes (Replicates)	Total blastocyst%±SEM (NO.)	Good blastocyst%±SEM (NO.)
Daylight	0 (control)	98 (5)	68.0±4.9 (67) [a]	55.8±4.5 (55)[a]
	0.5	63 (4)	77.5±2.1 (49) [a]	56.7±7.8 (37)[a]
	1	65 (4)	72.1±7.9 (47) [a]	38.3±5.7 (25)[b]
	4	42 (2)	58.3±8.3 (24) [a]	30.6±13.8 (12) [b]
	24	42 (2)	0 [b]	0 [c]
Lab light	0 (control)	114 (6)	69.2±4.2 (79)[a]	55.9±3.7 (64)[a]
	1	56 (4)	67.4±3.3 (38)[a]	46.4±5.5 (26)[ab]
	4	77 (5)	64.7±8.8 (49)[ab]	36.0±6.6 (27)[b]
	24	29 (2)	36.0±6.6 (14)[b]	24.8±6.0 (7)[b]

[a, b, c] Different superscripts in the same column indicate significant difference (*P<0.05*).

Notes

THE EFFECT OF EMBRYO CO-CULTURE WITH DIFFERENT TYPES OF BOVINE OVIDUCTAL EPITHELIAL CELLS AND CONDITIONED MEDIA IN VITRO ON EMBRYO DEVELOPMENT AND QUALITY

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Early embryonic development takes place in the oviduct, where the newly formed embryo experiences a changing environment during the reproductive cycle. Nowadays somatic cells are generally no longer used for bovine in vitro culture; however, they represent a suitable model for studying embryo–maternal interactions or improving embryo quality. The aim of the present study was to evaluate the developmental capacity of bovine zygotes co-cultured in vitro with bovine oviductal cells (BOEC) or their conditioned media (BCM) on the quality of the embryos produced. Presumptive zygotes (n=2504) were produced by in vitro maturation and fertilization of oocytes derived from ovaries of slaughtered heifers and cultured in groups of 20-25 in droplets of 25 µL under the following conditions: (i) SOF+5% FCS, Control group (used as a basic medium for the remaining groups-C), (ii) frozen line BOEC monolayer cells (BOEC-FzM), (iii) fresh BOEC suspension cells (BOEC-SC), (iv) BOEC conditioned media from fresh BOEC monolayer cells (BOEC-CM), and (v) BOEC-CM diluted 1:1 (BOEC-DM). BOECs were prepared 3-4 days before culture in a final concentration of $1x10^6$ ml^{-1}. Half of the media was changed every 48 hours. Cleavage rate was assessed on Day 2 and blastocyst development on Days 7, 8 and 9 of culture (Day 0=day of fertilization). Representative numbers of blastocysts on day 7/8 from each experimental group were used for quality evaluation through: (i) differential cell count and (ii) survival after vitrification/warming. In relation to embryo development, no differences were found between groups for either cleavage rate (range: 82.5±3.60 – 90.2±1.2%) or blastocyst yield on Day 7 (range: 21.6±3.2 – 26.0±2.3%), Day 8 (range: 31.0±4.0 – 36.0±1.8%) or Day 9 (range: 32.9±2.7 – 37.8±2.7%). Similarly, no differences were found between groups in terms of number of inner cell mass cells (range: 47.5±1.8 – 48.9±1.8). However, total cell number (166.4±4.6, 166.3±4.0 and 152.1±4.7, P=0.03) was higher in embryos derived from BOEC-DM and BOEC-CM compared to C embryos, and this was due to a greater number of trophectoderm cells (117.4±3.5, 118.3±4.3 and 104.3±4.2, respectively, P=0.02). Furthermore, after vitrification/warming, significantly more embryos survived at 24 hours for BOEC-CM and BOEC-FzM compared to C, BOEC-SC and BOEC-DM groups (72.8±4.7; 71.0±6.20 vs 50.9±2.2; 54.3±6.4; 55.2±3.6% respectively, P<0.05). With increasing time post warming, embryos from the BOEC-CM group had a significantly higher survival rate (41.1±8.5) at 72 hours compared to all other groups (C: 12.9±4.8; BOEC-SC: 19.2±7.0; BOEC-FzM: 9.5±4.2; and BOEC-DM: 18.2±4.8%, P<0.05). In conclusion, the use of conditioned media from BOEC has a positive effect on the quality of bovine embryos and constitutes a good alternative to the BOEC monolayer co-culture.

Notes

PSAMMAPLIN A INCREASES *IN VITRO* DEVELOPMENT AND QUALITY OF MOUSE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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Somatic cell nuclear transfer (SCNT) is still an inefficient technique in part due to an incomplete or incorrect reprogramming of the differentiated somatic nucleus to a totipotent embryonic state. Previous studies revealed that the treatment of SCNT embryos with epigenetic modifiers such as valproic acid (VPA), a histone deacetylase inhibitor (HDACi), significantly enhances embryonic development and cloning efficiency. Psammaplin A (PsA) is a natural and potent DNA methyltransferase inhibitor and HDACi that has never been used in nuclear reprogramming studies. The purpose of our study was to compare the effect of VPA and PsA treatments on the *in vitro* development and quality of mouse SCNT embryos.

Mechanically enucleated oocytes from B6CBAF1 (C57BL/6JxCBA/J) female mice were reconstructed with a cumulus cell nucleus, activated and then cultured in KSOM medium (37ºC, 5% CO_2). Embryos that reached the blastocyst stage at 96 h post-activation were differentially stained for counting of inner cell mass (ICM) and trophectoderm cells. In a first set of experiments, embryos were exposed to different concentrations of PsA (5, 10 and 20 μM) or VPA (2 and 4 mM) during 1-2 h after reconstruction and 6 h of activation (total 8-9 h). We found that only PsA 10 μM and VPA 2 mM significantly increased blastocyst rates (37.3 and 31 *vs.* 23.3 for the control group), although no differences were found in blastocyst quality (10.4-13.6 ICM cells). In a second set of experiments, we studied the effect of treatment duration by incubating the embryos in PsA 10 μM or VPA 2 mM during 8-9 h, 16 h or 24 h after reconstruction. With PsA, all treatments showed equivalent blastocyst rates (35.2-43.3%), which were higher than in the control group (20%), but only treatments for 16 h and 24 h yielded blastocysts with higher numbers of ICM cells (16.3 and 18.5 *vs*. 10 for the control group). With VPA, treatments for 8-9 h and 16 h were equivalent in terms of blastocyst rates (34.0 and 32.5%) and higher than the control group, but only VPA 16 h yielded blastocysts with a higher number of ICM cells (15.6).

In conclusion, PsA enhances *in vitro* development and quality of mouse SCNT embryos, to a higher extent than VPA.

Studies are currently being performed to determine whether this improvement in blastocyst rates by PsA treatment correlates with an increased development to term. So far, one live cloned pup has been obtained from PsA-treated embryos.

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Notes

MELATONIN EFFECT ON VITRIFIED OVINE BLASTOCYSTS DURING IN VITRO POST-WARMING CULTURE

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Cryostorage technology induces several types of injuries to cellular structures and biochemical molecules and reduces the developmental capability of vitrified embryos. Embryos that survive cryopreservation may accumulate reactive oxygen species which are known to have detrimental effects such as mitochondrial damage, ATP depletion, apoptosis and developmental block. Melatonin is a potent free radical scavenger and antioxidant and in contrast to the majority of other known radical scavengers, this indolamine is multifunctional and universal. The aim of this study was to assess the effects of various concentrations of melatonin during in vitro post-warming culture of vitrified ovine blastocysts. Differences in terms of embryo vitality, total cell number and DNA fragmentation were evaluated. In vitro produced and vitrified ovine blastocysts were randomly divided and cultured after warming in five different media: CTR)TCM 199 plus 10% fetal calf serum; CTRBSA)TCM 199 plus 0.04% bovine serum albumin (BSA); MEL10^{-3})TCM 199 plus 0.04% BSA and melatonin 10^{-3} M; MEL10^{-6})TCM 199 plus 0.04% BSA and melatonin 10^{-6} M; MEL
10^{-9})TCM 199 plus 0.04% BSA and melatonin 10^{-9} M. To assess the vitality post warming, blastocysts of all experimental groups were observed during 72 hours in vitro culture and the complete re-expansion of the blastocoel cavity and the hatching rates were recorded. Total cell number was evaluated fixing the expanded blastocysts in a solution of methanol (40% v/v) in PBS, stained with propidium iodide (10 µg/mL) and observed under a fluorescent microscope. Finally, TUNEL assay was used to detect DNA fragmentation and the expanded blastocysts were examined under fluorescence microscopy. Apoptotic nuclei stained yellowish green and normal nuclei stained orange-red. Data were analyzed using ANOVA and differences were considered to be significant when $P \leq 0.05$. MEL10^{-9} group showed a higher number of re-expanded embryos within 8 hour of culture post warming compared to CTRBSA and MEL10^{-3}; $P<0.05$), while no differences were observed among the other groups. Blastocysts cultured in media supplemented with MEL 10^{-9} M showed hatching rates comparable to those obtained in embryo cultured in serum supplemented medium, and these percentages were significantly higher than those obtained in the other groups ($P<0.05$). The total cell number was significantly lower in blastocysts cultured in CTR compared to CTRBSA and MEL10^{-3} media ($P<0.05$), while no differences were recorded among embryos cultured in CTRBSA, MEL10^{-3} and MEL10^{-9} media. The mean number of TUNEL-positive cells/blastocysts was significantly lower ($P<0.05$) in CTRBSA and MEL10^{-9} groups compared to the others. Thus, our data showed that the use of melatonin at 10^{-9} M concentration has a positive effect on in vitro development of vitrified ovine blastocysts during in vitro post-warming culture.

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Notes

IN VITRO EVALUATION OF A DIRECT TRANSFER WARMING PROCEDURE FOR VITRIFIED BOVINE EMBRYOS: ONE STEP TO DEVELOP A NEW VITRIFICATION DEVICE FOR DIRECT EMBRYO TRANSFER

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Vitrification is an attractive alternative to conventional cryopreservation due to the fact that it is a relatively rapid and inexpensive procedure, and it has been shown to be beneficial for embryos that have lower cryosurvival, such as in vitro-produced embryos. Numerous publications comparing conventional cryopreservation and vitrification of *in vivo*- or *in vitro*-produced bovine embryos report either similar or improved survival rates after vitrification. Although multiple formulations, methodologies and containers have been described in order to bring the vitrification procedure to the routine field use, there is a need for more practical and reliable methodology. Our team has developed a novel device that will allow direct warming and transfer of vitrified embryos directly at the farm but it is necessary to develop a one step dilution protocol for this support to be successful. The purpose of this study was to develop a simple, rapid and efficacious warming technique based in one step dilution for direct transfer of bovine embryos by using this new device.

Embryos produced *in-vitro* by standard procedures were vitrified at the blastocyst stage at day 7 post-insemination in a mixture of 15% ethylene glycol + 15% DMSO + 0.5M sucrose using cryotop devices. Embryos were warmed using an initial concentration of 1M sucrose with subsequent stepwise dilution (0.5M and 0M) (3 step dilution) or directly into 0M sucrose (one step dilution) and transferred to the SOF culture medium. Survival rates were assessed by blastocoel re-expansion and hatching at 3 and 24 h post-warming. Embryos warmed using a 3 step dilution showed significantly higher percentages of survival after 3 h than those blastocysts that were warmed directly at 0M sucrose. However, no significant differences were observed between dilution treatments in terms of survival rate at 24 h post-warming. Our results indicate that this simplified procedure of warming and diluting cryotop-vitrified embryos may enable 1-step bovine embryo transfer without the requirement of a microscope or other laboratory equipment.

Table 1. Effect of warming protocol on *in vitro* survival of *in vitro* bovine blastocysts after cryotop vitrification.

Warming procedure	Day 8 blastocysts, n (%)		
	No.	Re-expanded or hatching at 3h	Re-expanded or hatching at 24h
3 steps	74	67 (90.5)^{A}	63 (85.1)
1 step	146	97 (66.4)^{B}	103 (70.6)

Notes

THE BINDING OF RECOMBINANT PORCINE OVGP1 PROTEIN TO THE ZONA PELLUCIDA IS SPECIES-SPECIFIC

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The OVGP1 is the major non-serum protein present in the oviductal fluid. This glycoprotein is involved in the gamete interaction, blockage of the polyspermy and improvement of the *in vitro* embryo development. Previous studies have reported that this glycoprotein can bind to the ZP in different species. A recent study performed in our laboratory has observed that the prefertilization hardening of the zona pellucida (ZP) observed in the bovine and porcine species is mediated at least in part by OVGP1. Moreover, several studies detected the presence of this glycoprotein in different species; however, despite the similarity detected among species, some regions of the protein are species-specific. The aim of this study was the production of a recombinant porcine OVGP1 to define its molecular activity and to explore its use as a supplementary component for IVF medium to improve the IVF efficiency.

Total porcine oviduct RNA was isolated and cDNA was synthesized with oligo-dT as primer. The complete open reading frame of OVGP1 was amplified by PCR and cloned into pcDNA3.1-6xHIS expression vector by means of the Kpn I and Mun I restriction sites. OVGP1 construct was transiently expressed in human embryonic kidney cell line HEK 293T. The expression of OVGP1 protein was studied by Western-blotting in the conditioned media using a rabbit anti-His polyclonal antibody.

In vitro matured porcine oocytes, mouse oviductal oocytes and human oocytes which failed fertilization were incubated with the media from transfected and non-transfected cells for 1h. After washing, oocytes were fixed and imaged by confocal microscopy using a rabbit anti-His and a goat anti-rabbit IgG secondary antibody conjugated with FITC.

Western blot analysis demonstrated the existence of a major band in transfected cells media with an apparent molecular weight of 80 kDa. OVGP1 recombinant protein was immunolocalized in the ZP of porcine oocytes incubated with the transfected media; however, no specific immunolabelling was detected in the mouse and human ZP.

In conclusion, porcine OVGP1 was successfully expressed and secreted in HEK 293T cells. Porcine recombinant OVGP1 has the ability to bind to the porcine ZP and this binding is specie-specific. Future experiments are necessary to clarify the molecular mechanisms (ZP composition and/or OVGP1) involved in this interaction. Supported by MICINN (AGL2009-12512-C0201-02)

Notes

ULTRASTRUCTURE AND GENE EXPRESSION OF EQUINE OVIDUCT EXPLANTS DURING CULTURE

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The equine embryo remains for about 6 days in the oviduct. To study embryo-maternal interaction in vitro, culture of equine oviduct explants was optimized using markers for cell function such as ciliary activity, cell viability and cell differentiation. In a previous experiment, more than 98% of the explants were showing ciliary activity, but progressively more explants were showing central dark zones over a culture period of 6 days. We hypothesized that this was due to cell death and/or hypoxia, and to test this we evaluated ultrastructure and gene expression of explants at day 0 and day 6. Oviduct explants of mares were cultured in DMEM/F12 + 10% FCS. On day 0 and 6, explants were processed for fluorescence staining to detect apoptotic and necrotic cells, using either TUNEL-anticaspase-3-Hoechst, SYBR14/PI and trypan blue. Furthermore, explants were fixed for transmission electron microscopy (TEM). Next, mRNA expression of the hypoxia-related genes HIF-1a, GLUT-1, VEGF, uPA and PAI were quantified by RT-qPCR and normalized against the geometric mean of 5 validated reference genes (UBB, ACTB, 18S, RPL32, SDHA). Data were analyzed using the Wilcoxon-signed rank test or paired-samples t-test.

Trypan blue staining, SYBR14/PI and TUNEL-anticaspase-3-Hoechst revealed that less than 2% of the cells in the explants were apoptotic or necrotic. TEM demonstrated that the explants were bordered by highly differentiated epithelial cells. The cells in the central dark zones were showing nuclear and cytoplasmatic dark zones and vacuolization without any other signs of cell death, which are features of "dark cell degeneration" (DCD). Interestingly, none of the hypoxia-related genes were upregulated after 6 days of culture. Expression of HIF-1a and VEGF was not changed ($p>0.05$), uPA and PAI slightly were downregulated ($p=0.03$) and GLUT-1 was strongly downregulated ($p<0.0001$) on day 6.

After 6 days of culture, some of the explants were showing DCD, a hypoxia-mediated non-necrotic, non-apoptotic kind of cell death, up to now only described in Huntington's disease and in neuronal en cartilage cell degeneration. In the explants, the DCD present in the center could be caused by hypoxic culture conditions, although the hypoxia-induced genes were not upregulated. The slight downregulation of uPA and PAI may be explained by or the lack of hormonal stimulation in the culture system or the presence of inhibitors. The strong downregulation of GLUT-1 can be due to the high glucose concentration of the culture medium (17 mM). Ultrastructural analysis showed that the explants after 6 days of culture are still bordered by highly differentiated, intact epithelial cells of which less than 2% were necrotic or apoptotic. The oviduct explants, in contrast with monolayers, imitate thereby the in vivo situation as close as possible and are therefore an excellent tool to study sperm-oviduct and embryo-maternal interactions in the horse using an in vitro model.

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Notes

IDENTIFICATION OF BOVINE EMBRYOS CULTURED IN GROUP BY ATTACHMENT OF BARCODES TO THE ZONA PELLUCIDA

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The low number of oocytes collected from unstimulated donors by ovum pick-up means that embryos produced from each individual female have to be cultured individually or in very small groups. However, it has been demonstrated that single embryo culture is less efficient than embryo culture in group. For this reason, different *in vitro* culture systems have been designed where embryos can be tracked individually while sharing the same medium, keeping the benefits of collective culture. Nevertheless, all these systems prevent embryo free movements, which interfere in the transmission of paracrine factors and in the gradient changes that the embryo creates when it moves.

The present work presents an alternative *in vitro* culture method which allows the co-culture of embryos from different origins without movement restriction and preserving their pedigree by labelling the zygotes with polysilicon barcodes attached to the outer surface of the zona pellucida (ZP). These barcodes (10 x 6 x 1 μm) were fabricated using silicon microtechnologies and biofunctionalized by self-assembled monolayers with the lectin wheat germ agglutinin. They contain a total of 8 bits (256 possible combinations), which can be read under an inverted microscope.

To evaluate the efficacy of this system, presumptive zygotes from *in vitro* matured oocytes from slaughtered cows were encoded after denudation. Presumptive zygotes were individually rolled over barcodes under a stereomicroscope until 8 barcodes were attached. Four different barcodes, each with a different codification, were used to encode 25 embryos (6-7 embryos/barcode type), which were then cultured in the same drop. Cleavage, day 7 and day 8 blastocysts and barcode retention rates were assessed. In addition, day 7 blastocysts were vitrified and warmed by the cryotop method. Post-warming blastocyst survival was determined as re-expansion rate at 3h and 24h in culture. In all the experiments a control group without barcodes was cultured and vitrified-warmed.

Preliminary results show that barcode attachment to the ZP of bovine embryos did not affect *in vitro* embryo development, whereas post-warming survival seemed to be decreased (Table 1). All the embryos maintained barcodes attached until day 8 of culture (4.4 ± 1.8 barcodes/embryo) and could be identified.

Table 1. In vitro embryo development and post-warming survival of encoded and control embryos

Group	N	Embryo development, n (%)		N	Post-warming survival, n (%)	
		Cleavage 48hpi	Blastocysts		3h	24h
Control	70	51 (72.9%)	19 (27.1%)	13	13 (100%)	13 (100%)
Encoded	99	86 (86.9%)	39 (39.4%)	29	17 (58.6%)	19 (65.5%)

In conclusion, identification of co-cultured embryos by biofunctionalized barcodes attached to the ZP is feasible and allows to culture embryos from different donors in the same drop. However, further experiments are required, especially to elucidate the effect of barcodes on embryo vitrification.

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Notes

IN UTERO PROGRAMMING OF THE POSTNATAL GROWTH AND INSULIN SENSITIVITY AFTER BETWEEN-BREEDS TRANSFERS IN THE HORSE

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In equids, maternal size affects fetal intrauterine development, which in turn affects insulin sensitivity in the neonatal period and postnatal growth rate, but longer term effects have not been described. We induced intra-uterine growth restriction or enhancement through embryo transfer using Pony (P), Saddlebred (S) and Draft (D) horses and studied placental size, growth and insulin-sensitivity in foals from birth to 6 months of age.

Control pregnancies of S-in-S (n=18) and P-in-P (n=10) were obtained by AI. Lush *in utero* pregnancies were obtained by transferring S embryos (S-in-D, n=8) and P embryos (P-in-D, n=5) into D mares. Restricted *in utero* pregnancies were obtained by transferring S embryos into P mares (S-in-P, n=2). All conceptuses were conceived using semen from one S and one P stallion. At birth, placental mass and gross surface area were measured. Foal weight was recorded from birth to 6 months. At 3 days and 4.5 months of age, all foals underwent an intravenous glucose tolerance test (IVGTT). At 6 months, the euglycemic hyperinsulinemic clamp technique was used to determine the sensitivity and responsiveness of tissues to exogenous insulin in the foals. Data were analyzed using one-way ANOVA and Tukey *post hoc* tests.

Although they had a shorter gestation ($p<0.02$), S-in-S foals were significantly heavier compared to P-in-P ($p<0.0001$), with significantly heavier and larger placentas ($p<0.001$). Birth weight appeared to be lower, gestational length increased and placental mass and surface decreased in the two S-in-P, whereas there were no significant effect on gestational length, placental weight and surface, and postnatal growth in S-in-D compared to S-in-S. In contrast, P-in-D foals had a shorter gestation ($p<0.04$), heavier and larger placentas ($p<0.003$) and were significantly heavier compared to P-in-P until 6 months of age ($p<0.02$).

IVGTT at 3 days and 4.5 months revealed no difference in glucose clearance rates between groups. At 3 days, insulin response was not different in S-in-S and P-in-P, but was significantly higher S-in-D and P-in-D foals ($p<0.008$), indicating insulin resistance. Plasma insulin concentrations at 4.5 months remain to be assayed. Clamps at 6 months of age confirmed the development of an insulin resistant phenotype in S-in-D and P-in-D compared to normal insulin-sensitive S-in-S. P-in-P foals, however, had also become insulin resistant at 6 months of age compared to S-in-S, which is consistent with the known innate insulin-resistance of P.

In summary, enhanced fetal growth induced early insulin resistance in P. Moreover, although fetal and postnatal growth were not enhanced in S-in-D, they developed insulin resistance. More foals are to be born this year so as to increase group size and allow further conclusions regarding growth retarded foals.

Notes

EFFECT OF FOUR OVIDUCTAL GLYCOSIDASES ON PORCINE *IN VITRO* FERTILIZATION OUTCOME

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The oviductal fluid is the physiological milieu where the fertilization takes place. It is formed from a plasma transuded and *de novo* synthesized substances in the oviductal epithelium. Most of its components have been described (Aviles et al. 2010) and measured but their roles in the mechanisms that lead to fertilization are still unclear. It has been previously described the glycosidase activity in the oviductal fluid, with fluctuations along the estrous cycle (Carrasco et al. 2008). Among the described glycosidases, α-L-fucosidase (α-L-fuc), β-N-acetylglucosaminidase (β-N-gluc), β-D-galactosidase (β-D-gal) and α-mannosidase (α-man) showed different activity depending on the follicular or luteal phase of the estrous cycle. Possible roles for the presence of active glycosidases have been stated, i.e. interaction between spermatozoa and oviductal epithelium cells, *cumulus* cells dispersion, capacitation, binding of sperm to oocyte *zona pellucida* (ZP) and control of polispermy.

The objectives of the present study were to investigate the effect of four oviductal glycosidases on porcine IVF outcome**.** In order to imitate the oviductal conditions, similar enzyme concentration as described in late follicular phase, time when the oocyte reaches the oviduct, were used.

In vitro matured porcine oocytes were incubated for 30 min in TALP medium with α-L-fuc (2.84 EAU), β-N-gluc (36.29 EAU), β-D-gal (23.2 EAU) and α-man (34.91EAU) or without enzymes (control group) and were inseminated with $2.5x10^4$ sperm/ml. Gametes were cocultured for 18 h. Putative zygotes were fixed and stained to evaluate mean number of sperm bound to ZP (SPZ-ZP), penetration rate (PEN), mean number of sperm per penetrated oocyte (S/O) and monospermy rate (MON). The data were analysed by ANOVA ($p<0.05$) and showed as mean±SDM.

Results only showed significant differences for S/O data, being $3.77\pm0.29^{\mathbf{a}}$**,** $2.99\pm0.17^{\mathbf{a,b,c}}$**,** $2.77\pm0.2^{\mathbf{b,c}}$**,** $3.62\pm0.25^{\mathbf{a,b}}$and $2.48\pm0.18^{\mathbf{c}}$ for control, α-L-fuc, β-N-gluc, β-D-gal and α-man groups, respectively; data ranged from 72±4.03 to 86.67±3.12 for PEN; from 8.75±0.77 to 11.11±0.91 for SPZ-ZP and from 23.71±4.34 to 38.89±5.17 for MON, without any statistical significance.

Different roles for the glycosidase activities have been described. However, the present results show that IVF parameters are not affected, in general terms, by the addition, at physiological concentrations, of any of the tested enzymes. Further experiments should be performed to clarify the effect of these enzymes in sperm-oviductal epithelium interaction, ZP remodelation and early embryo development.

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Notes

ASSESSMENT OF OVIDUCTAL FLUID EFFECT ON THE ZONA PELLUCIDA, IN VITRO FERTILIZATION AND EMBRYO DEVELOPMENT IN PREPUBERTAL GOAT OOCYTE

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In mammals, fertilization and early embryo development occurs in the oviduct. Previous studies with porcine oocytes had shown that oviductal fluid (OF) has some compounds that interact with sperm and the oocyte influencing fertilization and improving embryo development. Its effect may be due to the hardening of the Zona pellucid (ZP), avoiding polyspermy.

The aim of this study was to use the oviductal fluid to improve blastocyst production derived from prepubertal goat oocyte. Cumulus oocyte complexes (COC) were exposed to prepubertal and adult goat oviductal fluid (gOF), and bovine oviductal fluid (bOF) for 30 minutes after in vitro maturation and prior to fertilization (*Reproduction* 2008 ***135*** *19-27*). Cleavage and blastocyst rates were recorded and analysed. Hardening of the ZP was analysed through its dissolution in a pronase solution.

Table 1. Embryo development of treated oocytes with oviductal fluid.

Group	N	Cleavage (%)	Blast/N (%)	Blast/cleav (%)
Control	331	240 (73.8)^{a}	91 (28.5)^{a}	91 (38.2)^{ab}
Adult gOF treat	272	166 (61.9)^{b}	71 (27.1)^{ab}	71 (43.7)^{a}
Prep gOF treat	175	101 (58.9)^{b}	33 (19.6)^{bc}	33 (32.5)^{ab}
bOF treat	276	146 (54.0)^{b}	45 (17.1)^{c}	45 (30.1)^{b}

a,b values in the same column represent statistically significant differences (Fisher test, P<0.05). N= total number of COCs treated; Blast: blastocyst; treat: treatment.

Table 2. Pronase disolution of the ZP.

	DISOLUTION TIME ± SD (seconds)			
N / GROUP	CONTROL	ADULT gOF	PREP gOF	bOF
40	105.7 ± 20.5^{a}	109.0 ± 46.2^{a}	84.53 ± 23.9^{a}	213.3 ± 86.2^{b}

a,b values in the same row represent statistically significant differences (Anova test, P<0.05). SD: standard deviation.

In conclusion, the treatment of prepubertal goat oocyte with OF before IVF did not improve embryo development, and only bOF treatment produce ZP hardening.

Notes

TRICHOSTATIN A USED AS AN EPIGENOMIC MODIFIER OF NUCLEAR RECIPIENT OOCYTES IN THE SOMATIC CELL CLONING OF PIGS

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The objective of our study was to examine the effect of trichostatin A (TSA; non-specific inhibitor of histone deacetylases) that was applied to epigenetic transformation of *in vitro*-maturing oocytes, subsequently enucleated and electrofused with adult dermal fibroblast cells, on the preimplantation development of porcine cloned embryos. Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 h in Tissue Culture Medium 199 (TCM 199) supplemented with 1 mM L^{-1} dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU mL^{-1} equine chorionic gonadotropin (eCG), 10 IU mL^{-1} human chorionic gonadotropin (hCG), 10% porcine follicular fluid (pFF), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF) and 0.6 mM L^{-1} *L*-cysteine. Afterwards, the COCs were incubated for 22 to 24 h in the db-cAMP- and eCG+hCG-depleted medium that was enriched with 80 nM L^{-1} TSA. The enucleated oocytes receiving fibroblast cell nuclei were subjected to delayed activation by two successive DC pulses of 1.2 kV cm^{-1} for 60 μs, and then cultured for 6 to 7 days up to morula and blastocyst stages. The TSA-mediated epigenetic modification resulted in acquiring the *in vitro* meiotic maturity by 195/202 oocytes (96.5%) as compared to 169/194 oocytes (87.1%) in a control, i.e., TSA-untreated, group. The percentages of dividing embryos (164/186; 88.2%), morulae (114/186; 61.3%) and blastocysts (63/186; 33.9%) descended from the nuclear-transferred oocytes that had been pre-modulated epigenomically by TSA were significantly higher than in the TSA-untreated group (102/158; 64.6%, 55/158; 34.8% and 27/158; 17.1%, respectively). In conclusion, improved capability of cloned pig embryos to complete the *in vitro* development to the morula/blastocyst stages seems to result from the increased reprogrammability of adult cutaneous fibroblast cell nuclei in an epigenomically-matured cytoplasm of recipient oocytes exposed to TSA.

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Notes

SEX DETERMINATION OF EQUINE EMBRYOS: TRANSLATING RESEARCH INTO A COMMERCIAL BREEDING PROGRAM

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In horses, pre-implantation genetic diagnosis (PGD) could be a very valuable technology for the equine breeding industry, as it would allow elimination of devastating genetic diseases and selection of genetic traits in the offspring produced. Unfortunately, so far, very few biopsied equine embryos have resulted in pregnancies and the genetic results were only determined after the embryos had been transferred into the recipients, which does not represent a real PGD program. In this study we evaluated the adequate timings to perform biopsy and sex determination in equine embryos before embryo transfer to apply this technology into a commercial breeding program.

To obtain embryos, donor mares were monitored using transrectal ultrasonography. When a follicle of > 35 mm in diameter was observed, 2,500 IU hCG were administrated, and mares were inseminated daily until ovulation was detected. Embryos were recovered nonsurgically on days 6.5 – 7 (day 0 = ovulation). A total of 27 embryos ranging from 150 to 560 μm were biopsied. Trophectoderm biopsies were performed in Syngro Holding Medium (Bioniche) using a blunt end pipette connected to a piezo-drill unit (PiezoXpert, Eppendorf). Trophectoderm biopsed samples were immediately processed for whole genome amplification and then sex determination was performed by PCR amplification of SRY, AMELX, AMELY or ZFY sex-horse specific genes.

Nineteen embryos were biopsied and transferred non-surgically into uteri of synchronized recipients at different time-points. Day 16 pregnancy rate for embryos < 300 μm transferred within 1 h post-biopsy was 100% (n = 2, 251 and 282 μm), which is not significantly different from that obtained for embryos transferred by 6 h (60%, 6 of 10, 151 - 239 μm) or 14h (20%, 1 of 5, 154 - 216 μm) post-biopsy. None of the embryos > 300 μm (n = 2, 347 and 560 μm) that were transferred by 6h post-biopsy resulted in pregnancies. Additionally, 8 embryos (168 - 337 μm) were vitrified by standard procedures after biopsy and later warmed and transferred directly. Day 16 pregnancy rate for embryos < 300 μm was 16.7% (1 of 6, 168 to 238), which is not significantly different from embryos of the same size transferred fresh. None of the embryos > 300 μm (n = 2) developed pregnancies after vitrification. Sex determination occurred for 88% of the biopsied samples processed in less than 6h. Results were later confirmed with an accuracy of 100% on ongoing pregnancies by fetal ultrasonography.

We concluded that biopsy, sex determination and direct transfer can be performed in real time on equine embryos < 300 μm without compromising pregnancy rates. Continued efforts in improving biopsy and vitrification of equine embryos > 300 μm is demanded, so that the PGD results can be successfully applied also in this particular group.

Notes

DEVELOPMENT CAPACITY OF OOCYTES FROM PRE- AND POST-PUBERTAL PIGS

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Introduction: In most *in vitro* work with oocytes from pigs, the source is often gilts rather than sows. However, gilts are slaughtered before puberty so gilt oocytes will not have completed their full follicular development, which may affect their usefulness. The aim of the study was to investigate the developmental capacity of gilt vs sow oocytes (pre- and post-pubertal, respectively) using inside-zona pellucida (ZP) diameter and morphological evaluation after *in vitro* maturation (IVM) as selection criteria. Materials and methods: Cumulus-oocyte-complexes were aspirated from gilt and sow ovaries and the oocytes were *in vitro* matured in groups under standard conditions (Zygote 2012 20:61). After IVM the gilt oocyte's inside-ZP diameter was measured ("Small": ≤110 µm; "Large": ≥120 µm), and both gilt and sow oocytes were morphologically evaluated as "Good" or "Bad". "Good" oocytes were defined by having even cytoplasm, smooth cell membrane and a visible perivitelline space. "Good" gilt and sow oocytes were checked for polar body (PB%) after IVM (15-25 s in pronase (10 mg/ml) for partial ZP digestion and visual inspection)."Good" and "Bad" gilt and sow oocytes were used for parthenogenetic activation (PA), using first electric and then chemical activation (Cryobiology 2012 64:60) before individual *in vitro* culture in WOWs for 6 days. Rates of cleavage (CL%) and blastocyst (BL%) were recorded on Day 2 and 6, respectively, and total cell number of all blastocysts was determined by staining with Hoechst 33342. PB%, CL% and BL% were analyzed by chi-square test, and total cell number in blastocysts by t-test. Results: Sow oocytes had higher PB% and cell number compared to "Large" gilt oocytes, and they also tended to have higher BL% (p=0.06). BL% in "Large" gilt oocytes showed larger variation between replicates (33-77%) compared to sow oocytes (60-79%), indicating reduced developmental capacity of some of the "Large" gilt oocytes. Maturational and developmental capacity of "Small" gilt oocytes was very low. Conclusion: Simple morphological evaluation combined with selection according to ZP-diameter is very useful to select the developmentally most competent gilt oocytes which have a developmental pattern comparable to that of sow oocytes.

Group (ZP-size)	**Polar Body**	**Cleavage**	**"Good" blastocysts**		**"Bad" blastocysts**
	PB (%) (n; N)	**Cl % (n; N)**	**Bl% (n; N)**	**No. cells (n; N)**	**Bl% (n; N)**
Gilt (Small)	0 (42; 2)	16.1 [a] (62; 4)	0 (62; 4)	Not determined	0 (7; 3)
Gilt (Large)	74.7 [a] (91; 2)	79.2 [b] (130; 4)	56.9 [a] (130; 4)	42 ± 2.2 [a] (44; 3)	0 (7; 4)
Sow	84.6 [b] (351; 5)	83.3 [b] (263; 7)	66.5 [a] (263; 7)	53 ± 1.7 [b] (116; 6)	0 (73; 7)

Values are the total observed frequencies from all replicates in the different groups.

Values are mean ± SEM

n: total no. of observations. **N:** no. of replicates. Values in the same column with different superscripts are significantly different: [a, b] ($p < 0.05$).

Notes

COMPARISON OF *IN VITRO* DEVELOPMENTAL CAPABILITIES OF PORCINE NUCLEAR-TRANSFERRED EMBRYOS DERIVED FROM SINGLE-COPY AND DOUBLE-COPY TRANSGENIC ADULT CUTANEOUS FIBROBLAST CELLS

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The present study was undertaken in order to determine the effect of two types of nuclear donor fibroblast cells, either mono-transgenic or bi-transgenic, on the preimplantation developmental outcome of porcine cloned embryos. The source of recipient cells for allogeneic genomic DNA in the somatic cell nuclear transfer (SCNT) procedure were the oocytes that had acquired the meiotic maturity state under *in vitro* culture conditions. The cumulus-oocyte complexes (COCs) were matured *in vitro* for 22 h in TC-199 medium supplemented with 10% foetal bovine serum (FBS), 10% porcine follicular fluid (pFF), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 0.6 mM *L*-cysteine, 0.1 IU mL^{-1} human menopausal gonadotropin (hMG) and 5 mIU mL^{-1} porcine follicle-stimulating hormone (pFSH). The COCs were subsequently cultured for a further 20 to 22 h in the maturation medium deprived of hMG and pFSH. The enucleated oocytes (ooplasts) were subzonally injected with cultured/trypsinised adult fibroblast cells that had been retrieved from ear skin explants of non-mosaic transgenic sows robustly and ubiquitously expressing either: 1. only one recombinant human immunoenzyme, designated as α-galactosidase A (α-GLA; Group I) or 2. two recombinant human immunoproteins, such as α-GLA and α-1,2-fucosylosyltransferase (H-transferase, α-1,2-FT/HT; Group II). Afterwards, reconstructed oocytes were artificially stimulated using the protocol of simultaneous fusion and electrical activation (SF-EA). The SF-EA was triggered by two successive DC pulses of 1.2 kV/cm for 60 µs. Post activation treatment, nuclear-transferred (NT) embryos were cultured in NCSU-23/BSA/FBS medium for 6-7 days up to morula and blastocyst stages. A total of 223 ooplasts were electrically fused with *α-GLA* single-transgenic fibroblast cells (Group I) and 234 were fused with *α-GLA* and *α-1,2-FT* double-transgenic fibroblast cells (Group II). In Groups I and II, 209/223 (93.7%) and 207/234 (88.5%) reconstituted clonal cybrids were selected for *in vitro* culture, respectively. Out of 209 and 207 cultured transgenic NT embryos, 166 (79.4%) and 145 (70.0%) were able to divide in Groups I and II, respectively. The percentages of cloned embryos that reached the morula and blastocyst stages were 118/209 (56.5%) and 61/209 (29.2%) or 85/207 (41.1%) and 36/207 (17.4%) in Groups I or II, respectively. Cumulatively, the competencies of *α-GLA* mono-transgenic adult dermal fibroblast cell nuclei to support the cleavage activity and *in vitro* development of porcine cloned embryos to morula and blastocyst stages were significantly higher than those for *α-GLA* and *α-1,2-FT* bi-transgenic adult dermal fibroblast cell nuclei.

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Notes

THE METHOD OF CHOICE FOR ICSI IN HORSES

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Due to the difficulties with conventional *in vitro* fertilization in horses, intracytoplasmic sperm injection (ICSI) is preferred for the *in vitro* production of equine embryos. Conventional ICSI, which involves mechanical penetration of the zona pellucida with a beveled injection pipette and breakage of the oolemma through aspiration, has been associated with variable results. Introduction of the less traumatic and more efficient piezo drill assisted ICSI coincided with improved cleavage rate, but this technique implies the use of mercury and has been associated with possible DNA damage. As an alternative method avoiding oocyte trauma, laser assisted ICSI is explored in this study. This technique, during which a hole is made in the zona pellucida prior to ICSI, is compared to piezo assisted ICSI.

Equine embryos were produced *in vitro* as described previously (Smits et al., 2012, Reproduction 143, 173-181). Oocytes were fertilized by piezo drill (Prime Tech, Ibaraki, Japan) or laser (XYClone, Hamilton Thorne, Beverly, MA, USA) assisted ICSI. During piezo drill assisted ICSI, a blunt injection pipette (piezo-6-25; Humagen, Charlottesville, VA, USA) was used. A progressively motile sperm was immobilized by piezo pulses. After penetration of the zona pellucida and the oolemma with a piezo intensity setting of 5 and 4, respectively, and a speed of 4 and 3, respectively, the sperm was injected into the ooplasm. For laser assisted ICSI, a progressively motile sperm was immobilized by crushing its tail. The laser device was used to create multiple adjacent small holes through the zona, using maximal power and a pulse time of 150 µs. The oolema was penetrated mechanically with a beveled injection pipette (MIC-50-50; Humagen, Charlottesville, VA, USA). Rates of cleavage (Day 2.5) and blastocyst formation (Day 9), were compared using a Pearson chi-square test. The mean injection times were compared using a *t*-test.

Of the 78 oocytes that were injected using the laser, 63 cleaved (81%), and four of these reached the blastocyst stage (6.3%). In the piezo group, 71 of the 104 oocytes cleaved, (68%); 31 of these cleaved piezo embryos were used in another experiment and five of the 40 remaining embryos, used in this study, developed to blastocysts (12.5%). Neither the cleavage rate nor the blastocyst rate differed significantly between the two groups ($p > 0.05$). The mean time for ICSI, including the handling of the oocytes before and after the procedure, was longer for laser assisted ICSI (4.0 min / oocyte) than for piezo drill assisted ICSI (2.9 min / oocyte) ($p > 0.05$). After laser assisted ICSI, a persistent hole in the zona pellucida was formed, which resulted in blastomere leakage at day 2.5 and hatching at the blastocyst stage.

The comparison of the two injection methods revealed no significant differences in resultant embryonic development. Piezo drill assisted ICSI appeared more sensitive to oocyte-positioning during injection, which might explain a slightly reduced cleavage rate. The creation of a hole in the zona during laser assisted ICSI was responsible for an increased oocyte manipulation time and the observation of blastomere leakage and prominent hatching. The mechanical breakage of the oolemma during laser assisted ICSI in conformity with conventional ICSI could be responsible for the tendency to lower blastocyst rates. In conclusion, both piezo drill and laser assisted ICSI appeared to have advantages and disadvantages and their value to equine ICSI requires further exploration.

Notes

IS THE DOUBLE BALLOON ENDOSCOPY USEFUL TO APPROACH THE PIG UTERUS AND OVIDUCT?

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The double balloon endoscopy (DBE) is a form of push-and-pull endoscopy which allows a complete exploration of the small intestine (Yamamoto et al. 2001). The equipment consists of an endoscope -200 cm length and 8.5 mm external diameter- and an overtube -145 cm length, 12.2 mm diameter- both of them with a latex balloon attached to the tip (Figure 1). The two balloons are inflated and deflated in an alternating sequence so as to allow the endoscope to progress (pushing phase) or fold the explored intestine behind the balloons (pulling phase). The anatomy of the sow's uterine horns is somehow similar to the small intestine (Nickel et al. 1979), and this could allow a complete hysteroscopic approach to the uterus and oviduct by DBE.

The aims of this work were: i) to evaluate if DBE could be used to explore the whole uterine lumen, and, if this objective is accomplished, ii) to cannulate the uterine foramen of the oviduct (UFO).

This experiment was carried out on 4 genital tracts from the slaughterhouse and 2 live sows. In the anaesthetized animals DBE was performed, and in order to standardize the exploration, registers of the time and number of push-and-pull maneuvers required to reach anatomical landmarks such as the internal foramen of the uterus or the UFO were annotated.

All the hysteroDBE were successfully performed in both postmortem genital tracts and in vivo (Figure 2). The average time to visualize the UFO in postmortem specimens and in vivo were 44.75 ± 9.98 and 72.5 ± 9.5 min, respectively. While the cannulation of the UFO was successfully accomplished in the postmortem models, in vivo it was impeded by the small diameter of the UFO and the forced position of the oviduct (acute angle) which is likely caused by the position of the inflated balloons inside the tip of the uterine horns.

This study demonstrates that pig hysteroDBE is feasible and could be a new minimally invasive method for obtaining uterine fluid samples or for blastocyst transfer. The approach of the oviduct from the uterus was very difficult and would probably require the development of new endoscopy devices for this purpose.

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Figure 1.

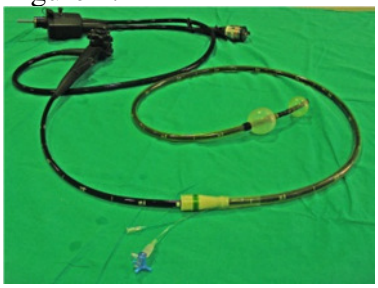
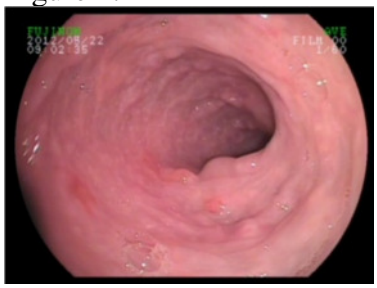


Figure 2.



Notes

PIG AND MOUSE MODELS RESPOND DIFFERENTLY TO THE INHIBITION OF THE PLASMINOGEN/PLASMIN SYSTEM DURING IN VITRO FERTILIZATION

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Plasminogen/plasmin (PLG/PLA) system has a role in fertilization recently described in detail for the porcine and bovine species (Coy et al., Hum Reprod 2012). Briefly, plasminogen, which is present in the oviductal fluid, binds to the oolemma and zona pellucida (ZP) of the oocytes. When the spermatozoon contacts the oolema, plasminogen activators are released and plasminogen is activated into plasmin. The last effect of this proteolytic enzyme is the decrease of sperm penetration into the oocyte. In the mouse model, previous studies indicated the opposite effect (Huarte et al., Dev Biol 1993), but they were not performed using the physiological concentrations of plasminogen that we quantified in the oviductal fluid. The objectives of the present study were i) to investigate the role of PLG/PLA system in mouse *in vitro* fertilization (IVF) by adding plasminogen at physiological concentrations and ii) to investigate the role of an inhibitor of the PLG/PLA system on the final output of IVF in the pig and mouse.

Porcine oocytes (N=346) were collected from ovaries by follicle aspiration (diameter, 2-6 mm). Murine oocytes (N=504) were obtained from oviducts after hormonal treatment. The oocytes were divided and treated 10 min before the IVF as follows: i) control group (C); ii) PLG group (150 µg/ml); iii) α-antiplasmin (α-AP) group (150 µg/ml); iv) α-AP+PLG group (150 µg/ml+150 µg/ml). The porcine and murine oocytes were fertilized using $25x10^3$ and $2x10^5$ spermatozoa/ml in TALP and M16 media, respectively.

The results show that addition of PLG in the IVF medium decreases the percentage of penetration in mouse as in pig (21.62±3.92 and 39.6±5.2, respectively, $P<0.05$) compared to the corresponding controls (87.02±2.94 and 65.1±5.2). However, α-AP had a different effect depending on the species: in mouse, the enzyme treated group did not show significant differences in the percentage of penetration compared to the control group (87.02±2.94), although α-AP decreased the effect of PLG when added in combination (59.54±4.30), indicating that the effect of PLG was partially inhibited by α-AP. In pig, α-AP reduced drastically the penetration (2.3±1.6), and again this strong effect was partially inhibited by the combination of α-AP with PLG (15.9±4.1).

In conclusion, PLG at physiological concentrations reduces oocyte penetration in the mouse as in the pig but the inhibitor α-AP has a different role depending on the species. Further studies are being performed to find out if this difference is related to the different gene expression pathway for PLG activators in the mouse and pig or to the different effect of α-AP on the sperm functionality in both species.

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Notes

RELATIONSHIP BETWEEN CIRCULATING AMH (ANTI-MULLERIAN HORMONE) AND EMBRYO PRODUCTION IN SUPEROVULATED HIGH PRODUCING DONOR COWS

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The main objective was to study the relationship between circulating AMH and superovulatory response of high producing donor cows at peak lactation. A secondary objective was to assess cow-related variables that influenced circulating AMH concentrations. Holstein cows (n=72), were milked twice daily and housed and fed individually in tie-stalls. All cows were synchronized and superovulated using a modified 5d-Double Ovsynch protocol with 4 d of decreasing FSH treatments. All animals were flushed at 65 to 75DIM, near peak production (39.6 kg/d). Non-sexed frozen semen ($15x10^6$ sperm/straw) were produced from single ejaculates of two high-fertility sires and cows were inseminated twice (12h and 24h after final GnRH treatment). To minimize variation, a single batch of FSH was used and two experienced AI technicians performed all breedings and flushings, which occurred 6d after synchronized ovulations. In addition, a single technician was in charge of searching and grading all embryos. Blood samples for AMH analysis (AMH-Bovine Assay, Minitube) were collected at 3 different times after calving at 56, 63, 74±3 DIM and in different stages of the synchronized estrous cycle (at random stage, pro-estrus, or diestrus). Body weights were measured weekly until embryo collection. The statistical analyses were performed with proc CORR and proc GLIMMIX of SAS®. The three AMH samples from individual cows were well correlated ($r>0.70$; $P<0.01$) and not influenced by day of the estrous cycle at sampling (AMH1=133pg/mL; AMH2=133pg/mL; AMH3=155pg/mL; $P>0.10$), and the within cow average was used as a final AMH score. Average AMH levels were correlated with parity ($r=0.23$; $P=0.05$). Surprisingly, AMH also tended to be negatively correlated with percent body weight loss from calving to embryo collection ($r=-0.22$; $P=0.06$). More importantly, averaged AMH was highly associated ($r=0.67$; $P<0.01$) with superovulation response (number of CL on the day of the flush), total structures collected ($r=0.50$; $P<0.01$), total transferable embryos ($r=0.28$; $P<0.02$), but not % fertilized embryos ($r=-0.16$; $P=0.18$), or degenerate embryos ($r=-0.10$; $P=0.43$). When cows were classified into 3 classes of AMH (<SD, Mean±SD, >SD), there was a remarkable difference ($P<0.01$) among AMH classes in superovulation response and total transferable embryos, respectively: <SD-AMH=10.6±1.8 and 2.2±0.8; Mean±SD-AMH=17.1±1.0 and 4.7±0.8; >SD-AMH=26.7±2.5 and 7.1±01.4. Circulating AMH postpartum was highly associated with superovulation response and embryo production and can be easily adopted in practice to improve efficiency of MOET. In addition, because of its association with body weight loss, it might also indicate level of negative energy balance and metabolic status in the postpartum of high producing cows.

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Notes

BOVINE IVP DERIVED EMBRYOS ARE AFFECTED BY C9T11- AND T10C12-CONJUGATED LINOLEIC ACIDS

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In dairy cows fertility problems are associated with a lack of energy especially during the post partal period. The oral supplementation with conjugated linolic acids (CLA) supposedly has a positive influence on fertility in high yielding dairy cows. Nevertheless, a direct influence on embryonic development has not yet been studied. The two CLA that are in the main focus are c9t11- and t10c12, the latter known to inhibit lipid synthesis in different animals. Therefore, the goal of the present study was to assess the effects of c9t11- and t10c12 CLA on early embryonic development in the *in vitro* model.

A total of n = 2089 oocytes were randomly allocated to seven groups (I: n=476, without supplementation; II: n= 205, 14 mmol L^{-1} DMSO (equivalent to 2.5µl), III: n= 252, 28 mmol L^{-1} DMSO (equivalent to 5µl); IV: n=300, 50 µmol L^{-1} t10c12 CLA in 2.5µl DMSO; V: n=291, 100 µmol L^{-1} t10c12 CLA in 5µl DMSO; VI: n=311, 50 µmol L^{-1} c9t11 CLA in 2.5µl DMSO; VII: n=263, 100 µmol L^{-1} c9t11 CLA in 5µl DMSO).

Cleavage and development rates were solely documented on day 8 with regards to the employed oil-free culture-system. Single expanded day 8 blastocysts were then snap frozen for later analysis through RT-qPCR.

Cleavage rates were not affected by supplementation with either linoleic acid in either concentration or the carrier substance. In contrast, the development rates were significantly higher in embryos of group II than in embryos of groups III through VII. In tendency, a higher number of embryos developed in group II than in group I.

No differences regarding the relative amount of gene transcripts could be detected among embryos derived from either group I, III, V, or VII.

The relative abundance of FASN, ACAA1, CPT2 and IGFBP2 transcripts was significantly up-regulated in embryos derived from group II compared to those from groups IV and VI. The mRNA expression of SCD1 and IGFBP4 was significantly higher for blastocysts cultured with 14 mM DMSO (group II) compared to those supplemented with 50 mMol c9t11-CLA (group VI).

The relative abundance of ACAA2 and IGF1R transcripts did not differ among blastocysts of all groups.

In conclusion, development and mRNA expression is affected by the supplementation of the culture media with conjugated linolic acids as well as DMSO. Changes in development rates and gene expression patterns that could be detected in embryos of group II were partially compensated by the addition of either c9t11-CLA (50µmol l^{-1}) or t10c12-CLA (50µmol l^{-1}).

Acknowledgements: *The authors would like to thank the DFG (PAK 286/1; Wr/54/1-1) for financial funding and Doris Müller for her assistance in the laboratory.*

Notes

ASSOCIATION BETWEEN TWO GLYCOSIDASES ACTIVITY (α-MANNOSIDASE AND β-N-ACETYLOGLUCOSAMINADES) AND IN VITRO FERTILIZING CAPACITY OF BOVINE OOCYTES

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The participation of NAGASE in sperm – oocyte interaction has been proposed in many species (mainly hamster, mouse, human), although it is still unclear at what stage. In mouse, oocyte cortical granule NAGASE is released at fertilization, where it inactivates the sperm 1,4-galactosyltransferase binding sites, accounting for the block in sperm binding to the zona pellucida. Furthermore, acrosomal NAGASE is released after initial sperm – oocyte binding and facilitates sperm penetration through the zona pellucida matrix. Bovine embryos release both NAGASE and α-MAN during their in vitro development and utilize α-MAN. Bovine oocytes recovered from small follicles develop to blastocysts in vitro at a significantly lower rate than those from larger follicles. The purpose of the present study was to evaluate the role of two glycosidases (α-mannosidase – α-MAN and β-N-acetyloglucosaminidase – NAGASE) during in vitro fertilization of bovine oocytes.

Approximately 2100 COCs were aspirated from abattoir ovaries and divided into three groups according to the follicular size evaluated by visual examination (Small: 2-5 mm, Large: 6-8 mm, Controls: 2-8 mm). Bovine embryos were produced using standard methods of IVM, IVF and IVC. Sperm of the same bull was used at all experimental replicates (n=10). Samples of the fertilization medium were stored at -20^{o} C until assayed spectrophotometrically for glycosidases activity; results were expressed in IU/l.

Cleavage rate for oocytes collected from large follicles was lower compared to controls (37.9% vs 52.0%, P <0.05). The activity of both glycosidases was higher (P<0.05) in IVF medium of oocytes derived from large follicles compared to small and controls (α-MAN: 47.5±2.4 vs 28.5±3.2 and 36.0±4.6 respectively, NAGASE: 106.1±22.8 vs 21.6±2.2 and 21.4±2.1 respectively). A negative relationship was detected between NAGASE activity in IVF medium and cleaved oocytes collected from large follicles and a positive one between α-MAN and cleaved oocytes collected from large follicles.

Our results showed an association between glycosidases activity in IVF medium collected after fertilization and the size of follicles. It seems that high amount of NAGASE released during IVF and NAGASE associated negatively to cleavage rate in the case of COC's collected from large follicles. These findings could be related to lower cleavage rate observed when COC's collected from large follicles. However, it is not clear if NAGASE is released from oocytes or cumulus cells or acrosome during sperm penetration through the zona pellucida.

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Notes

PANCREATIC β-CELL FUNCTION OF A NEWBORN BELGIAN BLUE CALF IS INFLUENCED BY ITS BIRTH WEIGHT AND PARITY OF THE DAM

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The association between birth size and activity of pancreatic β-cells was determined for 46 newborn double-muscled Belgian Blue calves out of 20 primiparous (PP) and 26 multiparous (MP) dams. All calves were born by elective Caesarean section and separated from the dam once they had received colostrum. After an overnight fast, the 3 day old calves were weighed and their withers height was measured to calculate the body mass index (BMI, body weight/height²). Blood samples were obtained immediately before and exactly 10 minutes after the intravenous infusion of a standardized glucose bolus (150 mg glucose/kg body weight). Calves from MP dams were significantly (P<0.001) larger than calves of PP dams, both in weight (56.6 ± 7.19 vs. 44.1 ± 6.15 kg) and in BMI (102.1 ± 9.38 vs. 87.1 ± 7.90). Male calves (n=20) of both PP and MP dams tended (P<0.1) to be heavier (53.5 ± 9.0 kg) than females (n=26, 49.0 ± 9.01). The ratio of basal insulin to glucose concentration (I_0/G_0), used as an indicator of insulin resistance, was similar in both parity groups and in both genders, with an overall mean of 1.1 ± 0.83.

Following the glucose administration, a similar increase in glucose was seen for female (1.8 ± 0.67 mmol/l) and male calves (1.8 ± 0.62 mmol/l). Calves of MP dams tended to have a slightly larger increase in glucose than calves of PP dams (respectively 2.0 ± 0.62 and 1.6 ± 0.64, P=0.09). Insulin levels increased significantly (P<0.001) following the glucose bolus in all calves, but achieved a higher level in MP calves in comparison to PP calves (P=0.001). In the PP group, insulin increased with only 9.7 ± 8.48 mU/L, compared to an increase of 24.9 ± 21.45 mU/L for the MP calves. The high insulin response in MP calves was unlikely to be due to insulin resistance, as I_0/G_0 was equal for all calves. An enhanced β-cell response, caused by a more efficient nutrient supply during their development *in utero*, may explain the higher insulin response in MP calves compared to the PP calves. In addition, as the BMI of the calf was significantly correlated with the insulin level after 10 minutes ($I_{10,}$ r=0.38, P<0.05), the larger birth weights of MP calves could partly explain their higher I_{10} levels.

Notes

ELEVATED CONCENTRATIONS OF SATURATED NEFA DURING BOVINE *IN VITRO* EMBRYO CULTURE COMPROMISE PRE-IMPLANTATION EMBRYO DEVELOPMENT

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Elevated non-esterified fatty acid (NEFA) concentrations represent a common feature of a distorted maternal metabolism, typically present in obese and type 2 diabetic patients, but also in high yielding dairy cows suffering a negative energy balance. There is growing evidence to assume that such elevated NEFA concentrations play a key role in the increased subfertility rates observed in women and cows suffering these lipolytic conditions. As such, recent research substantiated this, by showing that elevated concentrations of NEFA, with particular reference to the saturated, during oocyte maturation jeopardize further oocyte development and even impact on the resultant embryonic phenotype. Besides this information on elevated NEFA exposure at oocyte maturation level, very little is known about potential direct consequences of elevated NEFA exposure during embryo culture. In this study, we hypothesized that elevated concentrations of NEFA during bovine embryo culture negatively affect pre-implantation embryo development.

To investigate this, 780 bovine grade I cumulus oocyte complexes were matured in serum-free conditions (3 repeats), fertilized following standard procedures and cultivated for 6.5 days under control (72µM of total NEFA), HIGH SA (280µM stearic acid) and HIGH NEFA conditions (720µM, a combination of stearic, palmitic and oleic acid). Cleavage (48 h post insemination (pi)), and blastocyst rates (7.5 days pi) and the rates of blastocysts from cleaved zygotes were calculated. Developmental competence data were compared between the three treatments using a binary logistic regression model taking replicate, treatment and the interaction of both factors into account. Significant lower cleavage rates were observed for zygotes cultured under HIGH SA conditions (59.0%) compared to controls (71.3%) ($P<0.01$). Culturing zygotes in medium with HIGH SA or HIGH COMBI resulted in a significant reduction in the number of oocytes reaching the blastocyst stage at day 7.5 pi (1.5% and 11.4%, respectively) compared to control zygotes (21.8%) ($P<0.001$). Interestingly, a significant better development was observed for HIGH COMBI zygotes to develop into day 7.5 blastocysts when compared to HIGH SA zygotes ($P<0.001$). The capacity of cleaved zygotes to become blastocysts was drastically lower in the HIGH SA and HIGH COMBI group (2.2% and 18.3%, respectively) compared to the controls (30.4%) ($P<0.01$). Fascinatingly, a significant better development was observed for cleaved zygotes from the HIGH COMBI group to develop into day 7.5 blastocysts when compared to the HIGH SA zygotes ($P<0.01$).

In conclusion, our data show that direct embryonic exposure to elevated NEFA concentrations reduces the chance to develop until blastocyst stage and that the extent, to which development is impaired, is highly dependent on the type of NEFA, in which the mono-unsaturated oleic acid might compensate for the effects induced by the saturated NEFA. Moreover these data provide further evidence to assume that elevated NEFA concentrations might be responsible for the high rates of embryonic loss observed in women suffering lipolytic disorders.

Notes

IN VIVO PRODUCED EMBRYO-LEVELS AFFECTING ESTABLISHMENT AND MAINTENANCE OF PREGNANCY IN LACTATING HOLSTEIN RECIPIENTS

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The objectives of this retrospective study were to analyze some *in vivo* produced embryo-characteristics affecting the establishment and maintenance of pregnancy in lactating Holstein recipients. More specifically, variables such as the effect of embryo donor category (heifer, lactating, and non-lactating cows) and season in which embryos were produced (summer, autumn, winter, and spring) were taken into account when analyzing conception rate (CR) and late embryonic loss (EL) rates of the lactating Holstein recipients. The study was performed in a commercial dairy located in southwestern Brazil (22◦01'27''S/47◦53'19''W). The dataset available included 3,744 fresh embryos, produced (*in vivo)* and transferred from 2007 to 2010. Donors were superstimulated and inseminated at fixed time. All recipients were lactating dairy cows and received a single fresh embryo by non-surgical embryo transfer (ET) from 6 to 8 d after estrus detection. Pregnancy diagnosis was performed 23 and 53 d after ET by ultrasound. Statistical analysis was performed using the PROC GLIMMIX of SAS. Donor category tended to affect CR at 23 days after ET (P = 0.09), but not EL between 30 and 60 days of pregnancy (P = 0.19). However, embryos from heifers (28.1%^{b}; 363/1,292) resulted a lower (P = 0.04) CR at 53 days after ET than embryos from Lactating cows (32.3%^{a}; 463/1,433). In addition, CR of embryos from Non-lactating cows were intermediate (30.7%^{ab}; 301/982) and not different from other categories (Heifers or Lactating cows). The season in which embryo was produced had an important significant influence in the CR 23 [summer = 34.4%^{c} (173/503); autumn = 39.6%^{ab} (364/920); winter = 41.4%^{a} (544/1,315); spring = 35.9%^{bc} (350/976); P = 0.02) and 53 [summer = 27.4%^{c} (136/497); autumn = 31.6%^{ab} (290/919); winter = 32.6%^{a} (429/1,315); spring = 27.9%^{bc} (272/976); P = 0.04) days after ET. No statistical difference was found on late embryonic losses across seasons (P = 0.59). In addition, there were no significant interactions between donor category and season of the embryo production on CR at 23 (P = 0.43) and 53 (0.25) days post ET or late EL (P = 0.25). In conclusion, embryos from lactating Holstein donors seem to have greater CR at 53 days after ET in lactating Holstein recipients as compared to Holstein Heifer donors. Additionally, embryos produced and transferred during winter time had highest conception rate in lactating Holstein recipients.

Acknowledgements: Agrindus S.A.

Notes

SERUM-FREE INDIVIDUAL CULTURE YIELDS HIGH QUALITY CATTLE BLASTOCYSTS

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Although cattle embryos are considered to be a valuable model for human embryo development, there are two prominent differences in the way human and bovine embryos are being produced in vitro: human embryos are cultured individually, without serum, whereas in cattle embryos, group culture is the norm, often in presence of serum and individual culture is associated with inferior embryo development. Therefore we wanted to evaluate if individual serum-free culture system for bovine embryos can support embryo development and quality.

Bovine oocytes (n = 1320; 3 replicates) were matured in tissue culture medium (TCM199) supplemented either with 20% fetal calf serum (FCS) or serum-free with 20 ng/ml epidermal growth factor (EGF). After fertilization, half of the zygotes of each group were cultured individually in 20µl of synthetic oviductal fluid (SOF) supplemented either with 5% FCS or with 0.4% bovine serum albumin, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS). These four groups of individually cultured embryos (FCS-FCS; EGF-FCS ; FCS-ITS ; EGF-ITS) were compared with the control, group culture in serum conditions (GR). Blastocyst development was evaluated at 8dpi. Subsequently blastocysts were fixed for a differential apoptotic staining[1]. Developmental data were analyzed using a binary logistic regression model and data concerning the blastocyst quality using a linear mixed model analysis.

At 8dpi, no differences were found concerning blastocyst development for the embryos cultured individually in serum-free conditions compared to the control group (GROUP: 23.1%; FBS-ITS: 25.3%; EGF-ITS: 30.9%), but embryos cultured individually in serum containing medium showed a much lower blastocyst rate (FBS-FBS: 9.1%; EGF-FBS: 6.4%) indicating that the presence of serum is hampering individual embryo development. The TCN of the blastocysts collected at day 8 was lower for the group EGF-FBS compared to the control (GROUP: 165.0 ± 7.55; EGF-FBS: 107.8 ± 6.48), but no differences were found for the other groups compared to the control (FBS-ITS: 157.5 ± 5.54; EGF-ITS: 164.0 ± 7.13 ; FBS-FBS: 143.2 ± 6.93). The ICM ratio was higher for all groups compared to the control, except for the group FBS-FBS where no differences were observed (GR: 31.5 ± 1.23 ; FBS-ITS: 36.8 ± 1.01 ; EGF-ITS: 38.4 ± 1.30 ; FBS-FBS: 36.3 ± 1.55 ; EGF-FBS: 41.6 ± 2.73). There were no differences in apoptotic cell ratio (ACR) compared to group culture in serum conditions for all individually cultured embryos.

We demonstrated that by the simple act of replacing serum by ITS and BSA , we could culture bovine embryos individually just like in human, and obtain comparable development as in group culture, with regard to blastocyst formation and quality. Single culture of bovine embryos under serum-free conditions can be used as a valuable tool in order to further optimize and investigate the human IVF system.

[1]Wydooghe et al., (2011) Analytical Biology 416(2): 228-230

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