A.E.T.E. ASSOCIATION EUROPEENNE DE TRANSFERT EMBRYONNAIRE

EUROPEAN EMBRYO TRANSFER ASSOCIATION

23ème COLLOQUE SCIENTIFIQUE

23rd SCIENTIFIC MEETING

*

* *

Dr Steph Dieleman

Special Celebration

* *

*

Alghero, Sardinia, 7th and 8th September 2007

A.E.T.E. ASSOCIATION EUROPEENNE DE TRANSFERT EMBRYONNAIRE

EUROPEAN EMBRYO TRANSFER ASSOCIATION

Board Members

BRITTAIN Robert (U.K.)

BECKERS FRANK (Germany)

BRÜCK BØGH Ingrid (Denmark)

GUTIERREZ-ADAN Alfonso (Spain)

LACAZE Serge (France)

LAZZARI Giovanna (Italy) ***(President)***

LONERGAN Pat (Ireland) ***(Secretary)***

MERTON Sybrand (The Netherlands)

PONSART Claire (France) ***(Treasurer)***

RATKY Joszef (Hungary)

Thanks to all our sponsors!

BIONICHE
ANIMAL HEALTH EUROPE LIMITED

imv
TECHNOLOGIES
ITALIA S.r.l.
Via Salotti, 1 - San Bonico - 29100 Piacenza
Tel. 0523-360006 - Fax 0523-380723
E-mail: imvcbs@tin.it

CryoBioSystem

Nikon

Schering-Plough

HAMILTON THORNE BIOSCIENCES

CEVA
SANTE ANIMALE

CEVA VETEM

MILLIPORE

intervet
RESEARCH • PERFORMANCE • INTEGRITY

ARBOlatteREA
solo da allevamenti sardi

Pfizer

BODINCO

SELLA&MOSCA
CASA FONDATA NEL 1899

La Genuina ®

C O N T E N T S

Dr Steph J. Dieleman

A.E.T.E. Medalist 2007

Steph J. Dieleman
A.E.T.E. Medalist 2007

The A.E.T.E. Medalist 2007, Dr. Steph J. Dieleman was born in 1944 and grew up in Twisk, a village located at the southwest of The Netherlands, called Zeeland. This province formed for him the solid basis for an outstanding and fruitful scientific career. After finishing Gymnasium-β at the Dutch Lyceum in The Hague in 1962, he started his academic career at the University of Utrecht as a student by studying Chemistry with specialization in Organic Chemistry and Biochemistry. During his study he proved to be a promising scientist by receiving a prestigious price for a project performed in the company Shell/AKZO. In June 1969 he passed his final doctoral exams and started his career at the TNO chemical Institute, Utrecht. For a period of two years he worked on a project which studied biochemical aspects of the resistance against specific fungi infections in apples. In November 1971 he left the research on fruits and accepted a new employment and challenge at the Utrecht University at the Faculty of Veterinary Medicine, Department Obstetrics, Gynaecology and AI, together with Prof. Bert van der Weijden and Prof. Marcel Taverne. Their common task was to perform research on the hormonal processes around parturition. In this respect, Steph's first challenge was to develop the RIA technology for hormonal (steroid) analysis and to set up an endocrine laboratory for reproductive studies. In 1973, he became the head of this endocrine laboratory. During those days he went to Edinburgh, Scotland for a sabbatical to learn more about RIAs, and indeed he developed the first oestradiol RIA in Utrecht based on an antiserum which he received as a present during this visit. In 1975, Dr. Mart Bevers, mainly responsible for the development of the protein hormone assays, was employed and an intensive collaborative and productive research period started on the endocrinology of the bovine oestrous cycle and superovulation/embryo transfer in cattle. In 1984 the (biochemical and RIA) research of Steph resulted in his PhD Thesis entitled "*Steroids of preovulatory bovine follicles relative to the peak of lutenizing hormone"* which was succesfully defended at the Utrecht University. The results of this study showed that concurrently with changes in the micromorphology of the follicular wall, the steroidal micro-environment of the maturing bovine oocyte switches from predominantly oestrogenic at oestrus to one in which progesterone is the major steroid present shortly before ovulation. Through this milestone Steph created a basis for the introduction and further development of embryo technologies and hence a better understanding of the endocrinology during the normal oestrus cycle and the follicular development during superovulation in cattle. In this respect, the testing of the first monoclonal antibody in animals called 'anti-PMSG' was performed for the company Intervet, The Netherlands, is a good example.

Especially, the interest for follicular development in relation to (final) oocyte maturation have to be mentioned. Many (EU) projects and experiments have been performed, mainly in cattle, to get a better understanding on the communication between the oocyte and its microenvironment. Many laboratory tools have been developed and validated to support and prove the proposed experimental hypothesis: from hormonal analysis, staining techniques, the *in vitro* production of blastocysts to omic's. In this respect, Steph performed

research in the context of reproductive physiology hence he stimulated in vivo research being the bridge between fundamental, mainly in vitro studies and the final application in the field. To reach this goal, the multidisciplinary approach was an absolute prerequisite for him. He always strongly believed in his own originally scientific ideas and hypotheses although these ideas were always open for discussion and critical comments.

In the field of reproductive physiology, - technology and embryo production the national and international research achievements of Steph have been substantial: a very productive worldwide acknowledged multi-faced research: cows, pigs, horses, elephants, dogs, dolphins and ostriches. Through his work in collaboration with many research colleagues and international groups, he has contributed to more than 300 books, abstracts and full papers in regular and top scientific journals (see table). The supervision and professional guidance of many (post-doc's, PhD's, animal sciences and veterinary) research student's was an important achievement of Steph. Especially, the critical comments and notes on the English writing style of the manuscripts, papers and thesis have to be mentioned. Steph liked (t)his work, as a perfectionist and hard worker, and was therefore very stimulatory for all these students and colleagues that have spent time at his laboratory. They have learned the lab as a pleasant and stimulating place to be, filled with excellent research, lots of fun and good team work in a warm atmosphere.

During his scientific career, Steph received much recognition for his contributions and achievements. He has been awarded by academic honours and he has been invited as a main speaker and a chairman at many international scientific conferences and meetings. But also he exposed his financial expertise (in the role as 'the master of finance') in the board of the International Embryo Transfer Society (IETS). He has been member of the Board of Governors, Secretary Treasurer and President of this society. Moreover, Steph was Program Chair of the IETS meeting in Maastricht (2000), which was also organized by him. Further, he was President of the International Conference Animal Reproduction (ICAR). He organized many meetings, symposia and conferences, as there are the ICAR conference in The Hague (1992), the International Conference Pig Reproduction (ICPR) in Rolduc (2004), the AETE (European Embryo Transfer Society) meeting in Rolduc (2002) and finally, he organized the International Conference Farm Animal Reproduction (ICFAR), during springtime of this year in Rolduc, The Netherlands (June 2007), which was his final international recognition to his international friends.

For more than 30 years Steph Dieleman performed interdisciplinary, clinically orientated research and education in the field of reproduction. He was a true and driven scientist who exposed a serious attitude but also very much enjoyed the Burgundian life style. It is more than appropriate that the Board of Governors of the AETE has recognized the many international contributions and research efforts of Steph Dieleman and dedicate him with the Pioneer Award 2007.

Thanks for the numerous valuable contributions, friendship and the many stimulating discussions about science and life over the past decades! Steph will enjoy now the trips on his 'sloep' looking back to a successful and wonderful scientific career.

Peter VOS

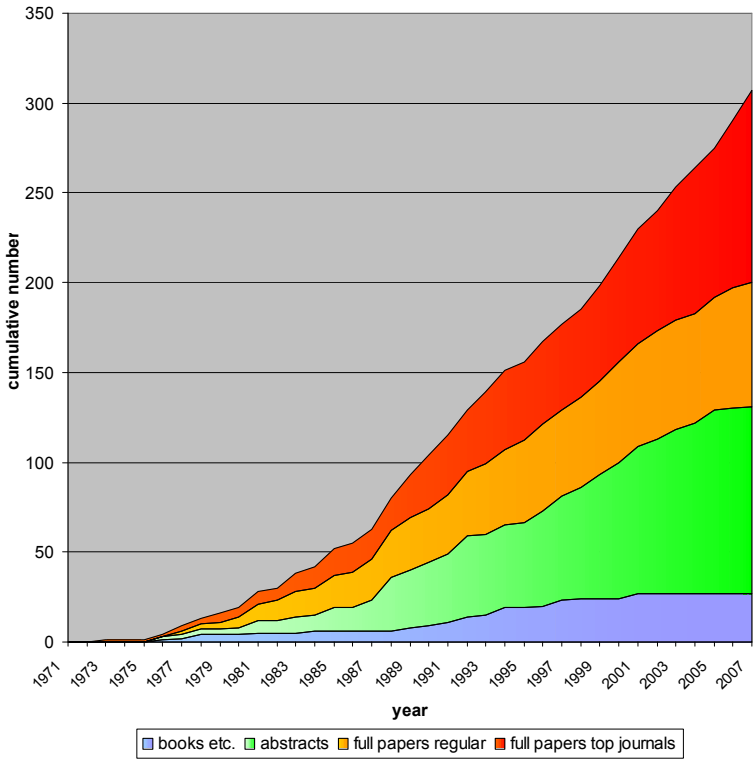

Figure 1. Publications by Dr S.J. Diememan.

SUPEROVULATION IN CATTLE: FROM UNDERSTANDING THE BIOLOGICAL MECHANISMS TO GENOMICS OF THE OOCYTE

DIELEMAN, S.J.

Department of Farm Animal Health, Faculty of Veterinary Medicine
Utrecht University, The Netherlands
E-mail: s.j.dieleman@vet.uu.nl

Abstract

Superovulation still constitutes the most widely used technique producing embryos in the cow for breeding but also to obtain large numbers of oocytes and embryos to investigate biological mechanisms in relation to competence of oocytes and embryos ultimately to develop into viable offspring. Of the variety of stimulation protocols that are applied for many decades those with a controlled release of the LH surge appear to be useful for research and practice.

The eCG/anti-eCG and a FSH protocol with norgestomet/GnRH-controlled LH surge are compared regarding effects on follicles, oocytes and embryos. In general, stimulation with gonadotropins also results in part of the oocytes and embryos being not competent or viable. Therefore, it is prerequisite to distinguish competent oocytes from non-competent. The steroid profile in the follicular fluid appears to provide reliable criteria assuming that functional preovulatory-sized primarily enclose competent oocytes.

Transcriptomics of the maturing oocyte have been studied following stimulation with gonadotropin using SSH and analysis by QPCR. The genes that were up-regulated at the start of resumption of meiosis related to different phosphodiesterases (PDE7), G-proteins, and regulators of G-protein signaling in meiotic resumption, molecular components involved in chromatids separation, regulation of Ca^{2+} oscillatory activity and cell cycle regulation. Most of these genes were identified for the first time in the mammalian oocyte. In addition, mRNAs were identified involved in correct translocation of organelles and segregation of chromosomes possibly explaining disturbed polar body emission and defects in cytoplasmic maturation as commonly observed in deviant oocytes from stimulated animals and in in-vitro matured oocytes. Finally, new information has been obtained regarding long chain fatty acid transport into the oocytes and regulation of energy requirement during maturation and blastocyst stage using lipid as a substrate, which may be used to adapt in-vitro culture of oocytes in many applications.

In conclusion, superovulation with a controlled LH surge is exceptionally useful to study regulation of oocyte competence in the cow but also as model for human IVF.

1. Introduction

Embryo transfer (ET) in cattle is used worldwide in cattle breeding for improvement of genetic quality and has recently been proposed anew to overcome fertility problems as for example caused by heat stress [1]. Although from the late 1980-ties on in-vitro produced embryos have been used successfully for ET superovulation still constitutes the major technique to obtain embryos [2,3]. Understanding the biology of treatment with

gonadotropins will not only improve application of superovulation in ET practice but also is essential to further knowledge on the basic mechanisms determining oocyte competence and embryo viability.

In follicles 2 to 3 mm in diameter the oocyte has acquired and stored most proteins and mRNA needed for further development and production of such compounds is largely terminated at this stage. Comparing the mRNA populations in oocytes collected from follicles < 2 mm to those from follicles > 5 mm [4] certainly contributes to identify genes involved in competence in broader sense. Following further development until (pre-) ovulation adds relatively few of these compounds. But they may be essential in determining the outcome of producing a competent oocyte to be fertilized and going through the first cell cycles until the transition from the maternal to the embryonic genome (MET) occurring between the 8- to 16-cell stage of early embryonic development. Global gene expression analysis during in-vitro maturation [5] will elucidate genes that are involved but may not pinpoint those that play a key-role in vivo. It appears, therefore, to be crucial to compare the transcriptome of in-vivo oocytes at onset of resumption of meiosis with that during final maturation using techniques such as suppression subtractive hybridization (SSH) which allows identifying up- or down-regulated low copy number mRNA transcripts.

It is evident that the single preovulatory oocyte and embryo of the untreated, normally cyclic cow remain the "golden" standard for any study on oocyte competence and embryo viability. However, to obtain for instance sufficient RNA simple calculation shows that approx. 1,000 cows/cycles are needed collecting round-the clock, while when using superovulation with a controlled LH surge some 50 cows will provide the required tissues and that during regular working hours. It is common knowledge that a substantial proportion of oocytes and embryos is not competent or viable following superovulation treatment, and although the sperm cell may contribute minute amounts of mRNA (See for review [6]) the origin of the oocyte determines the success of early embryonic development [7]. Therefore, discriminating follicles containing competent oocytes is a prerequisite to apply superovulation for research into topics such as nucleolar formation [8,9], metabolomics [10], mRNA expression [11], DNA methylation [12], proteomics [13], selective degradation of transcripts [14], and functional genomics [15] in relation to competence and embryo viability.

The aim of the present paper is to review briefly some superovulation protocols using eCG and FSH in relation to the effects on oocyte and embryo quality, and application of superovulation as a tool to study transcriptomics of competent oocytes.

2. Superovulation protocols

For many decades a variety of stimulation protocols with compounds containing FSH-activity have been used to collect large quantities of oocytes or embryos from the cow [16-18]. In general, the gonadotropin is administered at the onset of a follicular wave when FSH-activity is needed to start follicular development. This activity has to be present for several days to support further growth of the follicles, which is accomplished by gonadotropins with a long biological half-life such as eCG [19] or repeated administration of FSH preparations from ovine or porcine pituitary origin. Continued FSH-activity provides for signaling events at 2 different levels controlling the changes that must take place for follicular growth and attainment of oocyte developmental competence. The first signaling event comes from the

proper differentiation of the follicle as it normally occurs in the dominant follicle in preparation for ovulation. The second signaling event occurs as the process of follicle differentiation signals directly to the oocyte, possibly through the cumulus cells (See for review [20]).

The long-lasting eCG appears to have adverse effects on the competence of part of the oocytes possibly due to high estradiol concentrations in some of the preovulatory follicles affecting spindle formation [21] which can be suppressed by administering anti-eCG as originally developed by Bouters et al. [22]. It is evident that timing of the anti-eCG treatment in relation to the follicular development and the preovulatory LH surge is crucial [23]. When anti-eCG neutralized the FSH-activity before or at the onset of the LH surge the capacities to produce estradiol of the stimulated follicles was dramatically reduced, and release of the LH surge and thereby multiple ovulation did not occur in a majority of the animals. This makes the eCG/anti-eCG protocol practical primarily under laboratory conditions when e.g. the occurrence of the LH surge can be monitored using rapid radio immuno assay [24] facilitating anti-eCG administration shortly after the LH surge. Although almost 2-fold higher ovulation rates and yields of viable embryos were reported [24,25], in practice using fixed time protocols produced variable embryo yields (See for review [26]) leading to anti-eCG administration around ovulation which does not improve embryo yields but prevented formation of ovarian follicular cysts [27].

Following stimulation by gonadotropins, not all oocytes will show the same developmental competence due to deviations in preovulatory follicular development [28,29]. This heterogeneity in quality is probably due to intrinsic differences between oocytes originating from different follicular microenvironment as can be inferred from the considerable evidence for endocrine regulation changes after stimulation compared to normal cyclic cows. Firstly, a reduction occurs of endogenous basal secretion, pulse frequency and amplitude of FSH and of pulse frequency of LH by more than 50% [30,31], as well as a shortening of the period of preovulatory follicular development from 61 to 41 h in comparison to unstimulated cows [19]. Secondly, superovulation treatment has been shown to induce abnormal amounts of steroids in serum compared to the physiological levels seen during natural cycles [19,32-35]. Thirdly, different studies have also shown that follicular cells derived from stimulated cows have altered gonadotropin receptor mRNAs [36] and altered abundance of several transcripts for steroidogenic enzymes [37].

To explain and to improve the variability in oocyte competence to develop into viable embryos, the amount of required LH bioactivity in the follicle stimulating gonadotropin has been studied extensively. The eCG and FSH preparations with high LH bioactivity have been shown effectively to induce multiple follicle development, final oocyte maturation, ovulation and corpus luteum formation [38-40]. Currently, in clinical applications purified pituitary FSH is used either with added LH to a bioactivity ratio of 1:1 or with only a low remaining LH bioactivity, both products showing similar yields of viable embryos. Stimulation with FSH with low LH is now more common and has been proven to be an effective alternative to eCG protocols in terms of embryo quality [41-43]. However, in contrast to eCG, this type of FSH results in lower concentrations of estradiol in serum and follicular fluid, and of progesterone in serum [35,44]. When LH bioactivity is completely absent upon stimulation as with human recombinant FSH, development of preovulatory follicles still takes place but these follicles have a markedly reduced estradiol concentration and contain oocytes that lack

cytoplasmic maturation shortly before ovulation [45]. Therefore, balanced amounts of both FSH and LH are required for proper stimulation of follicles in the cow.

3. Discriminating competent follicles

The concept of developmental competence is not clearly defined since no particular mechanism is associated with it. But, it is believed that the acquisition of developmental competence is associated with different changes like, the synthesis and accumulation of specific RNAs and proteins, relocation of cytoplasmic organelles such as cortical granules, lipids and mitochondria. (See for review on intra-ovarian regulation [46]). The consequence of failing in any of these processes results in developmental failure. Assisted reproductive technologies routinely use controlled ovarian stimulation for oocyte recruitment and ovulation induction. Thus, an increased number of oocytes can be collected, though at the possible risk that not all gametes will show the same developmental competence [28,47]. This heterogeneity is probably due to intrinsic differences in the oocytes. It is well known that the hormonal milieu of the follicles is altered in cows stimulated with exogenous gonadotropin to a varying degree depending partially on the type of protocol and the hormonal treatment used [40,48-51]. We demonstrated that follicular concentrations of steroids are influenced by the size of the preovulatory follicle after oFSH stimulation [52]. However, as yet, the exact relationship between oocyte developmental competence and its respective intra-follicular environment is currently unknown. Nevertheless, in sheep, [53] and human [54], the physiological state of the follicles appears to affect subsequent oocyte maturation and competence in vivo. Asynchrony of follicle and oocyte maturation occurs after superovulation and may reduce oocyte developmental competence [28,47].

Although, we do not have proven reliable criteria to enable us to distinguish clearly between follicles with competent and non-competent oocytes, the steroid concentrations in the fluid of follicles can be used as indicator for competence. Oocytes collected from cows stimulated with recombinant human FSH have shown decreased concentrations of estradiol at onset of maturation, which were related to impaired distribution of cortical granules to the periphery at finishing of maturation. Normal distribution of these granules is considered a well known sign for developmental competence [45]. Steroid hormones are involved in a wide array of physiological responses, including regulation of glucose [55] and lipids, for instance, in aromatase-deficient (ArKO) mice, exogenous estradiol is necessary to maintain the gene expression and enzyme activity of the genes involved in hepatic lipid metabolism. Steroid hormones have been shown to regulate cell cycle progression [56,57], inhibition of apoptosis [58], and modulation of calcium release [59,60]. In the mammalian ovary, the follicle is the major site of synthesis and secretion of steroid hormones during preovulatory development and maturation of the oocyte. Regulation of steroid production by the ovarian follicular cells varies remarkably at different stages of development. During the preovulatory period, the selected dominant follicle is characterized by cyclical fluctuations in the levels of these hormones [61]. Before the preovulatory LH surge, granulosa cells synthesize and secrete estrogen, while after LH, granulosa cells luteinize and secrete more progesterone in concert with decreases in mRNA for 17alpha-hydoxylase and P450 aromatase [62]. The specificity of the steroid actions is due to the presence of intracellular receptor proteins. Despite the wealth of information about steroid receptors in different tissues and their importance in reproduction, only the receptor for estradiol ERβ mRNA has been identified in bovine oocyte [63]. Progesterone receptor mRNA in granulosa cells of the bovine preovulatory follicles is transiently induced within 5 to 7 h of the LH surge [64-66].

However, nothing is known about the expression of nuclear or membrane progesterone receptors in the oocyte of any mammalian species. In primates, androgen receptor (AR) mRNA activity is essential to early follicular development and oocyte quality [67], and in rats, complete disruption of AR activity is associated with intensive granulosa cell apoptosis in preovulatory follicles and poor quality cumulus oocyte complexes (COCs) [68]. Further, androgen receptors have been reported to translocate from the oocyte cytoplasm to GV, and then to the nucleolus suggesting a role as a ligand-activated, transcriptional factor [69]. In view of these observations, the identification and characterization of the patterns of mRNA changes, and functional analysis of the steroid hormone receptors that are expressed in the oocyte, if any, may provide a fundamental understanding of the critical roles of steroids during oocyte maturation in vivo. In clinical practice, there is a clear need to optimize the ovarian stimulation protocol, and proper design of superovulatory treatment should consider, LH concentration and half life in the FSH preparation, and steroid content and steroidogenic enzyme expression in the preovulatory follicles.

Therefore, it was assumed that functional preovulatory-sized follicles showing the changes in steroid concentrations as reported for untreated, normal cyclic cows primarily enclose competent oocytes.

4. Effects of superovulation on follicles and oocytes

Stimulation with gonadotropins not only affects the release patterns of endogenous hormones [30,31,70] but is also dependent of the developmental state of the follicles at onset of treatment. Since the cells of the follicular wall mediate the actions of the gonadotropin by an array of growth factors (For review see [71]) stimulating and inhibiting proliferation and differentiation of these cells, the effect of the gonadotropin on maturation of the oocyte varies markedly with size and state of atresia of the follicles (For review see [72]). Accordingly, the competence of oocytes to develop in vitro into blastocysts is related to the origin of the oocyte [73,74]. For example, oocytes collected from follicles in the presence of a growing dominant follicle (DF) show a reduced competence compared to oocytes recovered in the absence of a DF [75,76]. In practice, absence of the DF at onset of superovulation has been reported to increase the response [77-79] although removal of the DF by puncture at 38 to 46 h before stimulation did increase the number of viable embryos only in cows but not in heifers compared to animals that were not punctured [74]. Whether the DF exerts its effect on the remainder of the follicles > 2 mm by intra-ovarian or endocrine routes is not solved. In cows repeatedly treated with eCG/anti-eCG for 2 years the proportion of cows not showing a preovulatory LH surge was substantially reduced when the DF was removed (unpublished, PLAM Vos, B Aguilar, SJ Dieleman). Moreover, follicles < 4 mm may survive and participate in the next follicular wave [80].

Selection of animals showing a regular estrous cycle and timing of gonadotropin administration in relation to the follicular wave is prerequisite investigating biological mechanisms that determine the competence of the oocyte.

4.1. Superovulation with eCG/anti-eCG

In our early experiments, cows were administered 3,000 IU eCG (Folligon; Intervet International BV, Boxmeer, The Netherlands; heifers 2,500 IU) at Day 10 of a pre-

synchronized cycle and prostaglandin (PG) 48 h later. On average the endogenous LH surge occurred at 44 h (range 30 to 52 h) after PG, and anti-eCG (Neutra-PMSG; Intervet International BV) was administered i.v. at 6 h after the maximum of the LH surge in a dose sufficient to neutralize 3,000 IU eCG within 1 h [24]. In later experiments, norgestomet (Crestar; Intervet International) was implanted simultaneously with the administration of eCG [81] prolonging the period of stimulated follicular development by suppression of the LH surge. Subsequent administration of GnRH at 54 h after PG induced an LH surge at a controlled time which facilitates administration of anti-eCG and collection of oocytes and embryos at precisely defined stages of development.

The majority of the stimulated populations of follicles per cow showed a mixture of follicles with steroid concentrations in the fluid conform to or deviating from those reported for the preovulatory follicle of untreated cows during final maturation [61,82,83]. In cows treated with saline in stead of anti-eCG, significantly higher numbers of follicles were found with deviating, high estradiol concentrations in the fluid shortly before ovulation when the oocyte should have completed maturation. Neutralization of the eCG apparently did not affect the concentration of progesterone [83].

Oocytes from stimulated follicles at ovulation from cows with or without controlled LH surge only rarely lacked an expanded cumulus (3.5%). They also had a 2-fold higher competence to develop to the blastocyst stage after further in-vitro fertilization and co-culture compared to oocytes derived from 2 to 5 mm slaughterhouse follicles [81,84]. This difference may be due to the difference in origin of the oocytes: in vivo matured vs. immature. When in-vivo prematured oocytes were used for in-vitro maturation in stead of immature oocytes the blastocyst formation rate still remained significantly less than from in-vivo matured oocytes (26 vs. 41%, respectively; [85]). Although this finding strongly indicates that in-vivo maturation enhances competence, replacement in later experiments of the porcine FSH with rec hFSH in the maturation medium invalidated the hypothesis (unpublished, MM Bevers, SJ Dieleman).

Prolongation of the period of stimulated follicular development in the norgestomet/GnRH-controlled LH cows increased the ovulation rate but not the number of viable embryos [81]. Since a marked decrease or even absence of secretion activity was observed in the epithelium of the ampulla close to the junction with the isthmus, it was suggested that the milieu for early embryonic development was not optimum [81]. An explanation for this phenomenon could be the delayed switch from progesterone to estradiol dominance over the oviduct due to the norgestomet treatment. Products of the epithelium play a role in early embryonic development and their secretion is controlled by steroid hormone [86].

4.2. Superovulation using FSH with controlled LH surge

From 1999 onwards our research was continued using ovine FSH (Ovagen ICP, Auckland, New Zealand) as gonadotropin with a norgestomet/GnRH-controlled LH surge (Fig. 1). Only occasionally (13/185 animals) complete suppression of the release of the endogenous LH surge failed when the LH concentration in the peripheral blood started to increase 10 h before termination of suppression and administration of GnRH, and rarely (2/185) an LH surge occurred during suppression [87].

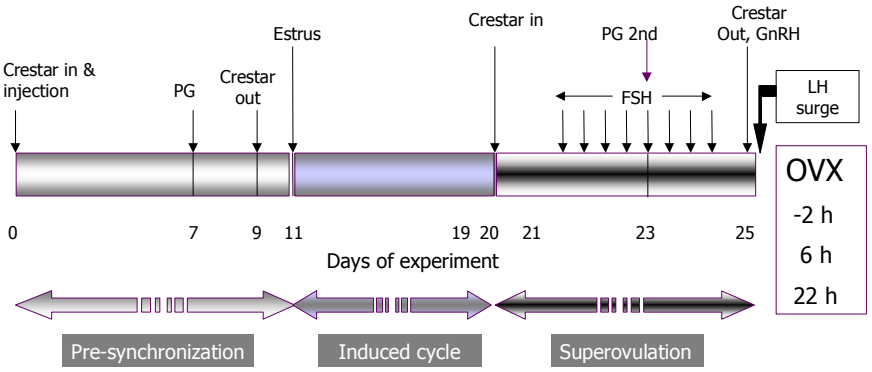

Figure 1. Schedule of treatment for pre-synchronization and superovulation with a Crestar/GnRH-controlled LH surge to obtain oocytes at specific times of development; PG=prostaglandin. The precise timing of the administration of PG during the FSH treatment was determined at intervals of 1 h to allow for periods of 1 h between each cow at ovariectomy, which is the time needed to collect all follicles. Similarly, removal of the 2nd earimplant (Crestar out) and administration of GnRH were carried out at 1 h intervals; a maximum of 4 cows was used every treatment run. (Adapted from [52]).

The number of stimulated follicles per cow varied substantially, e.g. 23.9 ± 12.1 SD (n=50 cows) upon ovum pick-up (OPU) shortly before or at 22 h after the induced LH surge [88]. A typical distribution per size category during final maturation is presented in Table 1 [89]. As with eCG/anti-eCG stimulation the oFSH protocol produces a mixed population of follicles with normal and deviating steroid profiles. Criteria to select follicles with presumably competent oocytes were derived comparing the follicular fluid concentrations in preovulatory-sized follicles with those of 5 to 8 mm follicles (Fig. 2; [52]) resulting in about 60% of the follicles > 10 mm with a normal steroid profile. However, in this normal category, the estradiol concentration before the LH surge was about half of the corresponding concentration in preovulatory follicles from non-treated normally cyclic cows. The lower estradiol concentration coincided with lower concentrations of its precursor androstenedione that is synthesized in the theca cell layer. It was suggested that this incident might be due to the low LH bioactivity of the oFSH preparation used. A major finding is the significant increase with size of the concentration of progesterone in particular shortly before ovulation when luteinization of the follicular wall should be completed. In Table 2 steroid concentrations are presented for normal and deviant follicles from which oocytes have been used to investigate the transcriptome [89].

Oocytes from non-selected stimulated follicles showed a marked competence for further early embryonic development in vitro (Fig. 3; [88]). Apparently, there was no difference between in-vitro and in-vivo routes for maturation with regards to the proportions of developed embryos which might be explained by the use of rec hFSH in the maturation medium as discussed above. However, significant improvement of traits was observed following in-vivo maturation such as numerical chromosome abnormality [88]. The degree of mixoploidy increases from Day 2 to 5 after insemination in embryos flushed from cows

stimulated with the oFSH protocol with controlled LH surge but levels out much earlier than in entirely in-vitro produced embryos [90].

High but variable numbers of embryos can be collected at specific stages of early development (11 per cow, n=99 cows; [87]) with high proportions characterized as viable (78% at the 8-cell stage and >50% at later stages after MET).

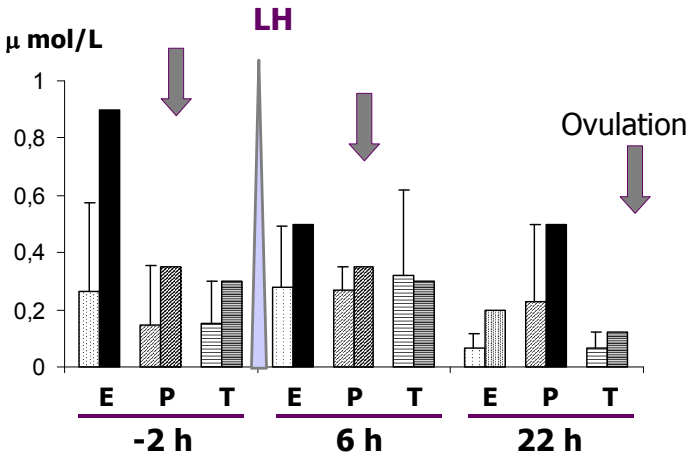

Figure 2. Threshold levels of steroid concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; E: estradiol, P: progesterone, T: testosterone. Of each pair of bars, the left bars represent the mean ± SD steroid concentration for follicles 5 to 8 mm, and the right bars are the threshold value for large preovulatory follicles with in black the predominant steroid value.

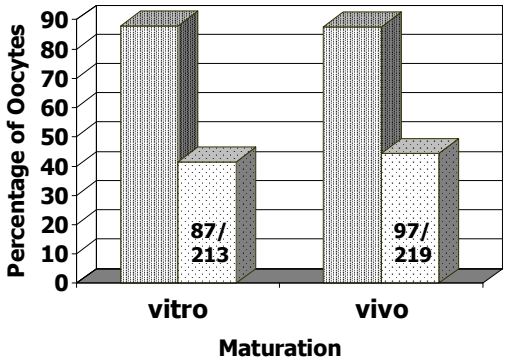

Figure 3. Competence of oocytes after in-vitro vs. in-vivo maturation. Oocytes were collected by OPU (n=4 experiments) from FSH-treated cows (n=36) with controlled LH surge at 2 h before and at 22 h after the induced LH surge, and matured in vitro or directly fertilized preceding further culture, respectively. Left bars: cleavage, right bars formation of morulae and blastocysts; numbers indicate number of embryos/oocytes collected. (Adapted from [88]).

4.3. Conclusions

Superovulation using norgestomet/GnRH to control the time of the LH surge facilitates collection of oocytes and embryos at defined stages of development although also non-competent and non-viable cells constitute part, requiring selection. In addition granulosa and cumulus cells can be recovered of interest investigating regulation and signaling between somatic cells and gamete or deriving non-invasive markers to establish competence and viability in IVF programs. Although regular numbers are obtained it is not yet clear whether the prolongation of stimulated follicular development would lead to higher yields of embryos in practice. Apparently the norgestomet/GnRH treatment does not affect in-vitro competence of oocytes but it remains to be resolved to what degree the oviductal milieu has become detrimental for early embryonic development.

The stimulation with controlled LH surge can be carried out with either eCG/anti-eCG or with FSH preparations and can be applied investigating biological processes in vivo such as apoptosis in embryos [91,92] and expression of genes [89,93]. At the molecular level differences in effect on reproductive cells can be foreseen between gonadotropin preparations due to mode and degree of glycosylation of the protein and to LH bioactivity.

5. Superovulation as a tool to study transcriptomics of competent oocytes

Follicle development was stimulated in Holstein-Friesian cows (n=40) using our standard protocol [93] with oFSH Ovagen ICP, Auckland, New Zealand) and a Crestar/GnRH-controlled LH surge (Intervet International B.V., Boxmeer, The Netherlands). Cows were allocated at random to three experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and completion (22 h after LH) of maturation to determine changes in mRNA expression related to resumption of meiosis in vivo (Fig. 1). Most of this study into the transcriptomics of the oocyte was done in the framework of the PhD-thesis of O.A. Algriany between 2003 and 2007 [89].

The growing mammalian oocyte, although morphologically simple, undergoes a series of discrete differentiation events. A relatively large number of genes are required to program its entire development. A small fraction of these genes are oocyte specific [94] while the large majority are expressed both in the oocyte and in somatic cells. Transcription and repression of genes is a dynamic process that can be expected to vary in the oocyte with the culture conditions. In order to assess which genes may be regulated by specific stimuli, it is necessary to have the capability of examining genes under a variety of exposure conditions. The triggers for change in gene expression in oocytes are critical for understanding the molecular mechanism of oocyte maturation. In fact, the information that is currently available on molecular mechanisms regulating oocyte maturation has been largely obtained from studies using in-vitro matured oocytes. Perhaps even more important, in the cow, most in-vitro studies have been carried out with oocytes collected from small or medium sized follicles (3 to 6 mm follicles), which lack the prematuration stage [73,88]. Prematuration begins at an average follicle diameter of 8.5 mm, that is the beginning of the difference in growth between the two largest follicles [95] and is associated with a differentiation of the concentration of estradiol [96].

Because in vivo oocyte maturation relies on a subtle balance between different follicular regulatory compounds, and also probably between different oocyte receptors, the molecular and biochemical alterations triggered by artificial ligands in vitro may not

necessarily reflect the normal in-vivo processes. The complexity of meiotic resumption regulatory mechanisms is also well demonstrated in bovine oocytes [97]. The bovine preovulatory follicle appears an attractive experimental model for study of the regulation of oocyte maturation and its ability to develop after fertilization. The preovulatory follicle contains sufficient follicular fluid for the analysis of steroid, proteins and various regulatory compounds. It contains also sufficient amounts of granulosa and cumulus cells, which offers an excellent opportunity to investigate functional interactions between various regulatory factors.

Differentially expressed genes between the oocytes exposed to LH and those collected before can help us understand the molecular basis of meiotic resumption in vivo. The identification and characterization of oocyte genes expressed exclusively or preferentially in the 6 h in-vivo matured oocyte will hopefully shed light on the mechanisms of the maturation process and provide useful information for the development of efficient maturation media. The suppression subtractive hybridization (SSH) method allows identifying overexpressed genes (designated forward +SSH) but also underexpressed genes (designated reverse -SSH) by exchanging the driver and tester populations during the procedure (Clontech, Palo Alto, CA, U.S.A.) [98-103]. SSH is a still widely used technique since it enables the recovery of abundant as well as low copy number mRNA transcripts. However, because it still needs a lot of initiating RNA which will burden using in-vivo matured oocytes, the use of the *S*witch *M*echanism *A*t the 5` end of the *R*everse *T*ranscript (SMART) amplification method overcomes this limitation.

The development of microarray technologies permits thousand of genes to be screened in a single experiment to establish differential gene expression in treated versus control cells and population. Consequently, the use of DNA microarray should significantly aid in minimizing the effort required to screen the many variables required to effectively examine gene expression patterns. Microarrays are developed to represent expressed mRNA transcripts (cDNA arrays), or distinguishable portion of an mRNA transcript (oligonucleotide arrays). The popularity in use of this technique is demonstrated by the exponential growth in publication using microarrays since its inception in 1995. Microarrays have been widely used to study issues in pathology, pharmacology, oncology, cell biology and recently, oocytes [94,104,105].

Different techniques have been used for gene discovery to design oocyte specific cDNA microarrays for possible use in assessing reproductive technologies performance. Two of these techniques employ a method for selectively segregating cDNA clones or fragments found in one cell or tissue population and absent in another. At present, the complete gene database for bovine is becoming available. Therefore, genomic information must be employed to construct a microarray to use in screening transcripts in the bovine oocyte. The ideal approach to gene expression profiling is to use full genome microarrays to identify genes up or down-regulated in response to certain treatment. However, because microarrays are not likely to become a routine test in the near future, reproductive biotechnology studies will probably require identifying a small subset of genes whose expression can be applied in the development of gene-based quality test. Therefore, we used SSH and microarray technique and oocytes matured in vivo to identify genes involved in regulating the maturation of bovine oocyte, suggesting that LH and maturation in vivo is instrumental in regulating several aspects of oocyte function.

5.1. Collection and processing of oocytes

Presumptive competent oocytes were selected on the basis of the steroid profile in the enclosing follicle. Pools of 10 to 14 denuded oocytes were assigned to replicates for Suppression Subtractive Hybridization (SSH) and validation by QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. For some studies QPCR analysis was also performed with "non-competent" in-vivo FSH-stimulated oocytes, in-vitro matured oocytes from slaughterhouse ovaries, and in-vitro [106] and in-vivo [107] produced expanded blastocysts. Total RNA was isolated using a microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit; Stratagen, San Diego, CA, U.S.A.). The SMART^{TM} PCR cDNA Synthesis Kit (Clontech) was used to maximize cDNA yields prior to the subtraction. The PCR-Select cDNA Subtraction Kit (Clontech) was used for SSH to isolate and enrich for gene sequences differentially-expressed between the two pools of oocytes: 1) collected before LH surge (-2 h, n=30) as driver and 2) exposed to LH (6 h, n=30) as tester. The subtracted material was then cloned as described by Algriany et al. [108]. Microarray preparation, hybridization and analysis as well as QPCR (Bio-Rad Laboratories, Hercules, CA, U.S.A.) were carried according to Sirard et al. [105,109]; for primers used see [108,110,111].

5.2. Differential expression at resumption of meiosis

Using SSH and defining a 1.3 fold difference as threshold, 115 regulated genes were identified from initially 945 DNA clones between the –2 h and 6 h after LH oocytes. This relatively small difference in the gene expression pattern due to LH surge may point to the fact that only a small subset of genes needed to regulate the meiotic resumption and developmental competence. Microarray analysis has uncovered novel mRNAs with potential roles in proper oocyte function, maturation and/or meiotic competence. We identified important changes in genes involved in cell cycle regulation, signal transduction, transcription and mRNA processing, cytoskeleton, cell adhesion, as well as in metabolism [108]. Real time QPCR analysis showed a significant 4- to 5-fold up-regulation for some genes: cAMP phosphodiesterase and Cytochrome c oxidase subunit VIII (Fig. 4). A tendency of approx. 3-fold up-regulation for: e.g. G-protein γ 12, metabotropic Glutamate receptor 5, PPAR binding protein, while the expression of some other genes was not different between "competent" oocytes before vs. after onset of resumption of meiosis [108].

Following the identification of these genes at the mRNA level, the challenge is to utilize efficiently this information to develop a better understanding of meiotic resumption mechanism. The proteomic approach may provide information that could not be obtained at the RNA level, due either to poor correlation between mRNA and protein levels or due to post-translational modifications that may result in several isoforms generated from one mRNA. For many genes identified, both the putative ligands that activate them and their targets of action remain unknown and represent challenges for future studies to unravel the mechanism of oocyte maturation and developing efficient IVM system.

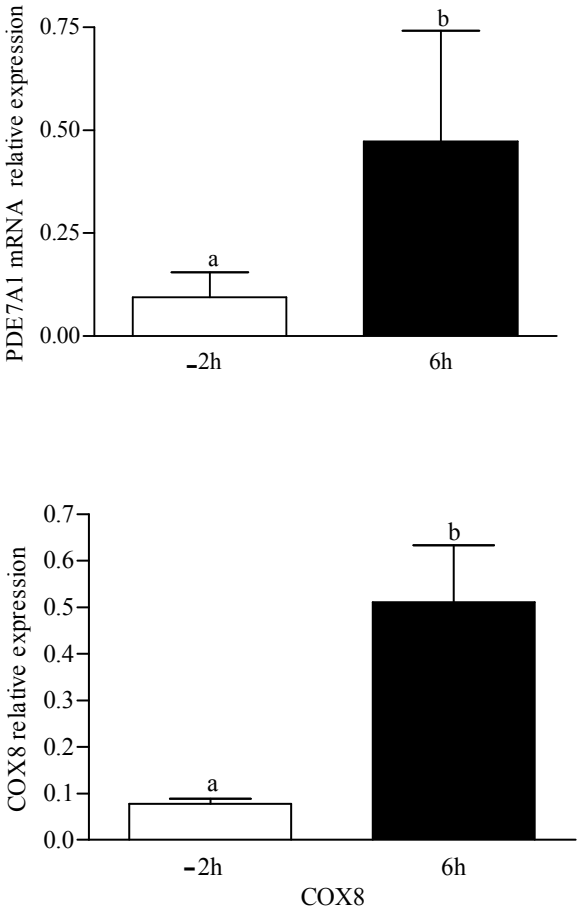

Figure 4. Real-time PCR analysis of mRNA expression of 2 of 10 genes selected from microarray results; data are shown as mean ± SEM of the mRNA level of the oocytes collected 2 h pre LH surge and the oocytes collected 6 h following the LH surge. Upper and lower panels: cAMP phosphodiesterase and Cytochrome c oxidase subunit VIII expression, respectively. Different letters indicate statistical significance between the two groups as determined by unpaired Student's *t*-test; $P<0.05$ was considered statistically significant. (Adapted from [108]).

5.3. Molecular motors and chromosome segregation

While the spindle and chromosome morphology of the meiotic events is known for over a century, most of the basic molecular mechanisms regulating the chromosome segregation in mammalian oocytes are widely unknown. Meiosis within the oocyte must be precisely regulated to ensure proper division of the genetic material. Missegregation of chromosomes results in aneuploidy and could lead to inviability. An estimated 10 to 30% of fertilized human eggs have the wrong number of chromosomes, with most of these being either trisomic or monosomic. This has profound clinical consequences: approximately one-third of all miscarriages are aneuploid, which makes it the leading known cause of pregnancy

loss and, among conceptions that survive to term, aneuploidy is the main genetic cause of developmental disabilities and mental retardation [112].

The real-time QPCR analysis of six of the genes having a significant role in the spindle formation and maintenance of accurate chromosomal segregation and construction of the cytoskeleton showed deregulated and aberrant mRNAs levels in, oocytes from follicles with deviant steroid profiles compared to normal [110]. In particular, the expression levels at onset of final maturation that is at 2 h before LH were significantly 5- to 10-fold lower in oocytes from follicles with deviant steroid profiles. After resumption of meiosis these differences were reduced to absent. In in-vitro cultured oocytes at corresponding times of maturation the expression levels were in general in between the levels of the in-vivo normal and deviant oocytes; the genes examined were KIF3A, Cytoplasmic dynein, Myosin regulatory light chain, Formin 2 like, Par3 and Aurora-A. Only Par3 and Myosin regulatory light chain were evidently expressed in expanded blastocysts, and were lower in in-vitro produced embryos than in the embryos obtained after flushing the superovulated cows [110]. This may explain the chromosomal abnormality frequently seen in the oocyte and early human preimplantation embryos cultured in vitro, which is commonly associated with impaired cleavage, poor embryo quality and increased fragmentation, all of which may compromise the implantation potential of the embryos [113-115].

While the polarity in mammalian embryos is a well-known phenomenon, existing polarity in mammalian oocyte is still controversial [116-118] and little is known about the genes regulating polarity and related activity in oocytes. We identified several transcripts in the oocyte known to play a role in polarity axis formation like par-3, formin, KIF3, β-catenin and CDC42 (unpublished, Algriany et al.). Organelles and cortical actin distribute asymmetrically in the oocyte of many species as the dorsal/ ventral axis forms [118]. The identification of genes regulating polarity in the oocyte and the recent finding that Par-3 protein is associated with meiotic spindles [119], may point to their important function during meiotic resumption and possible role in oocyte polarity.

Moreover, although most of the genes identified [110] are also common to somatic cells during mitosis, there is a fundamental difference. The chromatids are held together during prometaphase of meiosis II only at the centromere, whereas during prometaphase of mitosis they are joined (at least initially) along their entire length. This raises the interesting possibility that chromosome disjunction during mitosis also requires two different sets of machinery. One that is normally present during meiosis I that separates the chromatid arms, and another normally found in meiosis II (or during the preceding interphase) that leads to separation in the centromere region. In the future, it will be important to determine the differences between the two mechanisms that operate during meiosis to separate the chromosomes. The separation of sister chromatids is a complex process and there are certainly other factors involved in regulating the attachments and separation of sister chromatids.

5.4. Genes involved in lipid metabolism

Because the action of the products of the different transcripts identified in relation to resumption of meiosis, molecular motors and chromosome segregation, is ATP-dependent, and because the correlation of oocyte ATP content and developmental competence is well established, energy requirements constitute an important factor to accomplish competence

during maturation. In general, lipids form an energy source but there is a lack of information on the role of lipids as energy source in bovine. Therefore, based on information in somatic cells, we investigated the involvement of various pathways for lipid transport, β-oxidation and *de-novo* fatty acids synthesis during final maturation of bovine oocyte using QPCR.

A full understanding of the physiological effect of maturation in vivo on gene expression requires identification of the transcripts having an impact on metabolic pathways, their mode of action, and their consequences for growth, differentiation and survival. At a more practical level, gene identification is essential for formulating a successful maturation medium to support oocyte development after fertilization.

Changes associated with ultrastructure of the growing oocytes related to the accumulation of nutrients like lipids are prerequisite of energy for meiotic resumption and subsequent embryonic development. The origin of lipids reaching the oocyte is not fully understood. Lipids stored in the oocyte have been shown to be accumulated in the oocyte during follicular development [120] and start to decrease during the maturation process [121]. Kim et al. [122] showed that lipid content in bovine oocytes reflects the lipid content in the maturation medium, indicating that lipids accumulating in the oocytes must originate from the medium. It is not known whether these lipids pass via the junctions between the oocyte and its surrounding cumulus cells or are taken up directly from the follicular fluid. Since many genes are conserved across human and animal species, function of certain genes can be extrapolated. Therefore, mRNAs representing the major metabolic pathways involved in lipid metabolism were investigated in normal oocytes [111]. From the results, it is possible to suggest a model for long-chain fatty acid (LCFA) transport into the oocyte. The fatty acids are translocated from the extracellular environment to the cytoplasm by the fatty acids translocase (FAT/CD36) and then solubilized and transported by fatty acid binding proteins (FABPs) to the site where they are metabolized [123,124]. Once transported across the membrane, LCFA are targeted to specific metabolic fates. These findings together with the higher level of CPT-1 mRNA propose that fatty acid is directly required for meiotic resumption. Further, they indicate that β-oxidation is the major pathway contributing to the energy requirement during oocyte maturation and increased rate of lipogenesis at the blastocyst stage which may be needed to support earlier embryogenesis. It was clear that a switch from import of lipids to synthesis occurred between oocyte and blastocyst stage (Fig. 5) [125]. Then, the mRNA involved in lipid metabolism were compared to deviant oocytes collected from stimulated cows and those matured in vitro to pinpoint impairment of particular pathways of lipid metabolism. The aberrant levels of several mRNAs may indicate that intracellular fatty acid composition is not proper, decreased β-oxidation and may explain the lower progression of meiosis, lower ATP levels and lower developmental competence of these oocytes. The significant lower mRNA levels of Acetyl CoA carboxylase α (ACCα) the main enzyme controlling *de-novo* fatty acid synthesis may explain partially the lower developmental competence of in-vitro produced blastocysts. This may indicate a change in the quantity of synthesized lipid, resulting in a lack of certain LCFAs needed for membrane integrity and structure.

Although our study measured mRNA of the main pathway of lipid metabolism, the interpretation of the data on fatty acid metabolism has a number of limitations. For instance, considering that over 30 reactions are required to convert acetyl-CoA to triglycerides, there could be many steps or genes that control the yield of end product. Beside that, in addition to

fatty acids (FA), glucose is another main oxidized metabolic substrate, however, its role during oocyte maturation as energy source is controversial [126-128]. Interactions between

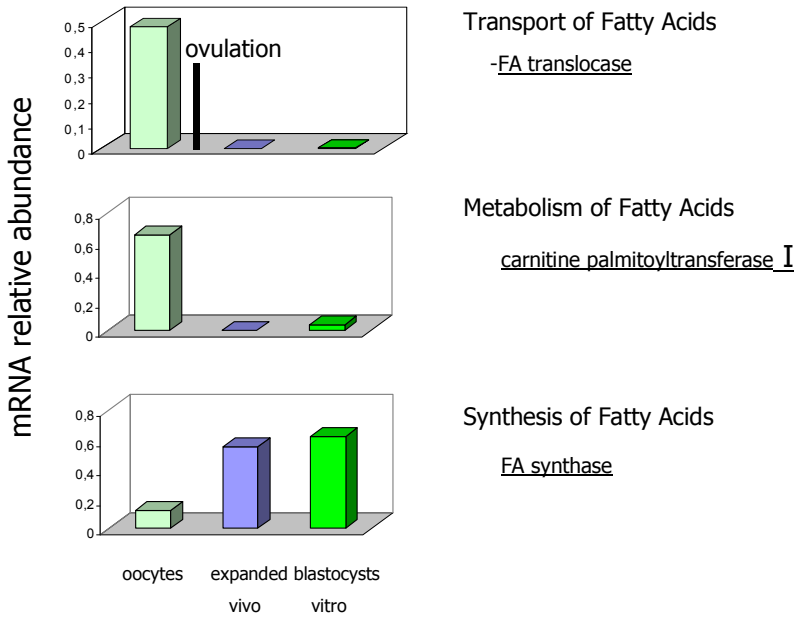

Figure 5. Switch from import of lipids into the oocyte (left bars) to synthesis of lipids in expanded blastocysts obtained in vivo after eCG superovulation (middle bars) and in vitro from slaughterhouse oocytes (right bars). (Adapted from [125]).

these substrates are thought to control the extent of their respective oxidation, i.e., to control the reciprocal relation between glucose and FA oxidation. However, which of the two substrates, glucose or FA, is the primary regulator of energy in the oocyte is not clearly known and needs further investigations.

5.5. Conclusions

Using oocytes from FSH-stimulated cows with a controlled LH surge has revealed many new basic properties of the bovine oocyte in relation to competence. We now know that mechanisms of meiotic arrest and resumption require different phosphodiesterases (PDE7), the involvement of G-proteins, and regulators of G-protein signaling (RGS) in meiotic resumption, molecular components involved in chromatids separation, regulation of Ca^{2+} oscillatory activity and cell cycle regulation. Most of these genes were identified for the first time in the mammalian oocyte. However, the challenge is to identify the potential ligands that activate these genes, which may provide an answer for how meiotic arrest is released. Future research will certainly provide answers to the open questions regarding these issues. In addition, the FSH-stimulated oocytes have provided crucial information regarding mRNAs involved in correct translocation of organelles and segregation of chromosomes possibly explaining disturbed polar body emission and defects in cytoplasmic maturation as commonly observed in deviant oocytes from stimulated animals and in in-vitro matured oocytes. Finally, new information has been obtained regarding long chain fatty acid transport into the oocytes and regulation of energy requirement during maturation and blastocyst stage

using lipid as a substrate, which may be used to adapt in-vitro culture of oocytes in many applications.

6. Perspectives

Although knowledge on oocyte maturation has grown rapidly during the last few years, the field needs to include completion of the molecular details, determination of key molecular structures, assignment of physiological functions, elucidation of physiological regulatory mechanisms, and exploration of interfaces with other cellular systems. As a result of our studies some routes for further investigation can be identified. First, we need proof that compounds such as steroids in the follicular fluid can be used as marker for oocyte competence for example by culturing single oocytes and determining rearrangement of organelles and competence to develop into viable blastocysts. Secondly, hybridization in microarrays of FSH-stimulated oocytes against oocytes derived from other routes such as in vitro, cross species [129] and last-but-not-least the "golden" standard from untreated animals might reveal eventual negative effects of the gonadotropin used. In this respect the cow can be used as model to improve IVF procedures in man. Moreover, comparing the expression of genes and using proteomics in the surrounding somatic granulosa and cumulus cells may lead to non-invasive clinical applications in human IVF.

Acknowledgements

It is impossible to be complete but here are some of the people I am most indebted to who have helped me with dedication round-the-clock which ultimately resulted in achieving the 2007 A.E.T.E. Pioneer award. I gladly accept the award on behalf of all of us. First of all the two colleagues who were always encouraging and interested to participate, the composed Mart Bevers with his great talent for in-vitro culture and Theo Kruip rich in ideas and always ready for a jest. Unfortunately, they both are not alive anymore to share this happening. We worked together for decades; that is anchored in memory! Next I should mention my much valued colleague and friend Peter Vos for his marvelous support and being a terrific sparring partner in science, and the post-docs Begoña Aguilar, Peter Hendriksen and Mitsuhiro Takagi for their specific insights in areas that were not directly my expertise. The team at the laboratory was without any doubt most essential to organize experiments and to get all analyses precisely done and in time!, in particular Thea Blankenstein, Christine Oei, Ad van de Poll, Leni van Tol and Elly Zeinstra. During their PhD study Ellen van de Leemput, Hiemke Knijn, and Omran Algriany, and many other PhD-students and guests from abroad were invaluable helping with collection of oocytes and embryos and with analysis; the many veterinary and biology and agricultural sciences Master students of whom Jan Joop Harkema, Bram van Schaik, Wilma Fokker, Elske de Boer, Henry Seinen, Sander van Gastel, Huijbert Groenendaal, Elsa van Rhenen and Annemarie Waijer are acknowledged. Last but not least, the animal caretakers and their supervisor Hans Lutz, and the surgeons Bert van der Weyden, Herman Jonkers, Maarten Pieterse and the surgery assistants are recognized.

Evidently this 'opus' could not have been accomplished without the generous collaboration of many people in other laboratories. I express my sincere appreciation to all of whom in particular Poul Maddox-Hyttel (Copenhagen, Denmark), Heiner Niemann and Christine Wrenzycki (Mariensee, Germany) and Marc-André Sirard (Quebec, Canada) and their colleagues. The generous supply of in-vitro embryos by Sybrand Merton (Holland Genetics, Harfsen, The Netherlands) and the donation of hormones by Intervet International

B.V. (Boxmeer, The Netherlands) is greatly appreciated. Finally, I heartily acknowledge the ever inspiring atmosphere and the tremendous collegiality I have experienced year upon year at the Annual Meetings of the A.E.T.E. and the I.E.T.S. It has been like a family.

References

1. Hansen PJ. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. Theriogenology 2007; [Epub ahead of print] PMID: 17482669.

2. http//www.iets.org/data_retrieval.htm
3. Proceedings of the 22nd Scientific Meeting of the Association Europeenne de Transfert Embryonnaire, Zug, Switzerland 2006.
4. Pfeffer PL, Sisco B, Donnison M, Somers J, Smith C. Isolation of genes associated with developmental competency of bovine oocytes. Theriogenology 2007; [Epub ahead of print] PMID: 17467046
5. Fair T, Carter F, Park S, Evans AC, Lonergan P. Global gene expression analysis during bovine oocyte in vitro maturation. Theriogenology 2007; [Epub ahead of print] PMID: 17512044.
6. Boerke A , S.J. Dieleman SJ, Gadella BM. A possible role for sperm RNA in early embryo development. Theriogenology 2007; in Press.
7. Van Soom A, Vandaele L, Goossens K, de Kruif A, Peelman L. Gamete origin in relation to early embryo development. Theriogenology 2007; [Epub ahead of print] PMID: 17467789.
8. Laurincik J, Schmoll F, Mahabir E, Schneider H, Stojkovic M, Zakhartchenko V, Prelle K, Hendrixen PJ, Voss PL, Moeszlacher GG, Avery B, Dieleman SJ, Besenfelder U, Muller M, Ochs RL, Wolf E, Schellander K, Maddox-Hyttel P. Nucleolar proteins and ultrastructure in bovine in vivo developed, in vitro produced, and parthenogenetic cleavage-stage embryos. Mol Reprod Dev 2003; 65: 73-85.
9. Maddox-Hyttel P, Svarcova O, Laurincik J. Ribosomal RNA and nucleolar proteins from the oocyte are to some degree used for embryonic nucleolar formation in cattle and pig. Theriogenology 2007; [Epub ahead of print] PMID: 17466364.
10. Singh R, Sinclair KD. Metabolomics: Approaches to assessing oocyte and embryo quality. Theriogenology 2007; [Epub ahead of print] PMID: 17490741.
11. Wrenzycki C, Herrmann D, Niemann H. Messenger RNA in oocytes and embryos in relation to embryo viability. Theriogenology 2007; [Epub ahead of print] PMID: 17524469.
12. Thurston A, Lucas ES, Allegrucci C, Steele W, Young LE. Region-specific DNA methylation in the preimplantation embryo as a target for genomic plasticity. Theriogenology 2007; [Epub ahead of print] PMID: 17482250.
13. Katz-Jaffe MG, Gardner DK. Embryology in the era of proteomics. Theriogenology 2007; [Epub ahead of print] PMID: 17477967.
14. Schellander K, Hoelker M, Tesfaye D. Selective degradation of transcripts in mammalian oocytes and embryos. Theriogenology 2007; in Press.
15. Habermann FA, Wuensch A, Sinowatz F, Wolf E. Reporter genes for embryogenesis research in livestock species. Theriogenology 2007; in Press.
16. Sirard MA, Lambert RD. In vitro fertilization of bovine follicular oocytes obtained by laparoscopy. Biol Reprod 1985; 33: 487-494.

17. Armstrong DT, Irvine BJ, Earl CR, McLean D, Seamark RF. Gonadotropin stimulation regimens for follicular aspiration and in vitro embryo production from calf oocytes. Theriogenology 1994; 42: 1227-1236.
18. Betteridge KJ. A history of farm animal embryo transfer and some associated techniques. Anim Reprod Sci. 2003; 79: 203-244.
19. Bevers MM, Dieleman SJ. Superovulation of cows with PMSG: variation in plasma concentrations of progesterone, oestradiol, LH, cortisol prolactin and PMSG and in number of preovulatory follicles. Anim Reprod Sci 1987; 15: 37-52.
20. Sirard MA, Désrosiers S, Assidi M. In vivo and in vitro effects of FSH on oocyte maturation and developmental competence. Theriogenology 2007; in Press.
21. Kruip TAM, van Beneden TH, Dieleman SJ, Bevers MM. The effect of oestradiol-17β on nuclear maturation of bovine oocytes. In: Proc of the 11th Intern Congr on Anim Reprod & A.I., Dublin, Ireland 1988; 3:336 [abstract].
22. Bouters R, Moyaert I, Coryn M, Vandeplassche M. The use of a PMSG antiserum in superovulated cattle: Endocrinological changes and effect of timing of ovulation. Zuchthygiene 1983; 18: 172-177.
23. Vos PLAM, van der Schans A, de Wit AAC, Bevers MM, Willemse AH, Dieleman SJ. Effects of neutralization of pregnant mares' serum gonadotrophin (PMSG) shortly before or at the preovulatory LH surge in PMSG-superovulated heifers on follicular function and development. J Reprod Fertil 1994; 100: 387-393.
24. Dieleman SJ, Bevers MM. Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG treated cows. J Reprod Fertil 1987; 81: 533-542.
25. Dieleman SJ, Bevers MM, Wurth YA, Gielen JTh, Willemse AH. Improved embryo yield and condition of donor ovaries in cows after PMSG superovulation with monoclonal anti-PMSG administered shortly after the preovulatory LH peak. Theriogenology 1989; 31: 473-488.
26. Dieleman SJ, Bevers MM, Vos PLAM, de Loos FAM. PMSG/anti-PMSG in cattle: a simple and efficient superovulatory treatment? Theriogenology 1993; 39: 25-41.
27. Bevers MM, Dieleman SJ, Gielen JTh, Wurth YA, Janszen BPM, van den Broek J, Willemse AH. Yield of embryos in PMSG-superovulated cows treated with anti-PMSG at 6 or 18 h after the LH peak. Vet Rec 1993; 132: 186-189.
28. Hyttel P, Callesen H, Greve T. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J Reprod Fertil 1986; 76: 645-656.
29. Moor RM, Kruip TAM, Green D. Intraovarian control of folliculogenesis: limits to superovulation. Theriogenology 1984; 21: 103-116.
30. Bevers MM, Dieleman SJ, van Tol HT, Blankenstein DM, van den Broek J. Changes in pulsatile secretion patterns of LH, FSH, progesterone, androstenedione and oestradiol in cows after superovulation with PMSG. J Reprod Fertil 1989; 87: 745-754.
31. Gosselin N, Price CA, Roy R, Carriere PD. Decreased LH pulsatility during initiation of gonadotropin superovulation treatment in the cow: evidence for negative feedback other than estradiol and progesterone. Theriogenology 2000; 54: 507-521.
32. Greve T, Callesen H, Hyttel P. Endocrine profiles and egg quality in the superovulated cow. Nord Vet Med 1983; 35: 408-421.
33. Mikel-Jenson A, Greve T, Madej A, Edqvist L-E. Endocrine profiles and embryo quality in the PMSG-PGF2 treated cow. Theriogenology 1982; 18: 33-34.
34. Ben Jebara MK, Carriere PD, Price CA. Decreased pulsatile LH secretion in heifers superovulated with eCG or FSH. Theriogenology 1994; 42: 685-694.

35. Alcivar AA, Maurer RR, Anderson LL. Endocrine changes in beef heifers superovulated with follicle stimulating hormone (FSH-P) or human menopausal gonadotropin. J Anim Sci 1992; 70: 224-231.
36. Soumano K, Lussier JG, Price CA. Levels of messenger RNA encoding ovarian receptors for FSH and LH in cattle during superovulation with equine chorionic gonadotrophin versus FSH. J Endocrinol 1998; 156: 373-378.
37. Soumano K, Silversides DW, Doize F, Price CA. Follicular 3 beta-hydroxysteroid dehydrogenase and cytochromes P450 17 alpha-hydroxylase and aromatase messenger ribonucleic acids in cattle undergoing superovulation. Biol Reprod 1996; 55: 1419-1426.
38. Mapletoft RJ, Pawlyshyn V, Garcia A, Bo GA, Willmott N, Saunders J, Schmutz S. Comparison of four different gonadotropin treatments for inducing superovulation in cows with 1:29 translocation. Theriogenology 1990; 33: 282 [abstract].
39. Goulding D, Williams DH, Roche JF, Boland MP. Factors affecting superovulation in heifers treated with PMSG. Theriogenology 1996; 45: 765-773.
40. Kanitz W, Becker F, Schneider F, Kanitz E, Leiding C, Nohner HP, Pohland R. Superovulation in cattle: practical aspects of gonadotropin treatment and insemination. Reprod Nutr Dev 2002; 42: 587-599.
41. Gonzalez A, Lussier JG, Carruthers TD, Murphy BD, Mapletoft RJ. Superovulation of beef heifers with folltropin: A new FSH preparation containing reduced LH activity. Theriogenology 1990; 33: 519-529.
42. Tribulo H, Bo GA, Jofre F, Carcedo J, Alonso A, Mapletoft RJ. The effect of LH concentration in a porcine pituitary extract and season on superovulatory response in Bos indicus heifers. Theriogenology 1991; 35: 286 [abstract].
43. Yamamoto M, Ooe M, Fujii C, Suzuki T. Superovulation of Japanese black heifers treated with FSH-P and FSH-R. J Vet Med Sci 1993; 55: 133-134.
44. Assey RJ, Hyttel P, Roche JF, Boland M. Oocyte structure and follicular steroid concentrations in superovulated versus unstimulated heifers. Mol Reprod Dev 1994; 39: 8-16.
45. Takagi M, Kim IH, Izadyar F, Hyttel P, Bevers MM, Dieleman SJ, Hendriksen PJM, Vos PLAM. Impaired final follicular maturation in heifers after superovulation with recombinant human FSH. Reproduction 2001; 121: 941-951.
46. Webb R, Garnsworthy PC, Campbell BK, Hunter MG. Intra-ovarian regulation of follicular development and oocyte competence in farm animals. Theriogenology 2007; [Epub ahead of print] PMID: 17540442.
47. de Loos FA, Bevers MM, Dieleman SJ, Kruip TA. Follicular and oocyte maturation in cows treated for superovulation. Theriogenology 1991; 35: 537-546.
48. Dieleman SJ, Bevers MM. Folliculogenesis and oocyte maturation in superovulated cattle. Mol Reprod Dev 1993; 36: 271-273.
49. Fortune JE, Hinshelwood MM, Roycroft J, Vincent SE. Superovulation in cattle: effects of purity of FSH preparation on follicular characteristics in vivo. Bull Assoc Anat (Nancy) 1991; 75: 55-58.
50. Keller DS, Teepker G. Effect of variability in response to superovulation on donor cow selection differentials in nucleus breeding schemes. J Dairy Sci 1990; 73: 549-554.
51. Kelly P, Duffy P, Roche JF, Boland MP. Superovulation in cattle: effect of FSH type and method of administration on follicular growth, ovulatory response and endocrine patterns. Anim Reprod Sci 1997; 46: 1-14.

52. Algriany OA, Vos PLAM, Groenendaal H, van Gastel ACTM, Sirard MA, Dieleman SJ. Effects of oFSH stimulation on steroid concentrations in the fluid of preovulatory follicles during final maturation in relation to the LH surge in the cow. Submitted (2007).
53. Moor RM, Trounson AO. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J Reprod Fertil 1977; 49: 101-109.
54. Tonetta A, diZerega GS. Intragonadal regulation of follicular maturation. Endocr Rev 1989; 10: 205-229.
55. Dumesic DA, Schramm RD, Peterson E, Paprocki AM, Zhou R, Abbott DH. Impaired developmental competence of oocytes in adult prenatally androgenized female rhesus monkeys undergoing gonadotropin stimulation for in vitro fertilization. J Clin Endocrinol Metab 2002; 87: 1111-1119.
56. Cheskis BJ. Regulation of cell signalling cascades by steroid hormones. J Cell Biochem 2004; 93: 20-27.
57. Skildum A, Faivre E, Lange CA. Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol 2005; 19: 327-339.
58. Goyeneche AA, Calvo V, Gibori G, Telleria CM. Androstenedione interferes in luteal regression by inhibiting apoptosis and stimulating progesterone production. Biol Reprod 2002; 66: 1540-1547.
59. Younglai EV, Wu YJ, Kwan TK, Kwan CY. Non-genomic action of estradiol and progesterone on cytosolic calcium concentrations in primary cultures of human granulosa-lutein cells. Hum Reprod 2005; 20: 2383-2390.
60. Tesarik J, Mendoza C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. J Clin Endocrinol Metab 1995; 80: 1438-1443.
61. Dieleman SJ, Kruip TA, Fontijne P, de Jong WH, van der Weyden GC. Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. J Endocrinol 1983; 97: 31-42.
62. Komar CM, Berndtson AK, Evans AC, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. Biol Reprod 2001; 64: 1797-1805.
63. Beker-van Woudenberg AR, van Tol HT, Roelen BA, Colenbrander B, Bevers MM. Estradiol and its membrane-impermeable conjugate (estradiol-bovine serum albumin) during in vitro maturation of bovine oocytes: effects on nuclear and cytoplasmic maturation, cytoskeleton, and embryo quality. Biol Reprod 2004; 70: 1465-1474.
64. Cassar CA, Dow MP, Pursley JR, Smith GW. Effect of the preovulatory LH surge on bovine follicular progesterone receptor mRNA expression. Domest Anim Endocrinol 2002; 22: 179-187.
65. Park O-K, Mayo K. Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. Mol Endocrinol 1991; 5: 967-978.
66. Natraj U, Richards JS. Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology 1993; 133: 761-769.

67. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest 1998; 101: 2622-2629.
68. Wu TC, Wang L, Wan YJ. Expression of estrogen receptor gene in mouse oocyte and during embryogenesis. Mol Reprod Dev 1992; 33: 407-412.
69. Szoltys M, Slomczynska M, Tabarowski Z. Immunohistochemical localization of androgen receptor in rat oocytes. Folia Histochem Cytobiol 2003; 41: 59-64.
70. Bevers MM, Dieleman SJ. Stimulation of follicular growth in the cow with pregnant mare serum gonadotrophin; impact on hormone levels in the peripheral blood. Current Trends in Experimental Endocrinology 1994; 2: 57-64.
71. Monniaux D, Monget P, Besnard N, Huet C, Pisselet C. Growth factors and antral follicular development in domestic ruminants. Theriogenology 1997; 47: 3-12.
72. Bevers MM, Dieleman SJ, van den Hurk R, Izadyar F. Regulation and modulation of oocyte maturation in the bovine. Theriogenology 1997; 47: 13-22.
73. Hendriksen PJM, Vos PLAM, Steenweg WNM, Bevers MM, Dieleman SJ. Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology 2000; 53: 11-20.
74. Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003; 59: 651-674.
75. Hendriksen PJM, Steenweg WNM, Harkema JC, Merton JS, Bevers MM, Vos PLAM, Dieleman SJ. Effect of different stages of the follicular wave on in vitro developmental competence of bovine oocytes. Theriogenology 2004; 61: 909-920.
76. Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, Peterson AJ, Schurmann A, Tervit HR. Development during sIVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. Mol Reprod Dev 1999; 53: 451-458.
77. Bungartz L, Niemann H. Assessment of the presence of a dominant follicle and selection of dairy cows suitable for superovulation by a single ultrasound examination. J Reprod Fertil 1994; 101: 583-591.
78. Huhtinen M, Rainio V, Aalto J, Bredbacka P, Maki-Tanila. Increased ovarian responses in the absence of a dominant follicle in superovulated cows. Theriogenology 1992; 37: 457-463.
79. Lussier JG, Lamonthe P, Pacholek X. Effects of follicular dominance and different gonadotropin preparations on the superovulatory response in cows. Theriogenology 1995; 43: 270.
80. Hendriksen PJM, Gadella BM, Vos PLAM, Mullaart E, Kruip TAM, Dieleman SJ. Follicular dynamics around the recruitment of the first follicular wave in the cow. Biol Reprod 2003; 69: 2036-2044.
81. Van de Leemput EE, Vos PLAM, Hyttel P, Van den Hurk R, Bevers MM, Van der Weijden GC, Dieleman SJ. Effects of brief postponement of the preovulatory LH surge on ovulation rates and embryo formation in eCG/prostaglandin-treated heifers. Theriogenology 2001; 55: 573-592.
82. Dieleman SJ, Bevers MM, Poortman J, van Tol HTM. Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood. J Reprod Fertil 1983; 69: 641-649.
83. Vos PLAM. Ovarian follicular function and development in cows following exogenous gonadotrophin. Thesis, Utrecht University 1994.
84. Van de Leemput EE, Vos PLAM, Zeinstra EC, Bevers MM, Van der Weijden GC, Dieleman SJ. Improvement of in vitro embryo development using in vivo matured

oocytes from heifers treated for superovulation with a controlled preovulatory LH surge. Theriogenology 1999; 52: 335-349.
85. Van de Leemput EE. Final follicular maturation in the cow and its effects on developmental potential of the oocyte. Thesis, Utrecht University 1999.
86. Gandolfi F, Brevini TA, Moor RM. Effect of oviduct environment on embryonic development. J Reprod Fertil Suppl. 1989; 38: 107-115.
87. Knijn HM, Fokker W, van der Weijden GC, Dieleman SJ, Vos PLAM. Effects of superovulation with oFSH and norgestomet/GnRH-controlled release of the LH surge on hormone concentrations, and yield of oocytes and embryos at specific developmental stages. Theriogenology 2007; submitted.
88. Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM, Vos PL. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology 2002; 57: 5-20.
89. Algriany OA. Expression analysis of genes implicated in meiotic resumption in vivo and developmental competence of bovine oocytes. Thesis, Utrecht University (2007) ISBN: 978-90-393-44835, 173 pp.
90. Viuff D, Hendriksen PJM, Vos PLAM, Dieleman SJ, Bibby BM, Greve T, Hyttel P, Thomsen PD. Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. Biol Reprod 2001; 65: 204-208.
91. Knijn HM, Gjørret JO, Vos PLAM, Hendriksen PJM, van der Weijden GC, Maddox-Hyttel P, Dieleman SJ. Consequences of in vivo development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos. Biol Reprod 2003; 69: 1371-1378.
92. Gjorret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI, Maddox-Hyttel P. Chronology of apoptosis in bovine embryos produced in vivo and in vitro. Biol Reprod 2003; 69: 1193-1200.
93. Knijn HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, van der Weijden GC, Niemann H, Dieleman SJ. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 2002; 124: 365-375.
94. Vallee M, Gravel C, Palin MF, Reghenas H, Stothard P, Wishart DS, Sirard MA. Identification of novel and known oocyte-specific genes using complementary DNA subtraction and microarray analysis in three different species. Biol Reprod 2005; 73: 63-71.
95. Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. Biol Reprod 1996; 55: 1187-1194.
96. Ginther OJ, Beg MA, Kot K, Meira C, Bergfelt DR. Associated and independent comparisons between the two largest follicles preceding follicle deviation in cattle. Biol Reprod 2003; 68: 524-529.
97. Vigneron C, Perreau C, Dupont J, Uzbekova S, Prigent C, Mermillod P. Several signaling pathways are involved in the control of cattle oocyte maturation. Mol Reprod Dev 2004; 69: 466-474.
98. Zahn PK, Lansmann T, Berger E, Speckmann EJ, Musshoff U. Gene expression and functional characterization of melatonin receptors in the spinal cord of the rat: implications for pain modulation. J Pineal Res 2003; 35: 24-31.
99. Robert C, Gagne D, Bousquet D, Barnes FL, Sirard MA. Differential display and suppressive subtractive hybridization used to identify granulosa cell messenger rna

associated with bovine oocyte developmental competence. Biol Reprod 2001; 64: 1812-1820.
100. Robert C, Barnes FL, Hue I, Sirard MA. Subtractive hybridization used to identify mRNA associated with the maturation of bovine oocytes. Mol Reprod Dev 2000; 57: 167-175.
101. Diatchenko L, Lukyanov S, Lau YF, Siebert PD. Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes. Methods Enzymol 1999; 303: 349-380.
102. Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 1996; 93: 6025-6030.
103. da Silva Xavier G, Leclerc I, Salt IP, Doiron B, Hardie DG, Kahn A, Rutter GA. Role of AMP-activated protein kinase in the regulation by glucose of islet beta cell gene expression. Proc Natl Acad Sci U S A 2000; 97: 4023-4028.
104. Dalbies-Tran R, Mermillod P. Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. Biol Reprod 2003; 68: 252-261.
105. Mourot M, Dufort I, Gravel C, Algriany O, Dieleman S, Sirard MA. The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. Mol Reprod Dev 2006; 73: 1367-1379.
106. van Wagtendonk-de Leeuw AM, Mullaart E, de Roos APW, Merton JS, den Daas JHG, Kemp B, de Ruigh L. Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. Theriogenology 2000; 53: 575-597.
107. Aguilar B, Vos PLAM, Beckers JF, Hensen EJ, Dieleman SJ. The role of the major histocompatibility complex in bovine embryo transfer. Theriogenology 1997; 47: 111-121.
108. Algriany OA, Dufort I, Vos PLAM, Sirard MA, Dielman SJ. Isolation and identification of differentially expressed genes involved in meiotic resumption of bovine oocytes in vivo. Submitted (2007).
109. Sirard MA, Dufort I, Vallee M, Massicotte L, Gravel C, Reghenas H, Watson AJ, King WA, Robert C. Potential and limitations of bovine-specific arrays for the analysis of mRNA levels in early development: preliminary analysis using a bovine embryonic array. Reprod Fertil Dev 2005; 17: 47-57.
110. Algriany OA, Waijer AJ, Blankenstein DM, Oei CHY, Knijn HM, Colenbrander B, Vos PLAM, Sirard MA, Dieleman SJ. Quantification of mRNAs encoding molecular motors and genes involved in chromosome segregation during final maturation of bovine oocytes in vivo. Submitted (2007).
111. Algriany OA, Vos PLAM, Sirard MA, Dieleman SJ. Alterations in the expression of genes involved in lipid metabolism during bovine oocyte maturation and at the blastocyst stage in vivo vs. in vitro, Submitted (2007).
112. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet 2001; 2: 280-291.
113. Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H, Thomsen PD. A high proportion of bovine blastocysts produced in vitro are mixoploid. Biol Reprod 1999; 60: 1273-1278.
114. Angell RR, Aitken RJ, van Look PF, Lumsden MA, Templeton AA. Chromosome abnormalities in human embryos after in vitro fertilization. Nature 1983; 303: 336-338.

115. Hassold T, MacLean C. Temporal changes in chromosome abnormality rate in human spontaneous abortions: evidence for an association between sex-chromosome monosomy and trisomy 16. Cytogenet Cell Genet 1984; 38: 200-205.
116. Plancha CE, Sanfins A, Rodrigues P, Albertini D. Cell polarity during folliculogenesis and oogenesis. Reprod Biomed Online 2005; 10: 478-484.
117. Albertini DF, Barrett SL. The developmental origins of mammalian oocyte polarity. Semin Cell Dev Biol 2004; 15: 599-606.
118. Edwards RG, Beard HK. Oocyte polarity and cell determination in early mammalian embryos. Mol Hum Reprod 1997; 3: 863-905.
119. Duncan FA, Moss SB, Schultz RM, Williams CJ. PAR-3 defines a central subdomain of the cortical actin cap in mouse eggs. Dev Biol 2005; 280: 38-47.
120. Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat Embryol (Berl) 1997; 195: 327-336.
121. Ferguson EM, Leese HJ. Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil 1999; 116: 373-378.
122. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction 2001; 122: 131-138.
123. Abumrad NA, Sfeir Z, Connelly MA, Coburn C. Lipid transporters: membrane transport systems for cholesterol and fatty acids. Curr Opin Clin Nutr Metab Care 2000; 3: 255-262.
124. Schaffer JE. Fatty acid transport: the roads taken. Am J Physiol Endocrinol Metab 2002; 282: E239-246.
125. Algriany O, Vos PLAM, Sirard MA, Dieleman SJ. Switch in the expression of genes involved in lipid metabolism for in vivo-matured bovine oocytes and blastocysts. Reprod Fertil Dev 2007; 19: 244 [abstract].
126. Sutton-McDowall ML, Gilchrist RB, Thompson JG. Cumulus expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle-stimulating hormone. Reproduction 2004; 128: 313-319.
127. Hashimoto S, Minami N, Yamada M, Imai H. Excessive Concentration of Glucose During In Vitro Maturation Impairs the Developmental Competence of Bovine Oocytes After In Vitro Fertilization: Relevance to Intracellular Reactive Oxygen Species and Glutathione Contents. Mol Reprod Dev 2000; 56: 520-526.
128. Downs SM, Humpherson PG, Martin KL, Leese HJ. Glucose utilization during gonadotropin-induced meiotic maturation in cumulus cell-enclosed mouse oocytes. Mol Reprod Dev 1996; 44: 121-131.
129. Niemann H, Carnwath JW, Kues W. Application of DNA array technology to mammalian embryos. Theriogenology 2007; in Press.

National Statistical Data of Bovine Embryo Transfer Activity in Europe in 2006

Table 1: Embryo Transfer Activity in 2006

COUNTRY: **AUSTRIA** ***A.E.T.E 2007***

Data collected by
Dr. Wetchy Gabi

Total number of approved E.T. teams in the country	15
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	154	B/A=	10.0
	Embryos collected	**B**	1541	C/A=	6.3
	Embryos transferable	**C**	964	C/B=	62.6%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	964	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	315		
In vivo	Frozen	**I**	629		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	944	H+I+J+K=	
Number of frozen stored embryos		**M**	744		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	66.6%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	155
Number of calves born from *in vitro* embryos	
Total	155

Table 2: Embryo Transfer Activity in 2006

COUNTRY: **CZECH REPUBLIC** ***A.E.T.E 2007***

Data collected by
Dr. Jirina Peteliková

Total number of approved E.T. teams in the country	6
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1210	B/A=	11.0
	Embryos collected	**B**	13357	C/A=	5.3
	Embryos transferable	**C**	6383	C/B=	47.8%
In vitro (*OPU*)	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro (*Slaughtered donors*)	Nb of transferable embryos	**E**	61		
Total i*n vitro* embryos		**F**	61	=(D+E)	
Total number of transferable embryos		**G**	6444	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3483		
In vivo	Frozen	**I**	2549	42.3% frozen	
In vitro	Fresh	**J**	13		
In vitro	Frozen	**K**	30	69.8% frozen	
Total embryos transferred		**L**	6075	H+I+J+K=	
Number of frozen stored embryos		**M**	2323		
% of *in vitro* embryos transferred		**N**	0.7%	(J+K)/L=	
% of frozen embryos transferred		**O**	42.5%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 3: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **DENMARK** ***A.E.T.E 2007***

Data collected by
Dr. Callesen Henrik

Total number of approved E.T. teams in the country	14
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	580	B/A=	11.8
	Embryos collected	**B**	6851	C/A=	7.5
	Embryos transferable	**C**	4377	C/B=	63.9%
In vitro (*OPU*)	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro (*Slaughtered donors*)	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	4377	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2107		
In vivo	Frozen	**I**	1363		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3470	H+I+J+K=	
Number of frozen stored embryos		**M**	2165		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	39.3%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	2019
Number of calves born from *in vitro* embryos	-
Total	2019

TABLE 4: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **ENGLAND** ***A.E.T.E 2007***

Data collected by
Dr. Anita Meacock

Total number of approved E.T. teams in the country	21
Number of teams providing data	10

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**		=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		(fresh + frozen)
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	4249	H+I+J+K=
Number of frozen stored embryos		**M**	8687	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L=

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 5: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: ESTONIA

A.E.T.E 2007

Data collected by Dr. Jaakma Ulle

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	0	B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B= %
In vitro (*OPU*)	Nb of oocyte donors		0	
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro (*Slaughtered donors*)	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
EMBRYO TRANSFER				
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	0	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L=

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 6: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **FINLAND** ***A.E.T.E 2007***

Data collected by
Dr. Mikkola Marja

Total number of approved E.T. teams in the country	8
Number of teams providing data	8

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	399	B/A=	11.7
	Embryos collected	**B**	4670	C/A=	7.0
	Embryos transferable	**C**	2798	C/B=	59.9%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	18		
Total i*n vitro* embryos		**F**	18	=(D+E)	
Total number of transferable embryos		**G**	2816	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	769		
In vivo	Frozen	**I**	1536	66.6% frozen	
In vitro	Fresh	**J**	13		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2318	H+I+J+K=	
Number of frozen stored embryos		**M**	1700		
% of *in vitro* embryos transferred		**N**	0.6%	(J+K)/L=	
% of frozen embryos transferred		**O**	66.3%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	963

TABLE 7: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **FRANCE**

A.E.T.E 2007

Data collected by
Dr. Guérin Bernard

Total number of approved E.T. teams in the country	27
Number of teams providing data	23

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5915	B/A=	9.6
	Embryos collected	**B**	56607	C/A=	5.6
	Embryos transferable	**C**	32899	C/B=	58.1%
In vitro	Nb of oocyte donors		84		
(OPU)	Nb of OPU sessions		307		
	Nb of transferable embryos	**D**	212		
In Vitro	Nb of transferable embryos	**E**	4017		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	4229	=(D+E)	
Total number of transferable embryos		**G**	37128	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	14938		
In vivo	Frozen	**I**	13504	47.5% frozen	
In vitro	Fresh	**J**	89		
In vitro	Frozen	**K**	2	2.2% frozen	
Total embryos transferred		**L**	28533	H+I+J+K=	
Number of frozen stored embryos		**M**	10815		
% of *in vitro* embryos transferred		**N**	0.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	47.3%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	6740
Number of calves born from *in vitro* embryos	47
Total	6787

TABLE 8: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **GERMANY** ***A.E.T.E 2007***

Data collected by
Dr. Cramer Hubert

Total number of approved E.T. teams in the country	
Number of teams providing data	24

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2445	B/A=	12.0
	Embryos collected	**B**	29232	C/A=	7.0
	Embryos transferable	**C**	17021	C/B=	58.2%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions		1459		
	Nb of transferable embryos	**D**	3586		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	3586	=(D+E)	
Total number of transferable embryos		**G**	20607	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5283		
In vivo	Frozen	**I**	7823	59.7% frozen	
In vitro	Fresh	**J**	1865		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	14971	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	12.5%	(J+K)/L=	
% of frozen embryos transferred		**O**	52.3%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 9: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: GREECE ***A.E.T.E 2007***

Data collected by
Dr. Samartzi Fonteini

Total number of approved E.T. teams in the country	2
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	4	B/A=	7.8
	Embryos collected	**B**	31	C/A=	4.5
	Embryos transferable	**C**	18	C/B=	58.0%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	18	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	10		
In vivo	Frozen	**I**	5		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	15	H+I+J+K=	
Number of frozen stored embryos		**M**	8		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	33.3%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	8
Number of calves born from *in vitro* embryos	
Total	8

TABLE 10: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **HUNGARY**

A.E.T.E 2007

Data collected by
Dr. Solti Laszlo

Total number of approved E.T. teams in the country	10
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	68	B/A=	11.1
	Embryos collected	**B**	756	C/A=	7.5
	Embryos transferable	**C**	508	C/B=	67.2%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	508	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	144		
In vivo	Frozen	**I**	136		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	280	H+I+J+K=	
Number of frozen stored embryos		**M**	283		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	48.6%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	736
Number of calves born from *in vitro* embryos	
Total	736

TABLE 11: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **IRELAND**

A.E.T.E 2007

Data collected by
Dr. Lonergan Pat

Total number of approved E.T. teams in the country	
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	271	B/A=	8.0
	Embryos collected	**B**	2177	C/A=	5.1
	Embryos transferable	**C**	1373	C/B=	63.1%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	1373	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	634		
In vivo	Frozen	**I**	889		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1523	H+I+J+K=	
Number of frozen stored embryos		**M**	739		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	58.4%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 12: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **ISRAEL**

A.E.T.E 2007

Data collected by
Dr. Zeron Yoel

Total number of approved E.T. teams in the country	1
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	115	B/A=	3.1
	Embryos collected	**B**	361	C/A=	2.8
	Embryos transferable	**C**	320	C/B=	88.6%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	320	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	269		
In vivo	Frozen	**I**	39		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	308	H+I+J+K=	
Number of frozen stored embryos		**M**	12		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	12.7%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	48
Number of calves born from *in vitro* embryos	
Total	48

TABLE 13: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **ITALY**

A.E.T.E 2007

Data collected by
Dr. Lazzari Giovanna

Total number of approved E.T. teams in the country	
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	725	B/A=	13.1
	Embryos collected	**B**	9484	C/A=	7.3
	Embryos transferable	**C**	5283	C/B=	55.7%
In vitro	Nb of oocyte donors		165		
(OPU)	Nb of OPU sessions		407		
	Nb of transferable embryos	**D**	1016		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	1823		
Total i*n vitro* embryos		**F**	2839	=(D+E)	
Total number of transferable embryos		**G**	8122	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3523		
In vivo	Frozen	**I**	1760	33.3% frozen	
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	2777	96.5% frozen	
Total embryos transferred		**L**	8160	H+I+J+K=	
Number of frozen stored embryos		**M**	3818		
% of *in vitro* embryos transferred		**N**	35.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	55.6%	(I+K)/L=	%

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 14: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **LITHUANIA** ***A.E.T.E 2007***

Data collected by
Dr. Kutra Jonas

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	0	B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B= %
In vitro	Nb of oocyte donors		0	
(OPU)	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro	Nb of transferable embryos	**E**		
(Slaughtered donors)				
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	0	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L= %

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 15: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **THE NETHERLANDS** ***A.E.T.E 2007***

Data collected by
Dr. Landman Bas

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2991	B/A=	9.1
	Embryos collected	**B**	27358	C/A=	5.5
	Embryos transferable	**C**	16512	C/B=	60.4%
In vitro	Nb of oocyte donors		193		
(OPU)	Nb of OPU sessions		1645		
	Nb of transferable embryos	**D**	2910		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	289		
Total i*n vitro* embryos		**F**	3199	=(D+E)	
Total number of transferable embryos		**G**	19711	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3366		
In vivo	Frozen	**I**	15569	82.2% frozen	
In vitro	Fresh	**J**	674		
In vitro	Frozen	**K**	1271	65.3% frozen	
Total embryos transferred		**L**	20880	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	9.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	80.1%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 16: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **POLAND** ***A.E.T.E 2007***

Data collected by
Dr. Jaskowski Jedrzej

Total number of approved E.T. teams in the country	1
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	20	B/A=	3.4
	Embryos collected	**B**	68	C/A=	2.9
	Embryos transferable	**C**	58	C/B=	85.3%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	58	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	23		
In vivo	Frozen	**I**	13		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	36	H+I+J+K=	
Number of frozen stored embryos		**M**	6		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	36.1%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE 17: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **ROMANIA**

A.E.T.E 2007

Data collected by
Dr. Zamfirescu Stela

Total number of approved E.T. teams in the country	2
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	22	B/A=	9.8
	Embryos collected	**B**	215	C/A=	4.7
	Embryos transferable	**C**	104	C/B=	48.4%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	104	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	25		
In vivo	Frozen	**I**	16		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	41	H+I+J+K=	
Number of frozen stored embryos		**M**	74		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	39.0	(I+K)/L=	%

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	17
Number of calves born from *in vitro* embryos	
Total	17

TABLE 18: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **SPAIN** ***A.E.T.E 2007***

Data collected by
Dr. De la Fuente Julio

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	339	B/A=	11.9
	Embryos collected	**B**	4040	C/A=	5.4
	Embryos transferable	**C**	1830	C/B=	45.3%
In vitro	Nb of oocyte donors		3		
(OPU)	Nb of OPU sessions		10		
	Nb of transferable embryos	**D**	10		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	10	=(D+E)	
Total number of transferable embryos		**G**	1840	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	375		
In vivo	Frozen	**I**	882	70.2% frozen	
In vitro	Fresh	**J**	9		
In vitro	Frozen	**K**	1	10.0% frozen	
Total embryos transferred		**L**	1267	H+I+J+K=	
Number of frozen stored embryos		**M**	975		
% of *in vitro* embryos transferred		**N**	0.8%	(J+K)/L=	
% of frozen embryos transferred		**O**	69.7%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	446
Number of calves born from *in vitro* embryos	2
Total	448

TABLE 19: EMBRYO TRANSFER ACTIVITY IN 2006

COUNTRY: **SWITZERLAND** ***A.E.T.E 2007***

Data collected by
Dr. Saner Rainer

Total number of approved E.T. teams in the country	6
Number of teams providing data	4

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	321	B/A=	11.7
	Embryos collected	**B**	3751	C/A=	7.7
	Embryos transferable	**C**	2467	C/B=	65.8%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	2467	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	532		
In vivo	Frozen	**I**	1427	72.8% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	1		
Total embryos transferred		**L**	1960	H+I+J+K=	
Number of frozen stored embryos		**M**	1633		
% of *in vitro* embryos transferred		**N**	0.1%	(J+K)/L=	
% of frozen embryos transferred		**O**	72.8%	(I+K)/L=	

Number of E.T. calves born (2006)

Number of calves born from superovulated embryos	1400
Number of calves born from *in vitro* embryos	1
Total	1401

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2006

I. EMBRYO PRODUCTION

(Data collected from 19 countries)

In vivo* produced embryos (superovulation)	
- number of flushed donors	15,579
- number of transferable embryos	92,915
- mean number per flushed donor	5.96
***In vitro* produced embryos:**	
From OPU	
- number of OPU sessions	3,828
- number of transferable embryos	7,734
- mean number per session	2.02
From slaughterhouse collected ovaries	
- number of transferable embryos	6,208
Total *in vitro*	13,942
Total number of transferable embryos	106,857

* Data from one country not available and not included.

(S. Merton, AETE, Alghero, Sardinia, Italy 2007)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2006

II. EMBRYO TRANSFERS

(Data collected from 19 countries)

***In* vivo produced embryos ***	**Number of embryos transferred** 83,936 (35,796 fresh / 48,140 frozen)
***In vitro* produced embryos**	6,845 (2,763 fresh / 4,082 frozen)
Total number of embryos transferred	95,030
Proportion of IVF embryos transferred	7.2%
Proportion of frozen embryos transferred	57.5%

***** Data from one country not available and not included.

(S. Merton, AETE, Alghero, Sardinia, Italy 2007)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2006

Species	Embryo Production	Embryo Transfers	Countries
Sheep	161	196	Greece Denmark Hungary Romania
Swine	296		Czech Republic
Goat	110	105	Romania
Cervidae	33	33	Austria
Horse	428* (incl. 17 IVP)	884 (incl. 12 IVP)	Austria Czech Republic Finland France Hungary Italy Netherlands Switzerland

* Data from one country not available and not included.

(S. Merton, AETE, Alghero, Sardinia, Italy 2007)

INVITED LECTURES

FROM RETRIEVAL TO BLASTOCYST CULTURE: FOLLOWING THE INDIVIDUAL OOCYTE

BOLS P.E.J., GOOVAERTS I.G.F., LEROY J.L.M.R.

Gamete Research Center, Laboratory for Veterinary Physiology,
University of Antwerp, Department of Veterinary Sciences,
Universiteitsplein 1, Gebouw U, B-2610, Wilrijk, Belgium
Peter.bols@ua.ac.be

Introduction

For several decades now, puncture of immature ovarian follicles has been used to retrieve oocytes for *in vitro* production of bovine embryo's (IVP). Various comprehensive updates reviewing IVP and embryo transfer in domestic animals indicate that the availability of 'good' quality oocytes is the pre-requisite for success (Hasler *et al*. 1998; Galli *et al*. 2001; Merton *et al*., 2003). Oocyte retrieval can be accomplished from different sources: post-mortem oocyte recovery from slaughterhouse ovaries as well as (repeated) transvaginal ultrasound-guided follicle aspiration (OPU) in living donors. Many important factors play a role in determining the final intrinsic developmental capacity and quality of the retrieved oocytes. Most of these are donor-related, as extensively reviewed by Merton *et al*. (2003): stage of the estrous cycle at the time of oocyte retrieval, follicular status in relation to oocyte growth and final maturation, donor condition and breed, the use of hormonal stimulation prior to OPU and the number of oocyte collections within a specific time span. These can all be considered as being 'biological factors' that can act separately or in combination for a specific donor. On the other hand, there is a group of more 'technical factors' (Bols, 2005) that are related to the retrieval procedure: adequate follicle visualization, needles and aspiration vacuum being used and cumulus oocyte complex (COC) processing before IVP.

Irrespective of which biological factors play the crucial role for a certain donor, there is a general agreement that the importance of an intact cumulus cell investment for oocyte maturation and *in vitro* development cannot be overestimated (Zhang *et al.*, 1995; Konishi *et al*., 1996, Boni *et al*., 2002). Because the method of retrieval clearly has an impact on COC morphology, and therefore also on subsequent developmental capacity *in vitro* (Takagi *et al.*, 1992; Hamano *et al*., 1993; Bols *et al.*, 1996; Bols *et al*., 1997), technical factors will always play a role, being inherently related to the retrieval procedure used.

Given this important role of the cumulus cells (for review see Tanghe *et al.*, 2002), immature bovine oocytes have since long been divided into different quality categories, based upon light microscopic evaluation of the compactness of the cumulus cell layers and transparency of the cytoplasm (de Loos *et al*., 1988; Hazeleger *et al.*, 1995). After all, it has been sufficiently documented that IVP using oocytes with an incomplete or damaged cumulus investment shows a significantly lower blastocyst outcome, compared to culture of COCs with a complete, dense cumulus, giving the highest cleavage and embryo production rates (Shiowa *et al*., 1988; Merton *et al*., 2003). Close contact between cumulus cells and the ooplasm is established through cumulus cell process endings (CCPEs), which penetrate the cortex and make gap junctions with the oolemma in the best quality category oocytes (de Loos *et al*., 1991), whereas this is not the case in low quality COCs. On the other hand, assessment of cumulus cell layer quality through morphological characteristics, has to be used with great care. Blondin and Sirard (1995) showed that oocytes with signs of beginning expansion in the outer cumulus cell layers and a slightly granulated ooplasm developed past the 16-cell stage significantly more than others. Boni and colleagues showed higher embryo

production efficiency with grade B (dark and compact cumulus and dark cytoplasm) COC quality (Boni *et al*., 2002).

Once the oocytes are retrieved and grouped for maturation, the immediate link between the individual oocyte and its specific follicular environment and physiological history is lost. Although this link with the originating follicle is undoubtedly extremely important for further oocyte development (Vassena *et al*., 2003), the only non-invasive quality and selection parameter left at this stage is indeed COC morphology. Tracking individual oocytes and studying factors that influence their quality from this point on necessitates two important things: individual or 'single' oocyte culture conditions to follow oocytes 'over time' and non-invasive (immature) oocyte quality assessment techniques to follow oocytes 'quality-wise'. Because in all routine IVP systems, group culture of COCs is a prerequisite to achieve acceptable blastocyst rates, current protocols do not permit to trace back the individual oocyte to the follicle it originates from. With all due respect for the excellent work of many researchers worldwide, using invasive technology to unravel factors determining oocyte quality (beyond the scope of this paper, but excellently reviewed by Krisher, 2004), there is an urgent need for additional non-invasive quality assessment procedures. Ideally, individual oocytes should survive quality assessment and be re-introduced into the IVP system to develop into a blastocyst and thereby eventually confirm the outcome of the previous quality assessment.

This paper offers a brief summary on the development of 'single oocyte' culture protocols, including some of our own research results. In addition, it reviews some of the scarce non-invasive oocyte quality assessment techniques currently available.

Developing a routine single oocyte culture system

The availability of a routine single oocyte culture system can serve many purposes. From a scientific point of view, tracing individual oocytes with a specific known background will definitely contribute to our knowledge on folliculo- and oogenesis, oocyte/embryo metabolism and gene-expression (Carolan *et al.*, 1996). Separate culture conditions are preferable to avoid conglomeration of oocytes of which the zona pellucida is removed (Vajta *et al.*, 2000), as well as to culture oocytes and embryos resulting from labor-intensive manipulative methods like nuclear transfer, where individual identification of oocytes is highly preferred (Mizushima and Fukui, 2001). As stated above, an *in vitro* system for growing embryos individually can be very useful to test the efficacy of non-invasive oocyte quality parameters in a proper way, because the individual oocyte can be followed from follicle until the blastocyst stage (Oyamada and Fukui, 2004). But also for commercial reasons, a routine and reliable single oocyte culture system has its benefits. Often, only one or a few oocytes can be obtained from a specific donor following ovum pick-up (Ward *et al.*, 2000; Bols, 2005), while these donors are of high genetic value or belong to an endangered species, in which case theoretically every oocyte represents a chance on a blastocyst (Carolan *et al.*, 1996; O'Doherty *et al.*, 1997).

Although there are several reasons to grow embryos individually, only a few research groups used single oocyte culture as a tool to link follicle environment and cumulus-oocyte complex characteristics to the developmental competence of the oocyte (Araki *et al.*, 1998; Hagemann *et al.*, 1999; Jewgenow *et al.*, 1999; Han *et al.*, 2006). It is a well documented observation that animal oocytes and embryos promote each other when they are cultured in group during *in vitro* development. Many research groups report low blastocyst rates following individual embryo production or even a complete failure to produce embryos (Paria and Dey, 1990; Ferry *et al.*, 1994; Donnay *et al.*, 1997; O'Doherty *et al.*, 1997; Hendriksen *et*

al., 1999; Jewgenow *et al.*, 1999; Fujita *et al.*, 2006). In addition, others found single cultured embryos with a lower cell number (Pereira *et al.*, 2005), a relative smaller inner cell mass (Ahern and Gardner, 1998), low hatching rates (Carolan *et al.*, 1996; Hendriksen *et al.*, 1999; Larson and Kubisch, 1999; Yuan *et al.*, 2000; Goovaerts *et al.*, 2007) and low tolerance to cryopreservation (Pereira *et al.*, 2005). Only a few groups achieved similar or even higher blastocyst rates following single compared to group culture (Hazeleger *et al.*, 1995; Carolan *et al.*, 1996; Vajta *et al.*, 2000; Han *et al.*, 2006), although the embryo quality was not always satisfactory.

In most domestic species, embryo development in the female tract occurs individually or at least separately, while this is obviously not the ideal condition *in vitro*. When oocytes and embryos are cultured in groups, they stimulate each other through paracrine secretions. These important growth factors that enhance *in vitro* development include, most probably among many others, insulin like growth factor I and II (IGF-I, IGF-II), transforming growth factor α and β (TGF-α, TGF-β), interferon τ (IFN-τ), epidermal growth factor (EGF), platelet-activated factor (PAF) and platelet derived growth factor (PDGF) (Paria and Dey, 1990; Thibodeaux *et al.*, 1995, Lim and Hansel, 1996; O'Neill, 1997). To mimic the *in vivo* situation, where the embryo is surrounded by small amounts of reproductive tract secretions, wherein growth factors are available in high concentrations, Vajta *et al.* (2000) constructed the 'well of the well' system (WOW) for *in vitro* culture. At the bottom of a regular 4-well plate, 10 to 15 V-shaped smaller wells were melted with a polished steel rod. The 4 large wells were filled with 500 µl of culture medium and covered with oil. In each V-shaped well a zygote, with or without the zona pellucida, was cultured. The volume of each V-shaped well was only 0.04 µl, which limits the dilution of autocrine factors, while the high amount of medium above the small wells provides nutrients and dilutes toxic metabolites. This culture system resulted in higher blastocyst rates than conventional group culture or single culture in droplets of 20 µl and the blastocyst cell number equalled the one of group culture embryos (Vajta *et al.*, 2000). Despite these good results, this system has not been used routinely afterwards. While Han *et al.* (2006) developed a well-in-drop (WID) culture system for single goat oocytes, other groups (Dode *et al.*, 2003; Pereira *et al.*, 2005) modified the WOW system slightly by covering each individual small well with 20 µl medium and using co-culture with cumulus cells at a high O_2 atmosphere. They didn't find higher blastocyst rates compared to an individual 20 µl regular droplet culture system, but the cell number of modified WOW cultured embryos was higher.

This brings up the debate again whether or not co-culture systems with somatic cells should be used in IVP systems. Despite the fact that the use of co-culture undoubtedly increases the amount of non-defined factors in the production system, the added cells can support embryo development and improve embryo quality, by the secretion of embryotrophic factors and by neutralizing embryo toxic components (de Wit and Kruip, 2001; for a recent review, see Orsi and Reischl, 2007). Individual culture was enhanced with co-culture of Buffalo rat liver cells (BRL) (Donnay *et al.*, 1997), cumulus cells (Donnay *et al.*, 1997) or granulosa cells (O'Doherty *et al.*, 1997). Recently, our group tested the effect of cumulus cell (CC) co-culture on group or single embryo culture (Goovaerts *et al.*, 2007). Therefore, cumulus oocytes complexes (COCs) from slaughterhouse ovaries were routinely matured and fertilized in groups and then assigned to 4 culture treatments: group or individual culture, with or without the addition of CC. Co-culture with CC was significantly ($p<0.001$) beneficial to blastocyst rates in single culture conditions, but not in group culture conditions. More embryos hatched when cultured in group than when cultured singly in the presence of CC. In conclusion, adding CC to culture medium only improves blastocyst rates of singly

cultured zygotes. Embryo quality is still under investigation. The results are summarized in Table 1.

Table 1. Cleavage, blastocyst and hatching rates after group and individual bovine in vitro embryo culture with or without cumulus cell co-culture (Goovaerts *et al*., 2007).

Culture Condition	Number of oocytes	Cleavage on day 3 (%)	Blastocysts on day 8 (%)	Hatched on day 10 (% of blastocysts on day 10)
Group – cumulus	189	144 (76.2)	58 (30.7)	50 (80.6)
Group + cumulus	187	137 (73.3)	46 (24.6)	34 (60.7)
Singly – cumulus	345	221 (64.1)	10 (2.9)	1 (8.3)
Singly + cumulus	307	188 (61.2)	67 (21.8)	35 (45.5)

An alternative way, though equally undefined, to achieve paracrine support and stimulation of singly cultured oocytes, is using conditioned media, in which cumulus-oocyte complexes or somatic cells were previously incubated. While the use of these conditioned media seemed not to be beneficial according to Hagemann *et al.* (1998), conditioned medium, derived from embryo culture in group, increased the blastocyst formation of individual cultured embryos for Fujita *et al.*, 2006.

In addition to conditioned media, several regular culture media, supplemented with a variety of additional growth factors, are tested during the different steps of (single embryo) *in vitro* production. While a detailed description is beyond the scope of this brief summary, one of the most discussed topics is the use of serum. According to Carolan *et al.* (1996), the use of serum during individual maturation is detrimental, while others didn't find this negative effect (Hagemann *et al.*, 1998). When cultured in serum free medium, a positive effect of the addition of amino acids and vitamins on the separate development of flushed sheep zygotes was found (Gardner *et al.*, 1994). Li *et al.* (2006) could enhance single bovine embryo development by adding amino acids, at concentrations found in the female reproductive tract, to a chemically defined culture medium.

Furthermore, the effect of the addition of several, more defined supplements was tested. Mizushima and Fukui (2001) added hypotaurine and β-mercaptoethanol to the maturation medium which resulted in significantly higher rates of normal fertilization and a lower rate of polyspermy compared to the supplementation of hypotaurine alone. Adding either β-mercaptoethanol or both showed no significant difference in cleavage, but improved blastocyst development compared to culture without additions. The presence of a high glucose concentration seemed to be beneficial for IVM under low oxygen tension, while the addition of EGF and/or cysteamine to the maturation medium improved fertilizability, developmental competence and cryoresistance (Oyamada and Fukui, 2004). This group also showed that the addition of EGF to a group-IVP system did not affect the blastocyst rate, whereas EGF addition clearly increased the number of blastocysts in the individual-IVP system (Oyamada *et al.*, 2004). The addition of gluthatione during single oocyte IVF

significantly increased the proportion of normal fertilization and decreased polyspermy (Fukui *et al*., 2000). Lim and Hansel (1996) demonstrated the positive effects of the addition of platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF $\beta_1\beta_2$) on the developmental capacity of singly cultured 8-cell embryos. Arachidonic acid, β-mercaptoethanol and glutathione stimulated the further development of 8-cell embryos in the presence of PDGF and TGF $\beta_1\beta_2$.

Apart from the more 'biological' influencing parameters discussed briefly above, some technical factors seem to influence the success rates of single oocyte culture as well. The ideal droplet size and medium replacement schedule, which is crucial to create equilibrium between the dilution of autocrine factors, the accumulation of toxic metabolites and the availability of nutrients during 7 days of culture, was tested by Fujita *et al.* (2006). They showed that medium replacement on day 5 or 6 yielded better results than a change on day 2 to 4 of culture. In addition, embryo development was shown to be compromised in droplet sizes smaller than 10 µl (Carolan *et al.*, 1996). An additional aspect is the possible influence of the gas atmosphere during development, which is linked to the embryo metabolism (Krisher, 2004) and the possible production of reactive oxygen species (ROS). Hagemann *et al.* (1998) didn't find a significant difference in developmental rates when using 7% or 20% O_2 during maturation or fertilization of single bovine oocytes, while according to Oyamada *et al.* (2004) cleavage and blastocyst rates were significantly higher following maturation at 20% O_2 compared to 5% O_2. Individual pig embryo development was improved in an atmosphere of 5% O_2 and 5% CO_2 (Berthelot and Terqui, 1996).

It is clear that individual culture of (bovine) oocytes is compromised, when compared to group conditions. To overcome low blastocyst rates and impaired embryo quality, many modifications on the regular *in vitro* production protocols are tested, with variable success. The method of choice, if it exists, depends on the indication for use of the single oocyte culture system. A chemically defined medium without co-culture, is most likely essential to study oocyte and embryo metabolism. If single oocyte culture systems are used in a commercial set up, where the aim is to produce the highest possible number of embryos with a very low amount of oocytes, the use of serum and co-culture systems can be beneficial. The more methods are tested to obtain an efficient single oocyte culture system, the more insights on the specific needs of a single oocyte or embryo will be generated.

The need for routine, non-invasive oocyte quality parameters

As stated above, the ideal single oocyte culture protocol should permit 'build in' oocyte quality assessment by which the oocyte is subjected to quality tests and immediately returned into, or better, not even removed from the IVP system. If this can be performed on a single oocyte, the retrieved information can be traced back to the oocyte's origin and projected to the (blastocyst) outcome of the IVP system.

However, alternative non-invasive oocyte quality assessment parameters need to meet several specific criteria in order to be usable in routine IVP systems. The major goal in a commercial IVP set up would be to sort out those oocytes with the highest developmental potential, preferentially before maturation. Thus, oocytes with a lower developmental potential can be discarded from the production system. This should benefit the developmental chances of the remaining oocytes and in the mean time, the economics of the procedure. The ideal quality parameters and/or techniques should therefore be simple to perform, cheap, quick, allow a high throughput of COCs, have an acceptable reliability and above all, be non-invasive so that the oocyte's developmental capacity is not hampered following quality assessment.

As stated earlier, although very valuable (Fair, 2003), keeping the link between the oocyte and its originating follicle is not an option in a commercial IVP environment. When looking at a cumulus oocyte complex through a binocular upon retrieval, bovine immature oocytes for IVP are usually selected on the basis of the cumulus investment morphology and the homogeneity of the cytoplasm (de Loos *et al*., 1988; Hazeleger *et al*., 1995). In the mean time, many studies have tried to link these morphological aspects to the oocyte's developmental capacity, with variable success (Bols, 2005). Indeed, even with the best quality COCs, on the basis of their morphology, we can only grow around 35-45% of blastocyst *in vitro*. This fact strongly suggests that there is a need for additional oocyte quality parameters. The use of a vital blue dye, brilliant cresyl blue (BCB), to select oocytes suitable for IVP is more and more documented in literature. One of the first reports describes the use of BCB for the selection of pig oocytes for IVM and IVF (Ericsson *et al*., 1993). Apparently, BCB can be a measure for the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that is synthesized within the oocyte during oogenesis as a part of the pentose phosphate cycle (Alm *et al*., 2005). It is particularly active in growing oocytes with a clear decrease in activity when the oocytes have finished their growth phase (Mangia and Epstein, 1975). BCB is a blue dye that is reduced to a colorless substance through the action of G6PD. Full grown oocytes show a decreased G6PD activity, meaning that their cytoplasm will remain blue, following the uptake of BCB. This specific characteristic makes BCB an indicator for G6PD activity and therefore indirectly indicates the growth stage of the oocyte. Rodríguez-González et al., (2002) used BCB to select more competent prepuberal goat oocytes for *in vitro* embryo production. They exposed oocytes to BCB diluted in PBS and subsequently classified them according to the color of their cytoplasm: oocytes with a blue cytoplasm or grown oocytes (BCB+) and oocytes with a colorless cytoplasm or growing oocytes (BCB-). They showed that BCB+ oocytes were significantly larger that BCB- oocytes, while a higher proportion of the BCB+ oocytes reached the MII stage compared to BCB- and non-treated oocytes. Several reports stress the importance of oocyte diameter in relation to the developmental competence (Lonergan *et al.*, 1994; Fair *et al*., 1995, for review see Fair, 2003). In addition, the proportion of embryos developing beyond the 8-cell stage and the number of morula plus blastocysts was higher for the BCB+ oocytes. Surprisingly, around 50% out of the group of oocytes graded as being the best quality, on the basis of routine morphological selection criteria, appeared to be BCB-. In other words, these oocytes have passed first selection on morphology, but should be excluded from IVP on the basis of the BCB test. Similarly, Pujol and co-workers (2004) used the BCB test to assess the developmental competence of heifer oocytes. While using a slightly modified oocyte quality grading system, they showed that nearly 79% of the morphologically classified grade 1 oocytes were BCB+ and confirmed that BCB+ oocytes were larger and more competent for IVP than control heifer oocytes. Alm *et al*. (2005) used the BCB test to increase the efficiency of *in vitro* blastocyst production by oocyte selection before maturation. They reported a significantly higher rate of maturation to metaphase II for control and BCB+ oocytes compared to BCB- oocytes. Interestingly, they only used COCs with a compact cumulus investment, retrieved by slicing slaughterhouse ovaries. This means that they in fact combined morphological characteristics and the BCB test in their oocyte selection protocol. About 58% of the oocytes from COCs thus retrieved turned out to be BCB+, which is on the low side when compared to 75.6% of BCB+ oocytes in pigs reported by Roca *et al*. (1998). The authors attribute this lower percentage of BCB+ oocytes to their oocyte retrieval technique, because slicing most probably leads to the collection of more growing oocytes, collected from very small follicles. Although the double COC screening resulted in a remarkable increase in the number of blastocysts from IVP with BCB+ oocytes,

compared to controls (34.1% vs 19.2% respectively), the final absolute number of blastocyst did not increase compared to other reports in literature, indicating the underlying importance of the oocyte retrieval technique (slicing vs aspiration) and the IVP culture system. Finally, the authors report no alterations in fertilization and cleavage rates following the use of BCB, which is a reassuring thought when considering the BCB test for a routine application. Additional recent research reports substantiate the value of the BCB test for the selection of developmentally competent oocytes (Sugulle *et al*., 2006) related to bovine nuclear transfer procedures (Bhojwani *et al*., 2007) and with COCs collected by OPU (Tagawa *et al*., 2006).

Finally, as stated earlier, an enormous amount of knowledge has been generated by the study of the interplay between the follicle as a whole, the cumulus cells in particular and the enclosed oocyte (Tanghe *et al*., 2002; Fair 2003). To go into detail is far beyond the scope of this paper. However, an interesting non-invasive approach to oocyte quality assessment is offered by the idea that knowledge on cumulus cells might tell us something about the developmental potential of the oocyte involved. Like for oocytes, we can distinguish morphological (non invasive) as well as biochemical and cytological (invasive) parameters. Whereas the first can be applied on a routine basis (Laurinčik *et al*., 1996), the latter most often involve a biopsy of the cumulus cells, to be non-invasive to the COC as a whole. While this procedure does not necessarily impair the COC developmental competence, it is very laborious and not applicable to a routine IVP system. Moreover, while it is still uncertain if cumulus cell characteristics are good predictors for the oocytes' developmental competence (Han *et al*., 2006; Anguita *et al*., 2007), the developmental stage of these cumulus cells and the species studied will play an important role (McKenzie *et al*., 2004).

Conclusion

The amount of knowledge on factors that determine the oocytes' *in vitro* developmental competence is increasing at an enormous speed. Looking specifically at bovine routine *in vitro* embryo production systems, which use immature oocytes, group culture is still a prerequisite to achieve acceptable blastocyst rates. As a consequence, it is still impossible to follow an individual oocyte through the IVP procedure and draw conclusions on the developmental capacity of the initial cumulus oocyte complex. Even if single oocyte culture would become possible in the (near) future, studying the factors that determine the oocytes' developmental ability would still be hampered because most of the competence studies include parameters which can only be investigated by sacrificing the oocyte. Only by combining research efforts on the development of single oocyte culture protocols and a search for additional non-invasive oocyte quality assessment techniques, it will be possible to trace individual oocytes through the IVP system and draw direct conclusions on factors that control oocyte developmental potential *in vitro*.

Once a routine single oocyte culture system is put in place, the combination with non-invasive oocyte and embryo quality assessment parameters will be a powerful tool to link the oocytes' follicular history to their developmental capacity. Hopefully this leads to a better selection of oocytes for *in vitro* production with less expensive, yet more and better quality embryos as a result.

References

Ahern TJ, Gardner DK. Culturing bovine embryos in groups stimulates blastocyst development and cell allocation to the inner cell mass. Theriogenology 1998;49:194.

Alm H, Torner H, Löhrke B, Viergutz T, Ghoneim IM, Kanitz W. Bovine blastocyst development rate in vitro is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. Theriogenology 2005; 63:2194-2205.

Anguita B, Vandaele L, Mateusen B, Maes D, Van Soom A. Developmental competence of bovine oocytes is not related to apoptosis incidence in oocytes, cumulus cells and blastocysts. Theriogenology 2007;67:537-549.

Araki N, Sato K, Hayashi K, Miyamoto A, Fukui Y. Relationships among follicular fluid estradiol-17 β concentration, morphology of cumulus-oocyte complex and developmental capacity of individual matured, fertilized and cultured bovine embryos *in vitro*. Journal of Reproduction and Development 1998;44:359-365.

Berthelot F, Terqui M. Effects of oxygen, CO_2/pH and medium on the *in vitro* development of individually cultured porcine one- and two-cell embryos. Reproduction Nutrition and Development 1996:36:241-251.

Bhojwani S, Alm H, Torner H, Kanitz W, Poehland R. Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. Theriogenology 2007;67:341-345.

Blondin P, Sirard MA. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 1995;41:54-62.

Bols PEJ, Van Soom A, Ysebaert MT, Vandenheede JMM, de Kruif A. Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. Theriogenology 1996;45:1001-1014.

Bols PEJ, Ysebaert MT, Van Soom A, de Kruif A. Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. Theriogenology 1997;47:1221-1236.

Bols PEJ. Puncture of immature ovarian follicles in bovine assisted reproduction. Verhandelingen 2005, LXVII, 3:177-02.

Boni R, Cuomo A, Tosti E. Developmental potential in bovine oocytes is related to cumulus-oocyte complex grade, calcium current activity, and calcium stores. Biology of Reproduction 2002, 66: 836-842.

Carolan C, Lonergan P, Khatir H, Mermillod P. *In vitro* production of bovine embryos using individual oocytes. Molecular Reproduction and Development 1996;45: 145-150.

de Loos F, Van Vliet C, Van Maurik P, Kruip ThAM. Morphology of immature bovine oocytes. Gamete Research, 1989;24:197-204.

de Loos FAM, Bevers MM, Dieleman SJ, Kruip ThAM. Morphology of preovulatory bovine follicles as related to oocyte maturation. Theriogenology 1991;35:527-535.

de Wit AAC, Kruip ThAM. Bovine cumulus-oocyte-complex-quality is reflected in sensitivity for α-amanitin, oocyte-diameter and developmental capacity. Animal Reproduction Science 2001;65:51-65.

Dode MAN, Pereira DC, Rumpf R. (2003). Evaluation of different methods for *in vitro* culture of a single bovine embryo. Theriogenology 2003;59:340.

Donnay I, Van Langendonckt A, Auquier P, Grisart B, Vansteenbrugge A, Massip A, Dessy F. Effects of co-culture and embryo number on the *in vitro* development of bovine embryos. Theriogenology 1997;47:1549-1561.

Ericsson SA, Boice ML, Funahashi H, Day BN. Assessment of porcine oocytes using brilliant cresyl blue. Theriogenology 1993;39:214.
Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Molecular Reproduction and Development 1995;42:437-442.
Fair T. Follicular oocyte growth and acquisition of developmental competence. Animal Reproduction Science 2003;78:203-216.
Ferry L, Mermillod P, Massip A, Dessy F. Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. Theriogenology 1994;42:445-453.
Fujita T, Umeki H, Shimura H, Kugumiya K, Shiga K. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. Journal of Reproduction and Development 2006;52:137-142.
Fukui Y, Kikuchi Y, Kondo H, Mizushima S. Fertilizability and developmental capacity of individually cultured bovine oocytes. Theriogenology 2000;53:1553-1565.
Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G. Embryo production by ovum pick up from live donors. Theriogenology 2001;55:1341-1357.
Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biology of Reproduction 1994;50:390-400.
Goovaerts IGF, Leroy JMLR, De Clercq JPB, Andries S, Bols PEJ. Effect of cumulus cell coculture on developmental competence during group or single bovine embryo culture *in vitro*. Reproduction of Domestic Animals 2007, in press (abstract).
Hagemann LJ, Weilert LL, Beaumont SE, Tervit HR. Development of bovine embryos in single *in vitro* production (sIVP) systems. Molecular Reproduction and Development 1998;51:143-147.
Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, Peterson AJ, Schurmann A, Tervit HR. Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. Molecular Reproduction and Development 1999:53:451-458.
Hamano S, Kuwayama M. In vitro fertilization and development of bovine oocytes recovered from the ovaries of individual donors: a comparison between the cutting and aspiration method. Theriogenology 1993;39:703-712.
Han Z, Lan G, Wu Y, Han D, Feng W, Wang J, Tan J. Interactive effects of granulosa cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction 2006;132:749-758.
Hasler JF. The current status of oocyte recovery, in vitro embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. Journal of Animal Science 1998,76 (3 Suppl):52-74.
Hazeleger NL, Hill DJ, Stubbings RB, Walton JS. Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential in vitro. Theriogenology 1995;43:509-522.
Hendriksen PJM, Bevers MM, Dieleman SJ. Single IVP using BRL cell co-culture and serum yields a lower blastocyst rate than a group culture. Theriogenology 1999;51:319.
Jewgenow K, Heerdegen B, Müller K. In vitro development of individually matured bovine oocytes in relation to follicular wall atresia. Theriogenology 1999;51:745-756.
Konishi M, Aoyagi Y, Takedomi T, Itakura H, Itoh T, Yazawa S. Presence of granulosa cells during oocyte maturation improved in vitro development of IVM-IVF bovine oocytes that

were collected by ultrasound-guided transvaginal aspiration. Theriogenology 1996;45:573-581.
Krisher RL. The effect of oocyte quality on development. Journal of Animal Science 2004; 82(E. Supl.):E14-E23.
Larson MA, Kubisch HM. The effects of group size on development and interferon-τ secretion by in-vitro fertilized and cultured bovine blastocysts. Human Reproduction 1999;14:2075-2079.
Laurinčik J, Hyttel P, Baran V, Schmoll F, Niemann H, Brem G, Schellander K. Corona radiata density as a non-invasive marker of bovine cumulus-corona-oocyte complexes selected for in vitro embryo production. Theriogenology 1996;46:369-377.
Li R, Wen L, Wang S, Bou S. Development, freezability and amino acid consumption of bovine embryos cultured in synthetic oviductal fluid (SOF) medium containing amino acids at oviductal or uterine-fluid concentrations. Theriogenology 2006;66: 404-414.
Lim JM, Hansel W. Roles of growth factors in the development of bovine embryos fertilized *in vitro* and cultured singly in a defined medium. Reproduction, Fertility and Development 1996;8:1199-1205.
Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture in vitro. Molecular Reproduction and Development 1994;37:48-53.
Mangia F, Epstein CJ. Biochemical studies of growing mouse oocytes: preparation of oocytes and analysis of glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. Developmental Biology 1975;45:211-220.
McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in woman undergoing IVF. Human Reproduction 2004;19:2869-2874.
Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003;59:651-674.
Mizushima S, Fukui Y. Fertilizability and developmental capacity of bovine oocytes cultured individually in a chemically defined maturation medium. Theriogenology 2001;55:1431-1445.
O'Doherty EM, Wade MG, Hill JL, Boland MP. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. Theriogenology 1997; 48, 161-169.
O'Neill C. (1997). Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos *in vitro*. Biology of Reproduction 1997;56:229-237.
Orsi N.M., Reischl J.B. (2007). Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. Theriogenology 2007;67:441-458.
Oyamada T, Fukui Y. Oxygen tension and medium supplements for *in vitro* maturation of bovine oocytes cultured individually in a chemically defined medium. Journal of Reproduction and Development 2004;50(1):107-117.
Oyamada T, Iwayama H, Fukui Y. Additional effect of epidermal growth factor during in vitro maturation for individual bovine oocytes using a chemically defined medium. Zygote 2004;12:143-150.
Paria BC, Dey SK. Preimplantation embryo development *in vitro* : cooperative interactions among embryos and role of growth factors. Proceedings of the National Academy of Sciences of the United States of America 1990;87:4756-4760.
Pereira DC, Dode MAN, Rumpf R. Evaluation of different culture systems on the *in vitro* production of bovine embryos. Theriogenology 2005;63:1131-1141.

Pujol M, López-Béjar M, Paramio MT. Developmental competence of heifer oocytes selectyed using the brilliant cresyl blue (BCB) test. Theriogenology 2004;61:735-744.
Roca J, Martinez E, Vazquez JM. Lucas X. Selection of immature pig oocytes for homologous in vitro penetration assays with brilliant cresyl blue test. Reproduction, Fertility and Development 1998;10:479-485.
Rodríguez-González E, López-Béjar M, Velilla E, Paramio MT. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 2002; 57:1397-1409.
Shioya Y, Kuwayama M, Fukushima M, Iwasaki S, Hanada A. In vitro fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured in vitro. Theriogenology 1988;30:489-496.
Sugulle A, Katakawa S, Yamamoto S, Oomori S, Itou I, Dochi O, Koyama H. Selection of bovine oocytes by brilliant cresyl blue before in vitro maturation improves blastocyst development. Reproduction, Fertility and Development 2006;19:273.
Tagawa M, Matoba S, Okada M, Metoki K, Imai K. Developmental competence of oocytes selected by the brilliant cresyl blue staining in prepubertal and adult cattle. Reproduction, Fertility and Development 2006;19:273-274.
Takagi Y, Mori K, Takahashi T, Sugawara S, Masaki J. Differences in development of bovine oocytes recovered by aspiration or by mincing. J Anim Sci 1992;70:1923-1927.
Tanghe S, van Soom A, Nauwynck H, Coryn M, de Kruif A. Mini-review: Functions of the cumulus oophorus during oocyte maturation, ovulation and fertilization. Molecular Reproduction and Development 2002;61:414-424.
Thibodeaux JK, Myers MW, Hansel W. The beneficial effects of incubating bovine embryos in groups are due to platelet-derived growth factor. Theriogenology 1995;43: 336.
Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, Callesen H. (2000). New method for culture of zona-included or zona-free embryos: the well of the well (WOW) system. Molecular Reproduction and Development 2000, 55:256-264.
Vassena R, Adams GP, Mapletoft RJ, Pierson RA, Singh J. Ultrasound image characteristics of ovarian follicles in relation to oocyte competence and follicular status in cattle. Animal Reproduction Science 2003;76:25-41.
Ward FA, Lonergan P, Enright BP, Boland MP. Factors affecting recovery and quality of oocytes for bovine embryo production *in vitro* using ovum pick-up technology. Theriogenology 2000;54:433-446.
Yuan YQ, Van Soom A, Laevens H, Coopman F, Peelman L, de Kruif A. (2000). Single embryo culture affects hatching rate in bovine *in vitro*-produced embryos. Theriogenology 2000;53:307.
Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA. Cumulus cell function during bovine oocyte maturation, fertilization and embryo development in vitro. Molecular Reproduction and Development 1995;40:338-344.

IN VITRO EMBRYO PRODUCTION IN BUFFALO (BUBALUS BUBALIS) SPECIES: POTENTIALS AND LIMITATIONS

GASPARRINI, B.

DISC IZIA, Faculty of Veterinary Medicine, "Federico II"Univeristy, Naples, Italy
Fax: +39 081 292981; e-mail: bgasparr@unina.it

Abstract

In the last few years, there has been an increasing interest in the in vitro embryo production (IVEP) technologies for faster propagation of superior germplasm in buffalo, due to the low efficiency of multiple ovulation (MO) and embryo transfer (ET) programs. Early attempts to produce buffalo embryos in vitro have been made by using procedures that were proven effective in cattle. However, the acquisition of more specific information on oocyte and embryo culture requirements in vitro in this species has resulted in an improved efficiency over the years. Although the IVEP efficiency has enhanced, as indicated by competitive embryo yields, pregnancy rate and development to term are still poor. Furthermore, the optimization of embryo cryopreservation methods in this species is critical for the diffusion of ET procedures in the field. The present review intends to describe the state of the art of IVEP in buffalo species, emphasizing the advances achieved and the limitations still to overcome.

Introduction

In the last few years interest in buffalo breeding has tremendously increased worldwide, due to the critical role that this species plays in many climatically disadvantaged agricultural systems. It is worth pointing out that environmental conditions, especially climate, make the River buffalo an irreplaceable dairy producer for developing countries situated in the tropics north of the equator. In these countries, the perfect interaction between the reproductive seasonality, environment, and forage availability during the year ensures that the buffalo compensates for the lack of bovine milk during the rainy season (winter-spring), and produces animal protein at competitive costs. The crucial role played by this species is evident when the increase in the world buffalo population during the past 40 years is compared to the increase in cattle population (86% vs 34 %, respectively). In Italy, however, the Mediterranean Italian buffalo is no longer considered a species of niche, but rather it is the only livestock species that has shown a growth trend. The Mediterranean Italian buffalo in Italy is an important economic resource due to the high market demand for mozzarella cheese, and its genetics is highly requested around the world due to its high milk production.

Successful buffalo breeding highly depends on the genetic improvement that can be achieved through the application of reproductive biotechnologies. The use of artificial insemination (A.I.) is critical to enhance the paternal contribution to genetic improvement. However, the use of A.I. in buffalo breeding is limited due to the low efficiency of A.I. in this species and to the complications related to seasonality (Campanile et al., 2005). As regards biotechnologies aimed to enhance genetic progress through the maternal contribution, in buffalo, due to the low and inconsistent response to multiple ovulation and embryo transfer (MOET) treatments (Misra et al, 1997; Zicarelli et al, 1997), there has been an increasing interest worldwide in the in vitro embryo production (IVEP) technology.

In addition to the limitations of MOET that have been demonstrated in this species regarding embryo output (Misra et al, 1997; Zicarelli et al, 1997), it is worth emphasizing that Ovum pick-up (OPU) can be performed on a wider typology of donors such as non-cyclic animals, pregnant cows, subjects with patent oviducts or genital tract infections, and animals that are not responsive to hormonal stimulation, the last representing a high proportion in buffalo. OPU has been successfully carried out in buffalo by Boni et al. (1996), and IVEP efficiency has greatly improved throughout the years (Neglia et al., 2003). The combined OPU and IVEP technology is currently the most promising tool for increasing the number of transferable embryos (TE) obtainable per donor over the long term in most species. In buffalo, this technology is even more competitive in terms of embryo yields compared to MOET: 11 TE were produced on average in a 6 month trial vs 5 TE theoretically obtainable with MOET (Gasparrini, 2002). It should be specified that the number of TE theoretically obtained by MOET was overestimated since multiple ovulations can be induced only in cyclic animals, and it is unlikely that buffalo cows are cyclic for 6 months because they tend to go into seasonal anoestrus. In addition, if donors are selected on the basis of their folliculogenetic potentials, the limitation due to the high variability of follicular recruitment, oocyte retrieval, and hence, blastocyst production (1-37 TE in 6 months, Gasparrini, 2002), can be overcome, thus resulting in a further increase of the embryo yield. This selection process only needs two weeks since the potential of animals to recruit follicles can successfully be predicted after the first 4 oocyte collection days (2 weeks), and as has been demonstrated, there is a definite correlation between the number of follicles and the number of oocytes and blastocysts (Gasparrini, 2002).

However, although the buffalo IVEP system has been greatly improved over the years, leading to high blastocyst yields (Gasparrini et al. 2006) and to the production of offspring (Neglia et al., 2004; Hufana-Duran et al., 2004; Sà Filho et al., 2005; Huang et al., 2005), this technology is still far from being commercially viable.

The low IVEP efficiency recorded in buffalo compared to cattle is in part due to peculiarities of the reproductive physiology of buffalo that are not easily modifiable, such as the low number of oocytes recruitable and their poor quality. Furthermore, it is worth remembering that scientific improvement in this species has been hampered by contingent factors. The major factor that has delayed scientific advances in buffalo IVEP is the scarcity of experimental material in all the countries in which the species is bred. This is in part related to the small number of buffaloes and a low culling rate compared to cattle. In Italy, for instance, the estimated population of buffaloes is 300.000 versus 7.2 million cattle, and the culling rate is < 15% vs > 35% for cattle. This scarcity is also due to differences in breeding systems, such as small farms scattered over large territories in the majority of the countries with high buffalo population (China, India). In addition, unlike cattle, the reproductive lifespan in buffalo is long (12 years in Italy and even longer in swamp buffalo in China and South East Asia), because, for economic reasons, buffalo cows are usually slaughtered when they are old or when their fertility and productivity are compromised. This results in a further decrease of the number of competent oocytes recoverable in the case of abattoir-derived ovaries as a source of gametes.

The secondary factors in this delay are: 1) the economic importance of the species has only been recently appreciated, and as a consequence, the first studies of advanced reproductive strategies in this species date back only to the 1990s; and 2) the majority of buffaloes is bred in developing countries where scientists often have to deal with poor resources and lack of facilities. The scarcity of experimental material for buffalo, together with the assumption that the reproductive biology in all ruminants is similar, led in the early attempts, to use the IVEP system in buffalo based solely on information acquired in cattle,

with the consequent result of low IVEP efficiency. However, despite these limitations, it has been demonstrated that improvements in IVEP are possible through the optimization of each procedural step; especially when taking into account species-specific differences, as shown by the higher blastocyst rates reported over recent years (Gasparrini et al., 2006).

Oocyte source and quality

The major intrinsic limitation of IVEP technology in buffalo lies in the low number of immature oocytes that can be recovered per donor. In our experience controlled follicular aspiration of abattoir-collected ovaries allows the retrieval of 2.4 good quality oocytes per ovary on average (Gasparrini et al., 2000). The slightly lower oocyte recovery reported in other studies (0.4 by Totey et al., 1992 and Madan et al., 1994; 0.6 by Das et al., 1996; 0.9 by Kumar et al., 1997; 1.8 Samad et al. 1998; 1.9 Mishra et al. 2007a) may be due to differences in breed, older age at slaughter, management and nutritional status (Goswami et al., 1992). The recovery rate is therefore much lower than in cattle in which 10 good quality oocytes are obtained on average per ovary (Gordon, 1994). Similarly, a low number of oocytes is recovered when OPU is performed in buffalo compared to cattle (4.5 vs approximately 10 respectively, Galli et al., 2000). It has also been reported that season affects the oocyte recovery rate in buffalo, with lower numbers of COC recorded during summer (Misra, 2005). However, it is worth noting that the effect of season varies according to latitude, and may be related to either day length and/or temperature. We speculate that the lower recovery found during summer by Indian authors is mainly due to heat stress rather than to day length because at latitudes in Italy the oocyte recovery rate does not change during the year (unpublished data). This limitation is currently the most insurmountable impediment for the diffusion of IVEP in the field, arising from physiological peculiarities of the species, such as the low number of primordial (Danell, 1987) and antral (Kumar et al., 1997) follicles present on the buffalo ovary, as well as the high incidence of follicular atresia (Palta et al., 1998), and as such, it is not easily improvable. In short, biology can be manipulated for human purposes, but only to a limited extent. However, it has been shown that the number of competent oocytes may be increased by selecting donors on the basis of their follicular population (Sà Filho et al., 2005). Interestingly a pretreatment of buffalo donors with BST has been found (Sà Filho et al., 2005) to promote follicular growth (12.2 vs 8.7 total follicles punctured; 9.1 vs 6.5 small follicles), without in parallel increasing the number of oocytes recovered (5.1 vs 4.5). However, these results are not conclusive because the study was only carried out on a limited number of donors and replicates, and it is known that the docility of an animal often affects recovery rate. Hence, it may be worth investigating different concentrations of BST or varying the administration protocol.

Oocyte quality, that is known to affect the IVEP efficiency in most species, plays a determining role in buffalo, further reducing the availability of the oocytes suitable for IVEP. The oocyte morphology can be used, with a certain reliability, to predict the gamete developmental competence; according to our classification, a progressive decrease of efficiency is recorded from Grade A to Grade D oocytes (Neglia et al., 2003), with Grade A and B considered suitable for IVEP. It results that the IVEP efficiency can be higher by only utilizing Grade A oocytes but this is not possible in the majority of the cases because the limited availability of oocytes often imposes the utilization of all the oocytes recruited, including Grade C, since the goal is to produce as many embryos as possible. It is worth pointing out that the percentage of good quality oocytes (Grade A and B), is lower in this species compared to others, not exceeding, in our experience 50 % of the total oocytes recovered. An analysis of the data collected over a 4 year period in our lab showed that from

a total number of 35.286 abattoir-derived oocytes (over 158 replicates) 47.8% were Grade A + B, 6.2 % were Grade C and 46.7 % were unsuitable for IVEP (unpublished data). A recent trial has shown an even lower proportion of good quality oocytes (33.7 % of Grade A+B), together with a higher incidence (37.9 %) of Grade C (Mishra et al, 2007a).

The oocyte quality may be affected by several factors, such as the aspiration pressure during collection, the source of gametes, the time between collection and processing, the temperature during transportation, season, etc. In our experience, the oocyte morphology varies with the source of gametes, with an apparent worse quality of OPU-derived oocytes, characterized by fewer layers of granulosa cells, compared to abattoir-derived ones. Interestingly, despite their worse morphological appearance, OPU-derived oocytes have a higher developmental competence compared to abattoir-derived ones (Neglia et al., 2003). This is because OPU resets the follicular population, subsequently increasing the follicular wave frequency and, as a result, follicles are aspirated before they become atretic and hence oocyte quality is improved. Therefore, we speculate that technical factors during OPU, such as the length of the needle, as well as that of the line connected to the suction unit, may result in a greater loss of granulosa cells (that in this species are not strongly adhered to the oocyte) and, hence in an underestimated evaluation of their quality.

Furthermore, when OPU is carried out in field conditions, with the donors often bred in farms distant from the laboratory, a significant improvement of blastocyst production can be achieved by reducing the time between oocyte collection and their maturation. In these situations, an increased efficiency is recorded with oocytes searched directly in the farm and immediately transferred in a hepes buffered in vitro maturation (IVM) medium in a portable incubator, compared with those searched in the laboratory, after many hours of their permanence in the follicular fluid (Gasparrini, 2006). It was speculated that the better developmental competence of OPU-derived vs abattoir-derived oocytes is related to the shorter exposure to environmental stress. Indeed, oocytes recovered from slaughterhouse ovaries undergo a longer time interval between excision of ovaries from the peritoneal cavity and laboratory processing and are probably affected by cellular damages due to autolytic processes, especially when they reside in excised ovaries for prolonged periods. It follows that, in the latter case, another important factor to consider is the time interval between ovary collection and processing in the laboratory. In our setting the time lapse between collection of ovaries at slaughter and their arrival at the lab usually varies between 3 and 6 hours. A retrospective analysis of data collected over the last 4 years in our lab showed that, although oocyte quality seems improved at the shorter time (3 h compared to 4, 5 and 6 h), as we would expect, as indicated by the higher percentage of grade A and B oocytes together with the lower incidence of degenerated oocytes, cleavage and blastocyst rates were not affected by extending the time interval up to 6 h (unpublished data).

Buffalo oocytes are very sensitive to shock temperature so it is important to monitor the temperature carefully during collection as fluctuations can easily occur. When OPU is carried out temperature is controlled by holding the collection tubes in suitable warm boxes. When abattoir-derived oocytes are utilized for in vitro embryo production, the ovaries are usually kept in physiological saline, under controlled temperature (30-37°C), during collection and transportation to the laboratory. It has been recently observed, that oocyte developmental competence, evaluated in terms of cleavage and blastocyst rates following IVF, is improved by lowering the temperature range during transportation to 25-29.5°C (unpublished data).

Finally, it is known that buffalo is a short-photoperiod species and that the efficiency of reproductive technologies such as A.I. and MOET is significantly affected by season (Campanile et al., 2005). A preliminary analysis of data collected in our IVEP laboratory

over the last 4 years has shown that the oocyte developmental competence is improved during the autumn months, as suggested by better cleavage and blastocyst rates (unpublished data). This finding is in agreement with the seasonality pattern exhibited by the species at our latitudes, with the fertility improved during short-day months. It has been also recently reported (Mishra et al., 2007b) that hot ambient temperature on day of slaughter negatively affects both cleavage (17.6% versus 36.6%, respectively for temperature > and < 40°C) and blastocyst development (0.0% vs 9.8%, respectively for temperature > and < 40°C) following IVF, confirming previous observations in buffalo (Nandi et al., 2001).

In vitro maturation (IVM)

An important requirement for a successful fertilization is undoubtedly the appropriate maturational status of the oocytes at the time they encounter the sperm. The oocyte maturation process, that invests both the nuclear and cytoplasmic compartments, triggered in vivo by the gonadotrophin surge, is imperative for the gamete to acquire full developmental competence. This highlights the importance of identifying optimal conditions for IVM in order to improve embryo development.

Buffalo oocytes can be matured in vitro in complex media**,** such as Tissue Culture Medium 199 (the most widely employed) and Ham's F-10, supplemented with sera, hormones and other additives. Different sources of serum have been utilized as supplements of IVM medium (Totey et al, 1993; Chauhan et al., 1998; Samad et al., 1998; Chuangsoongneon et al, 1991). Although it has been observed that buffalo oocytes can reach the maturation status even in the absence of hormones (Madan et al., 1994), higher maturation and fertilization rates have been recorded when oocytes are matured in the presence of gonadotrophins and 17β-estradiol (Totey et al., 1992; Totey et al., 1993). It is known that hormones interact with receptors located on the follicular cells, and that the signals are transduced into the oocyte through gap junctions or extracellular mechanisms. It results that the presence of cumulus cells is critical for the acquisition of developmental competence during IVM, as confirmed by the significantly reduced cleavage and embryo development of denuded vs cumulus-enclosed oocytes following IVF (Pawshe et al., 1993; Gasparrini et al., 2007a). This aspect is particularly important in buffalo because of the high proportion of totally or partially denuded oocytes usually recovered in this species. In order to rescue germinal material, it has been proposed to provide poor quality oocytes with the somatic support by performing IVM on a cumulus cells monolayer, obtaining improved maturation and fertilization rates (Pawshe et al.,1993).

In order to reduce IVEP costs, buffalo follicular fluid, a waste product of oocyte collection, has been used in replacement of expensive hormones and serum additives, obtaining comparable maturation, fertilization, and blastocysts rates (Chauhan et al, 1997).

The beneficial effects of several ovarian-derived growth factors, such as IGF-l, IGF-2 and insulin on oocyte maturation, fertilization and development to the blastocyst stage (Pawshe et al., 1998) have been also reported in this species. It has also been observed that supplementation of the IVM medium with EGF improves cumulus expansion, nuclear maturation, and cleavage rate of cumulus-enclosed buffalo oocytes without affecting the post-fertilization embryonic development (Chauhan et al., 1999).

Based on the assumptions that buffalo oocytes and embryos, because of their high lipid content (Boni et al., 1992), are particularly sensitive to oxidative damages, the IVM medium has been enriched by thiol compounds, known to act as antioxidants factors, by stimulating glutathione (GSH) synthesis. It is known that GSH plays a critical role in protecting mammalian cells from oxidative stress, that is a major factor affecting in vitro

mammalian embryo development. It has been demonstrated in other species (Telford et al., 1990; Gardiner et al., 1994), that the GSH reservoir formed during IVM is the only source of reducing power for the embryos before genomic activation occurs. It was previously demonstrated that cysteamine supplementation during IVM improves blastocyst yield in buffalo (Gasparrini et al., 2000), by increasing intracytoplasmic glutathione concentration (Gasparrini et al., 2003), without nevertheless affecting cleavage rate. The addition of cystine, in the presence of cysteamine, to the IVM medium (Gasparrini et al., 2006) has further increased the GSH reservoir of the oocytes and has significantly improved the proportion of oocytes showing normal synchronous pronuclei post fertilization (81 %), cleavage rate (78 %) and blastocyst yield (30 %).

Among factors affecting mammalian embryo development in vitro, the duration of IVM plays a critical role, since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First,1997), oocyte aging (Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). Furthermore, although sperm can penetrate oocytes prior to completion of oocyte maturation (Chian et al., 1992), subsequent development is generally reduced. Therefore, it appears that the optimum time for in vitro fertilization (IVF) is at completion of meiosis, that is known to occur at different times in different species, varying from 18-24 h in cattle (Sirard et al., 1989, Neglia et al., 2001) to 36-48 h in pig (Prather and Day, 1998). Although large variations in the timing of oocyte maturation have been reported in buffalo, with the highest proportion of MII oocytes observed between 16 and 24 h ((Neglia et al., 2001; Yadav et al., 1997, Gasparrini et al., 2007b), the majority of the authors inseminate buffalo oocytes in vitro 24 h after the start of IVM. The different oocyte maturation time-scale recorded among buffalo studies may be accounted for by different conditions of IVM and particularly by oocyte quality which, in this species, is also likely affected by seasonal factors. We have recently investigated both the kinetics of oocyte maturation and the influence of the duration of IVM on subsequent embryo development. In this study the attainment of the MII stage has commenced after 18-19 h maturation and the majority of oocytes completed nuclear maturation between 20 and 24 h; furthermore, it has been demonstrated that the duration of IVM affects buffalo oocyte developmental competence, with a progressive decrease of fertilization capability and embryo development as the IVM duration increases from 18 to 30 h (Gasparrini et al., 2007b). Therefore, the optimal time for IVF in buffalo appears to be at 18 h post-IVM or, in any case, not later than 24 h; in fact, delaying IVF over 24 h has resulted in a significant deterioration of oocyte developmental competence that could be predicted by the poor morphological appearance of oocytes matured for prolonged periods. An earlier aging of buffalo oocytes had been previously hypothesized based on the anticipated accomplishment of maturation, together with the increased incidence of degenerated oocytes at increasing times post-IVM (Neglia et al., 2001). The importance of oocyte aging in this species is also confirmed by activation studies that showed, in contrast to most other species, a deterioration of post-parthenogenetic embryo development at increasing times post-maturation (Gasparrini et al., 2004a).

In vitro fertilization (IVF)

Fertilization has often been considered the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other domestic species have been widely reported (Neglia et al., 2003, Gasparrini et al., 2004b, Galli et al., 2000). In our earlier studies, despite similar maturation rates (87% vs 94% respectively in buffalo vs cattle) significantly lower cleavage rates (65% vs 84%) were observed (Neglia et al., 2003). The

overall lower IVEP efficiency recorded in buffalo compared to cattle (26 vs 34 %, respectively) was mainly related to the poor cleavage rate; in fact similar blastocyst yields were obtained in buffalo and cattle (40 %) when the percentages were calculated in relation to the zygotes (Neglia et al., 2003).

What are the possible causes of a poor cleavage rate? Many factors may affect the in vitro fertilization efficiency, such as the sperm viability and capability, the adequate in vitro environment for gametes survival, the appropriate time of insemination, the duration of gametes co-incubation, the presence of cumulus cells and last but not least the acquisition of the oocyte developmental competence during the complex process of cytoplasmic maturation. In short, it is likely that the fertilization failure is related to inadequacies of the IVF system, but a previous inappropriate maturation of the egg should not be ruled out.

In earlier times the quality of the frozen semen was considered the major factor impairing in vitro fertilization (IVF), based on the demonstration of several damages of the male gamete occurring following cryopreservation (Meur et al., 1988), together with the drastic reduction of cleavage rate reported with frozen compared to fresh semen (Totey et al., 1992). Currently, the quality of frozen semen has improved, as indicated by similar fertility parameters, recorded for fresh compared to frozen semen (Wilding et al., 2003), suggesting that other factors may negatively affect fertilization.

However, the overall improvement of the quality of cryopreserved sperm has not eliminated another serious impediment, the so-called "bull effect", consisting in the high degree of variation between buffalo bulls in the fertilizing capability in vitro (Totey et al. 1993). It follows that, because only few bulls are characterized by good fertilizing capability in vitro (approximately 10 %), an accurate screening of sperm of several bulls is required in order to identify a suitable semen for IVF programs. Despite the availability of several fertility tests, it is known that currently the most accurate screening still goes through IVF trials, with different bulls tested on the same batch of eggs, that, in this species, because of the poor number of oocytes usable, are very time-consuming. Interestingly, it has been recently found that an easy, quick double staining technique with Trypan-blue/Giemsa (Kovács and Foote, 1992) can be used to predict the fertilizing capability in vitro of buffalo bulls, as shown by the correlation existent between the percentages of acrosome-intact viable sperm cells at thawing and the blastocyst yields (unpublished data).

Sperm need to undergo capacitation to acquire the fertilizing ability; this process, that in vivo occurs within the female genital tract, must be induced in vitro. Although several agents have been proven to induce sperm capacitation in vitro, heparin is still the most efficient method in the majority of the domestic species. It is not possible to rule out that the process of capacitation, required by spermatozoa to acquire the fertilizing ability is impaired in the currently used buffalo IVF system. In order to investigate whether the capacitation process in vitro can be improved by agents different than heparin, buffalo sperm have been incubated under different conditions and capacitation has been indirectly assessed by evaluating the capability of sperm to acrosome react following incubation with lysophosphatidilcholine, a fusogenic lipid, known to induce acrosome reaction in capacitated sperm without affecting motility. It has been demonstrated that progesterone induces buffalo sperm capacitation in vitro and may be considered as an alternative capacitating agent for buffalo IVF (Boccia et al., 2006a). Furthermore, it has been demonstrated (Boccia et al., 2007) that sperm treatment with sodium nitroprusside, a well known generator of nitric oxide in vitro, improves the efficiency of buffalo sperm capacitation in vitro compared to heparin, when the incubation is extended to 2 or 3 h (60.1 vs 44.1 % respectively at 2 h; 68.8 vs 36.6 % respectively at 3h). The most promising results have been obtained by incubating sperm with some biological fluids, such as buffalo estrus serum (BES) and the follicular fluid (FF)

recovered from a pool of dominant follicles (Boccia et al., 2005). In fact, sperm treatment with both BES and FF has resulted in a significantly higher incidence of AR sperm than with heparin treatment (84.3, 94.5 vs 50.1 % respectively), regardless of the incubation times. It is likely that factors derived by BES and FF, present in the oviduct environment around fertilization, play a critical role in processing the male gamete in vivo. These results show the possibility of significantly improving the efficiency of sperm capacitation in vitro in buffalo species and strongly suggest investigating the effects of BES and FF also on the fertilizing capability of buffalo spermatozoa.

The media commonly utilized for buffalo IVF are Tyrode's modified medium (TALP) and Brackett Oliphant (BO), supplemented by sperm motility inducing factors, such as combined hypotaurine-penicillamine or caffeine. However, significantly higher cleavage and blastocysts rates have been obtained, in a direct comparison trial, by using TALP medium, supplemented with heparin, hypotaurine and penicillamine (Gasparrini et al., 2004b).

A positive effect of cumulus cells at the time of IVF has been observed in buffalo, as in cattle, (Zhang et al., 1995), as demonstrated by the higher cleavage rate and embryo development obtained with cumulus-enclosed oocytes vs oocytes that were freed of their cumulus investment.

Another factor that may affect embryo development is the duration of gamete co-incubation during IVF. It has been suggested that prolonged gamete co-incubation under the conditions of IVF, in which high concentrations of spermatozoa are incubated in small volumes of medium, results in the production of high levels of hydrolytic enzymes (Rehman et al., 1994) and free radicals (Aitken, 1994) that damage the oocytes. It has been recently demonstrated that the optimal sperm-oocyte co-incubation time for maximizing the blastocyst yield in buffalo is 16 h (Gasparrini et al., 2007b). Shortening the gamete co-incubation length to 8 h has resulted in a significant reduction of oocyte cleavage, similar to that reported in cattle (Ward et al., 2002; Kochhar et al., 2003). Interestingly, the lower blastocyst development recorded at the shorter durations of sperm-oocyte co-incubation tested were mainly due to the lower cleavage rates, as suggested by the fact that the oocytes that had cleaved developed further and as fast as those in the 16 h group. On the contrary, extending gamete co-incubation to 20 h has been deleterious because, despite similar cleavage rates, the blastocyst production was reduced both when calculated in relation to COCs and to cleaved embryos. Furthermore, increasing the sperm-oocyte incubation time to 20 h has been found to be correlated to a higher incidence of polyspermy, confirming previous observations (Sumantri et al., 1997).

As previously mentioned, the poor cleavage rate may also be due to the lack of oocyte developmental competence, normally acquired during the maturation process. In order to investigate these aspects activation studies were carried out. The parthenogenetic activation of the oocytes represents a valid tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development, hence indirectly assessing the quality of the oocytes matured in vitro. In an earlier study we compared in vitro fertilization with parthenogenetic oocyte activation using two chemical agents such as ethanol and ionomycin, both followed by immediate exposure to 6-DMAP for 4 h (Gasparrini 2004a). A significant improvement of cleavage (71 % vs 56 %, respectively) and blastocyst yield (33 vs 23 %, respectively) was obtained with ethanol-induced activation vs IVF, indirectly suggesting that buffalo oocytes had acquired the developmental competence during IVM. However, cleavage and blastocyst rates obtained with ionomycin were intermediate (59 and 26 %, respectively). In addition, the improvement was not as such to make definite conclusions. It has more recently reported that activation with different methods give significantly higher cleavage and blastocyst rates compared to IVF, strongly suggesting that

the problem has paternal rather than maternal origin (Mishra et al., 2007a). However, we speculate that the evident difference shown by these authors may be due to serious problems with the quality of the sperm utilized in their system since very poor cleavage (< 37 %) and blastocyst rates (< 16 %) were obtained following IVF.

Finally, it is worth pointing out that, after many fruitless attempts to increase cleavage rate in this species, the fertilization efficiency has at last improved, reaching approximately 80 % of cleavage rate, by enriching the IVM medium with cystine and cysteamine. This improvement has been proven to be related to enhanced intracytoplasmic GSH levels (Gasparrini et al., 2006). This interesting finding would indicate that the poor cleavage rate of this species so far recorded was likely related to an inappropriate maturation of the female gamete rather than to the deficiencies of the IVF system. It has been, in fact, suggested that the GSH production is critical for the acquisition of developmental competence of oocytes at a cytoplasmic level (Eppig, 1996) and that the measurement of GSH at the end of IVM can be a reliable indicator of the cytoplasmic maturation (de Matos et al. 1997).

In vitro culture (IVC)

The in vitro culture system developments for buffalo embryos have imitated those for other ruminant species. Thus, the IVC started from in vivo culture in an intermediate host, such as ligated oviduct of sheep (Galli 98), which was replaced by the co-cultural systems, which was in turn substituted by defined media in the absence of serum and feeder cells. Buffalo embryos have been co-cultured with cumulus and oviductal cells (Totey et al., 1992; Madan et al., 1994; Chuangsoongneon et al., 1991) or with established cell lines such as BRL (Boni et al., 1999). Although many authors still prefer the co-culture system for embryo production in this species, the utilization of defined media for embryo culture has become necessary to comprehend the requirements of buffalo embryos in vitro which, in turn, would allow the formulation of an optimal species-specific culture system. A well known defined medium, such as the Synthetic Oviduct Fluid (SOF) has been utilized for embryo culture in this species since 1999 (Boni et al., 1999), obtaining higher blastocyst rates compared to the co-culture system with BRL cells (13.5 % vs 7 %). Subsequently, buffalo zygotes/embryos have been successfully cultured either in SOF and in another defined cell-free system, known as Potassium Simplex Optimized Medium (KSOM) with similar embryo development (Caracciolo di Brienza et al., 2001). The great improvement of blastocyst yields (35-40%) achieved in the following years is, according to our experience, due to the optimization of the IVM and in part of the IVF systems rather than to modifications applied to the IVC system. In fact, despite attempts to modify its original composition, at present the original version of SOF remains the most suitable medium for embryo culture in buffalo.

It has been demonstrated (Monaco et al., 2006) that, in contrast to sheep and cattle, the presence of glucose is absolutely required for in vitro culture of buffalo embryos, particularly during the early embryonic development (up to Day 4). In fact, the lack of glucose in the IVC medium both for the entire duration of culture or limitedly to early culture (up to Day 4) has seriously compromised blastocyst development (2.4 and 9.6 %, respectively). On the contrary, when glucose was provided during early culture and removed during late culture, blastocyst yields have been high and comparable to the control (SOF), in which glucose was present throughout culture (30.7 and 32.7 % respectively).

In order to reduce the accumulation of free radicals, ammonium and other catabolites that may affect embryo development, it has been suggested to use the easy expedient to change the medium more times during culture. However, no significant differences in buffalo

embryo development have been recorded by changing the IVC medium 3 (Day 1, 3 and 5) or 2 (Day 1 and 5) times during culture, with a tendency of improvement in the latter case (Boccia et al., 2006b) Therefore, in contrast to other species, the addition of fresh medium on Day 3 of culture in buffalo does not exert any positive influence. It is likely that this is related to the higher sensitivity of buffalo embryos to fluctuations of temperature and/or pH that normally occur during a culture change even if to limited extents; it results that it is advisable "not to disturb" buffalo embryos during culture!

Buffalo embryos in vitro develop approximately 12-24 h earlier than cattle embryos (Galli et al., 2000) and this pattern of development reflects that observed in vivo, with most of the blastocysts collected by uterine flushing in the hatched stage at 6.5 days after the onset of oestrus (Drost and Elsden, 1985). On Day 6 (Day = IVF) it is possible to find embryos in advanced stages of development, including hatched blastocysts but most embryos reach the blastocyst stage on Day 7. A small proportion of embryos are delayed, reaching the blastocyst stage on Day 8 but their quality and viability is poor, as demonstrated by their lower resistance to cryopreservation (Gasparrini et al., 2001).

Embryo cryopreservation

Embryo cryopreservation represents the best tool to overcome the major problem affecting the commercial application of embryo transfer (ET) procedures, i.e. the limited number of suitable recipients, that is particularly accentuated in buffalo because of the lower response to hormonal stimulation and hence to synchronization treatments. Furthermore, because of the seasonality of the species, it is advisable to carry out the transfers in the most favourable period for reproductive activity. Unfortunately, buffalo IVP embryos seem very sensitive to cryopreservation, probably due to their high lipid content. The tolerance to cryoprotectants may be increased by in vivo culture of the cleaved embryos in surrogate sheep oviducts (Galli et al., 1998), as demonstrated by improved development to term following transfers of embryos, which were frozen in 10 % glycerol with the slow-freezing method. Nevertheless, although this system yields embryos of quality comparable to MOET, it requires appropriate facilities and is more unpractical and expensive.

Buffalo embryos that were entirely produced in vitro have been successfully cryopreserved by vitrification, as demonstrated by their survival following in vitro culture (Gasparrini et al., 2001) and development to term after ET (Neglia et al.,2004, Sà Filho et al., 2005). Although development to term has been obtained, efficiency still needs to be improved for the diffusion of OPU-IVEP technologies in the field. A significant improvement of embryo survival rate following IVC of vitrified-warmed buffalo IVP embryos has been recently obtained by using minimum volume vitrification methods, such as Open Pulled Straw (De Rosa et al., 2006) and Cryotop (De Rosa et al., 2007) vitrification. It has also been demonstrated that the stage of development affects freezability of IVP embryos, with increased in vitro survival for the advanced embryo stages (De Rosa 2006, 2007), such as the expanded blastocysts, which are, in any case, better quality embryos since they develop faster in vitro. Unfortunately no data are as yet available regarding the pregnancy rate and the development to term following ET of embryos vitrified in such ways.

Conclusions

The acquisition of more specific information on buffalo oocytes and embryo requirements in vitro, has led to a significant improvement of the IVEP efficiency in this species. However, although blastocyst yield has greatly increased, pregnancy rates are still

low and only few calves have been produced after transfer of cryopreserved embryos from live animals (Galli, 1998; Neglia 2004; Sá Filho, 2005). In addition to the low number of oocytes recruitable, that seems to be a feature intrinsic to the species, an important limiting factor is the low resistance to cryopreservation of buffalo in vitro derived embryos that can be also considered a result of poor viability due to suboptimal culture conditions.

In conclusion, despite the encouraging results obtained in the last few years, further studies are needed to improve the in vitro system and hence the freezability of IVEP embryos for advanced reproductive strategies to become a routine procedure in buffalo breeding.

References

1) Aitken JR (1994) A free radical theory of male infertility. Reprod Fertil Dev 6, 19-24
2) Boccia L, De Rosa A, Attanasio L, Di Palo R, Zicarelli L, Gasparrini B (2005) Capacitation of buffalo spermatozoa in vitro. Reprod Fertil Dev 17 (1,2), 270
3) Boccia L, Attanasio L, Monaco E, De Rosa A, Di Palo R, Gasparrini B (2006a) Effect of progesterone on capacitation of buffalo (Bubalus bubalis) spermatozoa in vitro. Reproduction in Domestic Animals 41 (4), 311
4) Boccia L, Monaco E, Attanasio L, De Rosa A, Gasparrini B (2006b) Influenza del cambio di coltura (doppio vs singolo) sullo sviluppo di embrioni bufalini in vitro. "XII Giornate scientifiche", Università degli Studi di Napoli Federico II,, Polo delle Scienze e delle Tecnologie per la Vita; 15-16 Giugno, Facoltà di Medicina e Chirurgia, Napoli: 171
5) Boccia L, Attanasio L, De Rosa A, Pellerano G, Di Palo R, Gasparrini B (2007) Effect of sodium nitroprusside on buffalo sperm capacitation in vitro. Reprod Fertil Dev 19, 276
6) Boni R, Santella L, Dale B, Roviello S, Di Palo R, Barbieri V. (1992) An ultrastructural study of maturation in buffalo oocytes. Acta Medica Veterinaria 38, 153-l 61
7) Boni R, Roviello S, Zicarelli L. Repeated ovum pick-up in Italian Mediterranean buffalo cows. Theriogenology 1996;46:899-909
8) Boni R., Roviello S., Gasparrini B., Langella M., Zicarelli L. (1999). *In vitro* production of buffalo embryos in chemically defined medium. Buffalo Journal 1999, 1: 115-120
9) Campanile G, Neglia G, Gasparrini B, Galiero G, Prandi A, Di Palo R, D'Occhio MJ, Zicarelli L (2005) Embrionic mortality in buffaloes syncronized and mated by AI during decline in reproductive function Embrionic mortality in buffaloes syncronized and mated by AI during decline in reproductive function. Theriogenology 63 (8),2334-40
10) Caracciolo di Brienza V, Neglia G, Masola N, Gasparrini B, Di Palo R, Campanile G (2001) In vitro embryo production in chemically defined media. Atti 1° Congresso Nazionale sull'Allevamento del Bufalo, 3-5 ottobre, Eboli (SA), Italy, 341-344
11) Chauhan MS, Palta P, Das SK, Katiyar P, Madan ML (1997) Replacement of serum and hormone additives with follicular fluid in the IVM medium: Effect on maturation, fertilization and subsequent development of buffalo oocytes in vitro. Theriogenology 48, 461-469
12) Chauhan MS, Singla SK, Palta P, Manik RS, Madan ML (1998) In vitro maturation and fertilization, and subsequent development of buffalo (Bubalus Bubalis) embryos: Effects of oocytes quality and type of serum. Reprod Fertil Dev 10, 173-l 77.
13) Chauhan MS, Singla SK, Palta P, Manik RS, Madan ML (1999) Effect of epidennal growth factor on the cumulus expansion, meiotic maturation and development of buffalo oocytes in vitro. Vet Ret 144, 266-267.

14) Chian RC, Niwa K, Nakahara H (1992) Effect of sperm penetration in vitro on completion of first meiosis by bovine oocytes arrested at various stages in culture. J Reprod Fertil 96,73-78
15) Chuangsoongneon U, Kamonpatana M (1991) Oocyte maturation, in vitro fertilization and culture system for developing preimplantation Swamp buffalo embryos using frozen-thawed semen. Buffalo J 7,189-198.
16) Danell B (1987) "Oestrus behaviour, ovarian morphology and cyclic variations in follicular system and endocrine pattern in water buffalo heifers" PhD thesis, Uppsala, Sweden: Sveriges Lantbruksuniversiter
17) Das GK, Jain GC, Solari VS, Tripathi VN (1996) Efficacy of various collection methods for oocytes retrieval in buffalo. Theriogenology 46, 1403-l 411
18) de Matos DG, Furnus CC, Moses DF (1997) Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. Biol Reprod 57, 1420-1425
19) De Rosa A, Di Palo R, Attanasio L, Monaco E, Campanile G, Gasparrini B (2006) Open pulled straw vitrification for in vitro produced buffalo (Bubalus bubalis) embryos. Reprod Fertil Dev 18 (1,2), 153
20) De Rosa A, Attanasio L, Boccia L, Pellerano G, Campanile G, Gasparrini B (2007) Cryotop vitrification for in vitro produced buffalo (Bubalus bubalis) embryos. Reprod Fertil Dev 19, 174
21) Dominko T, First NL (1997) Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol Reprod Dev 47,456-67
22) Drost M, Elsden RP (1985) Blastocyst development in water buffalo (Bubalus bubalis). Theriogenology 23, 191
23) Eppig J (1996) Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev 8, 485-489
24) Galli C, Duchi R, Crotti G, Lazzari G (1998) Embryo production by Ovum Pick-up in Water Buffalo. Theriogenology 50, 259
25) Galli C, Crotti G, Notari C, Turini P, Duchi R,Lazzari G (2000) Embryo production by ovum pick-up from live donors. Theriogenology 55,1341-1357
26) Gardiner CS, Reed DJ (1994) Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. Biol Reprod51,1307-1314
27) Gasparrini B, Neglia G, Di Palo R, Campanile G Zicarelli L (2000). Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology 54, 1537-1542
28) Gasparrini B, Neglia G, Caracciolo di Brienza V, Campanile G, Di Palo R Zicarelli L (2001). Preliminary analysis of vitrified in vitro produced embryos. Theriogenology 55, 307
29) Gasparrini B (2002) In vitro embryo production in buffalo species: state of the art.. Theriogenology 57 (1), 237-256
30) Gasparrini B, Sayoud H, Neglia G, de Matos D, Donnay I , Zicarelli L (2003) Glutathione synthesis during in vitro maturation of buffalo (Bubalus bubalis) oocytes: effects of cysteamine on embryo development. Theriogenology 60, 943-952
31) Gasparrini B, Boccia L, De Rosa A, Di Palo R, Campanile G, Zicarelli L (2004a) Chemical activation of buffalo (Bubalus bubalis) oocytes by different methods: effects of aging on post-parthenogenetic development. Theriogenology 62/9, 1627-1637
32) Gasparrini B, Boccia L, De Rosa A, Vecchio D, Di Palo R, Zicarelli L (2004b) In vitro fertilization of buffalo (Bubalus bubalis) oocytes: effects of media and sperm motility inducing agents. Reprod Fertil Dev 16, 255

33) Gasparrini B, Boccia L, Marchandise J, Di Palo R, George F, Donnay I, Zicarelli L (2006) Enrichment of in vitro maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: Effects of cystine on glutathione synthesis and embryo development. Theriogenology 65(2), 275-287
34) Gasparrini B (2006) Advances on in vitro embryo production in water buffalo. Proc of the 5th Asian Buffalo Congress on "Social economic contribution of buffalo to rural areas", April 18-22, Nanning, China Vol 1, 15-21
35) Gasparrini B, Attanasio L, De Rosa A, Monaco E, Di Palo R, Campanile G (2007a) Cryopreservation of in vitro matured buffalo (Bubalus bubalis) oocytes by minimum volumes vitrification methods. Anim Reprod Sci 98 (3-4), 335-42
36) Gasparrini B, De Rosa A, Attanasio L, Boccia L, Di Palo R, Campanile G, Zicarelli L. (2007b) Influence of the duration of in vitro maturation and gamete co-incubation on the efficiency of in vitro embryo development in Italian Mediterranean Buffalo (Bubalus bubalis). Anim Reprod Sci (in press) PMID: 17481834
37) Gordon I (1994) Aspiration techniques: Old and new. In Laboratory Production of Cattle Embryos. Wallingford, UK: CAB International, 71-72.
38) Goswami SL, Manik RS, Balakrishnan CR (1992). Status of buffalo and goat ovaries collected from slaughterhouse. In: Proceedings of the Symposium on embryo transfer technology held at IVRI Izatnagar, April 16–17.
39) Huang Y, Zhuang X, Gasparrini B, Presicce GA (2005) Oocyte recovery by ovum pick-up and embryo production in Murrah and Nili-Ravi buffaloes (Bubalus bubalis) imported in China. Reprod Fertil Dev 17 (1,2), 273
40) Hufana-Duran D, Pedro PB, Venturina HV, Hufana RD, Salazar AL, Duran PG, Cruz LC (2004) Post-warming hatching and birth of live calves following transfer of in vitro-derived water buffalo (Bubalus bubalis) embryos. Theriogenology 61, 1429-1439
41) Hunter RHF, Greve T (1997) Could artificial insemination of cattle be more fruitful? Penalties associated with aging eggs. Reprod Domest Anim 32, 137-42
42) Kochhar HS, Kochhar KP, Basrur PK, King WA (2003) Influence of the duration of gamete interaction on cleavage, growth rate and sex distribution of in vitro produced bovine embryos. Anim Reprod Sci 77, 33-49
43) Kumar A, Solanki VS, Jindal SK, Tripathi VN, Jain GC. (1997) Oocytes retrieval and histological studies of follicular population in buffalo ovaries. Anim Reprod Sci 47, 189-195
44) Madan ML, Singla SK, Chauhan MS, Manik RS(1994). In vitro production and transfer of embryos in buffaloes. Theriogenology 41, 139–143.
45) Marston JH, Chang MC (1964) The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. J Exp Zool 15, 237-51
46) Meur SK, Roy SB, Mohan G, Dhoble RI (1988) Cryogenic changes in seminal proteins of cattle and buffalo. Theriogenology 30,1005-1010
47) Mishra V, Misra AK, Sharma R (2007a) A comparative study of parthenogenetic activation and in vitro fertilization of bubaline oocytes. Anim Reprod Sci, doi:10.1016/j.anireprosci.2006.12.019
48) Mishra V, Misra AK, Sharma R (2007b) Effect of ambient temperature on in vitro fertilization of Bubaline oocyte, Anim Reprod Sci doi:10.1016/j.anireprosci.2006.10.020
49) Misra AK (1997) Application of biotechnologies to buffalo breeding in India. Third Course on Biotechnology of Reproduction in buffaloes, Caserta, Italy,141-166
50) Misra AK (2005) Embryo transfer technology in buffaloes. Process and developments. In: National Seminar on Recent Advances in Conservation of Biodiversity and

Augmentation of Reproduction and Production in Farm Animals, Sardarkrushinagar, India, March 6–7
51) Monaco E, De Rosa A, Attanasio L, Boccia L, Zicarelli L, Gasparrini B (2006) In vitro culture of buffalo (Bubalus bubalis) embryos in the presence or absence of glucose. Reproduction in Domestic Animals 41 (4), 332
52) Nandi, S., Chauhan, M.S., Palta, P., 2001. Effect of environmental temperature on quality and developmental competence in-vitro of buffalo oocytes. Vet. Rec. 148, 278–279
53) Neglia G, Marino M, Di Palo R, Wilding M, Caracciolo di Brienza V, Dale B, Gasparrini B, Zicarelli L (2001) A comparison of in vitro maturation in buffalo (Bubalus Bubalis) and bovine oocytes using confocal microscopy. Theriogenology 55, 488
54) Neglia G, Gasparrini B, Caracciolo di Brienza V, Di Palo R, Campanile G, Presicce GA, Zicarelli L (2003) Bovine and buffalo in vitro embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology 59, 1123-1130
55) Neglia G, Gasparrini B, Caracciolo di Brienza V, Di Palo R, Zicarelli L (2004) First pregnancies to term after transfer of buffalo vitrified embryos entirely produced in vitro. Veterinary Research Communication (Special Issue) 28, 233–236
56) Palta P, Banzai N, Prakash BS, Manik RS, Madan ML (1998) Endocrinological observation of atresia in individual buffalo ovarian follicles. Ind J Anim Sci68, 444-447
57) Pawshe CH, Totey SM (1993) Effects of cumulus cells monolayer on in vitro maturation of denuded oocytes of buffalo (Bubalus Bubalis). Theriogenology39,281.
58) Pawshe CH, Appa Rao KBC, Totey SM (1998) Effect of insulin-like growth factor I and its interaction with gonadotrophins on in vitro maturation and embryonic development, cell proliferation, and biosynthetic activity of cumulus-oocytes complexes and granulosa cells in buffalo. Mol Reprod Dev 49, 277-285
59) Prather RS and Day BN (1998) Practical considerations for the in vitro production of pig embryos. Theriogenology 49(1), 23-32
60) Rehman N, Collins AR, Suh TK, Wright RW (1994) Effect of sperm exposure time on in vitro fertilization and embryo development of bovine oocytes matured in vitro. Theriogenology 41, 1447-52
61) Sá Filho MF, Carvalho NAT, Gimenes LU, Torres-Junior JR, Ferriera CR, Perecin F, Perini A P, Tetzner T A D, Vantini R, Soria G F, Garcia J M, Tonhati H, Zicarelli L, Gasparrini B, Baruselli P S (2005) Birth of the first buffalo calves after transfer of vitrified embryos produced in vitro in America. Atti del 3° Congresso Nazionale sull'Allevamento del Bufalo- 1° Buffalo Symposium of Europe and the Americas; October 12-15, Capaccio-Paestum (SA), Italy, 250
62) Samad HA, Khan IQ, Rehman NU, Ahmed N (1998) The recovery, in vitro maturation and fertilization of Nilli-Ravi buffalo follicular oocytes. Asian Aust. J Anim Sci 11, 491–497
63) Sirard MA, Florman H, Leibfried-Rutledge ML, First NL (1989) Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol Reprod 40, 1527-63
64) Sumantri C, Boediono A, Ooe M, Murakami M, Saha S, Suzuki T (1997) The effect of sperm-oocyte incubation time on in vitro embryo development using sperm from a tetraparental chimeric bull. Anim Reprod Sci 48, 187-95
65) Telford NA, Watson AJ, Schultz GA (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 26, 90-100

66) Totey SM, Singh G, Taneja M, Pawshe CH, Talwar GP (1992) In vitro maturation, fertilization and development of follicular oocytes from buffalo (Bubalus Bubalis). J Reprod Ferti1 95, 597-607
67) Totey SM, Pawshe CH, Singh GP (1993) In vitro maturation and fertilization of buffalo oocytes (Bubalus Bubalis): Effects of media, hormones and sera. Theriogenology 39, 1153-1171
68) Ward F, Enright B, Rizos D, Boland M, Lonergan P (2002) Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. Theriogenology 57, 2105-17
69) Wilding M, Gasparrini B, Neglia G, Dale B, Zicarelli L (2003) Mitochondrial activity and fertilization potential of fresh and cryopreserved buffalo sperm. Theriogenology 59, 466
70) Yadav BR, Katiyar PK, Chauhan MS, Madan ML (1997) Chromosome configuration during in vitro maturation of goat, sheep and buffalo oocytes. Theriogenology 47, 943-51
71) Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA (1995) Cumulus cell function during bovine oocyte maturation, fertilization and embryo development in vitro. Mol Reprod Dev 40, 338-344
72) Zicarelli L (1997) Superovulatory response in buffaloes bred in Italy. Third Course on Biotechnology of Reproduction in buffaloes, Caserta, Italy, 167-188

DEVELOPMENT OF THE RUMINANT EMBRYO FROM ELONGATION TO IMPLANTATION

GUILLOMOT, M.

Biologie du Développement et Reproduction
Institut National de la Recherche Agronomique – 78352 Jouy-en-Josas, France.

Abstract

During the pre implantation period the ruminant conceptus undergoes series of transformations which prepare it to the implantation process and the development of a viable foetus. Taking the sheep conceptus as a model, the present review describes the morphological, cellular and molecular changes relative to the elongation process and gastrulation which concern both the extra embryonic tissues and the embryo, respectively. In the elongating conceptus the parietal endoderm undergoes a syncytial transformation and elongates parallel to the long axis of the conceptus. Before the beginning of gastrulation the extra embryonic mesoderm differentiates from the ectoderm and migrates to form the yolk sac, a primitive placental annexe. All together the data reported underline the specific characteristics of the ruminant embryo by comparison with the most studied mouse model in developmental biology.

Introduction

In most mammals, the period of pregnancy from fertilization to implantation is critical in term of embryonic loss. This is particularly true in species, such as ruminants, in which the embryo remains free in the uterine cavity before the establishment of a placenta and thus depends on the uterine environment to sustain its development. The origins of such embryo mortality are not fully understood but it is obvious that both the embryo proper and the extra embryonic tissues are concerned since major differentiations of the embryo and of the extra embryonic tissues occur during that period of pregnancy. One of the main processes which concern the embryo is gastrulation, a common developmental process in all animals (for review see Gilbert, 1991). Gastrulation is one of the major steps during development since it gives rise to the three embryonic germ layers, i.e. ectoderm, endoderm and mesoderm, whose derivates will construct all the organs of the foetus and part of the placental annexes. It is also at this time that the antero-posterior and left-right axis of the embryo are established. The process begins by the formation of the primitive streak which extends from the posterior pole of the embryo towards the anterior ones. Ectoderm cells invaginate from the primitive streak to the ventral side and differentiate into mesoderm cells intercalated in between the ectoderm and the visceral endoderm. These mesoderm cells migrate to the extra embryonic areas and split in two layers. The inner layer forms form the yolk sac by fusion with the endoderm which is separated progressively from the trophectoderm and the outer layer lines the trophectoderm to constitute the chorion. This general scheme fits for all mammals, but the chronology of the events differs greatly among species. In rodents and man, gastrulation and differentiation of the extra embryonic mesoderm occur after implantation, only. On the contrary, in ungulates the process is initiated well ahead of implantation (Hue et al., 2001; Maddox-Hyttel et al., 2003; Flechon et al., 2004; Guillomot et al., 2004). The successive stages of the pre implantation period in sheep and cow are summarized in Figure1. Most of our knowledge on early differentiation of the embryo and the extra embryonic tissues arises

from studies in rodents. The obvious morphological differences between ruminant and rodent conceptuses and between chronologies of gestation imply to develop comparative studies on developmental biology in ruminants to avoid misleading by subjective extrapolations and assumptions. The aim of this contribution is to precise the main developmental processes which occur during the pre implantation period in ruminants. The data presented here were obtained from observations in sheep which, due to the close similitude with bovine embryology and smaller economic costs, represents a good model for developmental biology studies in ruminants. We will focus on the morphological and cellular transformations which appear in the extra embryonic tissues and the embryo itself during the elongation process and gastrulation. Although most of the differentiation processes occur concomitantly the changes which concern the various tissues are described separately. All observations were done on *in vivo*-produced embryos dated from the day of mating or artificial insemination (day 0).

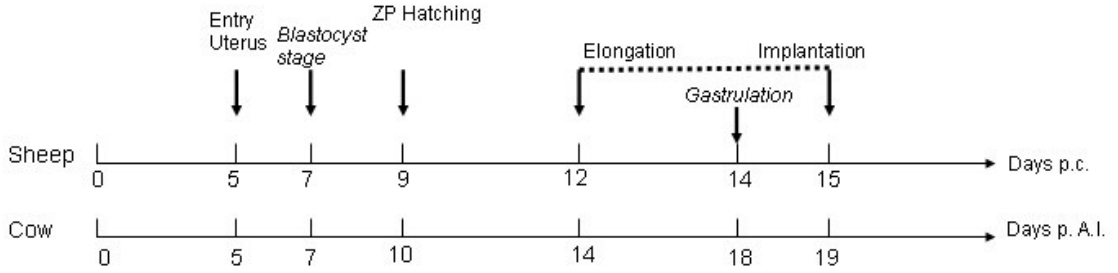

Figure 1. Chronology of the major events during the pre implantation period in sheep and cow. Days are calculated from the mating day (p.c.) or artificial insemination (p.A.I.) as day 0.

I. Elongation of the conceptus

This phase of development is very specific to the ungulates, excepted the equids. This process may be considered as a preparation of implantation and likely, is related to the gross anatomy of the diffuse and cotyledonary placenta which characterize the ruminant. Both the onset and rate of elongation vary among females at the same stage of pregnancy and even within the same litter of a given female (Figure 2). Thus, staging of the embryos based on the morphological aspect of the conceptus is more accurate than estimated by age post conception to compare rates of embryonic development in experimental studies, for example. According to the shape and size of the conceptus three stages can be characterized: ovoid, tubular and filamentous.

At days 9-10 the hatched blastocyst is constituted by the outer layer of trophectoderm, the inner cell mass (ICM) which is still covered by the Rauber's layer and the endoderm cells which underline both the inner faces of the trophectoderm and the ICM. Elongation starts around days 12 and 14 in sheep and cow, respectively (Fig. 1). The expanded blastocyst grows to become an ovoid conceptus of 2 mm in length and 1 mm width. One day later, the conceptus changes from ovoid to a tubular vesicle 0.3 to 1 cm long and 1 mm in width. The embryonic disc is located in the middle part of the tubular vesicle. Thereafter, the vesicle elongates very rapidly giving rise to a filamentous conceptus more than 10 cm long and 2 – 3 mm wide. The fully elongated conceptus fills the whole cavity of the uterine horn ipsi lateral to the corpus luteum and invade the lower part of the contra lateral horn in the case of a single pregnancy. At the end of the elongation process, the conceptus faces the implantation sites on which apposition of the trophoblast and the uterine epithelium begins by days 15 and 19 in sheep and cow, respectively (Guillomot, 1995). The elongation process must depend on uterine factors since it is not observed *in vitro* despite the use of sophisticated techniques. For

example, *in vitro* culture of blastocysts in gel tunnels to constraint elongation mechanically, was not fully successful since very few embryos elongated and none of them reached the size of *in vivo*-developed conceptuses (Brandao et al., 2004; Vajta et al., 2004). On the other hand, ovine trophoblastic vesicles produced after culture of fragments of filamentous conceptuses expanded without elongation but do elongate within a few days after uterine transfer in recipients female (Flechon et al., 1986). The direct role of the uterine secretions on the elongation process has been demonstrated in ewes whose uterine glands development was hormonally impaired (UGKO) before puberty. The UGKO ewes were fertile but the blastocyst did not elongate properly and elongation rate was proportional to the rate of uterine gland differentiation (Gray et al., 2001; Gray et al., 2002). Uterine secretory products likely act on the conceptuses tissues to induce the elongation process. The precise nature of these uterine factors and their target tissues in the conceptus remain to be determined. In pig, it has been suggested that elongation of the concetpus involves cellular remodelling of the trophectoderm rather than hyperplasia (Geisert et al., 1982; Mattson et al., 1990). This does not seem true in ruminants, since no major modifications of the trophectoderm cell shape could be observed during elongation. Whatever the stage of elongation, i.e. ovoid, tubular or filamentous, the trophectoderm retained a characteristic polygonal shape and presented no sign of elongation transformation (Wintenberger-Torres and Flechon, 1974; Guillomot, 1995). The main changes of the trophectoderm cell structure were correlated with the implantation process rather than with elongation of the conceptus (Guillomot et al., 1981; Guillomot and Guay, 1982; Guillomot, 1995).

Interestingly, by complementary techniques of electronic and light fluorescence microscopy, we have evidenced striking modifications of the endoderm layer in elongating ovine conceptuses (Flechon *et al*., submitted). The extra embryonic endoderm is the first layer which differentiates from the ICM. During the blastocyst growth the endoderm cells migrate beneath the trophectoderm to form an epithelial sheet all around the blastocoele. The primitive extra embryonic endoderm differentiates into the visceral endoderm (VE) located under the ICM which at least at the beginning may be considered as the area of cell proliferation and into the parietal endoderm (PE). Associated with the trophectoderm the PE forms a double epithelial barrier, namely the trophoblast, which separates the blastocoele from the external milieu. During the elongation process, the VE retained the cuboidal shape of typical epithelial cells whereas the PE cells were broader thin cells which became multinucleated as they migrate away from the embryonic area. In tubular and filamentous conceptuses the plasma membranes of the PE multinucleated cells stretched and aligned parallel to the elongation axis of the conceptus (Fig. 3A). The presence of pairs of nuclei linked by mid-bodies and numerous metaphase plates indicated that the syncytial differentiation of the parietal endoderm was due to karyokinesis which was not followed by cytokinesis rather than by cell fusion. Migration and spreading of the PE beneath the trophectoderm is facilitated by extra cellular components of the basal membranes (ECM) such as laminin which is specifically produced by the PE (Fig. 3B). Specific synthesis of the ECM constituents by the parietal endoderm has been reported also in cow (Vejlsted et al., 2005), pig (Richoux et al., 1989), rat (Notarianni and Flechon, 2001) and mouse (Gardner, 1983).

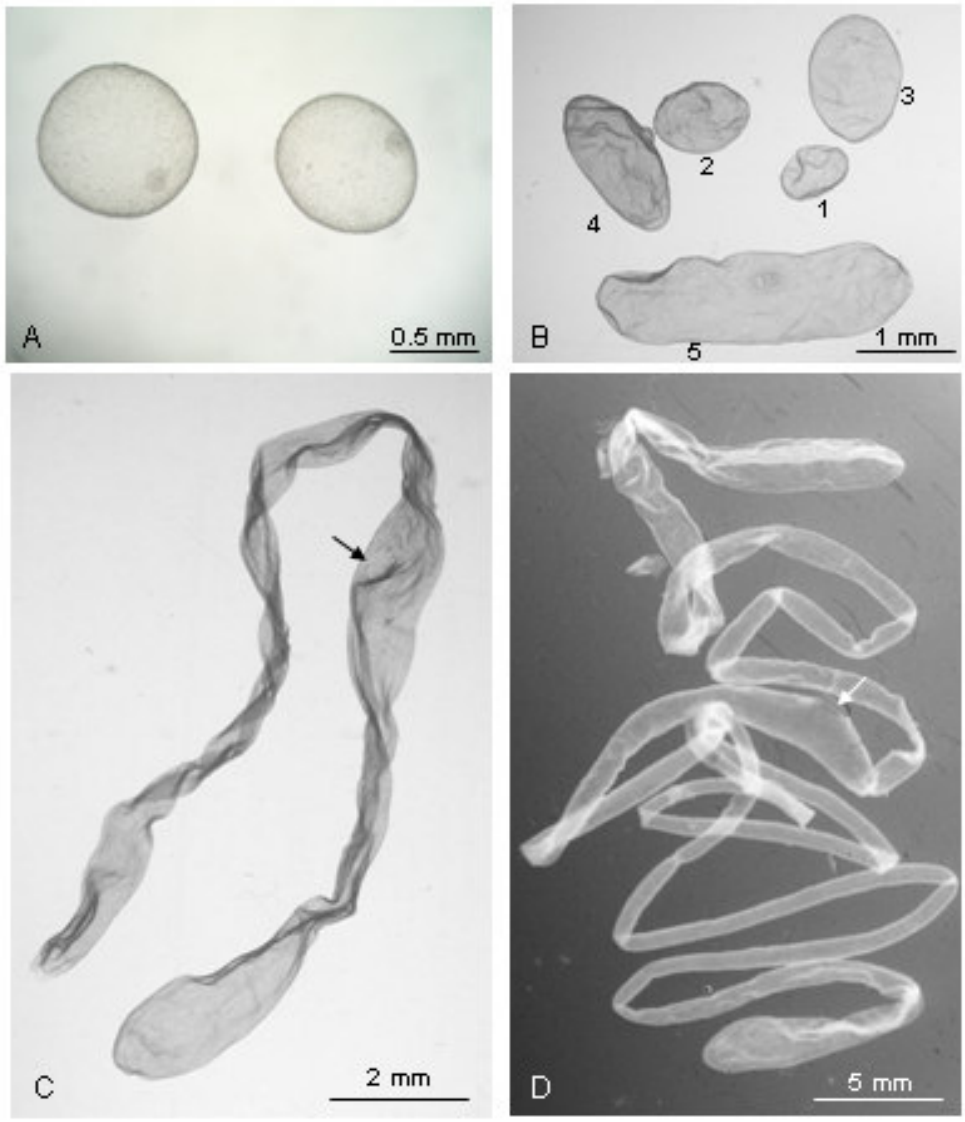

Figure 2. Ovine conceptuses collected at different stages of the elongation process. A) day-11 expanded spherical blastocysts; B) Ovoid (1 – 3) and tubular (4 and 5) conceptuses collected in the same ewe at day 12 of pregnancy; C) day-12 early filamentous stage; D) day-14 filamentous conceptus. Arrows in C and D point the embryonic discs.

The role of the syncytial structure of the parietal endoderm in the elongation process remains to be elucidated and whether this differentiation is specific to the ungulates or also occurs in other groups has to be specified. More recently, based on the analysis of the transcriptome in elongating conceptuses it has been shown that family of genes involved in cell remodelling and proliferation are differentially expressed during the transition from ovoid to tubular stages in sheep (Cammas et al., 2005) and cow (Hue et al., 2007). The cellular origin and the precise role of these groups of genes in the elongation process remain to be elucidated.

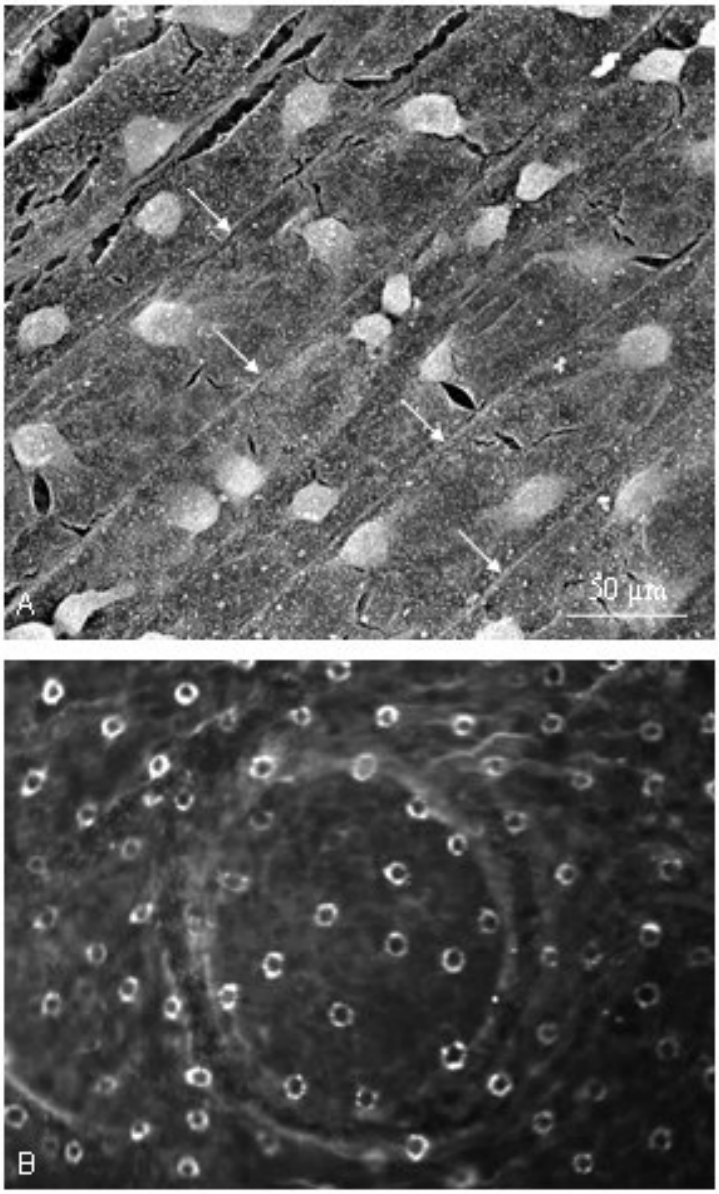

Figure 3. A) Scanning electron photomicrography of the parietal endoderm in an ovine filamentous conceptus. Note the parallel plasma membranes (arrows) which limit the plurinucleated cells (picture by J-E Fléchon). B) Immunocytochemical localization of laminin in the parietal endoderm cells viewed through the embryonic disc.

II. Embryo development

During the elongation of the conceptus, the embryo undergoes major differentiation processes which drive the patterning of the embryo and formation of the extra embryonic tissues which contribute to the placental structures. As mentioned above, the rate of embryo development differs greatly from one litter to another one at the same day of pregnancy. Thus, we propose a staging table which combines both the size of the conceptus and the differentiation of the embryo (Table 1) (Guillomot et al., 2004). The embryonic stages were classified according to morphological and histological criteria which included both the shapes of the conceptus and of the embryo, the formation of the primitive steak and

differentiation of the extra embryonic tissues. The development of the bovine embryo follows the same pattern but is delayed from one to four days according to the stage considered (see Fig.1).

Table 1. Staging and major features of the ovine embryos during the pre implantation period.

Stages	Days p.i.	Conceptus shape	Embryo	Embryonic layers	Placental annexes
PS-1	11-12	spherical	round	Ectoderm, endoderm	
PS-2	12-13	ovoid ≤0.5 cm	round	primitive mesoderm	
PS-3	12-13	tubular 1-3 cm	oblong		yolk sac
ES	13-14	elongated ≥10 cm	pear-shaped		
MS	13-14		primitive streak		amniotic folds
LS	14-15		notochord		
NP	15	*implanted*	1-3 somite pairs		amnion closure
HF	15-16		head folds		allantoïs bud

PS, pre-streak; ES, early streak; MS, mid-streak; LS, late streak; NP, neural plate; HF, head-folds.

Pre-Streak stages

These stages concern the expanded spherical, ovoid and the early tubular conceptuses. The Rauber's layer has completely disappeared so the rounded embryonic disc is exposed to the uterine environment and offers an easy access for direct observation (Fig. 4A). The outer ectoderm cells present the features of a polarized epithelium with an apical microvillous border (Fig 4B), typical epihtelial cell junctions, but lack expression of cytokeratins which are specific marker of epithelia.

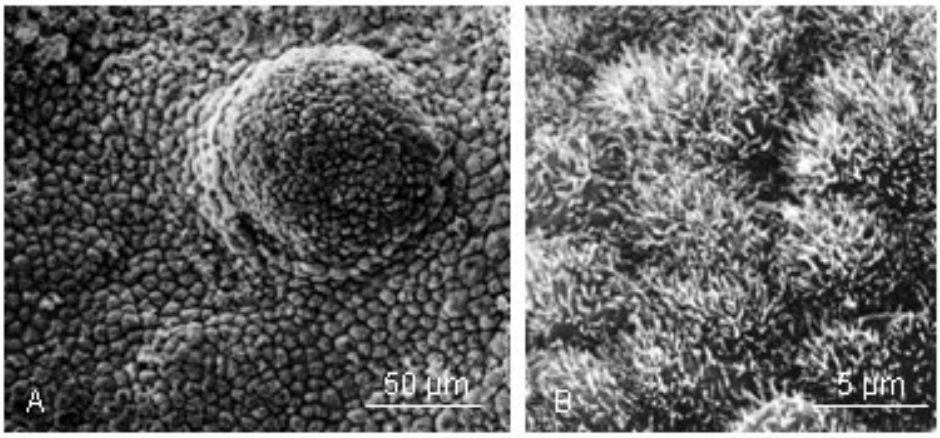

Figure 4. A) Ovoid day-12 ovine conceptus. Scanning electron micrography of the bulging embryonic disc free of the Rauber's layer at PS-2 stage. B) Apical cell surface of the ectoderm cells covered with microvilli.

Paradoxically, the ectoderm cells do express vimentin, a marker of mesenchymal cells (Fig. 5A). In PS-2 embryo a dense cellular area appears at one pole of the morphologically non polarized embryonic disc (Fig. 5B). Histological transverse sections of the embryos indicate that this area correspond to primitive mesoderm cells intercalated in between the visceral endoderm and the ectoderm (Fig. 5C). Since the pole from which the mesoderm cells emerge can be considered as the posterior pole of the embryo, that stage represents the earliest sign of the establishment of the embryonic antero-posterior (AP) axis which is firstly orientated perpendicularly to the elongation axis of the conceptus (Figs. 5B and 6A). Thereafter the embryo rotates so that the AP axis is parallel to the long axis of the whole conceptus. At PS-3 the embryo shape shifts from rounded to an oblong shape. The primitive mesoderm extends away from the embryonic area and the swollen yolk sac is apparent (Fig. 6A).

Primitive streak stages

In the filamentous conceptus the embryo is pear-shaped showing a rounded anterior edge and a constricted posterior pole. This morphological transformation characterizes the beginning of the primitive streak formation. In ES embryo AP polarity is evidenced by the loss of expression of vimentin in the posterior ectoderm cells which differentiate into mesoderm cells (Fig. 6B). The loss of vimentin expression is transient in the mesoderm cells since *de novo* expression is observed in the extra embryonic mesoderm which lines the yolk sac and the trophectoderm.

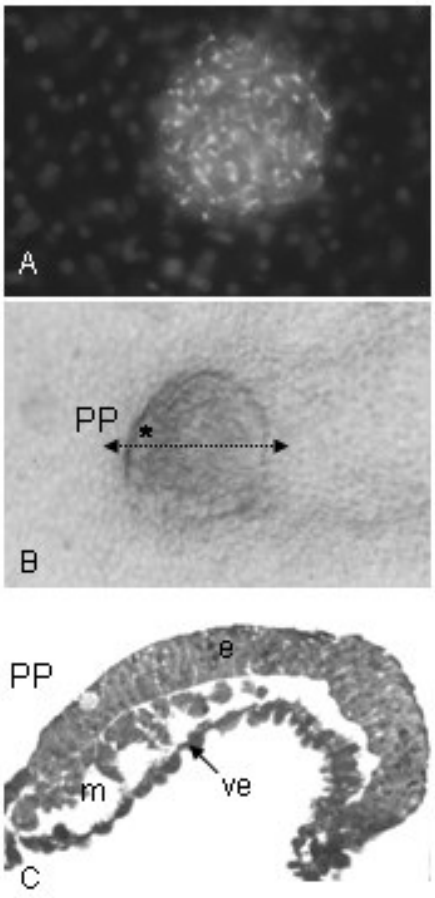

Figure 5. A) Immunolocalization of vimentin in the embryonic disc of a PS-1 embryo. B) Detail of the embryonic disc at PS-2 stage observed on the stereomicroscope. Note the thickening of tissue at one pole of the embryo (*). C) Transverse section through the plane indicated by the dottedarrow on figure B. Note accumulation of mesoderm cells (m) in between the ectoderm (e) and visceral endoderm (ve) at the posterior pole (PP).

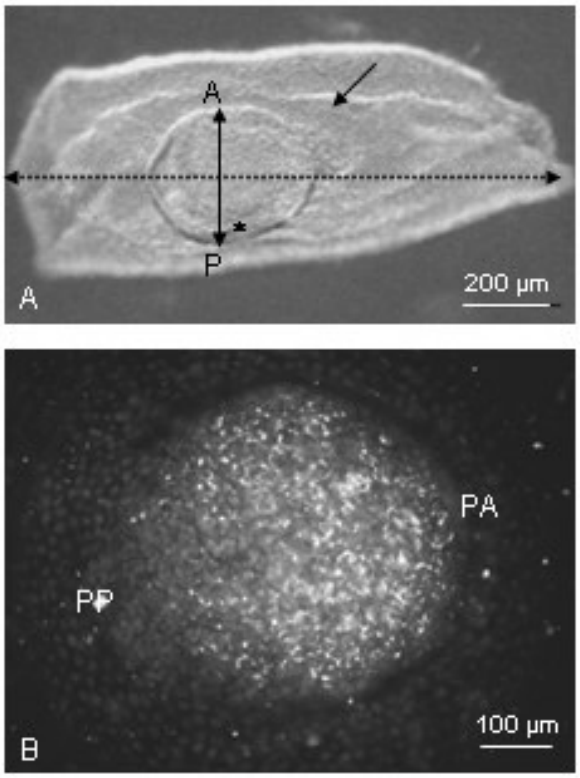

Figure 6. A) Embryonic area of a tubular conceptus (PS-3) showing the expanded yolk sac (arrow) viewed trough the trophoblast. Note the dense cellular area (asterisk) from which migrates the mesoderm. AP axis is the embryonic antero-posterior axis and the dotted line is the long axis of the conceptus. B) Imunolocalization of vimentin in ES embryo. Vimentin is not present in the ectoderm cells located at the posterior pole (PP) whereas the anterior pole (PA) is highly labelled.

At the same stage expression of *Brachyury* and *Eomesodermin* is detected in a clump of ectoderm cells located in the posterior pole of the embryo (Fig. 7A). Later on, the MS embryo completes the gastrulation process. The primitive streak is apparent as a groove which extends more than two-thirds from the posterior to the anterior poles of the embryo. The constricted posterior pole is surrounded by the amniotic folds (Fig 7B). Ectoderm cells along the primitive streak are vimentin negative but express *Eomesodermin* and *Brachyury* (Fig. 7C). These two genes belong to the T-box gene family and are key-factors in the gastrulation process in both vertebrate and invertebrate embryos (Herrmann and Kispert, 1994; Papaioannou and Silver, 1998). In mouse, *Brachyury* and *Eomesodermin* have been shown to play major roles during gastrulation and mesoderm patterning in the mouse (Herrmann et al., 1990; Ciruna and Rossant, 1999; Viebahn et al., 2002). Mutant mouse embryos lacking one of these genes fail to develop posterior parts of the body and extra embryonic tissues (Herrmann, 1991; Russ et al., 2000). Expression of *Brachyury* has been reported in bovine (Hue et al., 2001), rabbit (Viebahn et al., 2002) and pig gastrulating embryos (Flechon et al., 2004). In cow, ectopic expression of *Brachyury* has been observed in embryos produced by nuclear transfer cloning and could be one of the cause of for the arrest of development which occur during early pregnancy (Hue, unpublished data). As shown in sheep, *Brachyury* and *Eomesodermin* are expressed well after differentiation of the extra embryonic mesoderm which first occurs at the ovoid stage in PS-2 embryos. This clearly distinguishes again the ungulate embryo and this rises the question of the nature of the factors which act upstream of the T-box genes during mesoderm differentiation in ruminants.

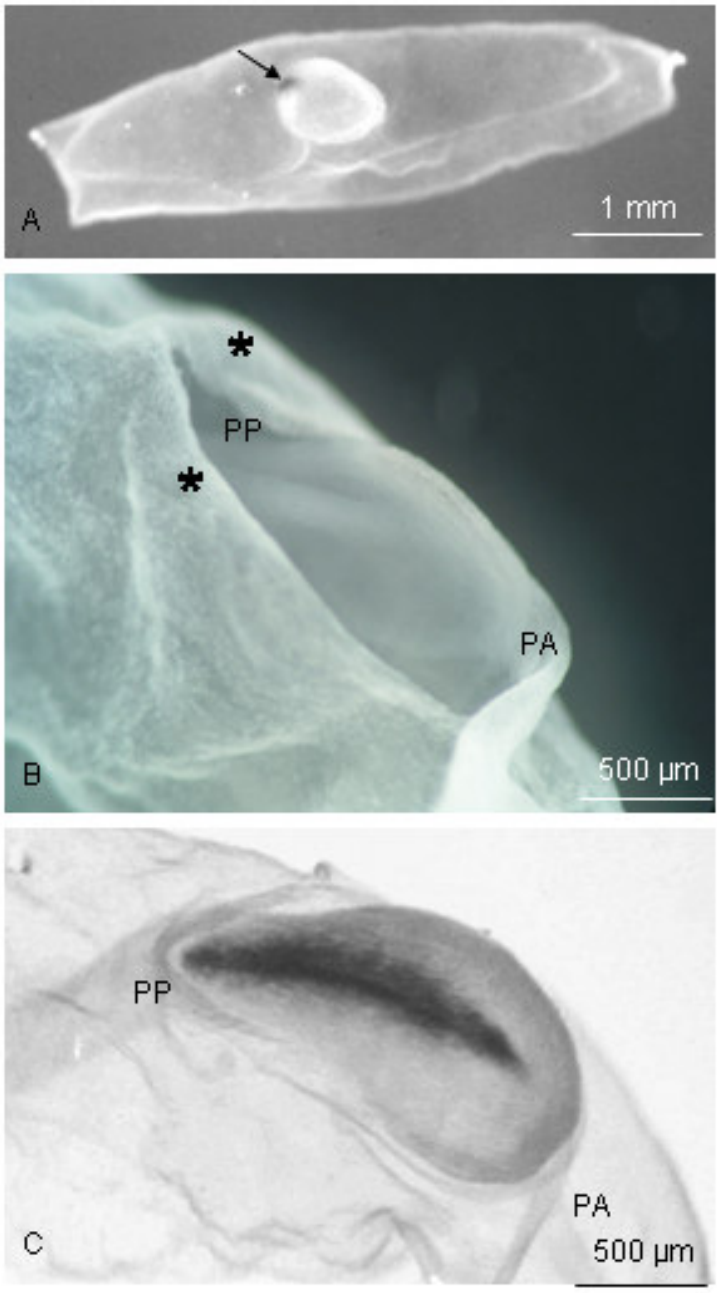

Figure 7. ES (A) and MS (B, C) ovine embryos. Expression of *Brachyury* in ES embryo (A) and MS embryo (C) analyzed by *in situ* hybridization with digogenin-labelled RNA probes. In ES embryo (A) a positive staining is detected in the posterior pole (arrow) of the embryo. Note the expanded saussage-like yolk sac viewed through the transparent trophoblast. In the MS embryos the primitive streak is apparent and the amniotic folds (asterix) cover the posterior pole. Expression of Brachyury (C) lines the primitive streak which extends from the posterior pole (PP) towards the anterior pole (PA).

Just before implantation the amnion folds fuse and enclose the LS embryo within the chorionic sac. At implantation the embryo enters the neural plate stage (NP) and one to three somite pairs are present. The yolk sac is fully developed and extends beneath the trophectoderm far away from the embryonic area. A vitelline vascularization appears, likely

ensuring the early exchanges between the embryo and the maternal tissues. The choriovitelline placenta which is formed soon after implantation, is transient since the yolk sac regresses and is replaced by the growing allantoïs giving rise to a definitive chorioallantoic placenta around day 35. In ruminants, failure of placental development is a common feature after nuclear transfer cloning (Hill et al., 2000; De Sousa et al., 2001; Chavatte-Palmer et al., 2002). Most of these failures are associated with transition of the choriovitelline to the allantochorionic placenta (De Sousa *et al.*, 2001). It is noteworthy that, although pathologies associated with embryo cloning affect both the fetal tissues and the placenta, most of them have a common origin, namely the extra embryonic mesoderm. Thus, the differentiation of the extra embryonic tissues in ruminants is of importance in the development of the placenta and requires further interest in future developmental studies.

Conclusions

In this review an attempt has been made to show how profound are the changes which affect the ruminant conceptus during the pre implantation period. Cell differentiations concern both the embryo itself and the extra embryonic tissues. It is obvious that complex interactions between the conceptus and the uterine milieu are required to drive these developmental processes but the precise nature of the factors and mechanisms involved remain to be elucidated. On the other hand, cellular interactions between the embryonic and extra embryonic tissues are also required. As evidenced in mouse (Beddington and Robertson, 1999) and rabbit (Idkowiak et al., 2004), experimental studies have established a key-role of the endoderm layer in mesoderm differentiation and axial patterning in the embryo. In view of these data, the cellular and molecular interactions between the various tissues which constitute the conceptus should be investigated more finely in the ruminant embryo.

References

Betteridge KJB, Flechon JE. 1988. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 29 :155-187.

Beddington RS, Robertson EJ. 1999. Axis development and early asymmetry in mammals. Cell 96:195-209.

Brandao DO, Maddox-Hyttel P, Lovendahl P, Rumpf R, Stringfellow D, Callesen H. 2004. Post hatching development: a novel system for extended in vitro culture of bovine embryos. Biol Reprod 71:2048-2055.

Cammas L, Reinaud P, Dubois O, Bordas N, Germain G, Charpigny G. 2005. Identification of differentially regulated genes during elongation and early implantation in the ovine trophoblast using complementary DNA array screening. Biol Reprod 72:960-967.

Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP. 2002. Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 66:1596-1603.

Ciruna BG, Rossant J. 1999. Expression of the T-box gene Eomesodermin during early mouse development. Mech Dev 81:199-203.

De Sousa PA, King T, Harkness L, Young LE, Walker SK, Wilmut I. 2001. Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. Biol Reprod 65:23-30.

Flechon JE, Degrouard J, Flechon B. 2004. Gastrulation events in the prestreak pig embryo: ultrastructure and cell markers. Genesis 38:13-25.

Flechon JE, Guillomot M, Charlier M, Flechon B, Martal J. 1986. Experimental studies on the elongation of the ewe blastocyst. Reprod Nutr Dev 26:1017-1024.
Gardner RL. 1983. Origin and differentiation of extraembryonic tissues in the mouse. Int Rev Exp Pathol 24:63-133.
Geisert RD, Brookbank JW, Roberts RM, Bazer FW. 1982. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 27:941-955.
Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. 2002. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction 124:289-300.
Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, Spencer TE. 2001. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod 64:1608-1613.
Guillomot M. 1995. Cellular interactions during implantation in domestic ruminants. J Reprod Fertil Suppl 49:39-51.
Guillomot M, Flechon JE, Wintenberger-Torres S. 1981. Conceptus attachment in the ewe: an ultrastructural study. Placenta 2:169-182.
Guillomot M, Guay P. 1982. Ultrastructural features of the cell surfaces of uterine and trophoblastic epithelia during embryo attachment in the cow. Anat Rec 204:315-322.
Guillomot M, Turbe A, Hue I, Renard JP. 2004. Staging of ovine embryos and expression of the T-box genes Brachyury and Eomesodermin around gastrulation. Reproduction 127:491-501.
Herrmann BG. 1991. Expression pattern of the Brachyury gene in whole-mount TWis/TWis mutant embryos. Development 113:913-917.
Herrmann BG, Kispert A. 1994. The T genes in embryogenesis. Trends Genet 10:280-286.
Herrmann BG, Labeit S, Poustka A, King TR, Lehrach H. 1990. Cloning of the T gene required in mesoderm formation in the mouse. Nature 343:617-622.
Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME. 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 63:1787-1794.
Hue I, Degrelle SA, Campion E, Renard JP. 2007. Gene expression in elongating and gastrulating embryos from ruminants. Soc Reprod Fertil Suppl 64:365-377.
Hue I, Renard JP, Viebahn C. 2001. Brachyury is expressed in gastrulating bovine embryos well ahead of implantation. Dev Genes Evol 211:157-159.
Idkowiak J, Weisheit G, Plitzner J, Viebahn C. 2004. Hypoblast controls mesoderm generation and axial patterning in the gastrulating rabbit embryo. Dev Genes Evol 214:591-605.
Maddox-Hyttel P, Alexopoulos NI, Vajta G, Lewis I, Rogers P, Cann L, Callesen H, Tveden-Nyborg P, Trounson A. 2003. Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. Reproduction 125:607-623.
Mattson BA, Overstrom EW, Albertini DF. 1990. Transitions in trophectoderm cellular shape and cytoskeletal organization in the elongating pig blastocyst. Biol Reprod 42:195-205.
Notarianni E, Flechon J. 2001. Parietal endoderm cell line from a rat blastocyst. Placenta 22:111-123.
Papaioannou VE, Silver LM. 1998. The T-box gene family. Bioessays 20:9-19.

Richoux V, Darribere T, Boucaut JC, Flechon JE, Thiery JP. 1989. Distribution of fibronectins and laminin in the early pig embryo. Anat Rec 223:72-81.

Russ AP, Wattler S, Colledge WH, Aparicio SA, Carlton MB, Pearce JJ, Barton SC, Surani MA, Ryan K, Nehls MC, Wilson V, Evans MJ. 2000. Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature 404:95-99.

Vajta G, Alexopoulos NI, Callesen H. 2004. Rapid growth and elongation of bovine blastocysts in vitro in a three-dimensional gel system. Theriogenology 62:1253-1263.

Vejlsted M, Avery B, Schmidt M, Greve T, Alexopoulos N, Maddox-Hyttel P. 2005. Ultrastructural and immunohistochemical characterization of the bovine epiblast. Biol Reprod 72:678-686.

Viebahn C, Stortz C, Mitchell SA, Blum M. 2002. Low proliferative and high migratory activity in the area of Brachyury expressing mesoderm progenitor cells in the gastrulating rabbit embryo. Development 129:2355-2365.

Wintenberger-Torres S, Flechon JE. 1974. Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 18. J Anat 118:143-153.

SHORT COMMUNICATIONS

SEXING OF OVINE EMBRYOS WITHIN A MOET SELECTION PROGRAM

ALABART J.L., DERVISHI E., COCERO M.J.[1], SÁNCHEZ P., ECHEGOYEN E., MARTÍNEZ-ROYO A., CALVO J.H., FOLCH J.

Unidad de Tecnol. en Prod. Animal, CITA, Av. Montañana 930. 50059-Zaragoza, Spain
[1]Depart. Reprod. Animal, SGIT/INIA. Av. Puerta de Hierro s/n. 28040-Madrid, Spain

In the selection scheme to improve prolificacy in the Rasa Aragonesa breed, the males to be tested are produced by a MOET program, using high merit ewes as donors. If only male embryos were transferred, costs of transfer procedures and maintenance of recipients would be reduced by about 50%. We present here preliminary survival rates of biopsed embryos for sex assessment within a MOET program.

One to ten cells from 97 embryos (compact morulas; grades 1 and 2) were aspirated through the zona pellucida using a micropipette. Fifteen embryos were severely injured during micromanipulation and were not transferred. The remaining 82 embryos were incubated in M199 at 38.5 ºC and 5% CO_2/air during the time required for genotyping (about 3 h) and transferred afterwards to FGA+eCG treated recipients (2 embryos/ewe). Fifty-two non-manipulated embryos from the same donors were simultaneously transferred and served as controls.

DNA was extracted from aspirated cells using the BLOODCLEAN DNA Purification kit (Biotools). Embryo sexing was performed by a Duplex-PCR, using one pair of ovine-specific primers (*PrnP* gene) and one pair of Y-chromosome-specific primers (GenBank Acc number U65982). Then, female samples show a unique band of 320 bp, while male samples exhibit an additional band of 186 bp. There was good agreement between the genotypic and fenotypic sex of the newborn lambs, even when only one cell was sampled. Survival rate of the biopsed embryos that were transferred was lower than that of controls, although statistical significance was not achieved (40.2% vs 55.8%; NS; Table 1). However, as 15 embryos were severely injured, overall survival rate was significantly lower compared with non-manipulated embryos (34.0% vs 55.8%; $P<0.05$).

Table 1. Embryo survival rates after embryo biopsy (a, b: $P<0.05$; chi-square test).

	N	Lambs born (%)
Embryos Biopsed		
Discarded	15	--
Transferred	82	33 (40.2) ab
Total	97	33 (34.0) a
Control	52	29 (55.8) b

In conclusion, this method is useful to determine the sex of embryos before transfer. However, the economical benefit of sexing in a MOET program may depend on whether the reduction of the survival rate due to biopsy (-21.8% at present) can be lowered or not.

Financed by Ministerio de Educación y Ciencia (Spain).

Notes

EFFECT OF SOMATIC CELL DONOR ON BOVINE NUCLEAR TRANSFER EFFICIENCY

AL-ROSTUM F.[1], BHOJWANI S.[1], POEHLAND R.[1], BECKER F.[1], VIERGUTZ T.[1], BRUNNER R.[2], KANITZ W.[1]

Research Unit Reproductive Biology[1] and Research Unit Molecular Biology[2] , Research Institute for the Biology of Farm Animals, Wilhelm-Stahl-Allee 2, D-18196, Dummerstorf, Germany

Since the first successful nuclear transfer (NT) experiments were carried out, various somatic cell types have been used as donor cells for the production of cloned animals. Recently, some researchers have shown that different cell cultures from different sources possess different capacities to support preimplantation development of NT embryos. The variation in blastocyst rates obtained in our previous studies led us to question whether the origin and culture conditions of the defined male and female fibroblast lines could be responsible for the differences in developmental potency.

In vitro matured oocytes were subjected to the Handmade Cloning (HMC^{TM}) procedure to produce cloned bovine embryos. For comparisons of somatic cell lines, ear fibroblasts from four bulls and two cows were utilized as nuclear donors and the blastocyst rate was determined after 7 days of culture. Prior to that, the cell cultures were subjected to flow cytometry (Beckman-Coulter EPICS Elite) and the cell cycle was analyzed using the Multicycle Program (Phoenix Flow Systems, San Diego, CA, USA). To measure the percentage of viable cells PI staining (30 µM, 10 min) was performed without fixation and RNAse treatment of the cells. The percentage of stained cells was analyzed by flow cytometer. Cytogenetic analysis was carried out to determine chromosomal abnormalities while karyotyping was performed using G-banding studies. To induce nonspecific DNA demethylation of somatic cells, 5-aza-2-deoxycytidine (5-Aza) was added to the cell culture and the subsequent DNA methylation measured by immunocytochemical staining using 5-methyl cytosine antibody. Semi-quantitative examination of the probes was conducted using a confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and image analyzing software (AnalySIS 3.3, SIS, Muenster, Germany).

Taking all our results into consideration, we conclude that different fibroblast lines recovered from the same tissue and cultivated under equal culture conditions could produce dramatically different blastocyst rates. The influence of cell line itself is higher than the influence of passage number. The observed effects of cell cycle stage, chromosomal aberrations, and diminished vitality are important but not sufficient to discriminate well-qualified nuclear donor cells. Notably, the blastocyst rates were dramatically decreased after induction of demethylation. We speculate that some epigenetically regulated deviations in the gene expression program are responsible for these phenomena.

Notes

EFFECT OF IGF-I AND EGF ON IN VITRO MATURATION OF SHEEP OOCYTES IN SEMI-DEFINED MEDIA

ALI A. BIN. T.[1,2], MAR L.[2], PILICHI S.[2], SANNA D.[2], DATTENA M.[2]

[1]*Dipartimento di Biologia Animale, Università di Sassari- Italia*
[2]*Istituto Zootecnico e Caseario per la Sardegna, 07040 Olmedo, Sassari - Italia*

The aim of this study was to determine the viability after transfer of fresh IVP blastocysts derived from oocytes cultured in media supplemented with insulin-like growth factor (IGF-I), epidermal growth factor (EGF), and a combination of insulin-like growth factor and epidermal growth factor (IGF+ EGF).

Ovaries of sheep of the Sarda breed were collected from a local slaughterhouse. Recovered oocytes were divided into four different maturation groups. The control group was matured in TCM199 supplemented with 4 mg/ml BSA, 100 µM cysteamine, 0.3 mM Na pyruvate, 0.1 UI/ml r-FSH (Gonal-F® 75), 0.1 UI/ml r-LH (Gonal-F® 75), and 1 µg/ml estradiol-17β. The IGF, EGF and IGF+EGF groups were matured in the same medium with the addition of either 100 ng/ml of IGF, 50 ng/ml of EGF, or a combination of 100 ng/ml of IGF-I plus 50 ng/ml of EGF.

Matured oocytes were fertilized with fresh Sarda semen in synthetic oviduct fluid (SOF) with 20% heat inactivated oestrus sheep serum. All the zygotes were cultured for 48 h in SOF supplemented with 1% BME and 1% MEM with 1 mM glutamine and 8 mg/ml BSA_{FF}. On the 3rd and 5th day, all culture groups were supplemented with 8 mg/ml fatty acid-free BSA with 6 mg/ml hyaluronan (HA).

Expanded blastocysts (6 to 7 days) were transferred in pairs directly into synchronized ewes. All pregnancies were confirmed by ultrasonography at 40, 60, and 80 days.Parturition was induced by corticosteroids with estradiol 17β (im.) on Day 146 of pregnancy. Cleavage rate, blastocyst rate, pregnancy, lambing, twinning, sex ratio and body weight were recorded.

There were no significant differences among the groups in cleavage, pregnancy rate at 40, 60 or 80 days, twinning rate, sex ratio or body weight. However, there were significant differences in blastocyst rate ($P<0.05$) between IGF and IGF+EGF and between the combination of the two growth factors and control group ($P<0.001$). In addition, there was significant difference in lambing rate ($P<0.05$) between IGF and EGF.

These results demonstrate the beneficial effects of growth factors (EGF, IGF+EGF) on the viability of ovine blastocysts.

Notes

THE VIABILITY OF VITRIFIED OVINE BLATOCYSTS PRODUCED IN THE PRESENCE OF IGF AND EGF

ALI A. BIN. T.[1,2], MAR L.[2], PILICHI S.[2], SANNA D. [2], DATTENA M.[2]

[1]*Dipartimento di Biologia Animale, Università di Sassari – 07100 Sassari - Italy*
[2]*Istituto Zootecnico e Caseario per la Sardegna, 07040 Olmedo, Sassari - Italy*

This study examined the viability, after transfer, of vitrified in vitro produced blastocysts derived from oocytes matured in medium supplemented with a combination of insulin-like growth factor and epidermal growth factor (IGF+ EGF).

Ovaries of sheep of the Sarda breed were collected from the slaughterhouse. Recovered oocytes were matured in TCM199 supplemented with 4 mg/ml BSA, 100 µM cysteamine, 0.3 mM Na pyruvate, 0.1 UI/ml r-FSH (Gonal-F® 75), 0.1 UI/ml r-LH (Gonal-F® 75), 1 µg/ml estradiol-17β, control group (C). Treatment group (IGF+EGF) was matured in same medium with the addition of 100 ng/ml of IGF-I plus 50 ng/ml of EGF. Matured oocytes were fertilized with fresh Sarda semen in synthetic oviduct fluid (SOF) with 20 % heat inactivated estrous sheep serum. All the zygotes were cultured for the first 48 h in medium consisting of SOF supplemented with 1% BME, 1% MEM, 1 mM glutamine and 8 mg/ml BSA_{FF}. On the 3rd and 5th day, the cultures were supplemented with 8 mg/ml fatty acid-freeBSA and 6 mg/ml hyaluronan.

Expanded blastocysts (6-7 days) were vitrified in 10% EG + 10% DMSO for 3/5 min, then placed into 20% EG + 20% DMSO + 0.5 M sucrose for < 40 sec., loaded into OPS, then immediately plunged into LN_2. Blastocysts were warmed by placing the OPS into a Falcon tube with TCM199 and 0.5 M sucrose solution at 37 °C for 3-5 min, and then into a drop of H-TCM199 and 20% FBS before transfer them into recipients ewes with the help of a tom-cat. The pregnancies were confirmed by ultrasonography at 40, 60 and 80 days. Cleavage rate, blastocyst formation, pregnancy, lambing, twinning rate sex ratio, and body weight were recorded.

There were no significant differences between the two groups in pregnancy rate at 40, 60, 80 days, lambing rate, twinning, sex ratio, and average body weight (Tables 1, 2). However, there were significant differences in cleavage rate ($P<0.05$), and blastocyst rate ($P<0.001$) between two groups (Table 1).Our data indicate that, vitrified ovine blastocysts produced in presences of IGF+EGF have similar viability after transfer compared to control group.

Table 1: Percentage of Cleavage, Blastocyst, Pregnancy Derived From Embryos Produced In Presence or Absence of Growth Factors.

Treatment	No. Oocytes	Cleavage rates (%)*	Blastocyst Rate (%)**	Pregnancy 40d (%)	Pregnancy 60d (%)	Pregnancy 80d (%)
IGF+EGF	97	88/97 (90.7) a	59/97 (60.8) a	6/12 (50.0)	6/12 (50.0)	6/12 (50.0)
Control	180	147/180 (81.7) b	72/180 (40.0) b	14/20 (70.0)	11/20 (55.0)	11/20 (55.0)

*a-b Values in a row of column with different letters differ ($P<0.05$).

**a-b Values in a row of column with different letters differ ($P<0.001$).

Table 2: Percentage of Lambing, Twinning, Sex Ratio, and Body Weight Derived From Embryos Produced In Presence or Absence of Growth Factors.

Treatment	Recipient	Lambing rate (%)	Twinning (%)	M (%)	F (%)	Body weight (Kg) Light (%)	Normal (%)	Heavy
IGF+EGF	10	7/20 (35.0)	1/6 (16.6)	5/7 (71.4)	2/7 (28.6)	0/7 (0.0)	6/7 (85.7)	1/7 (14.2)
Control	20	11/41 (26.8)	1/10 (10.0)	6/11 (54.5)	5/11 (45.4)	2/11 (18.2)	7/11 (63.6)	2/11 (18.2)

Notes

ROLE OF pZP1, pZP2, pZP3, AND pZP3-ALPHA TRANSCRIPT LEVELS IN FERTILIZING ABILITY OF PORCINE OOCYTES ISOLATED FROM CYCLING GILTS

ANTOSIK P.[1], KEMPISTY B.[2], BUKOWSKA D.[1], JACKOWSKA M.[2], GRÓDEK E.[2], JAŚKOWSKI J.[1], JAGODZIŃSKI P.P.[2]

[1]Department of Agricultural Veterinary, University of Agriculture, Poznań, Poland
[2]Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznań, Poland

Three major glycoproteins contribute to the formation of the protein structure of mammalian zona pellucida; ZP1, ZP2 and ZP3. Porcine zona protein 3 (pZP3) is responsible for major sperm receptor activity and plays a key role in the first step of the fertilization process. Porcine zona protein 2 (pZP2) is described as a secondary sperm receptor that binds sperm only after acrosome reaction. Both pZP2 and pZP3 are modified by the zona reaction soon after fertilization. pZP2 undergoes proteolytic cleavage and forms mature protein, and pZP3 loses the ability to activate the acrosome reaction. All of these mechanisms follow soon after fertilization and protect the oocyte from polyspermy.

The ovaries from cycling gilts (crossland-WBPxPBZ, n=14; age: 180 days; weight: 95-100 kg) were collected from a local slaughterhouse and transported to the laboratory at 35°C. Cumulus-oocyte complexes (COC) were isolated after scarification of the ovarian surface under a stereoscopic microscope. These isolated COC (n=50, from each gilt) were washed with NCSU37.

Total RNA was isolated from porcine oocytes according to Chomczyński and Sacchi (1987), treated by DNase I, and reverse-transcribed into cDNA. Quantitative analysis of pZP1, pZP2, pZP3, and pZP3-alpha cDNA was performed by RQ-PCR, which was conducted in a Light Cycler real-time PCR detection system Roche Diagnostics GmbH, (Mannheim, Germany) using SYBR® Green I detection dye. The pZP cDNAs were amplified using pairs of primers complementary to different exons. The quantity of mRNA products in each sample was adjusted to the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA or calculated per 10^3 oocytes. Quantification of copy number was derived from a standard curve of a known amount of synthetic DNA template. Each of these experiments was performed at least in triplicate. Results were estimated using Student's *t*-test and one-way analysis of variance (ANOVA) with Newman-Keul's *post-hoc* test. *P* value was determined by Student's *t*-test, with $P<0.05$ as the level of significance.

We found increased pZP2 and pZP3 transcript contents as compared to pZP1 and pZP3-alpha ($P<0.001$). We did not observe statistically significant differences between the levels of pZP2 and pZP3 mRNAs ($P=0.346$).

The zona pellucida proteins form oocyte-specific receptors, which mediate sperm-zona binding and process. The aim of the study was to characterize which of the proteins may play the most important role in this mechanism. Our observations suggest that pZP2 and pZP3 may contribute to successful fertilization and play a more important role in this process than pZP1 and pZP3-alpha. Increased levels of pZP2 and pZP3 mRNAs suggest that the oocytes are capable of fusion with sperm and protected from polyspermy.

Notes

DIFFERENTIAL EXPRESSION OF EPIDERMAL GROWTH FACTOR, TRANSFORMING GROWTH FACTORS, AND INSULIN-LIKE GROWTH FACTOR IN PORCINE ENDOMETRIUM

ANTOSIK P.[1], KEMPISTY B.[2], BUKOWSKA D.[1], JACKOWSKA M.[2], JAŚKOWSKI J.[1], JAGODZIŃSKI P.P.[2]

[1]Department of Agricultural Veterinary, University of Agriculture; [2]Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznań, Poland

Insulin-like growth factor (IGF) is a single-chain polypeptide structurally homologous to proinsulin and has insulin-like metabolic effect. IGF is expressed in the early proliferative phase of endometrium. The cyclic changes in IGF mRNA levels coincide with serum estradiol concentration. In most mammals, uterine epidermal growth factor (EGF) is expressed at the time of implantation. Transforming growth factor (TGF) is expressed in luminal epithelium throughout the uterus during the preimplantation period.

Endometrium was collected from multiparous (crossland-WBPxPBZ, n=5; age: 2.5-3.5 years; parity: 3-5; mean number of animals in litter: 10-12), cycling gilts (n=12, age: 180 days; weight: 95-100 kg), and prepubertal aimals (n=5; age: 180 days; weight: 95-100 kg) from a local slaughterhouse.

Each RNA sample was isolated from 0.5 g of endometrium according to Chomczyński and Sacchi (1987). Purity of the RNA samples was verified spectrophotometrically at 260 and 280 nm. RNA was treated with DNase I and reverse-transcribed into cDNA using random hexamer priming and reverse transcriptase (RT). Quantitative analysis of EGF, TGFβ1, TGFβ2, TGFβ3, and IGF cDNA was performed by RQ-PCR SYBR Green I analysis. Quantification of copy number was derived from a standard curve of known amounts of synthetic DNA template.

Real-time PCR analysis revealed a 6-fold higher expression of IGF in endometrium isolated from multiparous compared to cycling gilts and prepubertal gilts, although no significant difference was detected in expression between multiparous and prepubertal gilts ($P=0.436$). In addition, we did not observe statistical differences in TGFβ2 expression between the three groups of animals. Expression of TGFβ3 was 6-fold higher in prepubetral gilts compared to cycling gilts ($P<0.001$), and 8-fold higher compared to multiparous ($P<0.001$). We also observed an increased expression of EGF and TGFβ1 in multiparous compared to cycling gilts ($P<0.001$), and prepubertal gilts ($P<0.001$).

The aim of the study was to determine which of these growth factors plays the most important role in implantation. Increased expression of EGF and TGFβ1 in endometrium isolated from cycling and multiparous suggests that these factors are associated with the reproductive maturity of these animals. In addition, expression of these factors may be induced by hormones secreted during pregnancy and may stimulate successful embryo implantation. The lack of statistical differences in TGFβ2 expression between the studied groups of animals suggests that this factor is not associated with the process of sexual maturity. Decreased expression of TGFβ3 in endometrium of multiparous animals suggests that this gene plays a subordinate role in embryo implantation, and its expression may be inhibited by hormones secreted during sexual maturity and pregnancy. Estradiol is secreted by the ovary and stimulates endometrial sloughing and regeneration and may regulate IGF expression. Increased expression of IGF in cycling and multiparous animals suggests that this gene may be regulated by changes in estradiol secretion in sexually mature animals. Low IGF expression in prepubertal females may be associated with sexual immaturity and lack of ovulation in those animals.

Notes

RELATIONSHIP BETWEEN PROGESTERONE AND PREGNANCY ASSOCIATED GLYCOPROTEIN CONCENTRATIONS IN THE MATERNAL CIRCULATION DURING EARLY PREGNANCY IN DAIRY COWS

BARBATO O.[1], MERLO M.[2], SOUSA N.M.[3],TRENTIN E.[2], BECKERS J.F.[3], GABAI G.[2]

[1] Dip. di Scienze Biopatologiche ed Igiene delle Produzioni animali ed alimentari, Università di Perugia, via San Costanzo, 4 – 06126 Perugia, Italy;
[2] Dip. di Scienze Sperimentali Veterinarie, Università di Padova, Agripolis, 35020 Legnaro (PD), Italy;
[3] Laboratory of Endocrinology and Animal Reproduction, Faculty of Veterinary Medicine, University of Liege, B-4000, Belgium

The corpus luteum (CL) plays a major role for the successful establishment of pregnancy, and CL function during early embryonic development and implantation seems to be crucial for a correct foetus-maternal interaction. Pregnancy-associated glicoproteins (PAGs) represent a large family of glycoproteins expressed in the mononucleate and binucleate cells of the trophectoderm of several species. In the bovine, PAGs are detectable in the maternal circulation from D 24 after fertilization until parturition, and they are unquestionable indicators of the presence of a viable embryo. However, data about the biological function and the control of secretion of these glycoproteins are scarce. In this study, we tested the hypothesis that the progesterone (P4) secreted by the CL around the time of embryo implantation may influence PAG secretion during early pregnancy. The outcome of 84 artificial inseminations (AI) was studied in 56 pluriparous cows by measuring PAG and P4 (RIA) in maternal plasma samples taken at AI (D 0) and at 21, 28, 30, 45, 60 and 90 D after AI. Confirmation of pregnancy was performed by trans-rectal examination after D 45. Fourty-four AIs (52.4%) were positive, while embryo loss was observed in 10 cases (11.9%). On D 21, plasma P4 was higher, although not significantly, in positive AIs (6.09±0.39 ng/ml) than in AIs followed by embryo loss (4.88±0.79 ng/ml). Plasma PAG concentrations were significantly higher ($P<0.01$) in AIs performed before 80 days post parturition and were excluded from subsequent analysis. Plasma PAG concentrations were significantly lower in AIs followed by embryo loss ($P<0.001$) between D 30 and D 60. In most cases, embryo loss occurred between D 30 and D 45. Hormone data obtained from positive AIs performed after 80 days post parturition (N=40) were used to study the relationships between plasma progesterone measured on D 21 and PAG plasma concentrations. AIs were grouped on the basis of P4 concentrations observed on D 21 into Low P4 Group (P4<6.0 ng/ml at D 21) and High P4 Group (P4≥6.0 ng/ml at D 21), and the relationship between plasma P4 and PAG was analysed by ANOVA. On average, AIs of the High P4 Group showed higher levels of circulating PAG between D 30 and D 60 and, in particular, plasma PAG was significantly greater on D 60 (High P4 Group: 5.62±0.63 ng/ml; Low P4 Group: 4.00±0.57 ng/ml; $P<0.05$). The results of the present study confirm the hypothesis that circulating P4 around the time of embryo implantation can affect PAG secretion in early pregnancy.

Supported by Italian Ministry of University and Research, PRIN 2005

Notes

MELATONIN TREATMENT DURING ANESTRUS ENHANCES OOCYTE DEVELOPMENTAL COMPETENCE IN FSH-TREATED GOATS

BERLINGUER F., SPEZZIGU A., [1]SUCCU S., BEBBERE D., MADEDDU M., TEDDE A.[1], SATTA V.[1], LEONI G.G., NAITANA S.

Department of Animal Biology, [1]Department of Physiological Biochemical and Cellular Sciences, Faculty of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100 Sassari, Italy

Numerous studies have focused on developing hormonal treatments to improve follicular development and induce a fertile estrus in goats during the non-breeding season. Melatonin treatment has been shown to be an effective method for inducing estrous cycles and increasing ovulation and subsequent lambing rates during anestrus (Pellicer-Rubio et al., Anim Reprod Sci 2007 ; 98 :241–258). On the other hand, data concerning oocyte developmental competence are limited for anestrous goats. In sheep, it has been reported that administration of melatonin and CIDR devices in conjunction with FSH during seasonal anestrus had no effect on the number of antral follicles or the number of recovered and healthy oocytes, as evaluated by in vitro fertilization rates (Luther et al., Theriogenology 2005; 63:2136–2146).

The objective of the current study was to evaluate the effects of melatonin treatments on follicular development and oocyte quality in FSH-treated goats during seasonal anestrus. Follicular dynamics was monitored by daily ultrascound scanning, while oocyte quality was assessed by their in vitro developmental competence. During seasonal anestrus (March–May), 9 multiparous Sarda goats were randomly divided into two groups: MEL Group: goats received an implant containing 18 mg of melatonin (Melovine; CEVA VETEM, Milano, Italy) on Day 0 (n=5); CTR Group: untreated goats (n=4). Starting from Day 3 up to Day 30, total number of ≥3 mm follicles and size of the two largest follicles were assessed daily in all the animals by 7.5MHz transrectal ultrasonography (Aloka SSD-500, Multimage, Cagliari, Italy). All goats were given a total dose of 120 I.U. FSH (Folltropin, Bioniche Animal Health, Bio 98, Milano, Italy) im twice daily subdivided in 6 constant doses starting on Day 28. On Day 31, 12 hours after the last FSH administration, ovaries were collected at slaughter. Total follicles present on their surface were counted and oocytes were recovered after follicular aspiration with a 2.5 syringe fitted with a 22-G needle. Oocytes derived from the two different groups were kept separated throughout the in vitro procedures and in vitro matured, fertilized and cultured under standard conditions (Leoni et al., Anim Reprod Sci 2007; 92:373–383).

Ultrasound examination of the ovaries showed that melatonin treatment did not stimulate any change in follicular dynamics, nor in ovarian response to FSH treatment (39±20.9 vs 37.2±14.5 total follicles on Day 31 in MEL and CTR groups, respectively). On the other hand, its effect was evident on oocyte developmental competence, as shown in Table 1.

Table 1. Differences in in vitro maturation, fertilization and developmental capacity of oocytes collected from follicles of melatonin treated (MEL) or untreated (CTR) goats after FSH administration.

	IVM	IVF	CLEAVAGE			BLASTOCYST		
			26 h.p.f.	42 h.p.f.	Total	Day 7	Day 8	Total
CTR	88	80	9^{a}	37	46^{a}	12^{a}	4	16^{a}
MEL	128	116	25^{b}	59	84^{c}	30^{b}	6	36^{b}

h.p.f. = hours post fertilization. $^{a, b, c}$ Within the same column different superscripts indicate statistical difference: Chi^2 test a vs b $p<0.05$; a vs c $p<0.01$.

In conclusion, the results of this study showed that melatonin treatment during goat seasonal anestrus significantly enhances oocyte quality after FSH treatment, without causing any changes in ovarian follicular dynamics.

Supported by RAS (Special Funding De Minimis).

Notes

DIFFERENCES IN METHYLATION STATUS BETWEEN MALE AND FEMALE BOVINE BLASTOCYSTS PRODUCED IN VITRO

BERMEJO-ALVAREZ P.[1], RIZOS D. [1], RATH D. [3], LONERGAN P.[2], GUTIERREZ-ADAN A.[1]

[1]*Dpto. de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain*

[2]*School of Agriculture, Food Science and Veterinary Medicine, College of Life Sciencse, University College Dublin, Ireland*

[3]*Dept. of Biotechnology, Institute of Animal Breeding (FAL), Hoeltystr. 10, 31535 Neustadt, Germany*

Epigenetic differences provide a plausible link between physiological and gene transcription differences observed between male and female preimplantation bovine embryos. The aim of this study was to examine gender-related differences in methylation of different regions of the genome and mRNA transcription of genes related with cytosine methylation and histone methylation in bovine blastocysts produced in vitro.

Bovine cumulus oocyte complexes were matured in vitro for 24 h and inseminated with frozen-thawed sorted (X or Y) and unsorted (control) bull sperm. Zygotes were cultured to the blastocyst stage and were snap frozen in groups of 10. Analysis of methylation status was carried out by bisulphite-PCR technology and quantification of mRNA transcripts was done by quantitative PCR of DNA methyltransferase 1 (Dnmt1), DNA methyltransferase 3a (Dnmt3a), DNA methyltransferase 3b (Dnmt3b), HMT1 hnRNP methyltransferase-like 2 (Hmt1), and interleukin enhancer binding factor 3 (Ilf3).

The level of methylation in a sequence near a variable number of tandem repeats minisatellite region (VNTR) in males (39.8 ± 4.8) was higher than in females (23.7 ± 3.1). However, a stable maintenance of DNA methylation was revealed in a heterochromatic sequence (Satellite I) and in the promoter region of a single-copy gene (Cytokeratin). Transcription differences between genders were observed for Dnmt3a, Dnmt3b, Hmt1, and Ilf3.

Our results show epigenetic differences between genders in in vitro produced bovine embryos and suggest that before the sex determination initiation, epigenetic events may modulate the gender-related differences in speed of development, metabolism and transcription observed during the preimplantation period. Furthermore, to our knowledge, it is the first report of differences in methylation status between sexes during that period in any species.

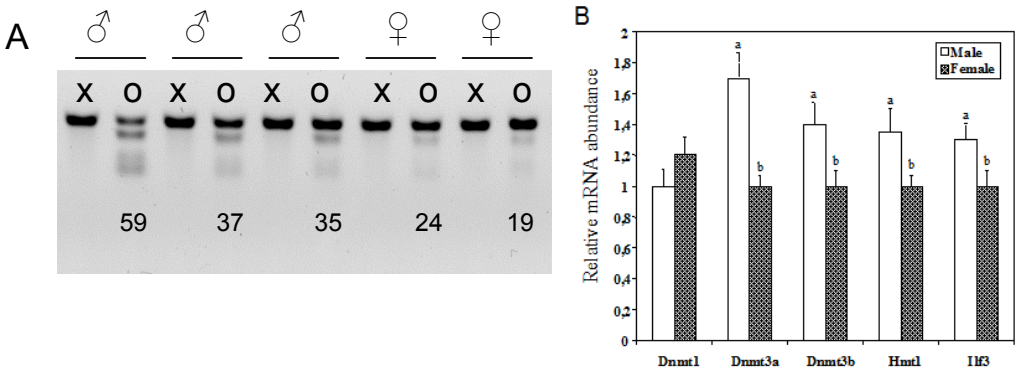

Figure. 1. (A) Representative result of differential methylation of a sequence near VNTR between male and female in vitro produced bovine blastocysts. PCR products obtained from bisulphite-treated DNA digested with *AciI* restriction enzyme. Intact undigested samples (X) versus digested samples (O). (Anova analysis, $P<0.05$). (B) Relative mRNA expression of genes related with cytosine methylation (Dnmt1, Dnmt3a and Dntm3b) or histone methylation (Hmt1 and Ilf3) in male and female bovine in vitro produced blastocyst. (Anova analysis, $P<0.01$).

Notes

OPTIMIZING BOVINE EMBRYO PRODUCTION BY SOMATIC CELL NUCLEAR TRANSFER

BHOJWANI S., TORNER H., ALM H., KANITZ W., POEHLAND R.

Research Unit Reproductive Biology, Research Institute for the Biology of Farm Animals, Wilhelm-Stahl-Allee 2, D-18196, Dummerstorf, Germany

The success of cloning an entire animal with the help of a differentiated somatic cell has been quite revolutionary with promises of a wide spectrum of biomedical potentials. Some of the challenges faced, however, are low and/or inconsistent blastocyst rates, variable blastocyst quality, low overall efficiency (in terms of live birth) and developmental abnormalities. Various strategies have been pursued in the past to overcome the different hurdles in the field of nuclear transfer (NT). The aim of the present investigation was to study the change in the efficiency of bovine NT embryo production (in terms of blastocyst rate) after modifications to the method of oocyte enucleation, electrofusion (cytoplast & somatic cell) and the gas-phase during embryo culture, in order to ultimately optimize the NT process.

For our experiments, compact cumulus oocyte complexes (COCs) were recovered from slaughterhouse-collected bovine ovaries by aspirating follicles greater than or equal to 5mm at the surface of the ovary. After IVM, oocytes were subjected to NT procedure (Handmade Cloning, HMC^{TM}) using ear fibroblasts from at least four different bulls (MF1 to MF4) as nuclear donors for the production of cloned bovine embryos which were then cultured for a period of 7 days to determine the blastocyst rate. Following modifications were carried out parallel to the standard HMC^{TM} protocol: (a) Chemically assisted enucleation using demecolcine (Vajta et al., Reprod Fertil Dev. 2005;17(8):791-7) vs. the standard enucleation by Hoechst staining & UV exposure; (b) Single-step electrofusion (Vajta et al., Reprod Fertil Dev. 2005;17(8):791-7) vs. the double-step fusion; (c) 20% oxygen tension during embryo culture vs. 5% oxygen in the gas phase. The data were evaluated by one-way analysis of variance (ANOVA). All results with $P < 0.05$ were considered statistically significant.

The different cell lines (MF1 to MF4) exhibited no significant differences in terms of the cleavage rate. Significantly different blastocyst rates were recorded between them with MF4 being the best of all. The difference in the blastocyst rates were maintained between the cell lines irrespective of the gas phase, though a low oxygen tension (5%) tended to favour blastocyst development rate significantly in all the cell lines. Chemically assisted enucleation utilizing demecolcine to form an extrusion cone resulted in significantly higher cleavage and blastocyst rates (in comparison to Hoechst selection) in the cell line which had earlier provided the best blastocyst rate (MF4; 40% vs. 27%). When the same line (MF4) was further tested for single-step electrofusion vs. double-step, a significantly higher blastocyst rate was achieved (42% vs. 30%) showing positive effects of reduced exposure to electric field. These results show that low oxygen tension, demecolcine-assisted enucleation and single-step electrofusion could increase the output of bovine embryo production after NT.

Notes

EFFECT OF ADDING SERUM OR FOLLICULAR FLUID TO THE MATURATION MEDIUM ON *IN VITRO* FERTILIZATION OF PORCINE OOCYTES

BIJTTEBIER J., VAN SOOM A., MATEUSEN B., MAES D.

Department of Obstetrics, Reproduction and Herd Health, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

Follicular fluid is a transsudate of serum but also contains locally produced molecules. Follicular fluid is usually added to the maturation medium to improve *in vitro* maturation and fertilization of porcine oocytes. In this study, the maturation medium was supplemented with sow follicular fluid on the one hand and with autologous sow serum on the other hand. The main purpose of the study was to investigate whether supplementation of follicular fluid has additional positive effects on maturation and fertilization outcome compared to serum. Special attention was paid to the incidence of polyspermic fertilization, which is one of the major problems of the *in vitro* fertilization system of porcine oocytes.

Three sows were slaughtered in the preovulatory stage of the oestrous cycle after hormonal treatment. Both the follicular fluid and the serum were collected, filtered (0.22µm) and kept at -80°C until use. Subsequently, oocytes of prepubertal gilts were obtained (n= 1302; 4 replicates) and subdivided in 7 groups according to the type of supplementation of the maturation medium. Oocytes were matured for 44 hours in 'North Carolina State University' (NCSU23) medium supplemented with 10% serum (S1, S2, S3) or 10% follicular fluid (F1, F2, F3) of the 3 sows. NCSU23 supplemented with 0.1% polyvinylalcohol was used as a negative control. A proportion of the oocytes was fixed and stained with Hoechst 33342 for evaluation of the nuclear maturation while the other part was fertilized. About 20 hours after the start of the oocyte-sperm incubation period, oocytes were fixed and stained (Hoechst 33342) for investigation of the fertilization parameters. The data were analyzed using logistic regression analysis including the effect of replicate. Treatment was included as fixed factor and the fertilization and maturation outcome (0-1) as dependent variable. Statistical analysis was performed using SPSS 14.00.

With regard to the nuclear maturation, a comparable number of oocytes reached the metaphase II (MII) stage and could be consequently considered as fully matured (86% for S1, S2 en S3, 85% for F1, F2 en F3) ($p > 0.05$). For the negative control group, nuclear maturation was inferior compared to the other groups (41% MII) ($p < 0.05$). Assessment of the fertilization parameters showed that more oocytes were penetrated in the groups matured in serum compared to those matured in follicular fluid (47% versus 34%) ($P < 0.05$). Moreover, of the penetrated oocytes, more polyspermic fertilization was seen in the groups matured in serum (43% versus 22%) ($P < 0.05$).

This study demonstrated a positive influence of both serum and follicular fluid on the nuclear maturation of porcine oocytes. Although normal fertilization was the same for oocytes matured in serum versus follicular fluid, the latter reduced the rate of polyspermy compared to serum. We hypothesize that a locally produced factor in the follicular fluid can protect the oocyte against polyspermic fertilization. The identity of this factor is the subject of current research.

Notes

EFFECT OF DIFFERENT VITRIFICATION PROTOCOLS AND SOURCE OF EMBRYOS ON VIABILITY AFTER VITRIFICATION OF OVINE BLASTOCYSTS

BOGLIOLO L., ARIU F., FOIS S., LEDDA S.

Department of Pathology and Veterinary Clinic, University of Sassari, 07100, Sassari, Italy.

The objective of this study was to assess the viability of ovine embryos produced by in vitro fertilization (IVF) or by parthenogenetic activation after vitrification with two different systems. For this purpose in vitro matured sheep oocytes were activated with ionomycin (5 μM, 5min)-6-DMAP (2mM, 3h) or fertilized in vitro with frozen/thawed ram semen and cultured in vitro. After 6-7 days of culture expanded blastocysts were divided in two different vitrification protocols as follows: (A) exposure to 10% glycerol, 3 min.; 10% glycerol and 20% EG, 3 min; 25% glycerol and 25% glycerol, 25 sec.; loading onto 0.25 ml french straw, plunging into N_2. (B) exposure to 7.5% EG and 7.5% DMSO, 3 min.; 16.5% EG, 16.5% DMSO, 0.5M sucrose, 30 sec; loading onto cryotop and plunging N_2.

For warming the straws were dipped into a 37°C water bath for 15 sec and embryos transferred into 0.25 M sucrose solution. Cryotops were directly inserted into 1.25 sucrose solution followed by stepwise diluition of cryoprotectants Blastocysts were cultured in TCM-199 with 10% FCS and examined at 8 h intervals for 24 h.The embryos that re-expanded and hatched were considered to be viable. Statistical analysis was done using the Chi-square test.

After 8 h culture, vitrification with system A induced a significantly ($P<0.05$) higher proportion of re-expansion in both parthenogenetic (76.2%) and IVF blastocysts (43.5%) compared to system B (43.5% and 25.7%, respectively). Re-expansion rate at 8 h was significantly ($P<0.05$) higher in parthenogenetic blastocysts vitrified with system A than in the other groups whereas any difference was recorded at 16 h and 24 h of culture. Hatching rates were significantly ($P<0.05$) increased in parthenogenetic and IVF blastocysts cryopreserved with A (61.9% and 54.2%, respectively) compared to B systems (26.1% and 25.7%, respectively).

In conclusion, the use of straw-vitrification protocol positively affects re-expansion and hatching rate of parthenogenetic and IVF embryos. Parthenogenetic blastocysts show a higher re-expansion within 8 h than IVF counterparts suggesting inherent differences between the two groups affecting embryo sensitivity to vitrification procedures.

Table 1. In vitro viability of parthenogenetic and IVF embryos after vitrification with different systems.

Vitrification system	Source of embryos	Vitrified embryos	Re-expanded (%)			Hatching (%)
			8 h	16 h	24 h	
A	Parthenog.	21	16 (76.2)[a]	16 (76.2)	16 (76.2)	13 (61.9)[a]
B		23	10 (43.5)[b]	13 (56.5)	13 (56.5)	6 (26.1)[b]
A	IVF	24	11 (45.8)[c]	13 (54.2)	13 (54.2)	13 (54.2)[a]
B		35	9 (25.7)[d]	15 (42.8)	16 (45.7)	9 (25.7)[b]

Different superscript are significant different ($P< 0.05$).

Notes

IDENTIFICATION OF INTEGRINS EXPRESSION IN DOG ENDOMETRIUM IN DIFFERENT PERIODS OF REPRODUCTIVE CYCLES

BUKOWSKA D.[1], KEMPISTY B.[2], ANTOSIK P.[1], REMBOWSKA M.[1], JACKOWSKA M.[2], JAGODZIŃSKI P.P.[2], JAŚKOWSKI J.M.[1]

[1]Department of Agricultural Veterinary, University of Agriculture, Poznań, Poland
[2]Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznań, Poland, University of Agriculture, Poznań, Poland

Integrins, described also as platelet cell adhesion molecule receptors have common heterodimeric structure with alpha and beta subunits. Integrins are generally expressed on the oocytes surface and are responsible for sperm specific interaction, including recognition and fusion. Our study first described the possible function of those proteins as dogs endometrial adhesion molecules involved in the process of embryo attachment during implantation.

The aim of the study was to evaluate the expression of integrins α2b, β2 and β3 in endometrium isolated from metoestrus and anoestrus bitches. Endometrium (0.5 g) was collected from metoestrus (n=5; age: 3-14, mean=7.5; weight: 12.6-26, mean=17.2; condition=3 points) and anoestrus (n=3; age: 3-8, mean=5.5; weight: 20-35, mean=27.5; condition=4 points) bitches after ovariohysterectomy. Total RNA was isolated from the bitches endometrium by the method of Chomczyński and Sacchi (1987), treated by DNase I, and reverse-transcribed into cDNA**.** Quantitative analysis of integrins α2b, β2 and β3 cDNA was performed by RQ-PCR, which was conducted in a Light Cycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I detection dye. The cDNA of the integrins was amplified using pairs of primers complementary to different exons**.** The quantity of mRNA product in each sample was adjusted to the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Quantification of copy number was derived from a standard curve of a known amount of synthetic DNA template. Each of these experiments was performed at least in triplicate. Results were estimated using Student's t-test and one-way analysis of variance (ANOVA) with Newman-Keul's post-hoc test. P value was determined by Student's t-test, with significance $P<0.05$.

We observed 6-fold higher levels of integrins β2 and β3 cDNAs in endometrium isolated from metoestrus as compared to anoestrus bitches ($P<0.001$). We also observed an increased content of integrin α2b in metoestrus bitches endometrium as in anoestrus animals ($P<0.001$). Integrins are described as leukocytes adhesion molecules, which are also involved in fertilization. It has been suggested that these proteins contribute in adhesion and fusion between the male and the female gametes, which is the first step to successful fertilization. Our results first demonstrated the specific expression of integrins α2b, β2 and β3 in endometrium isolated form metoestrus and anoestrus bitches. We postulated that those proteins might contribute not only to specific germ cell fusion but also to recognition and adhesion between early embryos and endothelial cell of endometrium. An increased expression of investigated integrins in endometrium isolated from metoestrus bitches suggested that this reproduction period might be responsible for successful embryo implantation and development. The low level of their expression in endometrium from anoestrus bitches is probably a result of the low level of ovarian activity and "reproductive cycles silence" in those animals.

Notes

QUANTITATIVE ANALYSIS OF EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTORS ALPHA AND BETA EXPRESSION IN BITCH ENDOMETRIUM

BUKOWSKA D.[1], KEMPISTY B.[2], ANTOSIK P.[1], REMBOWSKA M.[1], JACKOWSKA M.[2], JAGODZIŃSKI P.P.[2], JAŚKOWSKI J.M.[1]

[1]Department of Agricultural Veterinary, University of Agriculture, Poznań, Poland
[2]Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznań, Poland

Epidermal growth factor (EGF) is described as a potent mitogenic molecule that induces specific cell differentiation and growth. In the endometrium EGF is involved in the process of luminal epithelial cells proliferation, which is the initial step of preparing to embryo attachment during implantation. Transforming growth factor (TGF) is a multifunctional peptide that controls proliferation, differentiation, and metabolic functions in many cell types, including epithelium.

The aim of the study was to compare the differences in expression of EGF and transforming growth factors alpha and beta (TGFα, TGFβ1, TGFβ2, and TGFβ3) in endometrium isolated from metoestrus and anoestrus bitches. We also speculate on which of the growth factors may play the principal role in successful embryo implantation.

Endometrial tissue (0.5 g) was collected from metoestrus (n=5; age: 3-14, mean=7.5; weight: 12.6-26, mean=17.2; condition=3 points) and anoestrus (n=3; age: 3-8, mean=5.5; weight: 20-35, mean=27.5; condition=4 points) bitches after ovariohysterectomy. Each RNA sample was isolated from 0.5 g of endometrium according to Chomczyński and Sacchi (1987). Purity of the RNA samples was verified spectrophotometrically at 260 and 280 nm. RNA was treated with DNase I and reverse-transcribed into cDNA using random hexamer priming and reverse transcriptase (RT). Quantitative analysis of EGF, TGFα, TGFβ1, TGFβ2, and TGFβ3 cDNA was performed by RQ-PCR SYBR Green I analysis. Quantification of the copy number was derived from a standard curve of known amounts of synthetic DNA template.

Real-time quantitative PCR reaction revealed 8-fold higher expression of EGF, TGFβ1, TGFβ2 and TGFβ3 in endometrium isolated from metoestrus compared to anoestrus bitches ($P<0.001$). In the group of metoestrus bitches we observed the most increased expression of EGF and TGFβ1 compared to other genes. We did not find any statistical differences in TGFα expression between those two groups of animals ($P<0.085$).

Metoestrus is characterized as the last step of luteal period of reproductive cycles in bitches, which occurs quickly after successful fertilization. An increased expression of EGF and TGFs in endometrium from metoestrus bitches may result in high receptivity of uterus to receive the blastocyst. We suggest that the main role of this phase is to prepare the luminal epithelium for embryo implantation. We also postulate that from among all investigated growth factors, EGF and TGFβ1 may contribute to implantation more significantly than TGFβ2, and TGFβ3. Our results also confirmed that EGF as well as TGFs might be involved in regulation of serial events occuring during preimplantation and implantation periods. A decreased expression of EGF, TGFα,TGFβ1, TGFβ2, and TGFβ3 in endometrium from anoestrus bitches proved the hypothesis that this period was characterized as reduced reproduction activity in those animals.

Notes

EXPRESSION OF VASCULAR-ENDOTHELIAL GROWTH FACTORS A (164, 182, 188), AND B IN ANOESTRUS AND METOESTRUS BITCHES ENDOMETRIUM

BUKOWSKA D.[1], KEMPISTY B.[2], ANTOSIK P.[1], REMBOWSKA M.[1], JACKOWSKA M.[2], JAGODZIŃSKI P.P.[2], JAŚKOWSKI J.M.[1]

[1]Department of Agricultural Veterinary, University of Agriculture, Poznań, Poland
[2]Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznań, Poland

The most important step of successful blastocyst implantation in all mammalian species is forming specific connections of fetal and maternal blood supplies, which is involved in normal embryo development. Vascular-endothelial growth factor (VEGF) is a homodimeric glycoprotein described also as an endothelial cell mitogen due to its ability to induce vascular permeability. In dog three VEGF-A isoforms; VEGF164, VEGF182 and VEGF188 are generated through alternative splicing.

The aim of the study was to identify and evaluate the expression of vascular-endothelial growth factors; A (VEGF-A), A164 (VEGF164), A182 (VEGF182), A188 (VEGF188) and B (VEGF-B) in metoestrus and anoestrus bitches endometrium.

Endometrium (0.5 g) was collected from metoestrus (n=5; age: 3-14, mean=7.5; weight: 12.6-26, mean=17.2; condition=3 points) and anoestrus (n=3; age: 3-8, mean=5.5; weight: 20-35, mean=27.5; condition=4 points) bitches after ovariohysterectomy. Total RNA was isolated from bitches endometrium using the method of Chomczyński and Sacchi (1987), treated by DNase I, and reverse-transcribed into cDNA**.** Quantitative analysis of VEGF-A, VEGF164, VEGF182, VEGF188 and VEGF-B cDNA was performed by RQ-PCR, which was conducted in a Light Cycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I detection dye. The cDNA of the integrins was amplified using pairs of primers complementary to different exons**.** The quantity of mRNA product in each sample was adjusted to the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Quantification of the copy number was derived from a standard curve of a known amount of synthetic DNA template. Each of these experiments were performed at least in triplicate. Results were estimated using Student's t-test and one-way analysis of variance (ANOVA) with Newman-Keul's post-hoc test. P value was determined by Student's t-test, with significance $P<0.05$.

We observed 6-fold higher levels of VEGF-B, VEGF164, and VEGF188 cDNAs in metoestrus endometrium compared to endometrium isolated from anoestrus bitches ($P<0.001$). The expression of VEGF-A was three-fold higher in anoestrus endometrium compared to metoestrus bitches ($P<0.001$). We did not observe statistically significant differences in VEGF182 expression between those two groups of animals ($P=0.254$).

Metoestrus is described as the reproductive period, which occurs shortly after fertilization. An increased expression of VEGF-B, VEGF164, and VEGF188 suggested that these factors might contribute significantly to embryo implantation during metoestrus phase. The lower level of VEGF-A in endometrium from metoestrus bitches and no statistical differences in VEGF182 expression might be a result of a less visible effect of these factors on the mechanism of embryo attachment. We postulated that the most important role in growth and remodeling of the endometrial vasculature played VEGF-B, VEGF164, and VEGF188. Thus, these factors contribute to the process of connection of the fetal and maternal blood supplies.

Notes

GOAT CYTOPLASTS PREPARED BY DEMECOLCINE- AND NOCODAZOLE-INDUCED ENUCLEATION

COSTA-BORGES N., GONZÁLEZ S., PARAMIO M.T., SANTALÓ J., IBÁÑEZ E.

Dept. Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biociències. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

Demecolcine-induced enucleation has been shown to be an efficient method to prepare developmentally competent mouse and bovine cytoplasts for nuclear transfer. This technically simple approach has also been applied to goat oocytes, resulting in enucleation rates of 36%, measured as the proportion of activated oocytes that extruded all the chromatin within the second polar body (PB2) after exposure to demecolcine. This study was designed to evaluate the potential of another antimitotic drug (nocodazole, NOC), besides demecolcine (DEM), to induce enucleation of prepubertal goat oocytes.

Oocytes were recovered by slicing of slaughterhouse-derived ovaries from prepubertal goats and matured in vitro in a conventional medium (TCM199 with serum, hormones and cysteamine). At 27 h post-maturation, metaphase II oocytes were denuded, immediately activated in 5 µM ionomycin (5 min at 37 ºC), and then washed and cultured in KSOM medium supplemented with cycloheximide (5 µg/ml) at 38,5 ºC and 5% CO_2. At 30 or 90 min post-activation (p.a.), DEM (0.4 µg/ml) or NOC (0.3 µg/ml) were added to the medium and the oocytes were exposed to the antimitotic drug until 4 h p.a. At this time point, a group of oocytes was fixed (5 min in 2% paraformaldehyde, followed by 30 min in a microtubule stabilization buffer-extraction fixative), while the rest of the oocytes were removed from the antimitotic and cultured in drug-free KSOM for up to 14 h p.a. before fixation. A triple labelling protocol for microtubules, microfilaments and chromatin was applied to analyse oocytes (approximately 70 per time-point) by fluorescence microscopy. Oocytes were classified as completely or as partially enucleated when all the oocyte chromatin was located inside a completely or a partially extruded PB2, respectively. Experiments were repeated at least three times and data were statistically analysed by Chi-square or Fisher exact test.

At 4 h p.a., the percentage of DEM- and NOC-treated oocytes that were activated was high (82.3%-96.1%) and similar to that of the control group (91.7%). Rates of complete PB2 extrusion at 4 h p.a. were also equivalent between control oocytes (92.4%) and DEM- or NOC-treated oocytes (83.1%-93%). The highest rates of complete enucleation were achieved when activated oocytes were treated continuously with DEM (26.2%) or NOC (26.1%) from 30 min to 4 h p.a. Delaying the onset of the antimitotic treatment to 90 min p.a. resulted in a slight, though not significant, decrease in the proportion of completely enucleated oocytes for both DEM- and NOC-treatments (18.3% and 15.3%, respectively). The proportion of partially enucleated oocytes was significantly higher in the treatments with DEM starting at either 30 or 90 min p.a. (12.3% and 11.3%, respectively) than in the treatments with NOC (4.3% and 2.8%, respectively). Culture of oocytes after drug withdrawal for up to 14 h p.a. resulted in a significant decrease in the enucleation rates both for treatments with DEM (6.8% and 1.5%) and with NOC (8.1% and 5.4%). Because the proportion of DEM- and NOC- treated oocytes with a PB2 at 14 h p.a. (63.5%-71%) was significantly lower than at 4 h p.a., the observed decrease in enucleation rates could be caused by the reintegration of the chromosomes into the oocyte after incomplete PB2 extrusion, or by re-fusion of PB2 to enucleated oocytes.

Our results show that induced enucleation can be accomplished in prepubertal goat oocytes using both DEM and NOC, but further studies are required to optimize dosage and duration of exposure to the antimitotics in order to improve enucleation efficiency. In addition, to avoid reintegration of the chromosomes into the oocyte, PB2 should be removed by micromanipulation before the use of these cytoplasts for nuclear transfer procedures. Supported by the projects: MEC BIO 2006-11792; DGR 2004-XT00054; 2005-SGR00437, the Universitat Autònoma de Barcelona (EME2004-24) and the Fundação para a Ciência e a Tecnologia, Portugal (ref. 31263-2006).

Notes

HAS CLONING AN INFLUENCE ON EXPLORATORY AND SOCIAL BEHAVIOURS OF DAIRY CATTLE?

COULON M.[1,2], HEYMAN Y.[1], BAUDOIN C.[2], DEPUTTE B.L.[2]

1 INRA/ENVA, Biologie du Développement et Reproduction, 78352 Jouy en Josas, France
2 Université Paris 13, CNRS UMR 7153, Laboratoire d'Ethologie Expérimentale et Comparée, 93430 Villetaneuse, France

Behavioural studies on cloned animals are very scarce, especially in cattle (Savage et al., Theriogenology 2003; 60:1097-1110). To ensure the well-being of cloned animals and for a better knowledge of possible long lasting behavioural effects induced by cloning, these studies are needed. The aim of this work was to investigate whether somatic cloning procedure has an influence on behaviour of adolescent heifers.

Ten Holstein heifers 6-13 months old were used in this study. Five of them were clones derived by somatic nuclear transfer from three different donor cows and five others were age-matched AI controls born in the same experimental farm. All the heifers were individually reared under the same conditions up to 6 months and then grouped in the same cowshed for the study. Each heifer was observed in the social group during three 5-minute periods per day. The type of social behaviour and the identity of the social partner were recorded. Exploratory behaviour of heifers isolated in an unfamiliar environment was also recorded. Social behaviours of cloned and control heifers as well as their exploratory behaviour were compared using the Mann-Whitney U-test. A Hierarchical Index was calculated for each heifer and the correlation between this index and age and body mass was assessed using the Spearman rank correlation test.

The frequencies of agonistic and non-agonistic behaviours of clones and their controls were not significantly different (respectively $U=11.5$; $p=0.83$ and $U=10$; $p=0.6$; NS). However, cloned heifers showed significantly more non-agonistic and less agonistic behaviours towards other cloned partners than towards control ones. The results also stood for control heifers (non-agonistic: $U=698$; $p=0.01$; agonistic: $U=669$; $p<0.01$). As far as their Hierarchical Index was concerned, three cloned heifers were the highest ranking and two others the lowest ranking. The hierarchical index was positively correlated with body mass and age of heifers ($rhô=0.78$, $rhô=0.84$, $p<0.01$, respectively). In our experimental herd, social dominance appeared to be linked to body weight and age rather than to an effect of cloning. In an unfamiliar environment, cloned heifers showed more exploratory behaviour than did control ones ($U=2.5$; $p=0.03$). According to the limited number of individuals in each category and to the structure of the herd it was not possible to detect other behavioural differences between cloned and control heifers. The difference in exploration could be due to environmental factors during the developmental period rather than to cloning. Our mixed stable herd displayed a social organization, based on non-agonistic interactions, with two subgroups: clone and control heifers. Welfare consequences of this kind of social organization are discussed.

Notes

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IN BOVINE PREIMPLANTATION GENES (COX 2, CD9) AND THEIR ASSOCIATION WITH BULL SPERM QUALITY PARAMETERS

DAGHIGH KIA H., RINGS F., HÖLKER M., THOLEN E., SCHELLANDER K., TESFAYE D.

Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn 53115, Bonn, Germany

Fertility is highly influenced by management and environmental factors, but significant genetic differences exist in both male and female fertility. It has been proposed that candidate gene analysis can be used to identify individual genes responsible for traits of economic importance. In this study Prostaglandin G/H synthase-2 (PTGS-2/Cox 2) and CD9 genes were selected because they are indicative of various mechanisms in bovine preimplantation development. Prostaglandin synthesis is catalyzed by Cox 2. In pregnant mammals, Cox 2 is needed for pregnancy-associated events such as regulation of localized immune function, angiogenesis and regulation of blood flow, and development of implantation sites. CD9 is required for sperm-egg fusion. CD9 has been implicated in the regulation of cell-biological functions, including cell adhesion, motility, proliferation, differentiation, and signaling. This study was conducted to identify polymorphisms in the bovine Cox 2 and CD9 genes to perform an association study between polymorphisms of these genes and sperm quality parameters (volume of ejaculate, density, motility and survival after thawing). In this study, DNA was extracted from the sperm of 300 Holstein-Friesian bulls. Screening for polymorphisms was performed by PCR amplification and direct sequencing. The sequencing data were analyzed using multiple sequence alignment software (Multiple sequence alignment with hierarchical clustering). PCR-RFLP tests were created for each gene with gene-specific primers and corresponding restriction enzymes; AluI and DraI respectively for Cox 2 and CD9. The digested PCR products were separated on 2% agarose gels and stained with ethidium bromide. Sequencing results revealed the existence of two single nucleotide polymorphisms at position of 185T>C in intron 5 and 462A>G in exon 9 that corresponded to Cox 2 and CD9 respectively. There is no change in amino acid in CD9 gene. The data were analyzed with GLM procedure of SAS. Results for the association study of the polymorphisms in Cox 2 and CD9 with sperm motility was significant ($P<0.05$). The positive relationship between these SNPs and sperm characters indicate that these two genes can be use as potential molecular markers for semen quality.

Notes

THE EFFECT OF CYSTEAMINE ON LIPID PEROXIDATION DURING MATURATION AND SUBSEQUENT DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES IN VITRO

DE CLERCQ J.B.P., LEROY J.L.M.R., ANDRIES S., GOOVAERTS I.G.F., BOLS P.E.J.

Gamete Research Centre, Laboratory of Veterinary Physiology, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium

The production of reactive oxygen species (ROS) is a normal process during cell metabolism. As a consequence, lipid peroxidation, caused by ROS, usually implicates cell damage, which can impair the oocyte's developmental capacity. Cysteamine supplementation during *in vitro* maturation (IVM) improves embryo development by stimulating glutathione (anti-oxidant) synthesis and therefore can be used to prevent ROS damage to the embryo. However, it is not known whether the degree of lipid peroxidation after maturation is correlated with the *in vitro* developmental capacity of the oocyte. Therefore, the aim of this study was to investigate cumulus oocyte complex (COC) lipid peroxidation during IVM, either in the presence or absence of cysteamine, and to assess the subsequent oocyte developmental competence.

After puncturing 2-8 mm sized follicles of 139 slaughterhouse ovaries, only grade I COCs were selected for IVM (4 replicates). Half of the COCs were placed in a routine maturation medium (TCM 199 without phenol red, 20 ng/ml mEGF), with (n=342) or without (n=330) 0.1 mM cysteamine. After maturation and routine fertilisation, the presumptive zygotes were cultivated in groups of 25 in 50 µl culture medium droplets under mineral oil (SOF + 5% FBS, incubated in a modular incubator, 38.5°C, 5% CO_2, 5% O_2 and 90% N_2). Blastocyst rates (% of matured oocytes) were calculated at 8 days post fertilisation. Additionally, after 24 hours of maturation, the medium was tested on lipid peroxidation by measuring the concentration of malondialdehyde (MDA, end product of the lipid peroxidation), through an assessment of the levels of tiobarbituric acid reactive substances (TBARs). The TBAR levels were read with a spectrophotometer; based on the reaction that 1 molecule MDA and 2 molecules tiobarbituric acid (TBA) form a pink chromogen. A control maturation medium with or without cysteamine (placed for 24h at 5% CO_2 without COCs) was also measured. Data were analysed using a Paired Samples T-test (MDA concentrations) and a Binary Logistic Regression (blastocyst rates) (SPSS 13.0).

Maturation in the absence of cysteamine resulted in a significant ($P<0.05$) increase (66.9%) of MDA concentrations, while no significant MDA augmentation was detected in the medium after maturation of COCs in the presence of 0.1 mM cysteamine. Moreover, maturation with cysteamine resulted in a significant higher blastocyst rate (20.4%) while only 12.2% of the oocytes, matured without cysteamine, developed to the blastocyst stage.

These findings suggest that adding cysteamine during maturation reduces lipid peroxidation of COCs, hence facilitating further *in vitro* embryo development.

Notes

INHERITANCE OF EPIGENETIC ALTERATIONS PRODUCED BY IN VITRO CULTURE IN MICE

FERNANDEZ-GONZALEZ R., RAMIREZ M.A., PEREZ-CRESPO M., PERICUESTA E., BERMEJO P., HOURCADE J.D., PINTADO B., GUTIERREZ-ADAN A.

Departamento de Reproducción Animal, INIA. Carretera de La Coruña km 5,9. 28040 Madrid, Spain

The regulation of many genes expressed during preimplantation development is controlled by retrotransposable elements. Moreover, methylation is one of the most important processes to regulate gene expression. During preimplantation development in mammals occurs one of the main epigenetic reprogramming by demethylation. We have already shown that in vitro culture (IVC) of mice embryos in the presence of serum produces alterations in gene expression of imprinting and non imprinting genes, and also induces a long-term effect on postnatal development, growth, and behaviour (Fernandez et al. 2004). Altered foetal environment produces transgenerational effects like abnormal establishment of epigenetic patterns; however, no relationship has been established between alterations in the epigenetic reprogramming during preimplantation and transgenerational responses.

The objective of our study was to find any relationship between alterations in the preimplantational epigenetic reprogramming and the transgenerational responses, inducing epigenetic alteration in the Axin-fused ($Axin^{Fu}$) metastable epialleles. The presence or absence of $Axin^{Fu}$ phenotype, a kinked tail, correlates with differential DNA methylation at an IAP retrotransposon within $Axin^{Fu}$, and it is heritable transgenerationally.

To investigate if the inheritance of $Axin^{Fu}$ phenotype is affected we analyzed the proportions of offspring phenotypes arising from penetrant (kinky tail) and silent (normal tail) sires crossed with wild type dams under different foetal environment: super-ovulation, embryo transfer, IVC in KSOM, and IVC in KSOM+FCS.

The phenotype of the offspring was quantified according to the level of curvature and size of the tail from 0 to 5. No effects were observed due to the superovulation or the embryo transfer procedures; however, in vitro culture, and, more significantly, the presence of FCS increased the severity of the phenotype in the offspring generated ($p<0.01$, ANOVA one way analysis); and this severity of the phenotype was transmitted to the next generation. These results demonstrate that suboptimal IVC procedures induce epigenetic alteration in metastable epialleles that can be generationally inherited.

Notes

DEVELOPMENT AND QUALITY OF PORCINE BLASTOCYSTS CULTURED IN NCSU-23 MEDIUM WITH OR WITHOUT BOVINE SERUM ALBUMIN AND PHENAZINE ETHOSULFATE

GAJDA B., BRYŁA M., SMORĄG Z.

Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production, 32-083 Balice/Kraków, Poland

Basic culture media are usually supplemented with protein (serum or albumin), which contains amino acids that play an important role as energy sources, osmoregulators, and pH stabilizers. On the other hand, it is possible to produce in vitro bovine embryos in protein-free medium without affecting blastocyst yield and quality. Supplementation of culture media with phenazine ethosulfate (PES) may be a promising approach to improving in vitro production of embryos. The PES treatment increased glucose metabolism, tended to increase the pentose phosphate pathway flux of glucose and clearly reduced accumulation of lipids in bovine embryos produced in chemically defined media. It seems interesting, then, to determine whether supplementation with or without bovine serum albumin (BSA) and phenazine ethosulfate (PES), improved developmental competence and quality of cultured porcine embryos. The experiment was done on zygotes obtained from superovulated gilts. Morphologically normal zygotes were cultured *in vitro* in NCSU-23 medium containing: protein–free, 0.004 g/ml BSA, 0.004 g/ml BSA+ 0.05 µg PES. Embryo quality criteria were developmental competence (cleavage, morula and blastocyst rates), total cell number per blastocyst and degree of apoptosis as assessed by TUNEL staining. Data were evaluated by chi-square test. Results are shown in Table 1 and 2.

Table 1. Effect of BSA and BSA+PES supplementation on porcine embryo development

Treatment group	No. of embryos cultured	No. of cleaved embryos (%)	No. of morulae(%))	No. of expanded blastocysts (%)
protein-free	77	69 (89.6)	59 (76.6)[a]	17 (22.1)[d]
BSA	73	69(94.5)	66 (90.4)[b]	59 (80.8)[e]
BSA+PES	97	96 (99.9)	88 (90.7)[c]	49 (70.0)[f]
			a,b; a,c – P<0.05	d,e;d,f – P<0.01

Table 2. The quality of porcine blastocysts cultured in NCSU-23 medium supplemented with BSA and BSA+PES

Treatment group	No. of blastocysts assessed	Average number of cell/blastocyst	Average number of apoptotic nuclei/blastocyst	% TUNEL positive
protein-free	13	30.5	7.46[a]	24.46[d]
BSA	13	32.3	3.31[b]	10.25[e]
BSA+PES	24	30.5[c]	1.37[c]	4.49[f]
			a,b; a,c; e,f – P<0.05	d,e;d,f – P<0.01

These data demonstrate that the presence of BSA and BSA+PES in culture medium significantly increased morula and blastocyst production compared to protein-free group. The blastocysts cultured in protein-free medium had significantly higher average number of apoptotic nuclei and DNA fragmented nucleus index compared to BSA and BSA+PES group. Moreover the percentage of TUNEL positive nuclei per blastocyst were significantly lower in BSA+PES treated than BSA and protein-free group.

This work was supported by the Polish State Committee for Scientific Research. Project No. 2 P06D 003 26 from years 2004 to 2007.

Notes

IN VITRO DEVELOPMENT AND GENE EXPRESSION OF PUREBRED AND CROSSBRED EMBRYOS DERIVED FROM HOLSTEIN DONORS

GALLI C.[1,2], COLLEONI S.[1], DUCHI R.[1], LAZZARI G.[1]

[1]Laboratorio di Tecnologie della Riproduzione, Istituto Sperimentale Italiano Lazzaro Spallanzani, CIZ srl, via Porcellasco 7/f, 26100 Cremona, Italy, [2]Dip. Clinico Veterinario, Università di Bologna, via Tolara di Sopra, 50, 40064 Ozzano Emilia, Italy.

The reduction of fertility of dairy herds is one of the most important issues in cattle breeding. The multifactorial origin of this problem includes nutrition, milk production, environment, management and also genetic background. The aim of this study has been to investigate the effect of embryo genotype on a number of cellular and molecular parameters of the developing bovine embryo. For this purpose we have produced purebred and crossbred embryos by in vitro techniques using Holstein oocytes recovered from abattoir ovaries and fertilizing them with frozen semen of Holstein or Brown Swiss breed. The derived embryos, purebred and crossbred, have been analysed for cleavage and development to the morula/blastocyst stages. In addition, an RT-PCR study has been performed to investigate the expression of developmentally important genes (MnSOD, PED, Glut5, Gp130, FGF4, IGFr1, IF-tau, Cx43, Bax, G6PDH) in blastocyst stage embryos.

Table 1. Development of purebred and crossbred bovine embryos.

type of embryo	N. oocytes	N. cleaved (%)	N. Comp. Morulae D+6 (%)	N. blastocysts D+7 (%)	N. blastocysts D+8 (%)
purebred	1135	898 (78.8)	238 (26.01)a	170 (19.9)	262 (27.9)
crossbred	1139	847 (74.3)	257 (30.04)b	177 (24.1)	281 (34.8)

Values within columns with different letters are statistically different (T Student test, $p < 0.05$)

As shown in Table 1, the results indicate a significant difference between the two types of embryos at the level of morula compaction and also a tendency, although not statistically significant, for higher blastocyst development in the crossbred group. In addition our RT-PCR data have shown a significantly higher level of expression of three genes, MnSOD, gp130 and FGF4 in crossbred as compared to purebred embryos. In conclusion, this study provides some evidence that the genetic background of the embryo can influence the kinetics of development in vitro and the expression of specific genes.

Notes

FEATURES OF OVARIAN FOLLICULAR POPULATION OF NURSING RABBITS UNDER DIFFERENT REPRODUCTIVE RHYTHMS

GARCIA-GARCIA R.M.[1], ARIAS-ALVAREZ M. [1], REVUELTA, L. [1], REBOLLAR P.G.[2], LORENZO, P.L. [1]

[1]*Dpto. de Fisiología (Fisiología Animal). Facultad de Veterinaria- UCM. 28040-Madrid. Spain.*
[2] *Dpto. de Producción Animal. ETSIA-UPM. Madrid. Spain.*

The aim of current work was to analyze the ovarian follicular population and their quality in animals inseminated in intensive (4 days post-partum) and semi-intensive rhythms (11 days post-partum) after 5 or more parturitions; the effect of the use of eCG treatment was also studied. A total of 26 New Zealand white rabbits (5.4 parturitions), under intensive rhythm (Group A; n=15) or a semi-intensive one (Group B; n=11) were either treated with 20 IU eCG 48h h before euthanasia or not synchronized with hormonal treatment. Ovaries were recovered by laparotomy and the morphometrical parameters, the number of total follicles and the number of follicles ≥ 1 mm in size in the ovarian surface were recorded. Oocytes from follicles of one ovary were recovered and matured in TCM 199 supplemented with 100 ng/ml EGF, 10ng/ml IGF-I and 10% FCS. The counterpart ovaries were fixed in formaldehyde solution for histological studies. The detection of cell apoptosis was determined using TUNEL. The fixed ovaries of three untreated young animals (2.67 parturitions) were used as controls. ANOVA one-way statistical analysis was performed. The average height and width of ovaries, the mean of ≥ 1mm follicles and the number of total follicles were similar between groups (Table 1). The number of recovered oocytes (P=0.07) and oocytes selected for maturation (P=0.06) tended to be higher for untreated animals than for ecG injected ones in group A. The metaphase II rate was significantly lower in group A than B (43.5±8.4% *vs* 65.2±10.1%; P<0.01). On the other hand, the mean of primordial follicles was significantly lower for the group A than those of control group (P<0.05) but similar to group B; the number of total antral follicles also decreased for the group A in relation to group B (P=0.07). There were not found significant differences in mean apoptosis index of follicles between groups. Follicular population neither nuclear maturation rate was affected by the ecG treatment; however, apoptosis rate was higher for stimulated animals (P=0.07). In conclusion, rabbits in an intensive rhythm seems to be less receptive to eCG treatment after 5 or more parturitions, probably by depletion in their follicular population and decreased oocyte quality.

Table 1. Reproductive parameters of rabbit does in different productive rhythms

	Group A (intensive rhythm)		Group B(semi-intensive rhythm)		Control
	untreated	eCG	untreated	eCG	untreated
Ovary height (mm)	17.1±0.6	16.9±1.4	15.8±3.5	17.8±2.4	-
Ovary width (mm)	6.6±0.4	7.7±0.7	6.8±0.6	8.4±0.5	-
Mean of >1mm follicles	9.0±1.0	7.3±1.6	7.6±0.7	8.6±0.9	-
Mean of total follicles	10.6±1.9	11.4±2.2	10.8±1.8	13.4±1.5	-
Mean of haemorrhagic follicles	1.63±1.0	4.1±2.5	3.2±1.7	4.8±2.3	-
Mean of recovered oocytes	10.5±1.0	6.7±0.9	9.6±0.8	9.4±1.6	-
Mean of IVM oocytes	9.4±1.1	6.7±0.9	8.6±1.0	7.4±1.0	-
MII rate (%)	38.6±13.3	49.0±10.5	69.3±12.2	61.7±17.1	-
Mean of primordial follicles	75.7±17.3 [a]	79.0±30.7 [a]	97.7±43.3	87.3±22.0	186.7±62.0[b]
Mean of primary follicles	3.5±0.9	4.5±1.2	6.7±2.5	5.17±1.6	6.7±1.5
Mean of secondary fol.	6.0±0.8 [a]	10.8±2.1	12.5±4.8	10.8±2.7	20.0±6.5 [b]
Mean of antral follicles	3.6±0.6	3.3±0.8	6.83±2.0	4.6±3.3	4.7±3.3
Apoptotic follicles (%)	26.6±9.8	33.8±6.1	12.4±0.6	46.4±9.6	29.1±5.0

[ab] P<0.05

This work was supported by MEC project AGL 05-196. RMGG was supported by "Juan de la Cierva" MEC Program and MAA is granted by CM and FSE.

Notes

CULTURE AND CRYOPRESERVATION OF BOVINE EMBRYOS WITH VEGETAL PEPTONES

GEORGE F., VAN NUFFEL A., KERSCHEN D., DONNAY I.

Université catholique de Louvain, Institut des Sciences de la Vie, Veterinary Science Unit
Place Croix du Sud 5, B-1348 Louvain-la-Neuve, Belgium

Animal proteins (serum or BSA) are currently used for producing in vitro bovine embryos, which involves sanitary risks. We previously set up a whole culture system devoid of serum. The aim of the present study was to replace BSA with vegetal protein hydrolysates (peptones) in culture medium, without compromising the quality of the produced embryos in terms of developmental rate and resistance to cryopreservation.

In a first experiment, seven batches of vegetal peptones of different origins (cotton, soy, wheat, peas – Hypep®, Kerry) were checked for their antioxidant properties using two different tests: 1) capacity to neutralize reactive oxygen species generated by AAPH and 2) capacity to protect lipids against peroxidation. Two batches (cotton and wheat) showed a significant higher antioxidant power in comparison with the culture medium containing BSA and a mixture of insulin, transferrin and selenium (SOF-ITS-BSA).

In a second experiment, peptones were added to SOF-ITS. Oocytes were matured and fertilized in vitro and the resulting zygotes were cultured with different concentrations (0.18; 0.56; 1.8 mg/mL) of each peptone. The batch of wheat peptone at 0.18 mg/mL and the batch of cotton peptone at 0.56 mg/mL were found to give blastocyst rates at Day-7 higher than SOF-ITS but similar to SOF-ITS-BSA (containing 4mg/mL free fatty acid BSA – Table 1).

In a third experiment, expanded Day-7 blastocysts (≥160µm) produced with 0.18 mg/mL wheat peptones (SOF-ITS-PEP) or in SOF-ITS-BSA were frozen with 1.5 M ethylene glycol and 1.8mg/mL wheat peptones. After thawing, embryos were co-cultured for 48h on Buffalo Rat Liver cells. No significant difference was observed between SOF-ITS-PEP and SOF-ITS-BSA, neither in survival rates nor in hatching rates (Table 1).

In conclusion, a culture medium where animal proteins are replaced by vegetal peptones can sustain embryo development and give blastocysts resisting to cryopreservation.

Table 1: Development and resistance to freezing of bovine embryos cultured with vegetal peptones

Culture	Oocytes N	D6 blastocysts	D7 blastocysts	D8 blastocysts
		Mean % ± SEM		
SOF-ITS	126	4±1.5	21±3.8 [a]	35±5.2
SOF-ITS-BSA	127	14±1.9	37±3.9 [b]	39±5.1
SOF-ITS-wheat 0.18	165	16±3.1	40±3.9 [b]	44±4.3
SOF-ITS-cotton 0.56	162	17±2.9	37±4.7 [b]	44±4.9
Freezing	Frozen embryos	Survival at 48h %	Hatching at 24h %	Hatching at 48h %
SOF-ITS-BSA	102	96	59	88
SOF-ITS-PEP	95	94	47	80

[a,b] Values with different superscripts differ significantly (ANOVA2 – $P<0.05$)

Notes

COMPARISON OF DIFFERENT SYNCHRONIZATION PROTOCOLS ON OPU/IVF RESULTS IN FSH STIMULATED SIMMENTAL HEIFERS

GETZ I.[1], KARADJOLE M.[1], SAMARDŽIJA M.[1], MAKEK Z.[1], MATKOVIĆ M.[2], DOBRANIĆ T.[1], CERGOLJ M.[1]

[1]*Clinic for reproduction and obstetrics, Faculty of Veterinary Medicine, University of Zagreb, 10000 Zagreb, Croatia*
[2] *Centre for reproduction and animal breeding of Croatia, Zagreb, Croatia*

The success of ovum pick-up (OPU) and in vitro embryo production depends on the number and the quality of retrieved oocytes per session and also on the culture conditions. The aim of this study was to compare the effect of different synchronization protocols on oocyte retrieval and blastocyst production in vitro in FSH-stimulated Simmental heifers. Twenty-eight normal cyclic Simmental heifers, aged between 14 and 20 months were selected based on transrectal palpation for oocyte donors. They were randomly allocated to one of three synchronization groups. In the 1st (PGFx2) group heifers (n=10) were synchronized with two prostaglandin $F_2\alpha$ injections at an 11-day interval. In the 2nd (PGF + DFR) group heifers (n=8) were synchronized with two prostaglandin $F_2\alpha$ treatments at an 11-day interval and dominant follicles (>8 mm) were aspirated (DFR: dominant follicle removal) 36 hours before FSH stimulation. In the 3rd (Crestar + DFR) group (n=10) a norgestomet ear implant without the prescribed oestrogen injection (Crestar®, Intervet, Belgium) was inserted for 10 days. The implants were removed and dominant follicles were aspirated 36 hours before FSH stimulation. The stimulation with pFSH (Folltropin®-V, Vetrepharm Inc., London, Canada) was the same for all three groups and was administered twice a day, during two days beginning on the Day 9 or 10 of the oestrous cycle. The total amount of pFSH administered was 200 mg NIH-FSH-P1 in 4 equivalent doses. OPU was performed 48 hours after the last FSH injection. Only good quality oocytes were matured, fertilized and cultured in vitro in SOFaaBSA till the Day 9. Cultured embryos were evaluated morphologically according to IETS standards. The cleavage rates on Day 2, the total number of morulae (M) and blastocysts (Bl) on Day 7 and the number of hatched blastocysts (hBl) on Day 9 were recorded. The results were statistically analyzed with ANOVA (StatSoft, Statistic, version 7.1.) and with Tukey's tests post-hoc analysis.The results are presented in the table.

Table 1. Effect of different synchronization protocols on oocyte retrieval and IVF/IVC results in FSH stimulated Simmental heifers (mean±SD)

Synchronization protocol	Follicles aspirated	Oocytes retrieved	Oocytes in IVP	Cleavage (%)	M/Bl (%)	hBL (%)
PGFx2 group (n=10)	14.5 (±3.9)^{a}	10.3 (± 5.2)^{b}	9.5 (±5.6)^{a}	86.8 (±1.8)^{a}	41.1 (±2,2)^{a}	22.4 (±1.8)^{a}
PGF + DFR group (n=8)	13.3 (±3.9)^{a}	7.6 (±4.0)^{a}	6.9 (±4.5)^{ab}	91.0 (±1.3)^{a}	28.9 (±2.2)^{b}	12.4 (±1.4)^{b}
Crestar + DFR group (n=10)	13.1 (±4.1)^{a}	9.1 (±4.1)^{ab}	5.9 (±3.6)^{b}	94.7 (±0.8)^{a}	43.6 (±2.0)^{a}	23.2 (±1.5)^{a}

Values with different superscripts within the same columns differ significantly (P<0.05; ANOVA)

In conclusion, the results of this study demonstrate that synchronization protocol did not affect the number of follicles following superstimulation but it did affect the number and the quality of the retrieved oocytes and the blastocyst yield.

Notes

MATERNAL AND EMBRYONIC GENETIC EFFECTS ON FROZEN OVINE EMBRYO TRANSFER

GIMENEZ-DIAZ C.A., EMSEN E.

Ataturk University, Department of Animal Science, Erzurum, TURKEY

Factors specific to both embryos and recipients have been suggested to affect the survival of transferred embryos in cattle, sheep and goats. Knowledge of the effects of the embryo and the uterine genotype on fetal and placental development are helpful in better controlling those factors that are limiting both litter size and success of new reproductive technologies (Moce et al., 2004). The effect of the genotype of recipient and embryo on the lambing rate and survival rate at birth of frozen embryos was studied.

Embryos at the morula or blastocyst stages were obtained 6 d after artificially insemination from Romanov and Charollais donors. Morphologically normal embryos were frozen with a traditional freezing protocol (ethylene glycol) and stored in liquid nitrogen. After thawing, intact embryos were transferred (two embryos per animal) into the three different breeds of recipient - Awassi, Redkaraman and Tuj. Significant differences were observed in the number of corpora lutea observed at transfer. The recipient ewes of Redkaraman breed showed a significantly lower ($P < 0.01$) number of corpora lutea (1.14) than those of Awassi (2.0) and Tuj (1.8) ewes. When the lambing rate was analyzed in all recipient ewes, it was found that Awassi and Tuj had relatively but not significantly higher lambing rate than Redkaraman recipient ewes owing to their higher number of corpora lutea. However, when the survival rate was analyzed in lambed recipient ewes, no significant differences were found between embryo and recipient genotype; the average survival rate was approximately 66%. Recipient carried embryos from Romanov donors resulted with 13% higher lambing rate compared to those carried Charollais.

It was concluded that the major factor in the differential survival rate between Romanov and Charollais embryos was due to the genotype of recipient ewes. In general Romanov had higher prenatal survival rates.

Table 1. Reproductive performance of different genotype of recipient and embryos in frozen embryo transfer program.

Recipient genotype	n	Lambing rate (%)	Embryo survival (%)	Interaction	n	Lambing rate (%)	Embryo survival (%)
Awassi	10	70	50	Awassi *Charollas	5	60	50
Redkaraman	21	57	69	Awassi*Romanov	5	80	50
Tuj	20	63	79	Redkaraman*Charollais	7	57	63
Embryo genotype				Redkaraman*Romanov	14	57	75
Romanov	52	70	68	Tuj*Charollais	13	54	79
Charollais	50	57	64	Tuj*Romanov	7	71	80

Values in the same column in each category are not significantly ($P>0.05$) different

Notes

EHV-1 CAN BE REMOVED FROM EXPOSED EQUINE EARLY-BLASTOCYSTS (D6.5) BY A BATH OF TRYPSIN

HÉBIA I.[1], DUCHAMP G.[2], LARRAT M.[1], ROUX C.[1], PELLERIN J.L.[1], VAUTHEROT J.-F.[3], ZIENTARRA S.[4], FIENI F.[a], BRUYAS J.-F.[1]

[1]Sanitary Risks and Biotechnology of Reproduction, National Veterinary School, 44307 Nantes
[2]UMR INRA-CNRS-Univ. Tours-Haras Nationaux, PRC , 37 380 Nouzilly
[3]UR86 BASE INRA-Tours 37 380 Nouzilly, [d]AFSSA, 94703Maisons-Alfort, France.

Detection, by PCR, of EHV-1 DNA in an equine embryo collected from a healthy donor mare was reported by Carvalho et al. (Arq Bras Med Vet Zootec 2000; 52-54). In a previous study, we have demonstrated that, after an in vitro contamination of equine embryos, EHV1 cannot be removed from all (7/10) equine blastocysts (D7) by the washing procedure recommended by sanitary European regulations and IETS guidelines (Hebia et al, Theriogenology 2007; 67:1485-1491). As equine D7 blastocysts are surrounded by a capsule and a ZP or only by a capsule, the interactions between EHV1 and the capsule or the ZP could be different.

The objective of this study was to evaluate, in equine early-blastocysts (D6.5) surrounded by ZP, if the standard embryo washing procedure, or treatment with trypsin recommended by IETS guidelines for bovine embryos contaminated by BHV1, effectively decontaminate equine early-blastocysts exposed to EHV-1 in vitro.

Fifteen embryos were collected non-surgically on Day 6.5 after ovulation from pony mares previously inseminated with fresh semen from 3 stallions. The mares and stallions were regularly vaccinated against EHV1. The embryos were in a first step contaminated with the virus (strain Kentucky D) and in a second step randomly split into two groups: 5 embryos were individually washed in accordance with International Embryo Transfer Society (IETS) guidelines (Group 1), and 10 embryos (group 2) were individually, before the washing procedure, passed into a PBS without albumin bath and placed into a trypsin (0.25%) bath for 90 sec. The embryos (Groups 1 and 2), samples of wash media, semen, uterine flushes, and leukocytes from the mares and stallions, were then frozen and stored until DNA extraction using NucleoSpin®RNA Virus. The PCR used in this experiment was a type-specific PCR (Kirisawa et al, Vet Microbiol 1993; 36:57-67). The sequences of two primers were selected from the nucleotide sequence of EHV-1 and EHV-4 glycoprotein B genes.

EHV-1 DNA was not detected by PCR in any of the leukocyte samples from the mares and stallions. No EHV-1 DNA was found in the semen, uterine flushing media.
EHV-1 DNA was detected by PCR in 5/5 embryos (Group1) exposed to EHV-1 for 24 hours and only washed but not detected in the 10 embryos exposed to EHV1 and treated with trypsin. EHV-1 DNA was detected by PCR in the first 3 washes from all Group 1 embryos, in the 4th and 5th washes of 3 embryos (3/5), and in PBS bath, trypsin bath and two first washes from all Group 2 embryos, in the 3rd wash of 7 embryos (7/10) and in the 4th and 5th washes of 2 embryos (2/10); however no viral DNA was detected in the 6th to 10th washes from any embryos of 2 groups.

This study clearly demonstrates that the standard embryo-washing protocol as recommended by the European rules did not completely eliminate EHV1 after in vitro contamination of D6.5 equine embryos surrounded by ZP. The elimination of EHV-1 with the 10 successive washes and the absence of detection of viral DNA in the final washes of all embryos from group 1, and the efficacy of treatment with trypsin to remove EHV1 from all group 2 embryos demonstrate that the virus was adherent on the equine ZP.

Further studies are needed to evaluate the interaction between EHV1 and equine blastocysts surrounded only by the capsule and if the enzymatic treatment could effectively decontaminate them, as for equine early blastocysts (D6.5) in the present study and as for bovine embryos infected by BHV-1.

Notes

FREEZING OF SEMEN FROM ENDANGERED ASTURIANA DE LA MONTAÑA BULLS IN EGG YOLK FREE EXTENDERS

HIDALGO C.O.[1], TAMARGO MIGUEL C.[1], BELTRÁN BREÑA P.[2], DE LA FUENTE J.[2], PÉREZ-GARNELO S.S.[2] PALASZ A.[2]

[1]Área de Selección y Reproducción Animal Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) Camino de los Claveles, Spain, [2]Ministerio de Educación y Ciencia, INIA Ctra de la Coruña Km 5.9, Madrid 28040, Spain

A preliminary experiment was designed to test 3 different preparation protocols of zwitteronic lipids liposomes for use as an egg yolk replacement in bull semen extender. Liposomes were prepared with lipids of plant origin homogenized by high pressure homogenizer (Nira Saovi, Parma, Italy) using high (~900 bars) extender 1 (E-1); medium (~600 bars), extender 2 (E-2) and low (~300 bars) extender 3 (E-3) homogenization pressure. Extenders were prepared in Tris buffer containing 8% glycerol. The semen was collected from 3, endangered Asturiana de la Montaña bulls by the means of artificial vagina. Ejaculates with at least 70% motility were processed further by a standard freezing protocol used in our artificial insemination (AI) station: semen was diluted at 37^0C with each of the 4 extenders to a concentration of 92 x 10^6 spermatozoa per ml, cooled to 4^0C over 4 h, aspirated into 0.25 ml plastic straws (IMV, Aigle, France), frozen in bio-freezer (IMV Technologies) in 3 steps from 4^0C to -140^0C and then plunged into LN_2. Straws were thawed in a water bath at 37^0C for 30s. Sperm motility was analyzed microscopically, immediately after collection, after dilution with the extenders and after 4, 24, 48 and 72 hrs of storage at 4^0C. Post-thaw semen progressive motility was assessed microscopically, and sperm movement characteristics by CASA (SCA®, Microptic, Bacelona, Spain). Data were compared between extenders and bulls by Chi-square analysis. Percentage of sperm motility at 0 hr after dilution and stored at 4^0C ranged from 90 to 70% and were no different between extenders and bulls. However, after 72 h of cold storage progressive motility was significantly higher ($P<0.05$) for control extender (67.2%) than 3 liposome's extenders, E-1, (18.1%), E-2 (45%) and E-3 (7.7%) for bull 1, and for bull 2 in control extender (29%) and E-1 (8%), E-3 (13.7%). There was no difference in progressive motility between control (68.1%) and E-3 (70.5%) extenders.

Post-thaw sperm motility evaluated under microscope was 60% in Bioxcell (control), 55% E-1, 60% E-2 and 50% for E-3. Average post-thaw total motility for 3 bulls evaluated by CASA immediately and 1 hr after thawing were as follow; Control, 67.3 ± 6.8 and 66.0 ± 7.7; E-1, 78.5 ± 7.5 and 73.0 ± 15.1; E-2, 75.6 ± 5.5 and 70.3 ± 4.6; E-3, 65.6 ± 14 and 63.3 ± 17.3 accordingly and were no different between extenders. There was no differences between either bulls or extenders for 2 kinematics parameters evaluated by CASA that are correlated with field fertilization, sperm movements derived from heads (VCL; 93 ± 3.5) and linearity of sperm (LIN; 60 ± 3.0). Remaining parameters related to sperm movement were more profound between bulls than extenders and this observation should be tested on greater number of bulls.

In summary, zwitteronic plant origin lipids liposome's prepared by all 3 tested homogenization methods are equally effective and as good as Bioxcell extender to cryopreserve Asturiana de la Montaña bull semen.

Supported by: by grant # RZ2004-0031-C.02.00 and OT2006-002 from the Ministerio de Educación y Ciencia, Madrid, Spain.

Notes

VITRIFICATION OF BOVINE BLASTOCYSTS AND EXPANDED BLASTOCYSTS IN CRYOLOOP AND IN STRAW

IACONO E., MERLO B., MORGANTI M., MARI G.

[1]*Veterinary Clinical Department, University of Bologna, Ozzano Emilia 40064, Italy*

Vitrifcation in a cryoloop allows maximal freezing rate and reduction of vitrification solution volume. These factors minimize the cryoprotectant toxicity. Bovine blastocysts have been vitrified in cryoloop only by Lane et al. (Nature Biotechnology 1999; 17: 1234-1236). The aim of this study was to compare vitrification of bovine blastocysts in straw and cryoloop. Bovine oocytes, aspirated from abattoir-derived ovaries, were matured, fertilized and cultured in vitro. At Day 7 of IVC blastocysts and expanded blastocysts were divided in three groups: embryos vitrified in straw (S); embryos vitrified in cryoloop (CR); non vitrified embryos (C). For vitrification embryos were transferred (5 embryos/straw; 5 embryos/cryoloop), in a maximum volume of 1 µl, in 1 ml of V1 (EG 3.5 M in HSOF) for 3 min, then in 10 µl of V2 (EG 7 M, Galactose 0.5 M, Ficoll 70 18% in HSOF) for 20 sec; then the embryos were loaded in 0.25 ml straw (preloaded with 190 µl of dilution solution (D): Galactose 0.5M in HSOF) or cryoloop (Hampton Research, Laguna Niguel, CA, USA) and immediately plunged into liquid nitrogen. Blastocysts vitrified in straw were thawed in air for 10 sec, then in water at 37°C for 30 sec; the straw was shaken and emptied in a petri dish. Embryos vitrified in cryoloop were thawed by immersing the loop in 2 ml of D. In both groups, re-expanded embryos were washed three times in HSOF and cultured in SOFaaBSA (16 mg/ml) plus 5% FBS at 38.5°C in 5%O_2, 5%CO_2, 90%N_2. After 6 h and 24 h of IVC the expanded blastocysts were stained with Hoecsht 33342 and PI to evaluate the number of live and dead cells. The hatching rate was recorded after 48h of IVC. Chi Square test and ANOVA were used for statistical analysis (Statistica for Windows - Stat Soft Inc Tulsa USA). Results showed that the rate of re-expanded embryos was higher in cryoloop than straw. Hatching was higher in control than in embryos vitrified in straw but not in cryoloop (Table 1). Dead cells were higher in vitrified embryos than control 24 h post-thawing, but any difference was found for live and total cells (Table 2). In conclusion vitrification of bovine blastocysts and expanded blastocysts in cryoloop permits to obtain higher survival and hatching rates compared to vitrification in straw.

Table 1. Re-expansion and hatching rates of bovine vitrified blastocysts 48 h post-thawing.

Group	N°	% ZP damages	% re-expanded	% hatched
S	111	6.3	93.7[b]	41.0[b]
CR	100	4.0	100.0[a]	50.0[a,b]
C	90	/	/	68.3[a]

a vs b P<0.05

Table 2. Number of live, dead and total cells of vitrified bovine blastocyts 6 and 24 h post-thawing.

Group	N° cells 6 h post-thawing			N° cells 24 h post-thawing		
	Live	Dead	Total	Live	Dead	Total
S	57.7±8.8	9.1±4.7	66.8±12.3	68.5±9.8	29.6±14.1[b]	98.1±18.8
CR	61.0±6.6	9.1±6.3	70.1±10.2	70.8±16.0	22.4±8.0[b]	93.2±13.9
C	57.9±15.3	6.2±3.4	64.1±15.8	74.0±10.1	5.5±2.8[a]	79.5±11.5

a vs b P<0.05

Supported by Animal Stem Cells Laboratory, Regione Emilia Romagna, PRRIITT Project Number M-404AIWTSV.

Notes

THE EFFECT OF THE ORIGIN OF THE IMMATURE OOCYTES ON IN VITRO DEVELOPMENTAL COMPETENCE OF BOVINE EMBRYOS

KARADJOLE M.[1], GETZ I.[1], SAMARDZIJA M.[1], MATKOVIC M.[2], MAKEK Z.[1], KARADJOLE T.[1], BACIC G.[1], MACESIC N.[1], DOBRANIC T.[1]

[1]*Clinic for Reproduction and Obstetrics, Faculty of Veterinary Medicine, University of Zagreb, 10000 Zagreb, Croatia*

[2]*Center for Reproduction and Animals Breeding of Croatia, Zagreb, Croatia*

Numerous studies suggest that the intrinsic quality of the oocyte is the critical factor determining the proportion of the oocytes developing to the blastocyst stage, although culture conditions also affect developmental potential of the embryo. The aim of this research was to compare the developmental competence of immature oocytes recovered from slaughterhouse ovaries (SO) and those recovered in vivo by ovum pick up (OPU). Transvaginal ultrasound-guided (Pie Medical, Netherlands) oocyte collection was performed in 6 Simmental donor heifers. Heifers were synchronized with $PGF_2\alpha$ and stimulated with pFSH, twice a day during two days (Folltropin®-V, Vetrepharm Inc., London, Canada, a total dose: 200 mg NIH-FSH-P1). OPU was performed 48 hours after the last FSH injection and procedure was repeated once a week for six consecutive weeks. Oocytes were matured, fertilized and cultured in vitro in SOFaaBSA until Day 9. The cleavage rates on Day 2, the total number of morulas (M) and blastocysts (Bl) on Day 7 and the numbers of hatched blastocysts (hBl) on Day 9 were recorded. Differential staining of the inner cell mass (ICM) and trophectoderm cells were performed on random samples on Day 7 blastocysts (n=24). The results were analyzed with ANOVA (StatSoft, Statistic, version 7.1.) and with Tukey's post-hoc analysis. The results are presented in the table.

Table 1. Effect of bovine oocyte origin on in vitro development of embryos (mean±SD)

Oocyte origin	N	Cleavage (%)	M/Bl (%)	HBL (%)
Slaughterhouse oocytes	398	76.83 (±2.31)[a]	29.96 (±2.08)[a]	16.78 (±0.78)[a]
OPU-derived oocytes	212	81.29 (±8.83)[a]	44.71 (±7.20)[b]	35.57 (±5.87)[b]

Values with different superscripts within the same columns differ significantly ($P<0.05$; ANOVA)

The proportion of ICM cells in day 7 blastocysts was significantly higher in OPU derived embryos (29.36±1.16% vs. 26.05±0.8%, $p<0.05$).

These results demonstrate that the immature oocytes recovered in vivo after OPU are more competent to develop to the blastocyst stage than those derived from slaughterhouse ovaries. Also, the oocyte origin seems to have an effect on the quality of the blastocyst in terms of the proportion of ICM cell number.

Notes

ACTIVITY OF CASPASE-3 IN BOVINE BLASTOCYSTS DEVELOPED IN CO-CULTURE WITH VERO CELLS OR IN CULTURE WITH SOF MEDIUM

KATSKA-KSIAZKIEWICZ L., OPIELA J., RYNSKA B.

Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, 32-083 Balice, Poland

The aim of the experiment was to determine whether embryo culture system affects the quality of bovine blastocysts assessed on the basis of the activity of caspase-3, the quality marker of apoptosis. Bovine blastocysts were produced following IVM/IVF/IVC of immature oocytes selected for the experiment by combined evaluation of COC morphology and G6PDH activity (BCB staining). Embryo culture to the hatched blastocyst stage was carried out in two culture systems, i.e. 1) in modified SOF medium, at atmosphere of 5% CO_2, 5% O_2 and 90% N_2 or 2) in the co-culture with Vero cells, in B2 medium, at humidified atmosphere of 5% CO_2 in air.

A total number of 204 blastocysts (88 BCB+, 22 BCB- and 94 Control) developed in co-culture with Vero cells and 80 blastocysts (38 BCB+, 11 BCB- and 31 Control) developed in culture with SOF medium was used for assessment of caspase-3 activity.

There was a tendency towards growth of activity of caspase-3 progressively with blastocyst development. The highest activity of caspase-3 was assessed in the expanded and hatched blastocysts (70.5; 100 and 58.2% respectively for BCB+, BCB- and Control blastocysts developed in co-culture with Vero cells and 76.0; 100 and 62.5% respectively for BCB+, BCB- and Control blastocysts developed in SOF medium). Inverse relationships in the proportion of blastocysts with high activity of caspase-3 were observed for the early and middle stages of blastocyst (43.8; 20.0 and 29.4% respectively for BCB+, BCB- and Control blastocysts developed in co-culture system whereas for the blastocysts developed in SOF medium 50.0 and 46.2% respectively for BCB+ and Control blastocysts). In the late blastocyst stage, regardless of the origin, approximately 50% embryos showed high activity of caspase-3. The significant differences were observed between blastocyst rates with low and high activity of caspase-3, only in the BCB+ groups (Table 1). There was an tendency towards higher activity of caspase-3 in the BCB+ blastocysts developed in SOF medium in comparison to those from the co-culture system however the differences has appeared to be non-significant (Table 1).

Table 1. Comparison of low and high activity of caspase-3 in blastocysts following development of IVM/IVF oocytes BCB+, BCB- and Control in two culture systems

Culture system	Fluorescence intensity	Blastocysts BCB+ (%)	Blastocysts BCB- (%)	Blastocysts Control (%)
Vero cells, B2 medium, at 5% CO_2 in air	+	37 (42.0)$^{a\,*}$	8 (36.4)	45 (47.9)^{a}
	++	51 (58.0)$^{a\,*}$	14 (63.6)	49 (52.1)^{a}
SOF medium, at 5%CO_2, 5% O_2, 90% N_2	+	12 (31.6)$^{a\,**}$	1 (9.1)	15 (48.4)^{a}
	++	26 (68.4)$^{a\,**}$	10 (90.9)	16 (51.6)^{a}

Values with the same letters in the row do not differ statistically. Values with stars within the same column differ statistically (*P<0.05; **P<0.01) (test χ^2).

The tendency of growth in caspase-3 activity with the progression of embryo development, regardless of the type of oocytes used for fertilization, suggests that the activity of this apoptotic enzyme may be affected by suboptimal culture conditions.

Notes

EFFECTS OF OOCYTE QUALITY AND SEMEN DONOR ON THE EFFICIENCY OF IN VITRO EMBRYO PRODUCTION IN CATTLE

KATSKA-KSIAZKIEWICZ L., OPIELA J., RYNSKA B.

Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, 32-083 Balice, Poland

The aim of the present study was to determine whether a combined selection of immature oocytes by evaluation of cumulus-oocyte-complexes (COC) morphology and staining with brilliant cresyl blue (BCB) would be helpful to select developmentally competent oocytes and thereby increase the efficiency of blastocyst production from ovarian oocytes of slaughtered cattle. Additionally, interactions between oocyte quality and semen donors (3 bulls) were assessed. Although it has been already been shown that BCB staining is a useful tool to select more competent oocytes, still the test is not routinely included in laboratory selection protocol. The aim of our study was to present additional arguments for or against BCB test as a useful selection tool for oocytes recovered from ovaries of slaughtered cattle.

In the pool of morphologically normal COCs used for BCB staining (n=1668) the mean number and percentage of COCs classified as the BCB(+) was 91.9±41.6; 71.6% and the BCB(-) 36.4±23.6; 28.4%. Oocytes classified as grade 1 and BCB(+) (n=1151) showed similar developmental competence after in vitro maturation and fertilization compared with Control, unstained oocytes grade 1 (n=1052). The cleavage rates and blastocyst rates resulted 73.4%; 19.3 %; and 70.9%; 17.2% respectively for BCB(+)and Control oocytes. Significantly lower ($P<0.001$) developmental competence (58.5% and 11.6% respectively for cleavage and blastocyst rates) was noticed for oocytes grade 1 BCB(-) (n=412) compared with oocytes BCB(+) and Control.

Significant differences in the embryo cleavage and blastocyst formation rates among three bulls A, B and C were observed. Generally, for all tested bucks, differences in embryo production efficiency were related mainly to the oocyte quality. The cleavage rates and the blastocyst rates resulted respectively for bulls A, B, C and oocytes: 1) BCB(+) 86.0; 82.9; 51.8% and 19.0; 15.5; 9.0%; 2) BCB(-) 49.4; 66.8; 37.7% and 8.6; 7.2; 2.9%; 3) Control 70.4; 78.0; 60.0% and 13.5; 10.2; 8.3%.

These results show that the most important factor contributing to in vitro embryo production efficiency is oocyte quality. It may be concluded that while male variability can impact on developmental competence of embryos, the effect is not so drastic as in the case of the oocyte quality. Moreover, it may be concluded that the BCB test increases the precision of oocyte selection and can be of great value, especially for work relying on a single, high quality oocyte, such as somatic cloning.

Notes

EVALUATION OF INTEGRINS, LEUKOCYTE ADHESION MOLECULE CD18 AND TRANSMEMBRANE PROTEIN CD9 mRNA CONTENTS IN PORCINE OOCYTES USING REAL-TIME QUANTITATIVE PCR REACTION

KEMPISTY B.[1], ANTOSIK P.[2], BUKOWSKA D.[2], JACKOWSKA M.[1], GRÓDEK E.[1], JAŚKOWSKI J.[2], JAGODZIŃSKI P.P.[1]

[1]Department of Biochemistry and Molecular Biology, University of Medical Science;. [2]Department of Agricultural Veterinary, University of Agriculture, Poznań, Poland

The initial step of successful fertilization is characterized by species-specific gamete recognition, adhesion, and fusion. Several specific proteins are involved in this process. Integrins are the major receptors within the extracellular matrix (ECM) that mediate several functions connected with cell life and metabolism, such as cell adhesion, migration, cytoskeletal organization, proliferation, survival, and differentiation. CD9 is a member of the tetraspanin family, and interacts with integrins, immunoglobulins, proteoglycans, and growth factors. It has been suggested that CD9 protein forms an oocyte-specific receptor for sperm fertilin and contributes in sperm-egg binding and fusion. Leukocyte adhesion molecule CD18 belongs to the family of cell membrane glycoproteins called integrins, which are formed by alpha-beta heterodimers. Both CD9 and CD18 are candidate molecules involved in sperm-oocyte fusion.

The ovaries from cycling gilts (crossland-WBPxPBZ, n=14; age: 180 days; weight: 95-100 kg) were collected from a local slaughterhouse and transported to the laboratory at 35°C. Cumulus-oocyte complexes (COC) were isolated after scarification of the ovary's surface under a stereoscopic microscope. These isolated COC (n=50, from each gilt) were washed with NCSU37.

Total RNA was isolated from porcine oocytes according to Chomczyński and Sacchi (1987), treated by DNase I, and reverse-transcribed into cDNA**.** Quantitative analysis of integrins (β1, β6, αL, αM), leukocyte adhesion molecule (CD18), and transmembrane protein (CD9) cDNA was performed by RQ-PCR, which was conducted in a Light Cycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I detection dye. The cDNA of the integrins was amplified using pairs of primers complementary to different exons**.** The quantity of mRNA product in each sample was adjusted to the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA or calculated per 10^3 oocytes. Quantification of copy number was derived from a standard curve of a known amount of synthetic DNA template. Each of these experiments was performed at least in triplicate. Results were estimated using Student's *t*-test and one-way analysis of variance (ANOVA) with Newman-Keul's *post-hoc* test. *P* value was determined by Student's *t*-test, with significance $P<0.05$.

We observed 4-fold higher integrin β6 mRNA content compared to mRNA of integrin β1, and 6-fold higher compared to αL and αM ($P<0.001$). We did not observe statistically significant differences between the level of integrins αL and αM in porcine oocytes ($P=0.287$). An increase in CD9 mRNA level compared to CD18 was also observed ($P<0.001$).

The aim of the study was to evaluate the differences in levels of integrin mRNA, CD9, and CD18 in porcine oocytes isolated from gilts before the first pregnancy. We postulated that from all of the integrins that contribute in fertilization, integrin β6 may play the most important role in successful fertilization and in forming the reproductive ability of cycling gilts. In addition, CD9 may function as an oocyte-specific receptor that contributes to successful adhesion and fusion between the sperm and oocyte during fertilization. CD18 may have a less important role in this process.

Notes

SEXING AND DIRECT TRANSFER OF BOVINE BIOPSIED FROZEN-THAWED EMBRYOS UNDER ON-FARM CONDITIONS IN A COMMERCIAL PROGRAM

LACAZE S.[1], PONSART C. [2], HUMBLOT P.[2]

[1]*MIDATEST, , Domaine de Sensacq, 64230 DENGUIN , France*
[2]*UNCEIA, R & D Department, 13 rue Jouët, 94704 MAISONS-ALFORT, France*

Embryo sexing is used in France since the 1990's by using a PCR method based on a specific Y DNA sequence (INRA patent 1987). The sexing procedure was progressively simplified within the last 5 years by the R&D department of UNCEIA to facilitate its on-farm use. The present protocol (centrifugation of tubes containing biopsy cells, denaturation of DNA, addition of the reaction mix, amplification of sequences of interest by PCR, sample deposit on gel and electrophoresis) allows the identification of the sex of embryos within 140 min. This study describes on field results with this technique obtained by a large selection unit from the south of France (MIDATEST ET team) between January 2000 and February 2007 and the variation factors influencing pregnancy rates following transfer of biopsied frozen-thawed embryos.

A total of 979 Day 7 bovine embryos were biopsied by a single operator with a steel blade attached to a micromanipulator and frozen using ethylene glycol (1.5 M) plus fetal calf serum (40%, n=232, EG-FCS) from 2000 to 2004 or sucrose (0.1%, n=747, EG-S) since 2004. Embryos were collected from 171 sessions by conventional techniques (donor cows inseminated twice on observed oestrus following a standard superovulatory treatment and collected on Day 7). Embryos (n=319) were thawed (straws in air for 5-10 s and in a water bath for 30 s) and directly transferred. Pregnancy was assessed by ultrasonography following transfer of 246 biopsed frozen thawed embryos. Significant sources of variation of pregnancy rates were analysed with a multivariate logistic regression model (factors with $p<0.1$ from univariate analysis were kept for further multivariate analysis).

On average, 5.8±0.3 embryos were biopsed and frozen per session, corresponding to 41% of the total number of embryos (maximum of 19 biopsed embryos ; 1rst and 3rd quartiles averaged 3 and 7 embryos respectively). Most of the micromanipulated embryos were at the morulae stage (stage 4 : 69.2% ; 5 : 20.4% ; 6-8 : 10.2% according to IETS criteria). From those 44.8% were females and 50 % were males (5.2 % sex undetermined, low amplification of the autosomal sequence). The rate of determination was inversely related to the numbers of cells of the biopsy (≤3 cells (n=83) : 14.5% ; 4-6 cells (n=432) : 2.6% ; ≥7 cells (n=255) : 0% ; $p<0.05$), which averaged 5.8±0.6 and ranged from 1 to 10 cells. Embryos were frozen 150±1 minutes after recovery. Pregnancy rate averaged 47.2% and was mainly influenced by parity of recipients (heifers (n=205): 51.7% vs cows (n=38): 26.3%, $p<0.05$). The EG-S freezing protocol was found significant in increasing pregnancy rates from the univariate analysis (EG-S vs EG-FCS, + 13%, $p<0.05$). However, this factor was not found significant from the multivariate model. No significant effects of embryo stage (4 = 47.3%, 5 = 48.7%, 6-8 = 62.5%), interval between flushing and freezing (<120 min = 42%, 120-179 min = 47.6%, 180-300 min : 49.5%), number of micromanipulated embryos per flush (≤5 : 47.1%, 6-10 : 48.2%, ≥11 : 45.1%) were observed.

Altogether, these results confirm that this embryo sexing procedure is efficient when applied from biopsies containing 4 cells or more. Direct transfer of micromanipulated frozen-thawed embryos led to obtain similar pregnancy rates as intact frozen embryos. This confirms that heifers should be used preferentially as recipients. The positive effect of sucrose in the freezing medium remains to be confirmed under controlled conditions.

Notes

EFFECT OF ADDITION OF EICOSAPENTAENOIC ACID (EPA) TO CULTURE MEDIUM ON DEVELOPMENT OF BOVINE EMBRYOS IN VITRO

LAWSON C., WADE M., KENNY D., LONERGAN P.

School of Agriculture, Food Science and Veterinary Medicine, University College Dublin,Belfield, Dublin 4, Ireland.

There is emerging evidence that supplemental polyunsaturated dietary fatty acids (PUFA) increase cow fertility. For example, in vitro studies show that the ω-3 PUFA eicosapentaenoic and docosahexaenoic acids (EPA, DHA) have pivotal roles in the suppression of uterine prostaglandin $F2_{\alpha}$, a critical regulator of embryo survival, though the cellular mechanisms are as yet unclear. It is widely acknowledged that ω-3 PUFA are potent molecules supporting important cellular processes including membrane stability, gene transcription, cell adhesion and proliferation and inter- and intracellular transport. All these processes are critical to early embryo cellular proliferation and development and thus ω-3 PUFA could play important roles in embryo development and survival over and above their anti-luteolytic effects. To-date however, there are few published data on the specific effects of ω-3 PUFA supplementation on embryo development in cattle. The aim of this study was to examine the effect of EPA supplementation in vitro on cleavage rate and subsequent embryo development in cattle.

Presumptive zygotes produced by in vitro maturation and fertilization were randomly allocated to one of seven groups: (i) control medium alone or supplemented with, (ii) ethanol (vehicle control), (iii) 6.25 μM EPA, (iv) 12.5 μM EPA, (v) 25 μM EPA, (vi) 50 μM EPA or (vii) 100 μM EPA. Culture took place in an atmosphere of 5% CO_2, 5%O_2, and 90% N_2 at maximum humidity. Cleavage rate was recorded on Day 2 (day of IVF = Day 0) and blastocyst development recorded on Day 8. In addition, gas liquid chromatography (GLC) was used to assay the medium for fatty acids to ensure the presence of EPA throughout the duration of culture.

Despite the fact that the GLC results confirmed no significant depletion of EPA throughout the seven days of culture, addition of EPA did not improve either cleavage rate or blastocyst yield compared to the control. This would suggest that any positive effects of EPA on fertility are not directly on the embryo.

Table 1. Effect of addition of EPA to culture medium on development of bovine embryos in vitro.

Treatment		Cleaved		Day 8 Blastocysts	
	N	N	%	N	%
Control	190	138	72.6	64	33.7
Control +ethanol	188	133	70.7	59	31.4
6.25μM	190	151	79.5	48	25.3
12.5μM	189	139	73.5	56	29.6
25μM	190	145	76.3	51	26.8
50μM	189	149	78.8	64	33.9
100μM	164	122	74.3	37	22.6

Notes

EFFICIENCY OF WHOLE GENOME AMPLIFICATION FOR MULTI-MARKER DETECTION IN BOVINE EMBRYOS

LE BOURHIS D.[1-3] AMIGUES Y.[2], VANNIER A.[2], HEYMAN Y.[3], HUMBLOT P.[1], VIGNON X.[3]

[1]UNCEIA, Dept R&D, 13, rue Jouët, 94704 Maisons-Alfort, France, [2]LABOGENA Domaine de Vilvert, 78352 Jouy en Josas Cedex, France, [3]INRA/ENVA - Biologie du Développement et Reproduction, 78352 Jouy en Josas, France

Genomic tools have now become available for most livestock species and are being used routinely for marker-assisted selection (MAS) in cattle. One major challenge is the possibility to detect multiple markers from a minimum number of embryonic cells for the routine genotyping of preimplantation embryos for multiple markers. The detection of large number of markers that are widespread over the genome is generally limited by the amount of genomic DNA available in an embryo biopsy of a small size not to be detrimental to embryonic survival. Amplification of DNA from such a biopsy is then necessary. In this study, the efficiency of embryo genotyping for 42 markers (micro satellites) following whole genome amplification (WGA) was evaluated from samples of variable number of cells isolated from cattle embryos.

In vitro bovine embryos were produced according to the standard IVM-IVF protocol in use in the laboratory, fertilization being performed with the semen from a single bull. By Day 6, compacted morulae were selected from the culture dish and 1, 2, 5, 10 and 20 embryonic cells were removed from each morula. Cells were dry frozen in tubes before further processing. Whole genome amplification was performed using the commercial QIAGEN REPLI-g® Mini Kit according to the manufacturer instructions. WGA solution was then diluted, processed by PCR with 42 markers, and the resulting data were genotyped with GeneMapper® software (Applied Biosystems Europe). Accuracy and reliability of genotyping were assessed using different samples of cells from the same embryo (Table 1).

Table 1: Efficiency of bovine morulae multi-genotyping after WGA according to the number of cells

Nb of cells	Nb of embryo biopsied	% markers detected	% of error
1	10	45[a]	48[a]
2	10	75[b]	28[b]
5	10	98[c]	10[c]
10	10	98[c]	5[d]
20	5	93[c]	2[f]

Values with different superscript within columns are significantly different ($P<0.01$)

The results clearly indicate that a conventional biopsy of 5 to 10 cells is sufficient for multi-markers detection after whole genome amplification as 98% of the 42 markers were detected. This efficiency was not improved using a higher number of cells. These results illustrated that WGA could be a suitable method for recovery and amplification of DNA from embryo biopsy before routine genomic investigations. This WGA technique is now assessed on biopsies from superovulated bovine blastocysts that have been frozen and transferred into recipients. If confirmed under field conditions, these results may allow the development of robust methods for an optimal use of MAS within cattle populations.

This work has been done with the financial support from ANR-Genanimal and Apis-Gene, contrat Typagenae

Notes

EFFECT OF SEASON ON SUPEROVULATORY RESPONSE OF BOER GOATS

LEHLOENYA K.C.[1], GREYLING J.P.C.[1], SCHWALBACH L.M.J.[1], GROBLER S.[2]

[1]Department of Animal, Wildlife and Grassland Sciences, University of the Free State, Bloemfontein 9300, South Africa. [2]Department of Obstetrics and Gynaecology, Medical Faculty, University of the Free State, Bloemfontein 9300, South Africa

This study was conducted to evaluate the effect of season on the superovulatory response and embryo recovery rate in Boer goats. All does were synchronised for oestrus with controlled internal drug release dispensers (CIDR; Pharmacia & Upjohn, Auckland, New Zealand) for a period of 17 days and superovulated with porcine FSH (pFSH) (Folltropin®-Vetrepharm) during the natural breeding season (n=9) and outside the breeding season (n=11). The superovulation treatment was administered as a total dose of 200 mg pPFSH/doe given i.m. in 7 dosages, at 12h intervals, starting 48h prior to CIDR removal (the first dose being 50 mg and all others 30 mg each). Does were observed for oestrous behaviour 3 times daily at 8h intervals following CIDR withdrawal with the aid of teaser bucks to determine the onset and duration of the induced oestrous period. Cervical inseminations with o.1ml fresh undiluted semen were performed 36h and 48h following CIDR removal and the embryos were surgically flushed 6 days following the second AI. Recovered structures were microscopically evaluated and classified according to their morphological appearance as defined by Linder and Wright (1983). The total number of structures, unfertilised ova, fertilised ova, degenerated embryos and transferable embryos from each doe flushed were recorded. All data were analysed using ANOVA procedures of SAS (2004). All does showed signs of oestus. The mean onset of oestrus (24.9±4.8h) was significantly earlier during the natural breeding season, compared to outside the breeding season (30.5±9.1h) and the duration of the induced oestrous period significantly ($P<0.05$) longer during the natural breeding season (24.0±5.7h) than outside the breeding season (18.2±3.7h). The mean ovulation rate per donor, total number of structures (unfertilised ova and embryos) and the total number of embryos recovered per donor did not differ between seasons. The mean number of unfertilised ova per doe was significantly higher outside the breeding season (3.3±4.8), compared to during the natural breeding season (0.9±2.4). The total number of degenerated embryos and transferable embryos however, did not differ between seasons. Season influence the onset and duration of the induced oestrous period however, it did not affect the oestrous response. Although the number of unfertilised eggs increased outside the breeding season, superovulation was still warranted outside the natural breeding season in Boer goats as the number of transferable embryos were similar in and outside the natural breeding season.

Table 1. Effect of season on induced oestrus and superovulatory response

Parameters	Season	
	During breeding season	Outside breeding season
No. of does	9	11
Onset of oestrus (h)	24.9±4.8[a]	30.5±9.1[b]
Duration of oestrus (h)	24.0±5.7[a]	18.2±3.7[b]
No. of ovulations (total CL's/donor)	17.5±6.3	21.3±5.9
Total number of structures (unfertilised ova & embryos per doe)	18.0±5.8	17.5±4.8
Total number of embryos/donor	16.4±7.0	16.5±6.1
Total number of unfertilised ova/donor	0.9±2.4[a]	3.3±4.8[b]
Total number of degenerated embryos/donor	3.6±3.4	3.2±4.2
Total number of transferable embryos/donor	12.3±9.4	13.1±5.3

[a,b] Values with different superscripts within the same row differ significantly ($P<0.05$)

Notes

TIME-LAPSE STUDY OF THE EFFECT OF TRICHOSTATIN A ON *IN VITRO* DEVELOPMENT OF PORCINE HANDMADE CLONED EMBRYOS

LI J.[1,3], VILLEMOES K.[1], ZHANG Y.H.[1], DU Y.[1,3], KRAGH P.M.[1,3], PURUP S.[2], PEDERSEN A.M.[1], JØRGENSEN A.L.[3], BOLUND L.[3,4], YANG H.M.[4], VAJTA G.[1]

[1]*Institute of Genetics and Biotechnology;* [2]*Institute of Animal Health, Welfare and Nutrition, University of Aarhus, DK 8830 Tjele, Denmark;*
[3]*Institute of Human Genetics; University of Aarhus, DK 8000, Aarhus, Denmark*
[4]*Beijing Genomics Institute, Chinese Academy of Sciences, Beijing 101300, China*

Abnormal DNA hypermethylation is supposedly one factor accounting for the inefficient reprogramming of donor cells in the cytoplasm during somatic cell nuclear transfer. Both developmental competence and blastocyst quality in embryos were improved with trichostatin A (TSA) treatment in various species. Recently, high *in vitro* development of pig embryos with Handmade Cloning (HMC) was achieved with the treatment of 37.5 nM TSA for 22-24 h in our unpublished study. The present work was designed to examine the effect of TSA on *in vitro* development of pig HMC embryos with a time lapse system (Holm et al., Theriogenology 1998; 50:1285-1299). Reconstructed embryos with HMC method were randomly distributed into two groups, one with the treatment of 37.5 nM TSA and another without TSA treatment. After 22-24 h TSA treatment during activation and the first day of culture in porcine zygote medium 3 (PZM-3; Yoshioka et al., Biol Reprod 2002; 66:112-119) by using the well of the well system (WOWs; Vajta et al., Mol Reprod Dev 2000; 55:256-264), further culture was performed in PZM-3 without TSA. In three subsequent repeats using a total of 110 HMC embryos with or without TSA treatment, development was investigated under the time lapse system. Images of each embryo incubated in the WOWs were recorded sequentially every 30 min throughout the culture period (from Day 1 to Day 7). First appearances of the following developmental stages were recorded for each embryo: 2-cell, compacted morula (CM), blastocyst (BL). The first cleavage (2-cell stage) occurred earlier in the TSA treated than in the control (untreated) group (30 and 32 h, respectively) with similar peaks (82.7% vs. 81% of embryos), while the CM stages were achieved 15 later (103 and 88 h, respectively) with different peaks (60.3% vs. 48% of embryos, $P<0.05$). The highest rates of blastocyst (including expanded blastocysts) were registered 14 h later in control (untreated) group than in the TSA treated group (Fig. 1.). In conclusion, the time-lapse investigation revealed that TSA treatment has altered the developmental dynamics and resulted in eventually higher blastocyst rates after HMC.

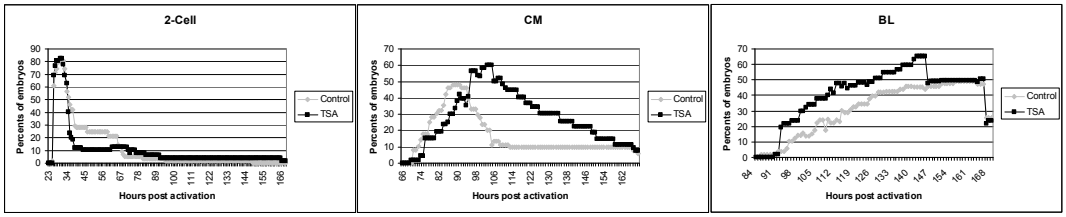

Figure 1. *In vitro* development of HMC pig embryos with or without TSA treatment.

Notes

A FIELD-MOET STUDYING PREGNANCY RATES OF BIOPSIED, FROZEN-THAWED EMBRYOS OF TOP BREEDING DAIRY CATTLE

LINDEBERG H.[1,2], KANANEN-ANTTILA K.[1], KAIMIO I.[2], VARTIA K.[2], HALMEKYTÖ M.[1]

[1]University of Kuopio, Department of Biosciences, FIN-70211 Kuopio, Finland;
[2]ProAgria Co-Operative Breeding Service; FIN-76101 Pieksämäki, Finland

A field MOET called "AATE project, Analysed embryos increase productivity of dairy herds in the Northern Savo" was organized by local and national breeding authorities in the Northern Savo, Finland during 1.11.05-30.4.07. The aims of the project were 1) to utilize embryo analytics in a field MOET, 2) to improve embryo logistics by developing storage techniques for biopsied embryos, 3) to obtain information on the pregnancy rates of biopsied and cryopreserved embryos of top breeding dairy cattle that were selected as resource animals and 4) to explore whether ascorbate exposure during biopsing and freezing would increase the viability of biopsied and cryopreserved embryos.

To induce superovulation, the resource animals were treated with FSH (Folltropin-V or Pluset) on decreasing regimen twice daily for four days (cows: 18 ml, heifers: 12 ml) and with 2.0 ml of prostaglandin $F_{2\alpha}$ (Estrumate, Dinolytic) on the third day of treatment to induce heat and ovulation. On the sixth day of treatment, the animals were inseminated two to three times on 12 h interval with frozen-thawed semen of top breeding bulls. The resource females were flushed seven days after the first insemination by four embryo transfer veterinarians. If one to three transferable embryos were recovered, the embryos were transferred fresh. In cases of four or more transferable embryos, the embryos were allocated in treatment groups and biopsied by a microblade. Grade I embryos were divided into two groups: control and 0.1 mM L-ascorbate exposure during biopsing, post-biopsy recovery and freezing. Time from embryo recovery to cryopreservation varied from 5 to 10 h. Grade II and III embryos were biopsied and stored overnight in culture using G-2™ version 3 medium (Vitrolife, Sweden). The Ampli-Y kit (Finnzymes Oy, Espoo, Finland) was used for sex determination. Only female embryos were transferred either the following day (fresh Grade II and III embryos) or after thawing (cryopreserved Grade I embryos). Pregnancies were diagnosed by rectal palpation two months after transfer.

A total of 49 embryo recoveries produced 454 transferable embryos from 26 cows (19 Ayshire and seven Holstein-Friesian cows that had calved on average two to three times) and 23 heifers (eight Ayshire and 15 Holstein-Friesian heifers). Three flushings resulted in no embryos or unfertilized oocytes (UFOs), one flushing produced only 2 UFOs, and in the remaining 45 flushings, the number of transferable embryos varied from one to 31. An average total number of all embryos and UFOs was 12.4 (608 embryos and UFOs/49 flushings) and an average total number of transferable embryos was 9.3 (454 transferable embryos/49 flushings) per resource female. A total of 427 transferable embryos were biopsied and the remaining 27 embryos from 16 flushes (33% of all flushings) were transferred straight after recovery without sex determination. Of the biopsied embryos, 46.4% were of male sex. 153 cryopreserved Grade I (update on the 15th of May 2007: 45 of 82 control and 44 of 71 ascorbate treated embryos) and 62 over-night cultured Grade II or III female embryos have been transferred into the uteri of recipient animals. Pregnancy rates are yet to be determined.

Notes

EFFECT OF OESTROUS CYCLE PHASE ON MORPHOLOGY AND MEIOTIC COMPETENCE OF PORCINE OOCYTES

MACHATKOVA M., HULINSKA P., HORAKOVA J., RECKOVA Z., HANZALOVA K.

Department of Genetics and Reproduction, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic

There is a general agreement that the meiotic competence of bovine oocytes is related to the size of follicles and the level of their atresia influenced by oestrous cycle stage. The objective of this study was to characterize the effect of oestrous cycle phase on morphology and meiotic competence of porcine oocytes derived from different-size follicles. A total of 46 cycling gilts, 8.5-9 month old, were used as oocyte donors. Oestrous cycles was synchronized by Regumate and the onset of oestrus was checked (Day 0). The gilts were slaughtered during the early (Days 1 - 5), middle (6 - 10) and late (11 - 14) luteal phase or the early (Days 15 - 16), middle (17 - 19) and late (20 - 21) follicular phase. Ovaries of each donor were evaluated for morphology and oocytes were isolated from small- (≤ 4 mm) and medium-sized (5-9 mm) follicles. Cumulus-oocyte complexes with a dark, evenly granulated cytoplasm and at least two compact layers of cumulus cells were selected as useful. Oocyte populations were matured by a standard protocol. After maturation, they were denuded from cumulus, fixed in glutaraldehyde, stained with 33258-Hoechst and examined by epifluorescence. The mean number (± S.E.M.) of oocytes collected per donor was significantly higher ($p<0.01$) but mean percentages (± S.E.M.) of useful and mature oocytes were significantly lower ($p<0.01$) for small than for medium follicles regardless of the phase (187.7 ± 48.4 vs 16.9 ± 6.1, 22.4 ±7.5% vs 80.2 ± 6.8% and 48.4 ±17.8% vs 79.9 ± 7.9%). In small follicles, the mean numbers (± S.E.M.) of useful oocytes collected per donor increased from the early to late luteal phase and decreased from the early to late follicular phase. In medium follicles, the mean numbers (± S.E.M.) of useful oocytes collected per donor were similar in the period from the middle luteal to middle follicular phase, when these follicles were present on the ovaries (Figure 1). In small follicles, the mean percentage (± S.E.M.) of mature oocytes increased during the luteal and decreased during the follicular phase and these differences were significant ($p<0.01$). Similarly in medium follicles, the mean percentages of mature oocytes increased during the luteal and decreased during the follicular phase but these differences were not significant (Figure 2). It can be concluded that populations of porcine oocytes from small and medium follicles were different in terms of their quantity, morphological quality and meiotic competence. The late luteal or the early follicular phases were most productive for oocyte collection because they provided more useful oocytes with greater meiotic competence than the other phases of follicular development.

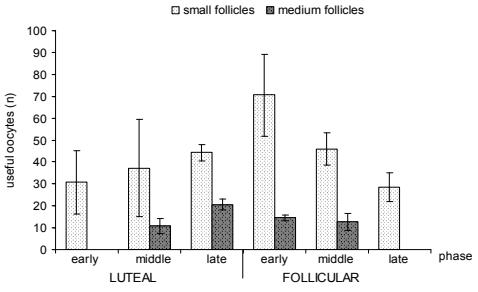

Figure 1 Mean No.± S.E.M. of useful oocytes collected per donor related to follicle size and phase

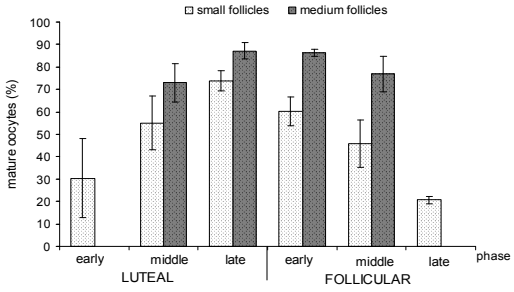

Figure 2 Mean % ± S.E.M. of mature oocytes related to follicle size and phase

Supported by Grants QG 50052 and QF 3218 of Ministry of Agriculture of Czech Republic

Notes

MONITORING OESTRUS IN SYNCHRONIZED SARDA EWES BY VAGINAL MUCUS IMPEDANCE ASSAY: A PRELIMINARY STUDY.

MASIA F. [1], MAYORGA I.[1,2], STELLETTA C.[2], MARA L.[1], CASU SARA [1], CHESSA F. [1], DATTENA M.[1]

[1] *Istituto Zootecnico e Caseario per la Sardegna, 07040 Olmedo, Italy*
[2] *Dip. Scienze Cliniche Veterinarie, Università di Padova, 35100 Padova, Italy*

A fixed insemination time is one of the factors contributing to the success of an artificial insemination (AI) programme in a synchronized sheep flock. However, in ewes synchronized with FGA sponges, the onset of oestrus varies from 24 to 48 h after sponge removal, and ovulation time may be distributed in the same way, affecting the success of AI. In addition, Robinson and Moore (1967) reported that 5-15% of synchronized ewes will not come into heat. Vaginal mucus electric impedance declines at the onset of natural oestrus (Bartlewski et al., 1999), and thus monitoring oestrus by a vaginal mucus impedance assay could be a cheap and easy way to determine the best time for insemination and to exclude ewes that did not come into oestrus. The aim of this work was to examine vaginal mucus impedance during oestrus in synchronized ewes. Sixteen Sarda ewes were treated for 14 days during the breeding season with FGA sponges (40 mg) and injected with 400 I.U. PMSG i.m. at the time of sponge removal . Electric impedance in ohms (EI) was monitored by intravaginal probe (*Draminsky ®, oestrus detector for sheep, Owcowa, Poland*) and oestrus behaviour was monitored using a vasectomized ram. These data were collected every 12 h starting at the time of sponge removal and continuing until insemination, a total of 55 hrs. Mean and standard deviation (SD) were calculated for all data recorded. Mean values of EI declined from the 12th (43 ± 5.7 ohm) to the 24th (30 ± 6.8 ohm) hour after sponge removal, remaining depressed until the conclusion of the experiment 55 hrs later (29 ± 9.8 ohm). The teaser ram identified ewes in oestrus at 24 (n=4), 36 (n=7) and 48 (n=5) hours after sponge removal (Fig 1). The time of peak numbers of ewes in oestrus corresponded to the time when minimum mean EI values were recorded, suggesting a relationship between EI and onset of oestrus. In conclusion, electric mucus impedance assay seems to be a reliable method of monitoring oestrus in ewes synchronized with FGA.

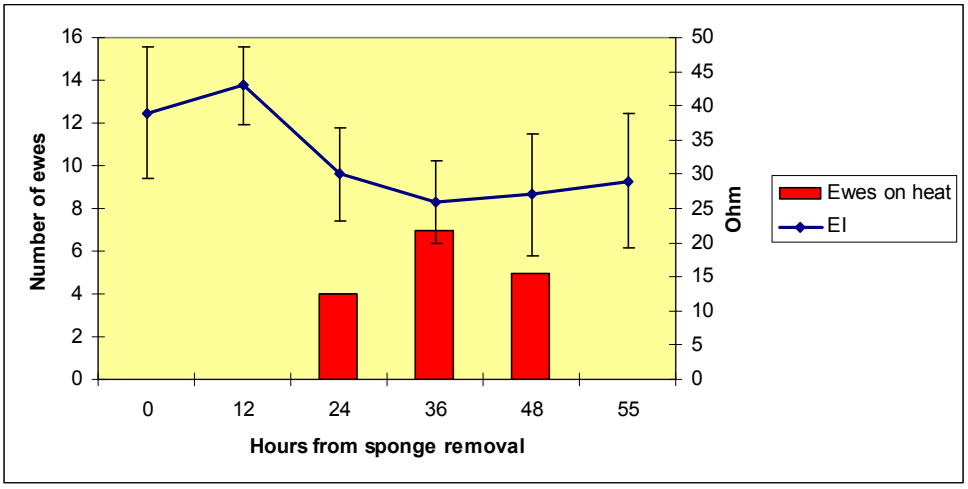

Figure 1. EI (mean and SD) and oestrus distribution in synchronized Sarda ewes.

Notes

SUPEROVULATION TREATMENT WITH FSH-p DURING NATURAL OESTRUS: COMPARISON WITH PROGESTAGEN SYNCHRONIZED SUPEROVULATION PROTOCOLS IN SARDA EWES

MAYORGA I.[1,2], MASIA F.[2], MARA L.[2], CHESSA F. [2], CASU SARA[2], DATTENA M.[2]

[1] *Dip. Scienze Cliniche Veterinarie, Università di Padova, 35100 Padova, Italy.*
[2] *Istituto Zootecnico e Caseario per la Sardegna, 07040 Olmedo, Italy.*

The aim of this study was to compare the response of superovulatory FSH treatment carried out during natural oestrus to more conventional progesterone FSH treatments. Thirty-four Sarda ewes were divided into 4 groups: single sponge (SS) 40 mg FGA for 12 days (n=10), double sponge (DS) 40 mg FGA; the sponge was replaced by a new one, maintained from day 7 to 14 (n=9), short treatment sponge (ST) 40 mg FGA for 7 days (n=9) and natural oestrus (NT) (n=6).

Superovulatory treatment per sheep consisted of 350 I.U. of porcine FSH (Folltropin®, Bioniche Animal Health, Ireland) administered in eight (i.m.) decreasing doses every 12 h (2 ml x 2, 1.5 ml x 2, 1.0 ml x 2 and 0.5 ml x 2) starting 48 h before sponge removal in the SS, DS and ST groups, and on day 4 after onset of oestrus (day 0) in the NT group. A single dose of 125 μg (i.m) cloprostenol (Estrotek®, Azienda Terapeutica Italiana, Italy) was injected at the same time as the first FSH dose in DS and ST groups and on day 6 after oestrus detection in the NT group. Ewes were naturally mated 24 h after sponge removal (SS, DS and ST groups) or cloprostenol injection (NT group).

Seven days after mating, inguinal laparotomy was performed and the number of corpora lutea (CL) were recorded; embryos were recovered by flushing each uterine horn according to the technique of Tervit and Havik (1976) with some modifications. The recovered embryos were evaluated according to their stage of development and their quality was scored on a scale of 1 to 3 (Niemann et al., 1981). Embryos with a score of 1 were considered of high quality. Length and cost of treatments for each ewe were also assessed. Data on number of corpora lutea (CL), embryos recovered (ER), embryos fertilized (EF), and high quality embryos (EQ_1) per ewe were analysed by ANOVA (GLM SAS procedure). Data on recovery (RR), fertility (FR) and embryo quality (Q_1) rates per treatment were analysed by a logistic analysis (SAS logistic procedure).

Although significant differences between treatments were detected only for RR and FR, results indicate that natural oestrus superovulatory treatment tended to be successful, as well as cheaper and less time consuming than other methods tested.

Table 1. Length of treatment, cost of treatment and responses to different superovulation protocols

Group	N° of ewes	Length of treat.	Cost of treat/ewe	CL/ewe	ER/ ewe	EF/ ewe	EQ_1/ ewe	RR %	F.R %	Q_1R %
NT	6	14 days	€ 101.80	11.5±3.5 (69/6)	7.3±5.0 (44/6)	7.3±5.0 (44/6)	5.5±4.9 (33/6)	63 [a] (44/69)	100[a] (44/44)	75 (33/44)
ST	9	15 days	€ 105.50	10.5±4.2 (95/9)	8.4±3.9 (76/9)	8.1±3.9 (73/9)	6±4.4 (54/9)	80 [b] (76/95)	96[a] (73/76)	74 (54/73)
SS	10	20 days	€ 103.80	7±3.2 (70/10)	5.6±3.2 (56/10)	4.5±3.5 (45/10)	4±3.0 (40/10)	80 [b] (56/70)	80[b] (45/56)	88 (40/45)
DS	9	22 days	€ 109.30	7.6±5.1 (69/9)	4.7±4.1 (43/9)	3.0±3.7 (27/9)	2.6±3.6 (24/9)	62 [a] (43/69)	62[c] (27/43)	88 (24/27)

Different superscripts indicates treatments with significant differences ($p<0.05$)

Notes

EFFECT OF VITRIFICATION USING TWO DIFFERENT CARRIER SYSTEMS ON EMBRYO DEVELOPMENT OF BOVINE OOCYTES

MORATÓ R.[1], IZQUIERDO D.[2], PARAMIO M.T.[2], MOGAS T.[1]

[1]Department of Animal Medicine and Surgery. [2]Department of Animal and Food Science. Faculty of Veterinary Science. University Autonomous of Barcelona,,E-08193 Bellaterra. Spain

The purpose of this study was to compare the effects of vitrification using Flexipet denuding pipette (FDP) or Open Pulled Straw (OPS) as carrier on the cleavage rate of bovine MII oocytes. In previous studies in our laboratory, the present authors have found that a Taxol pre-treatment during vitrification of mature bovine oocytes by OPS significantly improved spindle morphology and could promote embryo development after thawing. For this reason, 1μM Taxol™ was used in the vitrification solution. Oocytes were aspirated from slaughterhouse-derived ovaries and matured in TCM-199. Oocytes were randomly assigned to one of three experimental groups: (1) control oocytes matured *in vitro* for 24 h; (2) oocytes matured for 22 h and vitrified by the OPS method (Vajta et al. Mol Reprod Dev 1998; 51:53-58) and 1μM Taxol; (3) oocytes matured for 22 h and vitrified by the OPS method but using FDP as a carrier and 1μM Taxol. The oocytes were transferred back into the maturation dishes and matured for approximately an additional 2 hr before being subjected to fertilization. Fertilization was performed by using frozen-thawed Percoll-selected sperm. At 22 h after insemination, presumptive zygotes were pipetted and then cultured in drops of 25 μL SOF medium and 5% foetal calf serum.

Cleavage rates were significantly higher for untreated oocytes (72.6% and 72.8% for cow and calf, respectively) than for vitrified groups. In this preliminary study we observed that OPS group provided a significantly higher cleavage rate (46.8% and 42% for cow and calf, respectively) than FDP group (17.4% and 20.6% for cow and calf, respectively). Cow and calf oocytes vitrified by FDP were unable to develop to the blastocyst stage; whereas vitrification by OPS yielded 4.2% and 2% for cow and calf oocytes, respectively. Control group produced the highest blastocyst rate (18.4% and 17.5% for cow and calf, respectively). Note that not differences between cow and calf oocytes were observed between the different parameters studied.

In conclusion, the Flexipet denuding pipette and the OPS straws are both easy to perform. However, OPS resulted in higher cleavage and blastocyst rates. Therefore, the results presented in this study encourage us to develop a better protocol to increase its efficiency.

Notes

TYROSINE KINASE RECEPTORS, $p75^{NTR}$ AND FGFr2 EXPRESSION IN BOVINE EMBRYOS CULTURED IN VITRO

MUÑOZ M., RODRIGUEZ A., DIEZ C., CAAMAÑO J.N., DE FRUTOS C., FACAL N., GOMEZ E.

Genética y Reproducción SERIDA. Asturias. SPAIN.

The neurotrophin family of growth factors belongs to the group of tyrosine kinase ligands that mediate survival, growth and differentiation. Subtypes of neurotrophins act as anti-apoptotic factors by binding specifically to TrKA, TrKB, TrKC and the low affinity, non-specific p75 neurotrophin receptor ($p75^{NTR}$). Neurotrophins can cooperate both among them and with the basic fibroblast growth factor (bFGF). As embryo development could benefit from the presence of these growth factors in culture, we analyzed the temporal expression of the above receptors and FGFr2, a bFGF receptor, during the bovine early development in vitro. Slaughterhouse oocytes matured and fertilized in vitro were cultured in SOFaaci + 6g/L BSA under 5% CO_2, 5% O_2 at 38.7 ºC for 8 days. Pools of 37-50 immature oocytes, 20-35 zygotes, 10-35 2- to 4-cell embryos, 10-27 5- to 8-cell embryos, 8-17 morulae, 5-13 expanded blastocysts and 4-8 hatched blastocysts were placed in RNA-Later (Ambion, Austin, TX, USA), kept overnight at −4 ºC and stored at −80 ºC until RT-PCR analysis. All the above receptors were analyzed for mRNA expression; in addition, protein expression of TrKA, TrKB and TrKC was analyzed immunohistochemically. As seen in Table 1, we found mRNA for TrkA, TrkC and FGFr2 in immature oocytes, zygotes and 2- to 4-cell stage embryos; these stages, however, were negative for TrKB. At the 5- to 8-cell stage, TrKA, TrKB, TrKC and FGFr2, were not expressed, but their expression resumed at the morula stage onwards. We detected $p75^{NTR}$ mRNA and TrKA, TrKB and TrKC proteins in oocytes and all embryonic stages analyzed. Protein for TrKA, TrKB and TrKC was detected in all stages and samples analyzed from the immature oocyte up to the blastocyst stage and in mechanically isolated inner cell masses from Day-8 hatched blastocysts. This suggests that undetectable TrKB mRNA levels could be present in the bovine oocyte and subsequent maternal embryonic stages.

Table 1. Gene expression of tyrosine-kinase receptors (TrKA, TrKB, TrKC), fibroblast growth factor receptor-2 (FGFr2) and p75 in bovine immature oocytes and early embryos (positive samples / analyzed samples).

Gene	Immature oocyte	Zygote	2-4 Cell	5-8 Cell	Morula	Expanded blastocyst	Hatched Blastocyst
TrKA	2/4	2/5	1/4	0/4	2/5	3/6	3/5
TrKB	0/4	0/5	0/4	0/4	1/5	1/6	2/5
TrKC	2/4	1/5	1/4	0/4	1/5	1/6	2/5
FGFr2	3/4	3/5	3/4	0/4	1/5	3/4	¾
p75	3/4	3/5	2/4	1/4	1/5	4/5	¾

We have provided a detailed description of gene expression of some receptors involved in cell survival during bovine embryonic development, together with protein expression of the neurotrophin specific receptors TrKA, TrKB and TrKC. The information provided in our work can help to unravel mechanisms supporting cell proliferation during the early development.

Grant Support: Spanish Ministry of Science and Education. AGL-2005-04479. Marta Muñoz is sponsored by FICYT.

Notes

INFLUENCE OF BODY CONDITION ON QUALITY OF COC QUALITY AND CONCENTRATION OF LEPTIN, ACTIVE AND TOTAL GHRELIN IN BLOOD PLASMA AND FOLLICULAR FLUID IN DAIRY COWS

NOWAK T.A., SZCZEPANKIEWICZ D.[1], BŁASZAK B., JAŚKOWSKI J.M.

Department of Agricultural Veterinary, University of Agriculture, Poznań, Poland
[1]Department of Physiology and Biochemistry, University of Agriculture, Poznań, Poland

The influence of body condition on the number of follicles, number and quality of cumulus-oocyte-complexes (COC) and/or concentration of leptin (LEP), total and active ghrelin (TGHR and AGHR) in blood plasma and follicular fluids was the investigated.

The experiment was carried out on 100 donor dairy cows. Before slaughter all cows were divided into five classes according to body condition. For group I, all cows with BCS ≤ 2.5 were rated among groups II, III, IV and V respectively cows with BCS 2.51-3, 3.01-3.5, 351-4 and > 4.0 respectively. Follicular fluid was aspirated from follicles and COC were evaluated under the stereomicroscope. Oocytes were divided into four groups from oocytes of a very high quality to oocytes of insufficient quality (Grade 1-4) according to de Loos (1989). Results were analysed using one-way analysis of variance (ANOVA) with Newman-Keul's post-hoc test.

The mean number of oocytes was 1047, of which: oocytes of a very good quality - 210 (20%), good quality - 355 (33.9%), sufficient quality - 278 (26.6%) and insufficient - 204 (19.5%). The average total weight of ovaries was 20 and 19.9g in groups I and II, and was lower than in groups III, IV and V (24.2, 25.2 and 24.2g respectively). The mean number of follicles in cows with BCS = 1.5-2.5 was 12.1 (group I) and was lower than 13, 22.7, 22 and 17.7 in cows with BCS = 2.51-3.0 (group II), 3.01-3.5 (group III), 3.51-4.0 (group IV) and >4.0 (group V). From group I we aspirated 5.8 oocytes; this number was lower than 6.3, 14.1, 13.9 and 3,9 aspirated from follicles of cows from groups II, III, IV and V. The body condition had no significant influence on the weight of ovaries ($P>0.05$), the number of follicles on the surface of ovaries ($P>0.05$) and the number of aspirated oocytes ($P>0.05$). The mean number of oocytes of very good quality (Grade 1 and Grade 2) was 0.79 and 1.14 in cows with 2.51 and 2.51-3.0, and was lower than 2.69, 2.66 and 2.0 aspirated from follicles in cows from group III, IV and V. The body condition influenced the number of COC of a very good quality (Grade 1, $P<0.05$). There was no relation between the body condition and the number of oocytes (Grades 2–4). The collected bovine serum and follicular fluid were assayed for multi – species LEP, TGHR and AGHR by means of specific RIA kit according to the manufacturer's protocol. The body condition had no significant influence on the concentration of LEP and/or AGHR and TGHR in blood plasma and follicular fluid ($P>0.05$). The blood concentration of LEP was 0.462 nmol/l. Its content was the highest with BCS lower than 3.0 (0.467 nmol/l. In remaining groups its concentration was 0.379 to 0.382 nmol/l. The mean concentration of AGHR was 7.7 pmol/l and it was the lowest in BCS 3.51-4.0. Remaining groups were comparable and amounted 10.5 – 13.2 pmol/l. The concentration of LEP in follicular fluid was 0.362 (0.144 – 0.755) nmol/l and it was the highest in thin cows, and the lowest in the satisfactory condition (BCS = 3.51-4.0). In follicular fluids the concentration of AGHR and TGHR was 152.3 (9.7 – 580.9) pmol/l and 120.1 (36.2 – 417.4) nmol/l resp. The lowest concentration of AGHR in follicular fluid we observed in BCS 3.5 – 4.0, the highest in BCS > 4.0. – 126.9 and 178.4 pmol/l and/or 88 and 168.5 pmol/l.

Notes

EXPRESSION OF APOPTOTIC PROTEINS IN BOVINE IMMATURE AND MATURE OOCYTES WITH DIFFERENTIATED ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

OPIELA J., KATSKA-KSIAZKIEWICZ L., RYNSKA B.

Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production, 32-083 Balice, Poland

Brilliant cresyl blue (BCB) staining can be used to select oocytes developmentally competent for embryo production. BCB staining demonstrates the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD); the high concentration of G6PD reduces a blue compound in BCB to a colorless one (BCB-). However, there are no data indicating the correlation between activity of G6PD and the degree of apoptosis. Therefore, the aim of our study was: i) to estimate the level of apoptosis in immature and mature bovine oocytes on the basis of death promoting protein expression Bax and Bak and antiapoptotic protein Bcl-2; ii) to assess whether there is a correlation between the level of G6PD activity and the apoptotic process.

Bovine immature cumulus-oocyte complexes (COC) recovered from slaughtered ovaries were incubated in 26 μM BCB in PBS for 1 h at 37°C. Then COCs were separated according to the cytoplasm coloration (BCB+ and BCB-). COCs not subjected to BCB staining served as a control (C). The oocytes of all groups (n= 100 each) were stripped off cumulus cells, collected in minimal volumes of PBS (without Mg^{2+} and Ca^{2+}), snap frozen in liquid nitrogen and stored at –80°C for western-blotting analysis.

Oocyte samples were separated by SDS-PAGE. Bcl-2 and Bax purified proteins were used as a positive control. Then the proteins were transferred to PVDF membrane using a semidry blotting system (Bio-Rad Laboratories, Warsaw, Poland). Blots were incubated in 10% non-fat milk dissolved in 0,1% Tween-20 in Tris-buffered saline (TTBS), then hybridized with rabbit policlonal antibodies: anti-Bax and anti-Bcl-2 (2,4 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following washing with TTBS, second hybridization was done with peroxidase conjugated goat-antirabbit immunoglobulin G (0,3 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Finally, the membrane was washed in TTBS and TBS before proteins were visualized with help of a chemiluminescence kit (West Pico; Pierce Biotechnology, Inc. Rockford, IL, USA) on a X-ray films according to the manufacturer's instructions. Images were developed using developer and fixer (Kodak). The densitometric signal of Bcl-2 and Bax bands in each sample was calculated with Quantity One (Bio-Rad, Polska, Warszawa). The same blot was reprobed with anti-actin and anti-Bak antibodies(2,4 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

On the basis of densitometric signals, no statistically significant differences in the expression of Bax, Bak and Bcl-2 between BCB+, BCB- and Control groups both immature and mature oocytes were observed. The BCB+ group had the highest expression of Bcl-2, Bax and Bak proteins when compared to BCB- and C groups but no statistical differences were observed. In conclusion, no relation between expression of the Bcl-2 and Bax proteins in immature and mature oocytes and the activity of G6PD in immature oocytes was found. Therefore, the activity of G6PD cannot be used as a marker of apoptosis in bovine oocytes.

Research was supported by the State Committee for Scientific Research as a project 2PO6D 00728.

Notes

USE OF ALBUMIN-FREE SWIM-UP AND FERTILIZATION MEDIA FOR BOVINE OOCYTES

PALASZ A.T., BELTRÁN BREÑA P., PÉREZ-GARNELO S.S., DE LA FUENTE J.

Ministerio de Educación y Ciencia, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain

The phospholipids are predominant macromolecules in reproductive tract fluids of the cow that apparently lack of functions relative to embryo development. However spermatozoa require a high lipids content that proved to be effective surfactants (Palasz et al., Anim Reprod Sci 2000 ; 58:229-240) and provide the plasma membrane with the fluidity essential at fertilization (Wathes et al., 2007 ; Biol Reprod in press)). The objective of the present study was to examine whether liposomes made of Soya bean lipids or PVP can replace BSA in swim-up and fertilization media for bovine oocytes. Oocytes were collected from abattoir derived ovaries and matured in TCM-199 + 10% FCS and 10 ng/ml of epidermal growth factor. After 24 hr of incubation matured oocytes were randomly divided into 3 groups and fertilized with Fert-Talp medium supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 10 µg/ml heparin containing 0.6% BSA fatty acid-free (FAF) Group 1, (Control); 0.4 % PVP Group 2, and 0.6% liposomes of plant origin (LP) Group 3. Before fertilization, all oocytes were washed once in MOPS buffer and twice in the final Fert-Talp medium supplemented with one of 3 tested macromolecules. Oocytes were inseminated in Nunclon dishes in group of 50 in 250 µl Fert-Talp with $1x10^6$ ml spermatozoa selected after swim-up through hyaluronan (MAP-5, Bioniche, Belleville, ON, Canada). Swim-up medium was prepared in Fert-Talp with 1.0 mM of sodium pyruvate, 1 mg/mL HA and one of 3 tested macromolecules. Presumptive zygotes were cultured in 50 µl drops (~25 zygotes per drop) in SOF culture medium supplemented with 0.6% BSA-FAF under paraffin oil at 39^0C with 5% CO_2, 5% O_2 and 90% N_2 in humidified air. A total of 313 oocytes were used. All media in these experiments were supplemented with 50 µg ml of Gentymicina. Cleavage rates were recorded on Day 2, embryo development on Day 4 and number of blastocysts on Days 7, 8 and 9. Embryos development among groups was compared by Chi square analysis.

Cleavage rates were 67.6% in Control, 74.0% in Group 2 and 66.3% in Group 3 and were no different between groups. The proportion of embryos that developed to ≤8 cells at Day 4 was in Group 1, (Control) 34.2%; Group 2 (PVP) 40.2% and Group 3 (LP) 41.2%. The number of blastocysts that developed in Control, 31.5%; Group 2, 29.2% were significantly higher ($P<0.05$) than those in Group 3, 16.0%.

Based on the results of the present study we concluded that use of PVP or Soya bean liposomes in swim-up and fertilization media for bovine oocytes can eliminate the necessity of using BSA. However, whether lower rate of blastocyst development in media supplemented with zwitteronic lipids liposomes can be improved by selection of different preparations of lipids and different liposomes preparation remains to be seen.

Notes

FERTILITY RESULTS IN SHEEP AND GOATS AFTER ROUTINE USE OF A SIMPLIFIED INSEMINATION TECHNIQUE

PAULENZ H.

Team Semin, Department of Production Animals Clinical Sciences, Norwegian School of Veterinary Science, Box 8146 Dep, 0033 Oslo, Norway

In Norway, breeding in sheep and goats has, for many decades, been based on natural mating using a co-operative scheme, so-called “ram or buck circles”. During the mating season, the males are moved between the farms according to farms’ breeding requirements. As cervical artificial insemination (AI) in sheep and non-surgical uterine AI in goats was introduced as early as in the 1960s, AI has only been used as a supplement to natural mating, and only a few highly selected females have been inseminated artificially. Owing to the restrictions introduced more recently by the authorities to control diseases, especially scrapie and CAE, many farms are no longer allowed to send males to other farms, so reducing the effectiveness of the circles. Thus, there was a need to increase the use of AI. This required the availability of a cheap and effective insemination technique allowing the farmers to perform the inseminations themselves.

Vaginal insemination ("Shot in the dark"-SID) is a simple, less costly and time consuming technique compared to others, also bringing into focus the animal welfare aspects of the artificial insemination procedure. Therefore, from 2000, several field trials (8 in sheep, 7 in goats) have been conducted in Norway in order to establish the SID as a routine method in Norway. The effect on fertility of different routines for SID was evaluated using liquid as well as frozen-thawed semen. As a result, SID was introduced as the method of choice in 2002 for sheep and in 2006 for goats.

Norwegian crossbred sheep are a very fertile, and the lambing results were stable in the last four years varying from 72-74 % when using liquid semen and 59-64 % when using frozen-thawed semen. The difference between the non-return rate after 25 days and the lambing rate is only 2%. In goats, only frozen-thawed semen was used and the kidding rate increased gradually from about 25% in 2002 to about 50% in 2006. The difference between NRR and kidding rate is about 8-10%, mainly due to abortions.

Special conditions in Norway concerning the management and treatment of the ewes and goats may be of importance to produce good lambing and kidding rate after SID. Norwegian farmers have decided not to use any hormones associated with reproduction. It is known that AI after hormonal synchronization results in lower fertility rates compared to inseminations after natural oestrus.

The average flock size in Norway is rather small and varies in common between about 50 and 150 animals. The small flock size facilitates interaction between each individual ewe/goat and the farmer, thereby encouraging a positive relation between farmers and animals. This situation probably decreases the stress experienced by the ewes when handling them, especially at oestrus control and before, during and after AI.

In conclusion, SID is a simple AI technique in sheep and goats that could be applied by the farmers themselves and gives satisfactory fertility results and Norwegian conditions.

Notes

SOME WAYS TO IMPROVE THE EFFICIENCY OF AN EMBRYO TRANSFER PROGRAM IN SHEEP

RANGEL SANTOS R.

Universidad Autónoma Chapingo, Chapingo México. C.P. 56 230.
Email: rangelsr@correo.chapingo.mx

Mexico is a country with over seven million sheep from which about 70% are wool type and 30% are hair type. They are distributed over the whole country. Almost all of the sheep meat is consumed as typical dish call barbacoa with a cost of about 20.00 USD/kg. The use of reproductive technologies such as estrus synchronization, artificial insemination (AI) and embryo transfer (ET) is limited probably due to its high cost and low efficiency. The introduction of exotic breeds in small groups opens the possibility to apply some of those reproductive technologies to quickly increase their numbers. There is a great amount of information in the literature regarding to the use of such technologies in developed countries. In this case I would like to show you some information that we have generated in the last 10 years. It is well recognized that there are many factors that affect the efficiency of an ET program. Next I will show some results on the effect of age, breed and the use of two intravaginal sponges during the estrous synchronization scheme on the ovulatory response of donor ewes.

Age. Results from the literature have shown low superovulatory response and low survival of embryos coming from ewe lambs compared to embryos obtained from mature ewes. Given that there is interest to obtain offspring from young animals we evaluated the effect of the size of the uterus in Pelibuey 12 months-old ewe lambs. The results showed a significant effect of size of the uterus on the ovulatory response (OR) and the number of transferable embryos; ewe lambs classified as having larger uterine size showed higher responses.

Breed. There is information in the literature showing breed differences in the ovulatory response to the application of gonadotropins. In this case we found a higher OR and a larger number of transferable embryos in Charolais and East Friesian ewes compared to Dorper ewes.

Use of two sponges. The estrous synchronization programs for AI or superovulation in ewes normally include the use of a single sponge for 12 or 14 days. We have generated some results using two sponges is estrous synchronization programs for artificial insemination and embryo transfer work. The use of two sponges has significantly improved pregnancy rate and prolificacy in inseminated ewes, as well as ovulation rate in synchronized dairy goats. Particularly, in superovulated ewes the use of two sponges has consistently improved the number of transferable embryos.

The efficiency of our ET programs has been also improved through the use of embryo splitting producing on average 20% more lambs on the ground from which 25% are identical twins. Additional aspects will be considered in the presentation.

Notes

REPRODUCTIVE FITNESS OF SHEEP AND GOAT BREEDS ENDOGENOUS TO NORTH AFRICAN COUNTRIES: MAIN TRAITS AND WAYS OF IMPROVEMENT

REKIK M.[1], LASSOUED N.[2], BEN SALEM I.[1]

[1] Ecole Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisia ; [2]. Institut National de Recherche Agronomique de Tunisie, Rue Hédi Karray, 2049 Tunisia

This paper presents an overview of the state of knowledge regarding reproductive patterns of sheep and goat breeds native to North Africa that have evolved in low input systems and extends to complementary data reported in West Asia and Africa to encompass a larger arid part of the world where these species are key elements of the agricultural production systems.

In comparison to breeds commonly encountered in Europe that have served to establish biological models for reproductive rhythms of small ruminants, breeds of sheep and goats in the southern shore of the Mediterranean have their reproductive characteristics geared by complex genetic, social and environmental factors in particular their metabolic status which is an interaction between available natural feeding, supplementary feeding as well as live weight and body condition changes in the short and longer terms. In many instances and most particularly seasonal reproduction, published models, on their own, are insufficient to explain the observed patterns. Understanding integration of all these signals at the level of centres controlling reproduction could lead to new developments in the field of managing reproduction of sheep and goats in arid zones.

Obviously, one would think that under these constraining environmental conditions, there is little scope for introducing reproductive techniques and biotechniques to increase efficiency of the reproductive output. In its second part, this paper will discuss real opportunities and would present factual data original to the geographic area under study, placing some techniques and biotechniques at the forefront of any improvement scheme of reproduction for these breeds. Techniques with the minimum cost, yet effective in introducing sustainable improvements of the reproductive output will be stressed.

Notes

ANTI-MULLERIAN HORMONE AND FOLLICULAR RESPONSE TO SUPEROVULATORY TREATMENT IN THE COW

RICO C.[1], FABRE S.[1], BONTOUX M.[1], TOUZE J.L.[1], REMY B.[2], BECKERS J.F.[2], DI CLEMENTE N.[3], MONNIAUX D.[1]

[1]*Physiologie de la Reproduction et des Comportements, UMR6175 INRA – CNRS - Université de Tours - Haras Nationaux, 37380 Nouzilly, France*
[2]*Laboratory of Endocrinology and Animal Reproduction, Faculty of Veterinary Medicine, University of Liege, B-4000 Liege, Belgium*
[3]*Endocrinologie et Génétique de la Reproduction et du Développement, INSERM UMR S-782, 92140 Clamart, France*

In cattle, embryo production following FSH simulation is a choice method to amplify the lineage of chosen females and to preserve the genetic diversity. However, despite improvements in superovulatory treatments, ovarian responsiveness to gonadotrophins remains highly variable between individuals and difficult to predict. It has been recently established that Anti-Müllerian Hormone (AMH), secreted by granulosa cells of growing follicles, is the best endocrine marker of the FSH-responsive follicular populations in women submitted to in vitro fertilisation protocols. The objective of this study was to test whether AMH might reflect the ovarian follicular status and be predictive of ovarian response to superovulation in the cow.

Nine Prim'Holstein cows with progesterone implants (Crestar) were submitted to a standard superovulatory FSH treatment (Stimufol, two daily i.m. injections at decreasing doses during 4 days, total FSH per cow = 32 mg) and received a prostaglandin (Prosolvin) i.m. injection on the third day of treatment, before implant removal. Blood samples were collected before FSH treatment (T1), and after treatment at the time of oestrus (T2). Ovarian response to treatment was assessed by ultrasonography performed 7 days after oestrus. After a 2 months-resting period, the cows were treated with 2 injections of prostaglandin spaced 11 days apart to induce luteolysis and synchronize occurrence of oestrus, and slaughtered 8 days after the second injection. Daily blood sampling was performed between the first injection and slaughtering. At slaughtering (T3), ovaries were recovered and all the follicles larger than 3 mm diameter of the first wave of the luteal phase of the synchronized cycle were dissected and measured. Granulosa cell smears were performed for each follicle and the quality of follicles (healthy or atretic) was assessed later on by microscopic analysis of the Feulgen-stained smears. Plasma concentrations of AMH were measured by an ELISA human AMH kit.

Results showed that AMH concentrations were not different between T1 and T3, but increased between T1 and T2 (59.6 ± 46.4 vs. 120.6 ± 85.7 pg/ml, $p < 0.01$, t-paired test). Concentrations at T1 were highly correlated to concentrations at T2 ($r = 0.90$, $p = 0.001$) and at T3 ($r = 0.95$, $p<0.001$). Daily measurements during the 20 days that preceded slaughtering showed that concentrations were quite steady for each animal (intra-animal variations < 6%). The number of corpora lutea + large follicles visualized by ultrasonography after FSH treatment was correlated to AMH concentrations at T1 ($r = 0.74$, $p < 0.05$) and T2 ($r = 0.77$, $p < 0.05$). The number of healthy small antral follicles (3 to 7 mm diameter) present on ovaries at T3 was highly correlated to AMH concentrations at T3 ($r = 0.86$, $p < 0.01$) and T1 ($r = 0.92$, $p < 0.001$). Altogether, these results suggest that in the cow, as in the woman, plasma concentration of AMH would be a very good marker of the status of the follicular populations able to respond to FSH treatment. It is suggested that endocrine levels of AMH might allow to predict the ovarian response to superovulation and particularly to identify high as well as poor potential responders).

Notes

TARGETED KNOCKDOWN OF BIRC6/APOLLON RESULTS IN THE DEVELOPMENTAL ARREST OF BOVINE PRE-IMPLANTATION EMBRYO

SALILEW D.[1], TESFAYE D.[1], RINGS F.[1], SCHEPERS U.[2], SCHELLANDER K.[1]

[1]*Institute of Animal Science, Animal Breeding and Husbandry group, University of Bonn, Endenicher Allee 15, D-53115 Bonn.* [2]*Kekulé institute of Organic Chemistry and Biochemistry, University of Bonn, Gerhard Domagk Str. 1, 53121 Bonn, Germany*

Baculoviral inhibitor of apoptosis repeat-containing (Birc)6 gene (BIRC6/APOLLON) inhibits apoptosis by targeting key cell death proteins. A deletion of the C-terminal half of the mouse apollon, Bruce, has been reported to activate caspases and apoptosis in the placenta and yolk sac resulting in embryonic lethality. However, its involvement in regulation of apoptosis and cell survival in the bovine preimplantation embryo is not documented. Therefore, this experiment was conducted to investigate the role of BIRC6 in bovine preimplantion embryos using RNA interference (RNAi). For this, gene-specific long double stranded RNA (dsRNAi) and short hairpin RNA (shRNAi) targeting BIRC6 were in vitro synthesized. Following this, in vitro fertilized zygotes were categorized into three groups, namely, those injected with dsRNAi (n = 193), shRNAi (n = 211) uninjected control group (n = 166). The proportion of uncleaved zygotes, 2, 4 and 8 cell embryos were assessed at 96 hours post insemination (hpi). To investigate the level of knockdown following RNAi microinjection, the mRNA of BIRC6 was determined using quantitative real time PCR (qPCR) in uncleaved zygotes 2, 4 and 8 cell embryos. To investigate the effect of BIRC6 knockdown on anti and pro apoptotic genes, survivin and Bax mRNA levels were quantified in 4 and 8 cell stages. The specificity of RNAi was evaluated by quantifying GAPDH in each treatment groups. The results showed that the proportion of uncleaved embryos (mean ± SEM) was 31.7 ± 1.8, 39.2 ± 3.4, 25.9 ± 2.7 in dsRNAi, shRNAi and control group respectively and 2 cell stage embryos were significantly ($P < 0.05$) higher in dsRNAi (18.2 ± 2.7) and shRNAi (17.6 ± 6.5) compared to control groups (9.4 ± 2.2). The proportion of 4 cell stage embryos was not significantly different between the dsRNAi, shRNAi and the control groups. However, the proportion of 8 cell stage embryos was significantly higher in control groups (46.0 ± 6.4) compared to dsRNAi (34.4 ± 3.7) and shRNAi group (25.83 ± 6.0). The BIRC6 mRNA in uncleaved embryos was decreased by 80% in dsRNAi and by 70% in shRNAi compared to control groups and in 2 cell embryos, it was reduced by 85% in dsRNAi and by 50 % in shRNAi. Furthermore in 4 cell embryos, the mRNA of BIRC6 was reduced by 70% in dsRNAi and 80% in shRNAi injected groups compared to the control. In 8 cell stage embryos it was reduced by 50 % in dsRNAi and by 40% in shRNAi groups compared to the control. Furthermore, the mRAN expression level of Bax was increased by 50% and 45% in dsRNAi groups in 4 cell and 8 cell embryos respectively. The transcript level of GAPDH was similar between the treatment groups revealing the specificity of knockdown. Similarly, the expressional level of survivin tends to be constant in dsRNAi and control group revealing that suppression of BIRC6 didn't affect other inhibitor of apoptosis families. In conclusion, higher proportions of uncleaved embryos and 2 cell embryos followed by lower proportion of 8 cell embryos at 96 hpi accompanied by reduction of mRNA level BIRC6 in RNAi groups may reveal the involvement of BIRC6 in bovine embryo development.

Notes

OCCURRENCE OF APOPTOTIC CELL DEATH IN PORCINE CLONED BLASTOCYSTS FOLLOWING SIMULTANEOUS FUSION AND ACTIVATION OF OOCYTES RECEIVING ADULT DERMAL FIBROBLAST CELL NUCLEI

SAMIEC M., SKRZYSZOWSKA M.

Department of Animal Reproduction Biotechnology,
National Research Institute of Animal Production,
32-083 Balice/Kraków, Poland

The purpose of the study was to examine the effect of simultaneous physical fusion and activation of porcine somatic cell nuclear-transferred oocytes on the preimplantation development of cloned embryos and the induction of apoptotic cell death processes at the blastocyst stage. The source of recipient cells for allogeneic nuclear and mitochondrial genome in the somatic cell cloning procedure were *in vitro*-matured oocytes. The enucleated oocytes were reconstructed with the genomic DNA of non-apoptotic adult ear skin-descended fibroblast cells. Then, nuclear transfer-derived oocytes were artificially stimulated with the use of simultaneous physical fusion and activation (SP-F/A). In the SP-F/A protocol, electric pulses which induced a fusion of cytoplast-somatic cell couplets were simultaneously the stimuli initiating the activation of reconstructed oocytes. The complexes of enucleated oocytes and fibroblast cells were subjected to plasma membrane electroporation by application of two successive DC pulses of 1.2 kV/cm for 60 μsec. The electropermeabilization of cell plasma membranes was performed in an isotonic dielectric solution with concentration of calcium cations increased up to 1.0 mM L^{-1}. After activation treatment, cloned embryos were cultured *in vitro* in the NCSU-23 medium for 6-7 days. At the end of the *in vitro* culture period, embryos at the blastocyst stage were analyzed *intra vitam* on proapoptotic symptoms in inner cell mass and trophectoderm cells using the conjugate of annexin V with enhanced green fluorescent protein (eGFP), which has the high affinity to phosphatidylserine residues exposed onto the surface of plasma membrane. A total of 294/342 (86.0%) reconstituted zygotes were selected to *in vitro* culture. Out of 294 cultured nuclear-transferred embryos, 203 (69.0%) were cleaved. The percentages of cloned embryos, that reached the morula and blastocyst stages, yielded 148/294 (50.3%) and 48/294 (16.3%), respectively. As measured in relation to a total number of nuclear transfer-derived blastocysts analyzed on apoptosis, the frequency of the embryos, in which annexin V-eGFP-positive cells were detected, was 26.5% (9/34), respectively. In conclusion, using the SP-F/A protocol the abilities of adult dermal fibroblast cell nuclei to support the *in vitro* development of porcine cloned embryos to morula/blastocyst stages yielded high rates. It has been also found that the relatively low percentage of blastocysts, in the cells of which the proapoptotic symptoms were diagnosed using the annexin V-eGFP, was obtained.

Notes

EFFECT OF BSA AND HYALURONAN DURING IN VITRO CULTURE OF OVINE EMBRYOS AND THEIR QUALITY IN TERMS OF CRYOTOLERANCE, GENE EXPRESSION, LAMBING RATE AND BIRTH WEIGHT

SANNA D.[1], RIZOS D. [2], ALI A. BIN. T., MARA L. [1], BERMEJO-ALVAREZ P.[2], GUTIERREZ-ADAN A.[2], DATTENA M. [1]

[1]Department of Biotechonology of Reproduction, Istituto Zootecnico e Caseario per la Sardegna 07040 Olmedo (SS) Italy

[2]Dpto. de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain

It is well known that modification of the post fertilization culture environment of mammalian pre-attachment embryos can affect blastocyst quality, manifested in terms of morphology, cryotolerance and relative abundance of certain gene transcripts. Therefore, any improvement in the quality of blastocysts produced in vitro is likely to derive from the modification of post fertilization culture conditions. The objective of this study was to examine the effect of BSA or HA during the period of embryo culture in vitro of ovine embryos on 1) embryo development, 2) blastocyst quality, assessed by cryotolerance and relative transcript abundance, 3) pregnancy rate and 4) lamb body weight after transfer of fresh or vitrified embryos. Ovine cumulus oocyte complexes were matured in vitro in TCM199 supplemented with 4 mg/ml BSA for 24 h and inseminated with fresh ram sperm. Zygotes were cultured for 6-7 days in 20 µl droplets of SOF supplemented with 8 mg/ml fatty acid free BSA at 39° C in an atmosphere of 5%CO2, 7% O2 in air. On the third and fifth day of culture (day 0=day of fertilization) 8 mg/ml fatty acid-free BSA was added to the BSA group and 8 mg/ml fatty acid free and 6 mg/ml HA was added to the BSA+HA group. Blastocysts on Day 6-7 either transferred to recipient ewes fresh or after vitrification/warming using the OPS technique, or snap frozen in LN_2 for gene expression analysis by quantitative RT-PCR in groups of 10. Pregnancy was confirmed by ultrasonography at 40 days and pregnancies were allowed to go to term. The relative abundance of mRNA was investigated for genes implicated in resistance to apoptosis (Survivin), response to stress (heat shock binding protein 1 -Hsbp1), metabolism (Glut-1), compaction (Cx43), methylation (Dnmt1, Dnmt3a and Dnmt3b), related to pluripotency (Oct-4 and FGF-4), modulate cellular binding (Hyaluronan Mediated Motility Receptor -RHAMM), cell to cell adhesion (E-cadherin -E-cad), fetal growth factor and cellular proliferation (IGF-II), preimplantation development, implantation, or early postimplantation development (Interleukin-6 -IL-6) and a transcription factor for endoderm differentiation (SOX17). Cleavage rate and blastocyst yield was similar in both groups (Cleavage: 88.0 vs 85.6%; Bl yield: 44.2 vs 45.5% for BSA and BSA+HA respectively). Pregnancy and lambing rate from fresh or vitrified blastocysts were also similar in both groups (Pregnancy rate: fresh-75.0 vs 73.9%, vitrified-78.2 vs 63.6%; Lambing rate: fresh-53.6 vs 57.4%, vitrified-46.9 vs 43.7% for BSA and BSA+HA respectively). However, as the birth weight from fresh transferred embryos was similar for both groups, more heavy lambs were born from those vitrified from BSA (39.1%) compared to those from BSA+HA (15.8%). The level of expression of RHAMM**,** FGF-4, E-cad, Cx43, IL6 and SOX17 was significant higher on blastocyst cultured in presence with HA. In addition, the level of expression of Hsbp1 was decreased in the presence of HA. However, the level of expression of Dnmt1, Dnmt3a, Dnmt3b, IGF-II, Oct-4, Glut-1 and Survivin was not significant between groups.

In conclusion, using a combination of measures of developmental competence and quality to give a more complete picture of the consequences of modifying medium composition on the embryo, we have shown that conditions of post fertilization culture can affect the quality of the resulting ovine blastocysts and more importantly can produce heavy lambs after vitrification and transfer.

Notes

TESTICULAR ENDOCRINE FUNCTION IN MANGALICA BOARS – PRELIMINARY STUDY

SARLÓS P., EGERSZEGI I., NAGY S.Z.., HUSZÁR S.Z., RÁTKY J.

Department of Reproductive Biology and Cell Biology, Research Institute for Animal Breeding and Nutrition, Gesztenyés u. 1. 2053 Herceghalom, Hungary

The proper assessment and prediction of the fertility of male animals are very important tasks in controlling reproduction. The individual testosterone production of the male animals markedly determines their reproductive. The GnRH test gives extra information about the testicular endocrine function of the males.

There are no previous data about reproductive performance in native Hungarian Mangalica boars; therefore, the testicular endocrine function was investigated with GnRH treatment in 48 Mangalica boars (18 Swallow-belly, 20 Blonde and 10 Red Mangalica). Blood samples were taken from each animal to determine basic testosterone levels. 90 minutes after iv. application of 100 μg GnRH (Fertagyl, Intervet) a new blood-sampling was done to analyze hormone response. After the GnRH test, animals were castrated and volume of testes was measured. The testosterone concentration was analyzed by RIA method (Testosterone RIA Kit, Isotope Institute Ltd.). Statistical analysis was performed with STATISTICA 6.0 Software (StatSoft, Inc. 2001).

The mean basic testosterone level was 9.91 nmol/l, whereas 90 minutes after treatment the hormone concentrations increased to 20.0 nmol/l with high standard deviations. Mean testis volume was 514.2 cm^3 at the age of 25.3 months. Moderate correlation was observed between age and basic testosterone level (Tb) ($r=0.47$ $P<0.05$); furthermore, it was the same between age and increase of testosterone concentration (Tincr%) after treatment ($r=–0.57$ $P<0.05$). The relationship between live weight of the animals and Tb ($r=0.50$ $P<0.05$) and between live weight and Tincr% ($r=–0.58$ $P<0.05$) was nearly the same as above. Strong negative correlation was found between Tb and Tincr% ($r=–0.75$ $P<0.05$), nevertheless in contrast to earlier publications weak correlation was detected between testis volume and Tb and Tincr% respectively.

The effect of age, live weight, testis volume and Tb as independent factors was investigated on Tincr%. The combination of these factors had a high effect on the level of testosterone response ($r^2=0.61$, $P<0.0001$), thereafter it can be used for prediction of Mangalica boars' reproductive performance.

The study was supported by fund OMFB-602/2004 and Hungarian OTKA T048847

Notes

THE USE OF VITAL PLASMA MEMBRANE DIAGNOSTICS OF EARLY APOPTOSIS IN PORCINE FOETAL FIBROBLAST CELLS IN THE NEGATIVE SELECTION TO SOMATIC CELL NUCLEAR TRANSFER

SKRZYSZOWSKA M., SAMIEC M.

Department of Animal Reproduction Biotechnology,
National Research Institute of Animal Production,
32-083 Balice/Kraków, Poland

One of the most important factors which determine the development of mammalian cloned embryos is structuro-functional quality of nuclear donor cells. Therefore, a system of early apoptosis diagnostics-mediated pre-selection would allow the sorting of donor nuclei with the high morphological, biochemical and biophysical susceptibility to somatic cell cloning. The aim of our study was to determine the *in vitro* developmental capability of porcine nuclear-transferred (NT) embryos reconstructed with foetal fibroblast cells, which had been analyzed on apoptosis through the live-plasma membrane fluorescent labelling. Frozen/thawed fibroblast cells, which had been *in vitro* cultured up to a total confluency, were used for analysis. To detect the biochemical and biophysical early-apoptotic changes in the plasma membrane involving the externalization of phosphatidylserine molecules and the subsequent loss of lipid composition asymmetry, the fibroblasts were tagged using the conjugate of annexin V with enhanced green fluorescent protein (eGFP). In the somatic cell cloning procedure, enucleated *in vitro*-matured oocytes were reconstituted with non-apoptotic foetal fibroblast cell nuclei. Afterwards, NT-derived oocytes were artificially stimulated with the use of sequential physico-chemical activation. The activation protocol was combined from two steps involving simultaneous physical fusion and activation (SP-F/A) and sequential chemical activation *de novo* of reconstituted oocytes. The SP-F/A was induced by application of two successive DC pulses of 1.2 kV/cm for 60 μsec. Two-grade chemical reactivation was initiated with 1.5-2-h delay after the first step including SP-F/A. The nuclear-cytoplasmic hybrids were exposed to 15 μM L^{-1} calcium ionomycin for 5 to 7 min and then incubated in the culture medium supplemented with 10 μg mL^{-1} cycloheximide for 3 h. Reconstructed embryos were *in vitro* cultured in the NCSU-23 medium for 6-7 days. Fluorescent analysis of the foetal fibroblast cells revealed the relatively high proportion of donor cells exhibited proapoptotic changes in the plasma membrane. The percentage of late-apoptotic cells with advanced morphological transformations did not exceed 30%. Moreover, the extremely low rate (ranging from 0 to 2%) of the early-apoptotic cells with normal plasmolemma ultrastructure, which emitted the green eGFP-derived chemiluminescence, was detected. A total of 207 enucleated oocytes were subjected to reconstruction and 179/207 (86.5%) were successfully fused with non-apoptotic nuclear donor cells. Out of 179 cultured NT embryos, 131 (73.2%) were cleaved. The frequencies of cloned embryos, that reached the morula and blastocyst stages, were 103/179 (57.5%) and 41/179 (22.9%), respectively. In conclusion, a sufficient selection factor for detection of apoptosis in the cultured foetal fibroblast cells turned out to be morphological criteria of their classification to the somatic cell cloning. Moreover, it was found that the annexin V-eGFP is able to detect the early phases of apoptosis, because the insignificant proportion of morphologically normal cells also emitted the annexin V-eGFP–derived biochemiluminescence. Nonetheless, the probability of their random erroneous selection to the somatic cell nuclear transfer appears to be extremely low.

Notes

THE EFFECT OF SEASON ON THE RESULTS OF SUPEROVULATION AND EMBRYO TRANSFER IN BEEF CATTLE

SLEZAKOVA M., HEGEDUSOVA Z., ŘÍHA J.

Research Institute for Cattle Breding, Ltd., Rapotin, Czech Republic

The aim of the study was to evaluate the influence of the year, the season on the gain and quality of embryos after superovulatory treatment. Further the conception rate after transfer of fresh and frozen embryo was evaluated. The beef cattle breeds were monitored during 1991–2004. The superovulatory treatment was carried out with twice application of the PGF2α in 11-days intervals, following by FSH administration. The non-chirurgical recovery of embryos was conducted every 7th day after the 1st insemination by the standard metod (Říha *et al.,* 1988, Petelíková, 2004) and embryo were transferred into synchronized recipients. 487 realised embryo recoveries, 2008 realised transfers during 14 years were involved into the statistic evaluation and analayzed by method of least squares. Statistical processing was made with program Unistat 4.53. The highest conception rate was in 2002 (31.54%), the least it was in 1994 (30,00%). There was obvious the improving of the pregnancy rate in 1995–1999, in the other years the pregnancy was on the acceptable level from 50% to 61.54%. The results showed the improving tendency in pregnancy development after embryo transfer (from 32.84% in 1991 to 60.40% in 2004), though in some years the worse results (51.92% in 1999, 50% in 2001, 54.21 in 2003) occurred temporarily. The positive influence of the season on the embryo gain and their quality was documented in summer (suitable 3.68 ± 3.65, degenerated 0.88 ± 3.18, unfertilised 1.74 ± 2.54; the ratio of the suitable and flashed in total 59.3%) and in autumn (suitable 3.54 ± 3.80, degenerated 0.88 ± 3.18, unfertilised 2.08 ± 3.03; the ratio: 54.48%). A little different results from the summer were achieved in spring (suitable 2.99 ± 3.56, degenerated 0.75 ± 1.57, unfertilised 2.65 ± 3.52; the ratio: 46.81%) and the worse results were in winter (suitable 2.53 ± 2.80, degenerated 0.98 ± 2.01, unfertilised 3.00 ± 5.45; the ratio: 38.59%). In summary, the season influence on the superovulation efficiency is variable and probably depends on the donor individuals and other factors (nutrition level, welfare, breeding level etc). The spring, summer and autumn season were optimal time for embryos recoveries and embryo transfer.

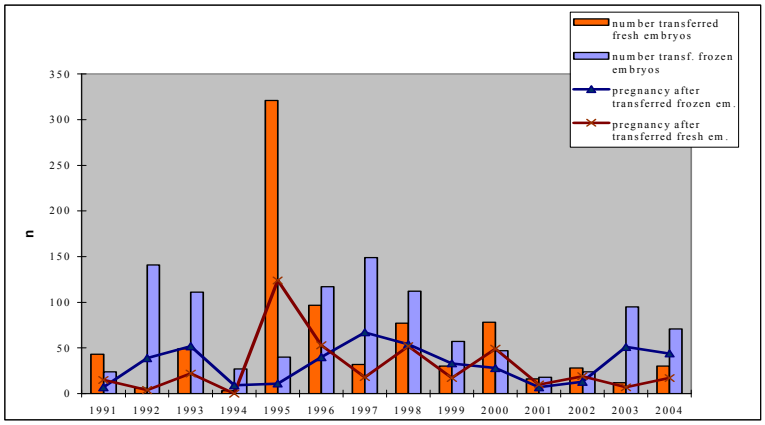

Figure 1. Number transferred fresh embryos and influence of season on transfer efficiency of fresh and frozen embryos.

The research was supported from Ministry of education by projects MSM2678846201; LA 239; Ministry of agriculture by projects QF 302; NAZV 1B44035.

Notes

CALCIUM FREE VITRIFICATION MEDIUM IMPROVES DEVELOPMENTAL COMPETENCE OF VITRIFIED OVINE OOCYTE.

SUCCU S., BERLINGUER F., MADEDDU M., SATTA V., BEBBERE D., TEDDE A., LEONI G.G.[1], NAITANA S.

Department of Animal Biology, [1]Department of Physiological, Biochemical and Cellular Science, Veterinary Medicine Faculty, University of Sassari, 07100 Sassari, Italy

Despite some success with different species and the publication of several studies on gamete cryobiology, oocyte cryopreservation is not recognised as a well established procedure. Vitrified-warmed oocytes show impaired survival, loss of developmental competence and low offspring generation rates. These morphological and functional alterations could also be related on species involved and on cryopreservation procedures utilized. Ovine oocytes vitrified using dimethyl-sulfoxide (DMSO) and ethylene glycol (EG) as cryoprotectants show lower fertilization rate compared with fresh oocytes (Succu et al., 2007). These two cryoprotectants, commonly used during vitrification, have been shown to cause a large transient increase in intracellular calcium in mouse metaphase II (MII) oocytes. This increase is sufficient to induce cortical granule release and subsequent zona hardening which impairs the ability of fertilization (Larman et al., 2006).

The aim of this study was to evaluate if the removal of calcium from vitrification medium improves the developmental capability in vitrified ovine oocytes. Cumulus oocytes complex (COCs) were in vitro matured in standard conditions (Leoni et al., 2007). MII oocytes were vitrified in cryotop (Kuwayama and Kato, 2000) using as base medium (BM) Dulbecco PBS Ca^{++} free (TREATED). Oocytes vitrified using hepes-buffered TCM 199 as BM were used as control (CTR). Oocytes of two experimental groups were firstly exposed to equilibration solution containing 7.5% DMSO, 7.5% EG and 20% fetal calf serum (FCS) in BM for 3 minutes, then transferred to vitrification solution containing 0.5 M sucrose, 16.5% DMSO, 16.5% EG and 20% FCS in BM for 25 sec, before being immersed into liquid nitrogen. After warming CTR (n= 108) and TREATED (n=138) oocytes were fertilized and cultured in vitro up to blastocyst stage in standard conditions (Leoni et al. 2007). Data were compared using chi square and fisher exact test when appropriate.

A significantly higher rate of cleaved oocytes was pointed out in TREATED group compared to CTR (57.24% versus 37%; $P<0.01$). TREATED group evidenced also a higher embryo development than CTR ($P<0.05$). Blastocyst rate in TREATED group was in fact 11.39% of cleaved oocytes. No blastocysts were produced in the control group.

Our data showed that removal of calcium from vitrification medium improves in vitro development, especially through an increased fertilization rate, suggesting that also in sheep oocytes EG and DMSO increase the uptake of extracellular calcium.

References: Kuwayama and Kato, 2000. J Assist Reprod Genet 17: 477; Larman et al. 2006. Reprod 131: 53-61; Leoni et al. 2007. An Reprod Sci 92: 373-383; Succu et al. 2007. Mol Reprod Dev, published online 8 Feb 2007, DOI: 10.1002/mrd.20693.

Supported by RAS (Special project Biodiversity)

Notes

ASTURIANA DE LA MONTAÑA CATTLE BREED GENETIC RESOURCE BANK: LONG-TERM STORAGE OF SEMEN AND SPERM CHARACTERIZATION

TAMARGO C.[1], CARBAJO M.[2], DÍEZ C.[1], MARTÍN D.[1], RODRIGUEZ A.[1], HIDALGO C.O.[1]

[1]SERIDA, 33203-Gijón, Spain; [2]Patología Animal-Sanidad Animal, Facultad de Veterinaria, 24071- León, Spain

Asturiana de la Montaña, a local cattle breed, has been suffering a considerable reduction on its population size, being classified as in risk of extinction. Semen cryopreservation with high recovery rates on thawing is a valuable tool in the conservation of endangered populations The aim of this study was to evaluate the fresh and frozen-thawed semen quality for the establishment of a germplasm bank. Ejaculates (n=145) from 8 bulls, aged 21 to 29 months, were weekly collected by means of artificial vagina along the year. Immediatly after collection, routine parameters (volume, concentration and mass motility) were determinated. Semen was extended with a commercial extender (Bioxcell, IMV Technologies, France), loaded into 0.25 ml plastic straws at a concentration of 23 ×1 per straw, frozen and stored for further analysis.

Three straws per ejaculate were thawed and pooled for evaluation. Motion characteristics were assessed by using a CASA system (SCA® 2002 Microptic, Spain) added to an optical, phase contrast microscope with heatable plate. Immediately after thawing we analyzed the percentages of motile spermatozoa (MS) and progressively motile spermatozoa (PMS). Membrane functionality was evaluated by the Hypoosmotic Swelling test (HOST) to detect the presence of swollen tails in a 100 mM citrate–fructose solution at 37 ºC during 30 minutes (percentage of HOST-positive spermatozoa, HOST-PS). Membrane integrity (percentage of viable spermatozoa, VS) was evaluated by fluorescent microscopy with a dual staining system (propidium iodide PI and 6-carboxyfluorescein diacetate CFDA). Primary (head, HA) and secondary (midpiece, MA, and tail, TA) abnormalities were determined by direct count of 100 cells from random fields of an immobilized smear, using 1000x magnification and the same for acrosome integrity (altered acrosomes, AA). Statistical analysis was carried out by means of the GLM and Duncan test for means (SAS Institute, Inc., Cary, NC, USA).

A significant ($p<0.05$) decrease was observed after freezing/thawing, both in the sperm motility (MS: 87.29±0.82 vs 52.68±1.15; PMS: 71.24±1.20 vs 40.74±1.33) and membrane status (HOST-PS: 67.30±0.73 vs 38.93±0.61; VS: 64.96±0.60 vs 41.82±0.57), for fresh and frozen/thawed semen, respectively. Also, the frozen/thawed sperm showed increased percentage of HA, MA and AA ($p<0.05$).

Conservation and development of local breeds are important because they represent a unique source of genes for improving health and performance traits of industrial breeds. These conservation initiatives, together with modern reproductive technologies, are essential to avoid the risk of loss of genetic diversity.

This work was performed in collaboration with ASEAMO and supported by RZ2004–00031–C02–01.

Notes

INVESTIGATION OF DIFFERENTIAL REGULATION OF MICRORNAS DURING BOVINE OOCYTE MATURATION BY HETEROLOGOUS APPROACH USING ARRAY ENRICHED WITH HUMAN, MOUSE AND RAT PROBES

TESFAYE D.[1], WORKU D.[1], SALILEW D.[1], HOELKER M.[1], RINGS F.[1], UN C.[2], SCHELLANDER K.[1]

[1]*Institute of animal Science, Department of Animal breeding and Husbandry, Univeristy of Bonn, Endenicher Allee 15, 53115 Bonn, Germany*
[2]*Yıldız Technical University, Faculty of Science and Arts, Department of Biology. 34210-Esenler,Istanbul- Turkey*

MicroRNAs (miRNAs) are members of noncoding small RNAs (~21-25 nucleotides long) inhibit translation and/or induce degradation of protein-coding mRNAs that contain complementary sequences to miRNAs by interacting with the 3´ untranslated region. They are known to influence the expression of hundreds of genes in numerous biological processes including development. In order to get insight into the regulation of miRNAs during bovine early development, here we apply a heterologous approach to identify and predict bovine miRNAs differentially regulated during oocyte maturation. For this miRCURY^{TM} locked nucleic acids (LNA) array (Exiqon, Vedbaek, Denmark), consists of 454 capture probes for human, mouse and rat miRNAs as registered and annotated in the miRBase release 8.0 at The Wellcome Trust Sanger Institute, was used to compare the miRNAs expression profile between bovine immature and matured oocytes. Total RNA with miRNAs were isolated from three independent pools (each containing 200 oocytes) of immature and in vitro matured oocytes using miRNeasy mini kit (Qiagen, Hilden, Germany). RNA samples from immature and matured oocytes were labelled either with Hye3^{TM} or Hye5^{TM} dyes und hybridized on three independent slides. After 16 hrs incubation at 60 ^{0}C, slides were sequentially washed. Array scanning and image analysis was performed using Axon GenePix 4000B scanner and GenePix Pro-analysis software (Axon Instruments, Foster City, CA), respectively. For the data analysis species-specific GenePix® Array Lists (GAL) files of human, mouse and rat were used independently. From the total miRNAs probes (454) spotted on the array about 400 were detected in bovine oocytes. Statistical analysis of the array data using a human, mouse and rat files indicated that a total of 33, 46 and 76 clones to be differentially regulated during oocyte maturation respectively. Out of these 11 miRNAs were found to be common between all the three array analyses. Of these immature oocytes were found to be enriched with miR-208, miR-125a, miR-130b, miR-25, miR-375 and miR-382, while matured oocytes were enriched with miR-122a, miR-101, miR-206, miR-515-5P and miR-101_MM1. Validation of the expression profile using a quantitative real-time PCR will supplement the results of this study. Moreover, investigation of the corresponding target mRNAs and protein products during oocyte maturation will enable to get insight into miRNAs controlled regulation of maternal gene expression.

Notes

EXPRESSION OF FIBRONECTIN ON MALE AND FEMALE GAMETES IN BOVINE

THYS M., AZEVEDO S., NAUWYNCK H., VAN SOOM A.

Reproductive Biology Unit; Depart. of Reproduction, Obstetrics and Herd Health; Depart. of Virology, Parasitology and Immunology; Ghent University; 9820 Merelbeke; Belgium

Carbohydrates and glycoproteins modulate several adhesion and binding events during reproductive processes. Their involvement in sperm-oviduct adhesion, sperm-oocyte interaction and embryo implantation has already been established. When glycoproteins fibronectin (FN) and vitronectin (VN) are supplemented to the fertilization medium, they cause a high respectively moderate inhibition of sperm penetration during bovine in vitro fertilization (Tanghe et al., 2004). In human, both molecules are expressed on the cell surface of capacitated and acrosome reacted spermatozoa respectively, and FN is secreted during cumulus expansion. The present experiment was conducted to determine if FN is also expressed on the gametes of cattle.

Cumulus oocyte complexes (COCs) were obtained from follicles from bovine ovaries collected at a local slaughterhouse. One group of COCs (n=10) was immediately fixed in 4% paraformaldehyde (1 h, 4°C). The remaining complexes were matured *in vitro* for 24 h. Part of the matured COCs were fixed without further treatment (n=16), whereas the other ones were vortexed to remove the cumulus cells. Fifteen denuded oocytes were instantly fixed, while the remaining 18 cumulus-denuded oocytes were incubated with 0.1% pronase to dissolve the zona pellucida (ZP). After fixation, all oocyte groups and an *in vitro* grown bovine cumulus monolayer were treated with 0.5% Triton X-100 (permeabilization: 30 min, 22°C). Afterwards, they were subsequently incubated with 10% goat serum (blocking: 30 min, 37°C), monoclonal mouse anti-FN antibodies (1 h, 37°C) and goat anti-mouse FITC antibodies (1 h, 37°C). Following staining with 2% propidium iodide, the oocyte groups and the cumulus monolayer were evaluated for the presence of FN by means of fluorescence and confocal microscopy. Additionally, fresh as well as frozen-thawed semen from the same bull was collected. Both samples were divided into 3 fractions: a non capacitated fraction, a capacitated fraction (30 min, 20 µg/ml heparin) and an acrosome reacted fraction (15 min, 5 mg/ml lysophosphatidyl choline). The 6 fractions were fixed in 1% paraformaldehyde and allowed to dry on glass slides overnight. Subsequently, they were processed as described above.

Both immature and mature COCs contained FN positive cells (42.3% resp. 58.2%). Fibronectin production seems not to be homogeneous in the cumulus cell population, since a higher percentage of positive cells was observed in the outer layers of the cumulus oophorus. Cumulus-denuded oocytes (not treated with pronase) displayed a fine fluorescent band underneath the ZP. However, the oolemma of the ZP-free oocytes did not show any staining. Of the cumulus monolayer 64.5% of the cells were FN positive and a mesh work of fluorescent structures was found in the extracellular matrix. We were not able to detect FN expression on the surface of bovine sperm cells of fresh nor frozen-thawed semen regardless of the functional status (capacitation – acrosome reaction).

We were able to demonstrate the presence of fibronectin at the level of the cumulus oophorus and in the perivitelline space or at the oolemma of ZP-intact bovine oocytes. The absence of fluorescence at the oolemma of ZP-free oocytes is most likely due to the pronase treatment. This enzyme has been shown to remove or damage certain protein molecules at the surface of the oolemma. Fibronectin is not expressed on the surface of the bovine sperm cell.

Notes

NORMAL AND CYSTIC FOLLICULAR DEVELOPMENT IN THE COW

TOUZE J.L. [1], FABRE S.[1], BELVILLE C. [2], BONTOUX M.[1], DI CLEMENTE N. [2], MONNIAUX D. [1]

[1]Physiologie de la Reproduction et des Comportements, UMR6175 INRA – CNRS - Université de Tours - Haras Nationaux, 37380 Nouzilly, France
[2]Endocrinologie et Génétique de la Reproduction et du Développement, INSERM UMR S-782, 92140 Clamart, France

In cattle, terminal follicular development is characterized by recruitment of a cohort of 3-5 mm antral follicles, followed by selection of a single dominant follicle that will either ovulate if the hormonal milieu permits or regress by atresia. In some cases, the dominant follicle fails to ovulate or regress but continues to grow into an ovarian follicular cyst. Formation of follicular cysts is a frequent ovarian dysfunction and a major cause of reproductive failure in cattle. The exact pathogenesis of cysts is unclear and early functional changes in the growing follicles of the cohort that precede formation of cysts are unknown. Our objective was to characterize growth and intrafollicular changes in steroids and Anti-Müllerian Hormone (AMH), chosen as functional follicular markers, during normal and cystic follicular development in the cow.

Seventeen Maine-Anjou cows, originating from a herd selected for twinning, were treated with 2 i.m. injections of prostaglandin spaced 11 days apart to induce luteolysis and synchronize occurrence of estrus. From the day of the second injection (D0) to the day of slaughtering (D8), ovarian follicular growth was followed by daily ultrasound scanning and individual follicular growth curves were reconstituted. The presence of cysts (follicles larger than 20 mm diameter present at D0 on ovaries) was detected on 8 cows (cows with cysts). Cyst regression was induced by puncture and aspiration of follicular fluid (n = 2 cows) or by i.m. GnRH injection (n = 2 cows), or not induced (n = 4 cows). At D8, follicles larger than 3 mm diameter were dissected and measured, follicular fluid (FF) of follicles larger than 5 mm were recovered individually, and a pool of FF was performed from small (S) 3-5 mm follicles for each cow. In FF, concentrations of estradiol-17□ (E2), progesterone (P) and testosterone (T) were measured after extraction by specific RIAs and concentrations of AMH were measured by a human ELISA kit.

As expected in cows without cysts, emergence of a follicular wave after ovulation at D3 was followed by the development of 1 or 2 large (L) 10-15 mm follicles at D8. In contrast, cysts present on ovaries at D0 did not ovulate or regress spontaneously until D8 and no follicular wave emerged. When cyst regression was induced, emergence of a follicular wave occurred and was followed by rapid development of 1 or several very large (VL) 15-20 mm follicles at D8. Daily follicular growth rate of all the largest follicles (L + VL + cysts, n = 44) was positively correlated to E2 ($r = 0.41$, $p < 0.01$) and T ($r = 0.43$, $p < 0.01$), and negatively to P ($r = -0.39$, $p < 0.01$) concentrations in FF. E2 ($p < 0.001$), P ($p < 0.001$) and T ($p < 0.05$) concentrations increased between L and VL follicles, and P concentrations were the highest in cysts ($p<0.01$ vs. VL). Interestingly, in cows with cysts (regressed or not) compared to cows without cysts, S follicles had higher E2 (2.40 ± 0.57 vs. 0.32 ± 0.10 ng/ml, $p < 0.05$) and T (88.1 ± 19.7 vs. 47.9 ± 9.2 ng/ml, $p < 0.001$) concentrations in FF. AMH concentrations decreased during follicular development (790.4 ± 97.1 vs. 51.8 ± 5.8 ng/ml, S vs. L, $p < 0.001$) and remained low in VL follicles and cysts. AMH concentrations did not differ between cows with and without cysts.

These results suggest that cyst formation would be preceded by enhanced steroidogenesis in small antral follicles of the cohort and high follicular growth rate. In contrast, intrafollicular AMH would not be a marker of cystic development in cow follicles.

Notes

USE OF PEROXIDE AS APOPTOSIS INDUCER IN BOVINE OOCYTES

VANDAELE L., DE FREITAS SANTOS R., MAES D., VAN SOOM A.

Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium

It has been shown in cattle and mice that treatment of zygotes or embryos with peroxide can induce mitochondrial dysfunction, developmental arrest and apoptosis. The aim of the present study was to detect at which concentration peroxide can induce apoptosis at the blastocyst stage without affecting embryo development.

A total of 2460 immature bovine cumulus-oocytes-complexes (COCs) (4 replicates) were matured *in vitro*. Oocytes were exposed to different peroxide concentrations (group 1: $0.5x10^{-4}$ to 10^{-4}M, group 2: 10^{-6} to 10^{-5}M, group 3: 10^{-8} to 10^{-7}M) in maturation medium without pyruvate for 1 hour just before fertilization. Meanwhile two control groups were kept in maturation medium without pyruvate (C-) and normal maturation medium (C+), respectively. After incubation, COCs were washed twice in HEPES-TALP and fertilized *in vitro*. All presumed zygotes (n=2296) were denuded 24h post insemination (hpi) and cultured in modified SOF medium with 10% FCS at 39.0°C in 5%CO_2. Embryos were evaluated for blastocyst development and all blastocysts were subsequently fixed in 4% paraformaldehyde. After TUNEL staining, total cell number (TCR) and apoptotic cell ratio (ACR) were determined in all groups. Univariate analyses of variance were used with blastocyst yield and TCR as dependent variables, group as fixed factor and replicate as random factor (Mixed model ANOVA). Differences in ACR were analyzed by a non-parametric Kruskal-Wallis test.

Table 1. Number of zygotes (zyg), number of blastocysts (blast), mean blastocyst yield in % (%± sem), mean total cell number (TCR ± sem) and apoptotic cell ratio (ACR ± sem) for different concentrations of peroxide during 1 hour before fertilization.

group	zyg	blast	% ± sem	TCR ± sem	ACR ± sem
C –	582	226	38.5 ± 2.7^{ab}	110.0 ± 3.2	3.9 ± 0.4^{a}
C +	190	58	31.8 ± 4.7^{a}	116.7 ± 8.5	5.5 ± 0.9^{a}
$0.5x10^{-4}/10^{-4}$M	569	267	47.3 ± 2.6^{b}	109.0 ± 3.3	5.3 ± 0.4^{a}
$10^{-6}/10^{-5}$M	398	168	41.9 ± 3.5^{ab}	104.3 ± 4.4	6.6 ± 0.5^{b}
$10^{-7}/10^{-8}$M	557	246	42.5 ± 2.7^{ab}	105.7 ± 3.7	7.0 ± 0.4^{b}

^{ab}within a column, values with a different superscript differ significantly ($P<0.05$)

The blastocyst rate was very comparable between groups although the highest concentration of peroxide resulted in a significantly higher blastocyst yield (47.3%) in comparison with control medium with pyruvate (Table 1). There were no differences between groups for TCR while ACR was increasing with decreasing peroxide concentration (Table 1).

In conclusion, a pulse of peroxide (ranging from 10^{-8}M to 10^{-4}M) before fertilization has a minor influence on blastocyst yield and TCR. Unexpectedly, exposing oocytes to the lowest concentrations (10^{-5} to 10^{-8}M) significantly increased ACR at the blastocyst stage. We are at present investigating the possible causes of this intriguing finding.

Notes

EFFECT OF SUPPRESSION OF DNA (CYTOSINE 5)-METHYLTRANSFERASE 1 (DNMT1) IN BOVINE OOCYTES AND EMBRYOS USING SHORT INTERFERENCE (siRNA)

WILAIPHAN P., RINGS F., HÖLKER M., THOLEN E., TESFAYE D., SCHELLANDER K.

Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn 53115 Bonn, Germany

DNA (cytosine 5)-methyltransferase 1 (DNMT1) is believed to be involved in DNA methylation which is the well-characterized epigenetic modulator that has been shown to have essential functions in germ line and embryo genomic imprinting. In the present study we aimed to investigate the consequences of suppression of bovine DNMT1 transcript using sequence specific siRNA. For this siRNA designed to target the bovine Dnmt1 and its scrambled sequence were used for microinjection at immature oocytes and zygotes stage of bovine development. In the first experiment immature oocytes (n=850), aspirated from slaughterhouse ovaries were collected and categorized into three groups namely; those injected with siRNA (n=299), those injected with scrambled sequence (n=297) and uninjected control (n=254). Similarly, in the second experiment *in vitro* fertilized zygote stage embryos (955 zygotes) were categorized into three groups namely; those injected with siRNA (n=335), those injected with scrambled sequence (n=364) and uninjected controls (n=296). In both experiments consequences of microinjection of siRNA on developmental phenotypes, mRNA and protein levels were determined. The protein expressions were determined at matured oocyte stage in the first experiment and at 8-cell stage for the second experiment. Microinjection of siRNA at immature oocyte stage has resulted in reduction of matured oocytes with extruded first polar body 62.7% compared to those injected with scrambled sequence (74.1%) and uninjected controls (75.6%). Similarly, microinjection of siRNA at the zygote stage has significantly reduced the cleavage rate 63.64% compared to those injected with the scrambled sequence (75.0%) and uninjected control (88.64%). Real time quantitative analysis of the mRNA level at 8-cell stage has shown that microinjection of siRNA at zygote stage has resulted in suppression of the DNMT1 mRNA by 50 and 70% compared to scrambled sequence and uninjected controls, respectively. However, in both experiments no significant effect was observed in protein level as determined by western blotting. Further studies on the involvement of DNMT1 in the regulation of the expression of other genes will be determined by a combination of suppression of this transcript followed by large transcriptional analysis using microarray.

Notes

MEMBRANE PERMEABILITY OF IVP BOVINE MORULAE FOR WATER AND GLYCEROL; IMPLICATIONS FOR THE FREEZING PROTOCOL

WOELDERS H.[1], MULLAART E.[2], MERTON S.[2]

[1] *Animal Sciences Group, P.O. Box 65, 8200 AB Lelystad, The Netherlands*
[2] *HG R&D, P.O. Box 5073 6802 EB Arnhem, The Netherlands*

Slow freezing of bovine IVP blastocysts gives good post thaw survival and development. However, the 'freezability' of morulae is much lower. Slow-freezing protocols have largely been developed on an empirical basis and have little been tailored according to the fundamental cryobiological properties of the embryos. Woelders and Chaveiro (Cryobiology 2004; 49:258-271) have developed a theoretical model that calculates a non-linear freezing protocol in which at every subzero temperature the freezing rate is maximized (to prevent 'slow-cooling damage'), while too high cooling rates that could lead to intracellular ice formation are precluded. In order to apply this model, one needs to know the membrane permeability coefficients for water (L_p) and for the cryoprotectant (P_s), the respective activation energies (E_a), the aqueous volume of the embryo, and the membrane surface area.

In the present study, we have measured these properties for bovine morulae in Emcare medium plus 5% (v/v) glycerol. Morulae were produced in vitro (IVP) from oocytes obtained from slaughterhouse ovaries (SH, n = 32) and from Ovum Pick Up (OPU; n=8). Shrink-swell cycles induced by suddenly changing from 0 to 5% glycerol were recorded on video. At 10-13 time points of each recorded shrink-swell cycle, the cross section surface of the embryo was measured by image analysis software, and the corresponding embryo volume was calculated. L_p and P_s were estimated by fitting the volume excursions with a two-factor description of membrane transport of water and glycerol A typical example is shown in figure 1 below. This was repeated at 25, 13, and 3 °C in order to make Arrhenius plots to calculate the E_a values.

No significant differences were seen between SH and OPU embryo's in isotonic volume (V_{iso}), L_p, P_s, or E_a, so all data were pooled. Mean values (± s.d.) were as follows: L_p at 25 °C was 2.75 ± 0.8 $\mu m.min^{-1}.atm^{-1}$; P_s was 20.7 ± 14.0 $\mu m.min^{-1}$; E_a values were 6.5 and 7.3 $kcal.mol^{-1}$ for L_p and P_s, respectively.

These values were applied to the model to generate theoretically optimal freezing programmes. The cooling rate very little depended on the permeability for cryoprotectant (P_s and E_a of P_s), but strongly depended on the permeability for water (L_p and E_a of L_p). The predicted freezing programmes have much higher average cooling rates than the presently used (linear) standard freezing programme. We will now empirically compare the predicted freezing programmes with the standard protocol.

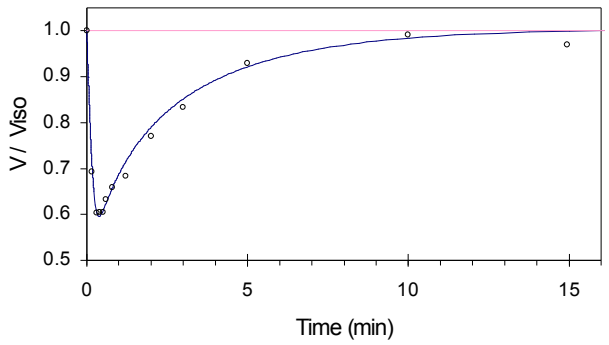

Figure 1. Typical example of the volume excursion of bovine morulae in Emcare medium after mixing 1:1 with medium with 10% glycerol. The permeability parameters were determined by fitting the theoretical curve (solid-line) to the experimental data points (circles).

Notes

AUTHOR INDEX

AUTHOR INDEX

E

F

G

H

I

J

K

S

T

U

V

W

Y

Z