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24ème COLLOQUE SCIENTIFIQUE

24th SCIENTIFIC MEETING

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Dr Gottfried Brem

Special Celebration

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PAU, France, 12th and 13th September 2008

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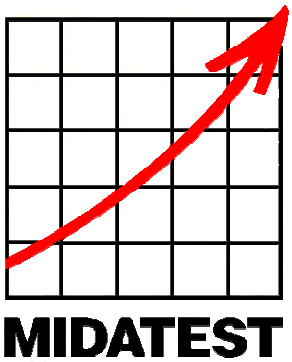

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Dr Gottfried Brem

A.E.T.E. Medalist 2008

Gottfried Brem
A.E.T.E. Medalist 2008

Pioneer of Animal Biotechnology and Outstanding Science Mentor

Gottfried Brem, the A.E.T.E. Medalist 2008, was born in 1953 and grew up as first son of a farmer in Lauterbach near Rosenheim, Bavaria. This environment provided ample opportunities to observe the plethora of biological processes involved in animal breeding and also to see them in an economical context. After finishing the Finsterwalder-Gymnasium in Rosenheim with the "Abitur", Gottfried Brem studied Veterinary Medicine (1972-1977) and Economy (1978-1980) at the Ludwig-Maximilians-University (LMU) Munich, and Agricultural Sciences (with specialization in animal production) at the Technical University of Munich (1976-1979). Obviously, multi-tasking was never a problem but is rather a passion of him.

After finishing his studies with the "Staatsexamen" and Approbation in Veterinary Medicine, a Diploma in Agricultural Sciences, and a Bachelor in Economy, Gottfried Brem started his academic carrier at the Institute of Animal Breeding and Hygiene at the LMU Munich and finished his habilitation in 1985. Based on his innovative concept to merge quantitative genetics, molecular genetics and reproductive biotechnologies, he created an entirely new field in animal breeding. Consequently, he obtained multiple offers of professor positions at renowned universities, including the Chair for Biotechnology in Animal Breeding at the Christian-Albrechts-University Kiel (1986), the Chair for Molecular Animal Breeding at the LMU Munich (1987), the Chair for Female Reproductive Biology within the Center for Reproduction at the University of Veterinary Medicine Hannover (1991), and the Chair for Animal Breeding and Genetics at the University of Veterinary Sciences Vienna (1992). Gottfried Brem held the position of the Ordinarius for Molecular Animal Breeding at the LMU Munich from 1987 to 1993 and is since then Ordinarius for Animal Breeding and Genetics at the University of Veterinary Sciences in Vienna.

The record of Gottfried Brem's research activities is outstanding and includes a number of pioneering developments and discoveries. He was the first to generate transgenic large animals in Europe (Brem et al., Reprod Dom Anim 20: 251-52, 1985) and used this technology extensively for both basic research (e.g. Masu et al., Nature 365: 27-32, 2003; Förster et al., Cell 87: 1037-47, 1996) and for animal biotechnology (e.g. Müller et al., Gene 121: 263-270, 1992; Brem et al., Gene 149: 351-355, 1994; Coulibaly et al., FEBS Lett 444: 111-116, 1999). He very substantially contributed to the development of embryo biotechnologies, including embryo splitting, cryopreservation, in vitro production of embryos, and nuclear transfer cloning. Gottfried Brem generated the first set of bovine clones in Germany and uncovered important characteristics of cloned embryos, such as mitochondrial DNA heteroplasmy (Steinborn et al., Nat Genet 55: 255-257, 2000). Moreover, he used nuclear transfer to generate the first transgenic bovine clone producing a bispecific antibody for tumor therapy (Grosse-Hovest et al., PNAS 101: 6858-6863, 2004). Altogether the publication record of Gottfried Brem counts 325 papers in peer-reviewed international journals, 96 publications in German journals, and 70 papers in other languages. Gottfried

Brem is author of 26 books and monographies (2 in English, 15 in German, 9 in other languages) and contributed chapters to more than 150 books.

His outstanding achievements were honored by multiple awards, such as the Uovo d'Oro (Premio Internazionale per la Zootecnia, 1995), the Martin-Lerche-Research Award by the German Veterinary Association (1995), honorary doctorates (Dr. h.c.) by the Veterinary University Budapest (1995) and by the LMU Munich (2003), and the prestigious Walter-Frei-Prize of the University of Zurich (2008), to name but a few. Moreover, he was elected by a number of renowned scientific societies, e.g. as Member of the Russian Academy of Agricultural Sciences (1991), as Member (1997) and Senator (2002) of the German Academy of Sciences *Leopoldina*, as Corresponding Member of the Austrian Academy of Sciences (since 2003), and as Honorary Member of the Hungarian Academy of Sciences (since 2004).
However, the scope of Gottfried Brem's activities goes far beyond excellent papers and prestigious academic awards. He always had an intrinsic interest to translate his discoveries into practical application. Thus – in parallel to his multiple academic activities – he is an ambitious entrepreneur and founded or co-founded a number of biotech companies, such as the Agrobiogen Biotechnologie GmbH, Larezhausen (1997; www.agrobiogen.de), the Xenogenetik Biotechnologie GmbH, Vienna (1997; www.xenogenetik.at), the apoGene Biotechnologie GmbH & Co KG, Larezhausen (2000; www.apogene-gmbh.de), the PAKTT Gesellschaft für die Produktion von Antikörpern für die Tumortherapie mbH, Larezhausen (2001), and the nexttec^{TM} Biotechnologie GmbH, Leverkusen (2001; www.nexttec.biz).

In spite of his multiple activities in academia and biotech, Gottfried Brem always found time to mentor and promote young scientists. It is spectacular how quickly he can switch between rather different scientific topics and always come up with a brilliant idea. So far, Gottfried Brem supervised 56 doctoral theses and mentored 8 habilitations. With his unmet creativity and enthusiasm, and with a unique ability to motivate young scientists, Gottfried Brem – who always had to deal with pedigrees during his career – in fact set up his own academic pedigree, which is now already in the second generation and starting the third generation (Fig. 1). His outstanding performance in the promotion of young scientists characterizes Gottfried Brem as one of the most influential animal scientists in Europe. We are very glad and proud to be part of this pedigree which follows Gottfried Brem's attitude "*vivat*, *crescat, floreat*". We are very pleased that the A.E.T.E. selected Gottfried Brem as winner of the A.E.T.E Medal 2008, another prestigious award honoring his outstanding achievements.

We congratulate our mentor and look forward to further fruitful scientific interactions and continued friendship.

Bernhard Aigner, Munich
Urban Besenfelder, Vienna
Bertram Brenig, Göttingen
Mathias Müller, Vienna
Eckhard Wolf, Munich

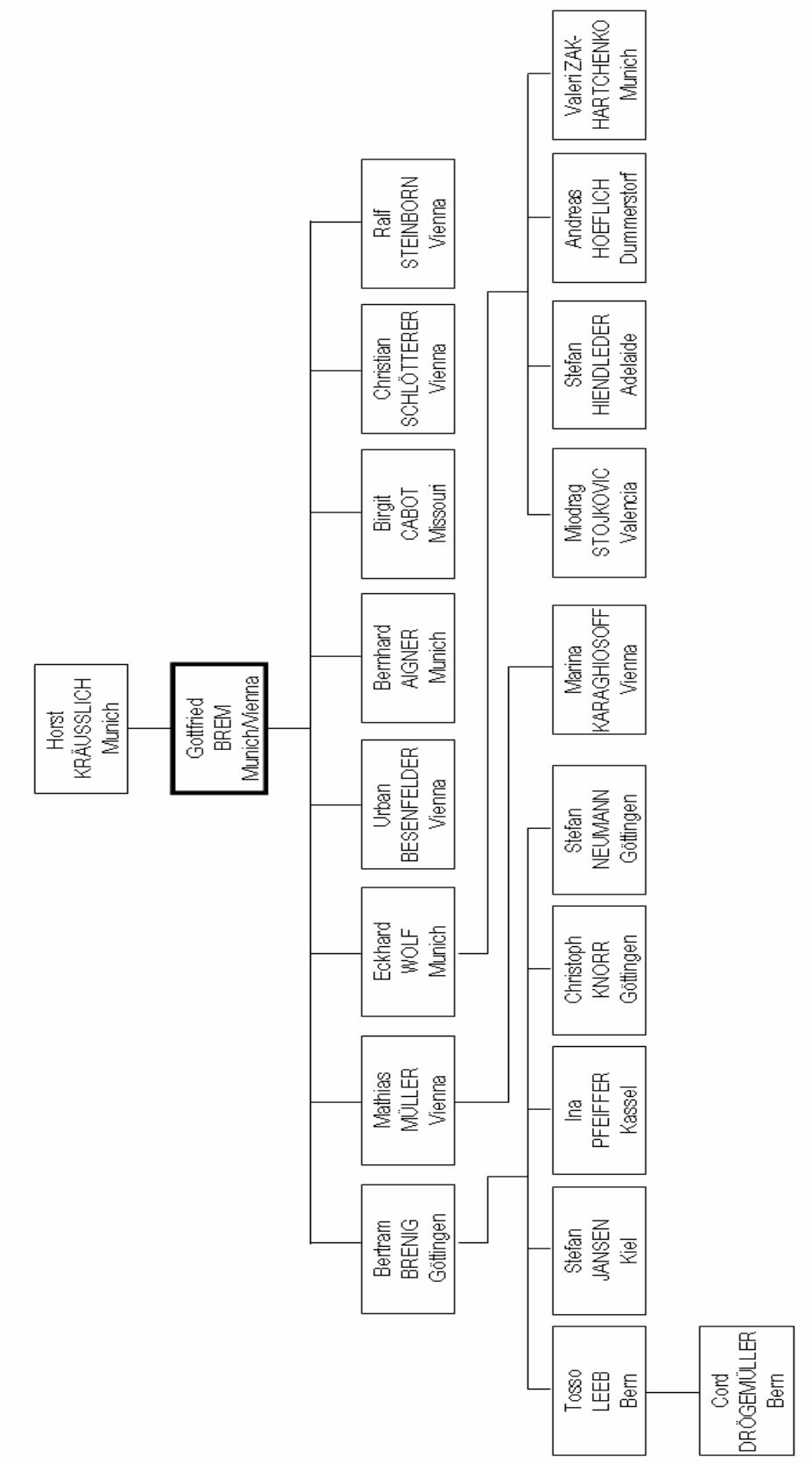

Figure 1: Academic Pedigree of Prof. DI Dr. Dr. h.c. Gottfried Brem. The diagram shows academic offspring who have finished their habilitation and are working in an academic environment.

Generation of Recombinant Antibody Transgenic Farm Animals

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Introduction

The pioneer work of Emil von Behring led to the definition of the *antitoxins*. Since that time serum and serum constituents of animal blood has been widely used in diagnosis and treatment of human diseases. Physiologically, B lymphocytes are responsible for the humoral immune response by producing and secreting antibodies. Most immunoglobulins consist of two light and heavy chain polypeptides. The genes encoding these chains are composed by several exons corresponding to the constant and variable regions of the antibody. The variable portions are not fixed genetic entities but are joined somatically from separated gene segments by recombining during B cell maturation. Although B cells are diploid and hence all gene segments involved in immunoglobulin rearrangement exist in two copies, only one allele is rearranged and expressed. The hybridoma technology (Köhler and Milstein, 1975) has extremely simplified the cloning of functional rearranged antibody encoding genes.

Immunoglobulin transgenes also cause allelic exclusion of the endogenous counterpart and this mechanism seems to operate even if the difference between the transgenic and the endogenous isotypes is quite pronounced. Since the early 1990's, several transgenic animal systems have been developed to produce recombinant therapeutic proteins using different naturally produced body fluids from various farm animal species. The mammary glands of cattle, goat, sheep, rabbit and pig have been developed as bioreactors for the production of recombinant therapeutic proteins. We have used B-cells of transgenic animals as a production compartment for recombinant antibodies.

The process known as gene farming can result in effective bioreactors for large-scale production of antitumor drugs. In order to preserve healthy cells antitumor treatments can be targeted by using antibodies that recognize proteins found only on cancerous cells. The effectiveness of an antitumor antibody can be increased by fusing it to a second antibody capable of activating immune cells. However, such "bispecific" antibodies are difficult to be produced in *in vitro* systems.

The successful expression of antibodies in milk and serum of transgenic livestock has been described before and recently cloned transgenic calves were generated that carry a human artificial chromosome vector with the entire human IgH and IGλ loci (Kuriowa et al., 2002). In all these cases polyclonal antibodies in a "physiological" format were produced in sufficient yield albeit with the inherent/general problem of generating hybrid antibodies if animal IgG loci remain active.

Compared to antibodies in a conventional format, bispecific antibodies generated by combining only the variable fragments (single chain Fv fragments) as currently used in

experimental cancer therapy are generally much more difficult to produce in good yield and high quality.

Gene transfer in farm animals by DNA microinjection

The integration of a segment of foreign DNA, the transgene, into the germline of animals results in so called transgenic animals. The generation of the first transgenic farm animals was reported 23 years ago (Hammer et al. 1985, Brem et al. 1985). Production of transgenic farm animals provides a technology to introduce new gene constructs into the genome to be inheritated like a Mendelian gene. The mainly used technique was the pronuclear microinjection of DNA.

As an example the generation of transgenic rabbits is described shortly. The whole process can be subdivided in six steps:

1. Preparation of the DNA solution for microinjection.
2. Collection of rabbit zygotes.
3. DNA microinjection into pronuclei.
4. Transfer of injected embryos.
5. Detection of the transgene in the born offspring.
6. Establishing a transgenic line that carries the hereditary transgene.

Ad 1. The DNA construct (w/o cloning vector backbone) is isolated, purified and diluted with injection buffer. The concentration should be about 1µg/ml (about 1000 copies of a gene construct with a length of less than 10 kb). This concentration has shown the best results as far as integration frequency and efficiency are concerned. The DNA microinjection solution has to be absolutely free from contaminants in order to avoid clogging of the injection needle or harming of the injected zygotes. All solutions that are used prior to adding the DNA have to be sterile filtered.

Ad 2. Adult rabbits are housed in single cages. At least 17 days prior to superovulation or synchronisation they are kept individual to avoid pseudopregnancy. Rabbits are housed under conditions of 14 hours light and 10 hours dark and are fed with a combination of concentrate (about 80 g per day), water, and hay ad libitum. Donor rabbits receive intramuscular injections of 20-30 IU PMSG (pregnant mares serum gonadotropin)/kg body weight 3 days prior to ovulation. An alternative superovulation scheme uses 6 subcutaneous injections of FSH every 12 hour prior to insemination. Ovulation is induced by intravenous injection of 180 IU HCG. Immediately after the HCG application the donor animals are artificially inseminated twice. For embryo collection the donors are sacrificed 19 hours after insemination. The mesosalpinx and the fat tissue are removed from the oviducts which are subsequently rinsed with PBS. Storage and short-term in vitro culture of embryos is done in PBS supplemented with 20% fetal calf serum. Zygotes of good quality are free of cumuls cells and show a bright cytoplasm with two easy detectable large pronuclei placed near together in the centre of the cell.

Ad 3. Zygotes are washed with medium before DNA microinjection. Microinjection is carried out at 400 X magnification using an inverted microscope. Zygotes are placed on a depression slide in a drop of medium with a top layer of paraffin. For injection a zygote is fixed on the holding pipette by suction, and the injection pipette is inserted carefully through the zona pellucida and the cell membrane until the tip is positioned within a pronucleus. Microinjection is done by air pressure.

The indication for a successful injection of DNA is the visible swelling of the pronucleus. After injection the zygotes are stored for short term before being transferred to oviducts of recipients. Surviving embryos are washed again before transfer into oviduct.

Ad 4. Recipient rabbits are caged individually for at least 3 weeks. Synchronisation of recipient rabbits is achieved by the induction of ovulation with 120 IU HCG. We have developed a laparoscopic embryo transfer technique (Besenfelder and Brem, 1993). The recipients are anesthetised by i.v. injection of xylacine-ketamine and then fixed on a movable table in a vertical position. After making a small incision (<1cm) an endoscope trocar is introduced through the abdominal wall. After removal of the trocar, the abdomen is inflated with air and the endoscope is inserted. A transfer capillary carrying the microinjected embryos is then inserted through a vein catheter into the oviduct and the embryos are transferred into the ampulla via the infundibulum. Between 10 and 15 injected embryos are transferred to each of the oviducts. Afterwards the air is removed from the abdominal cavity and the incision is closed using a clamp. The laparoscopic transfer of rabbit embryos into the Fallopian tubes of recipients is less time consuming than other techniques and requires minimal operative procedure as well as manipulation of the reproductive organs. The recipient animals are examined for pregnancy by palpation on day 12 after ovulation.

Ad 5. From born offspring hair roots, buccal swabs, tail or ear biopsies or blood samples are collected. DNA is isolated and PCR analyses identify transgenic founder animals.

Ad 6. Transgenic founders are reared and 4 to 5 months later they can be mated to non transgenic rabbits. Offspring is investigated whether the gene construct has been transmitted. It has to be considered that some founders are mosaics. Transgenic mosaics carry the transgene only in some of their cell populations. Thus about 30% of the primary transgenic animals will not pass the transgene to their offspring at an expected rate of 50%.

Significant progress has been made in the use of transgenic animals as bioreactors for the production of protein-based therapeutics.
The basic concept developed in the early 1980 was improved by the production of livestock offspring by somatic cell nuclear transfer.

Gene transfer in cattle by nucleus transfer of transformed somatic cell

Pioneering experiments carried out at the Roslin Institute (Cambell et al. 1996) demonstrated for the first time that cells from adult animals can be used successfully for cloning (Wilmut et al. 1997). The successful generation of offspring in livestock animals derived after nuclear transfer depends upon a wide variety of factors like activation of the oocytes and coordination of the cell cycle (Renard 2005). Although it is now possible to produce cloned mammalian offspring from differentiated cells after transfer of nuclei, the overall success rate is still low. Wilmut et al. (1997) had proposed a so called serum starvation of donor cells as a method of choice for arresting the cells in the GO status of the cell cycle which seems to be ideal for successful reprogramming of the donor chromatin from differentiated cells. Calves have been born after nuclear transfer of serum starved fetal fibroblasts however live calves have also been born after nuclear transfer of non-starved fetal fibroblasts (Cibelli et al. 1998, wolf et al, 1998, Zakhartchenko et al. 1999a).

We have evaluated the developmental potential of fetal fibroblast using nuclear transfer techniques. Nuclei of starved (8 day culture, 0.5% fetal calf serum) fetal fibroblasts

from a 37-day-old fetus transferred to enucleated oocytes developed to blastocysts at a rate of 39%. Nuclei from non-starved cells showed only a development rate of 20%. Fusion rates (81%) obtained with starved fibroblasts tended to be higher than those obtained with non-starved cells (72%), cleaved at a higher rate (77% and 66%) and also the developmental capacity to blastocysts was better (39% and 20%). After transfer of blastocysts derived from non-starved and starved fibroblasts 33% and 78% of recipients were pregnant on day 30. Two live calves were born from cloning non-starved fetal fibroblasts.

In a nuclear transfer program from 223 primary mammary gland cells only 63% of fused and 26% of the fusion complexes developed to the blastocyst stage (Zahkartchenko et al. 1999a). After transfer of 4 blastocysts to two recipients two day 90 pregnancies were detected and one calf was born. This calf was the first successful experimental repetition of the sheep cloned by Wilmut et al (1997) using mammary gland cells.

Consequently, as a result of the fusion of the cytoplasms of different origin cloned cattle show mitochondrial DNA heteroplasmy, which was first described by our group (Steinborn et al. 2000).

Completely reprogramming of nuclei from differentiated cells occurs only after nuclear envelope breakdown and chromosome decondensation. Reprogramming is initiated by a high level of maturation promoting factor (MPF) activity. Thus the activation of the oocyte should not be induced prior or soon after nucleus transfer. Primary cultures of bovine ear skin fibroblasts were established from tissue samples of a 3-year-old cow. The tissues were cut into small pieces, treated with trypsin and transferred to culture. The skin fibroblasts did not change homogenous size and morphology till passage 10 and expressed vimentin at all passage numbers. After nuclear transfer using 92 primary ear fibroblasts 89% fused, 60% of theses developed to blastocysts and after transfer of 16 blastocysts to 12 recipients 3 day 90 pregnancies and one born calf were observed (Zahkartchenko et al. 1999b). Somatic cumulus and oviductal cells were used as donor cells for cloning by Kato et al. (1998). Eight calves were derived from these differentiated cells demonstrating that bovine cumulus and oviductal epithelial cells of the adult have the genetic capacity to direct the development of newborn calves (Brem 2006).

Adult somatic cell nuclear transfer was used to determine the totipotent potential of cultured mural granulosa cells. Nuclei were exposed to oocyte cytoplasm for prolonged periods by electrically fusing quiescent cultured cells to the enucleated metaphase II cytoplasts 4-6 h before activation. After the transfer of 100 blastocysts survival rates on day 60, 100, 180 and term were 45%, 21%, 17% and 10%, respectively (Wells et al. 1999). Ten calves were born and reared.

Schnieke et al (1997) demonstrated for the first time the successful transfection of ovine fetal fibroblasts and the generation of transgenic sheep by nuclear transfer. For the generation of human factor IX transgenic sheep on average 21 sheep were required for the generation of one transgenic sheep. Using DNA microinjection more than 50 sheep were required for the production of one transgenic sheep.
Actively dividing fetal fibroblasts were genetically modified with a marker gene, a clonal line was selected and the cells were fused to enucleated mature ooyctes. Out of 28 embryos transferred to 11 recipients three transgenic calves were born (Cibelli et al. 1998). Furthermore transgenic goats were generated by nuclear transfer techniques with transgenic

fetal fibroblast. For the generation of transgenic calves we have transfected and selected fetal fibroblasts with different gene constructs. Performing more than 500 nuclear transfers the fusion rate was 85%, the developmental rate was 33% and after transfer of 95 blastocysts into 49 recipients 8 transgenic calves were born. Two of them survived till adulthood.

Gene targeting has also been achieved by nuclear transfer from cultured somatic cells which had been gene targeted during in vitro culture. McCreath et al. (2000) described an efficient and reproducible gene targeting in ovine fetal fibroblasts. A therapeutic transgene (alpha 1 antitrypsin) was placed into the ovine procollagene locus and live sheep were produced by nuclear transfer. The target locus was chosen because the gene is highly expressed in fetal cells which allows an efficient selection process. One of the surviving lambs was hormonally induced to lactate and showed high expression of alpha 1 antitrypsin in the milk.

Kuriowa et al. (2002) prepared a human artificial chromosome vector containing the entire unrearranged sequences of the human immunoglobulin heavy and light-chain loci and introduced this vector into bovine primary fetal fibroblasts. Selected cells were used to produce cloned fetuses and healthy transchromosomic calves in which human immunoglobulin proteins could be detected.

The generation of transgenic livestock animals using transfected cells provides numerous advantages compared to the classical methods (Wolf et al. 2000):

- Transgene integration and in some cases even expression can be evaluated in vitro
- No mosaics or chimeras are produced if recloning is used
- The germ line transmission rate of homozygote founders is 50%
- Transformed cells can be stored frozen
- Sex selected cells can be used
- Time schedules are reduced
- Costs and efforts are reduced

Transgenic expression of recombinant monoclonal antibodies

Expression of cloned genes of monoclonal antibodies has been extensively investigated in transgenic mice (Storb, 1986). In most of the studies only genes for single immunoglobulin chains were introduced by gene transfer. In an attempt to extend theses studies we have introduced genes for the light and heavy chain of a mouse antibody into the germ-line of rabbits and pigs. These experiments were designed to evaluate whether antibodies of diagnostic and therapeutic interest could be produced in large amounts in the serum of transgenic livestock. In addition the expression of antibody genes could be a first step towards in vivo immunisation against bacterial and viral diseases.

The Ig heavy- and light-chain genes were isolated from the monoclonal Igγ1 antibody secreting hybridoma cell line A20/44 (Weidle, Lenz and Brem, 1991). This monoclonal antibody was directed against 4-hydroxy-3-nitro-phenylate (NP). The κ--encoding (5,5 kb) and the γ-1-encoding (9,25 kb) genes were subcloned in head-to-tail and head-to-head fashion in a prokaryotic vector and used for microinjection into pronuclei of mouse, rabbit and pig zygotes. Three transgenic founders were generated in mouse and rabbit and two in pig. DNA

from transgenic animals contained one to three copies in mice, 40-60 copies in rabbit and about 50 copies of the construct in pig.

The expressions of the anti-NP antibody into the blood were quantified by ELISA. In two mouse lines the synthesis of A20/44 antibody was detected (20 µg and 38 µg/mL). The sera of two transgenic rabbits showed higher titers (300 µg and 140 µg/mL). In the serum of one out of the three transgenic pigs a surprisingly high titer of more than 1 mg/mL was analysed. The secretion of the anti-NP antibody into the milk of founder animals was examined during the second lactation (Müller, Weidle and Brem, 1997). Titers of more than 100 µg /mL mouse milk during the first three days of the lactation were detected. In the remainder of the lactation the titer rapidly went down 10-3 µg /mL on days 4-6 and to 1 µg /mL on day 12.

Transgenic antibodies were purified from serum of transgenic rabbits and pigs and were shown to have two intact binding sites for the antigen when analysed in ELISA. However, in isoelectric focusing only a small fraction of the transgenic product matched to the mouse monoclonal antibody. This could be due to heterologous antibodies by association of endogenous light chains with the mouse transgene heavy chains and vice versa or attributed to species- and cell-type specific posttranslational modifications.

Potential use of transgenic antibodies in farm animals

The possibility of expressing foreign genes in mammals by gene transfer has opened new dimensions in animal breeding and husbandry (Müller and Brem, 1991).

An important aspect is the improvement of animal health by transgenic means. Approaches to reduce disease susceptibility of livestock will be a benefit in terms of animal welfare and will also be of economic importance. The costs of disease have been estimated to account for 10-20% of total production costs. Historically vaccines and drugs, quarantine safeguards and eradication programmes have been and still are used. Attempts to breed animals with improved disease resistance are hampered by different problems. Novel immunisation strategies based on nucleic acid technologies focus on two main issues: additive gene transfer and the development of nucleic vaccines. The aim is to stably or transiently express components known to provide or influence non-specific or specific host defence mechanisms against infectious pathogens.

Using antibody encoding genes two approaches are possible for the improvement of health, the congenital and intracellular immunisation. „Congenital“ immunisation of animals utilises the transgenic expression and germ line transmission of a gene encoding an immunoglobulin specific for a pathogen and therefore providing congenital immunity without prior exposure to that pathogen.

„Intracellular“immunisation was originally used for the overexpression in the host of an aberrant form of a viral protein that is able to interfere strongly with the replication of the wild type virus. This definition was then extended to all approaches based on intracellular expression of transgene products which inhibit the replication of pathogens in host organisms. Intracellular antibodies, also termed intrabodies, represent a versatile tool to modulate the function of selected intracellular gene products and also promise to be powerful in the defence against infectious pathogens.

Production of bispecific antibodies in transgenic animals

Treatment of diseases with human polyclonal and monoclonal immunoglobulins is generally considered of high profit. Different modifications of the antibodies have been suggested for the improvement of the therapeutic purposes. One of these is the generation of bispecific antibodies. Single-chain Fv proteins are recombinant antibodies derived from monoclonal antibodies and are able to bind to their antigens with similar affinities as the parental antibodies. They only contain the variable light chain and the variable heavy chain domains covalently linked by an engineered polypeptide linker. Due to their small size, the lack of assembling requirements and the easy determination they are well suited for the design of new therapeutics.

Bispecific antibodies comprising two target specificities can direct effector cells of the immune system towards therapeutic targets. So far, approaches to construct bispecific antibodies include hybrid hybridomas, chemical coupling, renaturation from bacterial inclusion bodies, or the use of noncovalent coupling in a diabody format and suffer from occurrence of undefined by-products, laborious procedures or low yields in expensive production of bulk quantities. The latter is known for all kind of immunoglobulin fusion proteins and recombinant single chain variable region fragments (scFv) and derivatives thereof, like a bispecific scFv (bi-scFv).

Despite improvements in molecule design and expression systems the quantity and stability of single chain antibodies produced in cell culture is often insufficient for treatment of human disease and the costs of scale up, labor and fermentation facilities are prohibitive. The ability to routinely yield mg/ml levels of antibodies and the scale-up flexibility make transgenic production an attractive alternative to mammalian cell culture as a source of large quantities of biotherapeutics.

We took advantage of the B cell compartment as the native immunoglobulin factory and reasoned that the secretion of bispecific antibodies from circulating blood cells would yield a stable and continuous production system. We introduced rearranged variable heavy and variable light genes of a bispecific single chain antibody (bi-scFv) in a format that does not disrupt endogenous immunoglobulin gene rearrangements into transgenic farm animals.
The bi-scFv molecule with anti-human CD28 x anti-human melanoma specificity (r28M) was previously shown to induce “supra-agonistic” stimulation of resting human T cells by triggering the co-stimulatory CD28 molecule without any engagement of priming-signals (TCR/CD3) and subsequent tumor-cell killing at nanomolar concentrations (Grosse-Hovest et al. 2003).

To select the relevant promoter sequences and intronic transcription elements essential for high-level expression of bi-scFv r28M, four different expression cassettes were microinjected into the male pronucleus of rabbit zygotes. First we tested two promoter regions, namely the immunoglobulin heavy-chain promoter region and the kappa-light chain promoter region. With these constructs altogether 23 and 4 founder rabbits were generated, respectively. Using both constructs only an expression level was achieved similar to *in vitro* expression systems, since the expression level of these animals was only up to 1 mg/liter serum. Nevertheless these transgenic rabbits already produced recombinant antibodies allowing testing the functionality in *in vitro* tests.

Next we changed the size of the immunoglobulin 3' γ1-switch sequence. The expression level did not increase substantial, as was shown in 3 transgenic lines. The next attempt for improving the expression cassette was to place the complete γ1 intron (5.5kb) as an artificial intron between the 3' of the variable heavy regi on and 5' of the C_{H1} – linker. Using this construct the bispecific single-chain antibody r28M was expressed in supernatants of transfected Sp2/0 myeloma cells in the range of 1-3 mg/liter. 24 transgenic rabbits were generated with this construct and 4 of them showed a dramatically 100- fold increased transgene expression compared to the previously used transgenics. Due to mosaicism in some of the founders the F1 offspring showed even higher expressions.

To extend the production capacity we decided to use the latter tested construct for generating transgenic cattle using nuclear transfer of transformed fetal fibroblasts. Bovine fetal fibroblasts were collected from pure bred German Fleckvieh cows that were slaughtered 42 days after artificial insemination. Fibroblasts were cultured in Dulbecco's modified Eagles medium. Subconfluent cells were transfected with a linearized 13.58 kb r28M expression cassette including the cloning vector backbone, using lipofectamine. 48 hours after transfection, the cells were split 1:10 and geneticin was added to a final concentration of 1mg/ml. At subconfluence, the selected cells were passaged in duplicates for DNA analysis and further expansion. Cells were exposed to geneticin for an additional 15 days. Positive integration of the bi-scFv r28M DNA was verified by PCR (Grosse-Hovest et al. 2004).

The transfer of recombinant DNA containing fibroblast nuclei into enucleated oocytes was carried out as described previously (Zakhartchenko et al. 1999c). Day 6 or day 7 *in vitro* cultured nuclear transfer blastocysts were then transferred non surgically to estrus synchronous recipients. Altogether 10 cloned calves were born and reared. Unfortunately we failed to detect the integrated gene construct.

The next round of transfection brought better results. Out of 309 nuclear transfers, 96 (31%) developed till blastocyst stage *in vitro*. 77 blastocysts were transferred into 31 synchronized heifers and resulted in 13 pregnant recipients. 2 animals were sacrificed on day 46 of pregnancy for collecting fetal cells and analysing expression of the targeted gene; 2 pregnancies were lost on days 41 and 83. The remaining 9 pregnant recipients delivered 11 calves, 9 of them were born alive and healthy, while 2 calves died around parturition.
All cloned foetuses and calves were analysed transgenic.

To quantify the amount of bi-scFv r28M and to monitor the expression levels in the blood at different time points, specific ELISA measurements were made of sera taken from cloned and control calves. To analyse the binding capacity of bispecific antibody scFv fragments, cells expressing CD28 and the melanoma-associated proteoglycan (Jurkat and Sk-Mel63, respectively) were incubated with sera or purified material at various concentrations, washed and stained with phycoerythrin-labeled $F(ab')_2$ fragments of a goat anti-mouse IgG antibody and then analyzed in flow-cytometry based binding assays.

Each cloned calf showed a significant increase in bi-scFv r28M production with age, starting from 0.5-5 to 8-10 months post-partum. This delay in transgene expression of about 5 months is consistent with that reported for the maturation of splenic B cells in cattle and the increased immunoglobulin titer observed in mature calves compared to suckling newborns.

The binding capacities of serum, bi-scFv r28M purified from serum, and the purified r28M standard were similar on Jurkat T cells and Sk-Mel63 melanoma cells. The activity of the diluted serum, when compared to the standard protein preparation is consistent with serum concentrations of 0.5-1 g/liter as determined by ELISA. No detectable binding was found on cells incubated with serum of a control calf or with transgenic serum incubated with an antigen negative cell line (Grosse-Hovest et al. 2004).

With respect to the pharmacokinetics of the bispecific single chain antibody fragments, it was of interest to know whether or not the cloned calves might excrete the 57 kDa bi-scFv r28M molecule. None of 5 tested animals had detectable levels of recombinant protein in the urine, whereas bi-scFv fragments were measurable in r28M spiked control urine. In rabbit we could also not detect the recombinant protein in the urine in former investigations.

T-cell mediated tumor cell killing by the recombinant bispecific antibody r28M

To purify bi-scFv r28M from animal blood, serum was passed over a protein L agarose column and bound material was eluted at pH3 in 0.1M glycine. The advantage of calf serum for protein L affinity purification of bi-scFv r28M is that the kappa light chains of bovine immunoglobulins, unlike rabbit immunoglobulins interact only weakly with protein L. SDS-PAGE analysis and immuno blotting indicated that the 57 kDa bi-scFv r28M derived from cloned calves is of 50-70 percent purity and is identical to that of r28M standard from supernatant of the transfected murine myeloma cell line Sp2/0 after a single affinity chromatography step. Mass-spectrometry analysis further revealed 100% identity to the aminoacid-sequence predicted from the cDNA sequence.

Unpurified serum derived from transgenic animals as well as protein L purified material induced a strong dose-dependent proliferation of PBMC (peripheral blood mononuclear cells) comparable to that of the pan clonal stimulator phytohemagglutinin (PHA). No stimulatory effect of bi-scFv r28M was observed on PBMC co-cultured with a mammary carcinoma cell line T47D lacking expression of the HMWG proteoglycan, which underlines the target cell-dependence of r28M induced T-cell activation. No T-cell proliferation was detectable with serum of a control animal. In all experiments, T-cell proliferation was target cell-restricted over at least a two log concentration range.

Cytotoxicity assays demonstrated furthermore that the r28M induces not only a pan-clonal T-cell activation, but leads also to an effective dose-dependent tumor cell killing. Using either the serum or the purified material, melanoma cells were almost completely killed – whereas no cytotoxic effect was seen by using a bovine control serum. Tumor-cell destruction was complete after 3 days of co-culture with PBMC and bi-scFv r28M containing sera of either transgenic rabbit serum or cloned transgenic calf serum.

The same was seen after 5 days of co-culture with PBMC and the r28M protein, U87MG glioblastoma cells were killed almost completely. HMWG-negative mammary carcinoma cell line SKBr3 cells were not affected although the sensitivity of these cells towards killing by activated NK- and T-cells is comparable to that of U87MG. When T-cell depleted PBMC were used in this assay, no significant killing of the U87MG cells was observed whereas purified T cells were highly active.

Lytic activity of rM28 activated PBMC is mediated by T cells as well as NK cells whereby the latter need activation by T cell derived cytokines, e.g. IL-2. In fact, the amount of IL-2 generated during r28-mediated T cell activation well exceeds the concentrations necessary to induce lymphokine activated killer (LAK) activity in both cell populations. It is

noteworthy that the secretion of IL-2 and other cytokines like IFN, TNF and IL-4 is strictly depending upon the presence of the U87MG target cells over a wide range of antibody concentration. Thus one may speculate that upon clinical application of r28M a cytokine release syndrome may be avoidable.

A major concern for the clinical application of antibodies in a non-physiological, recombinant format is their serum half-life. To determine the *in vivo* half-life of r28M molecules, 25 µg of purified protein in a final volume of 150 µl PBS was injected into the peritoneum of C57BL/6 mice. Collection of blood samples started 1 hr after injection and was repeated over 130 hrs. Blood was drawn by retro-orbital sinus puncture and concentration of the r28M protein was determined by flow cytometry. To this end, CD28-positive Jurkat cells were incubated with 1:10 diluted serum samples, washed and stained with phycoerythrin-labeled $F(ab')_2$ fragments of a polyclonal goat anti-mouse IgG antibody. Analysis of stained cells was performed in a flow cytometer. For quantitation, a standard curve was generated using different concentrations of purified r28M protein diluted 1:10 in serum of C57BL/6 mice.

The half-life of the r28M after i.p. injection of 25 µg protein in serum of healthy C57BL/6 mice was around 12 hrs (Grosse-Hovest et al. 2005). More than 4 days after injection, the protein was still clearly detectable in mouse serum at concentrations that are biologically highly active (0,4-0,8 µg/ml). Thus, the pharmacokinetic of r28M appears to be considerably more favourable than previously reported for other bispecific single chain antibodies. This might be attributed, at least in part, to the tendency of the r28M protein to form dimers. In fact, we noted that more than 50% of the material purified from the serum of cloned cows is in a dimeric form, as revealed by analytical size exclusion chromatography. This is in accordance with previously published results using material isolated from tissue culture supernatant. Thus, concerns that a short half-life precludes clinical application of bispecific single chain antibodies do not appear valid as far as the r28M antibody is concerned. Rather, the data accumulated so far indicate that r28M may be capable of inducing an effective T-cell response against HMWG-positive tumors *in vivo*.

Next, we examined whether the process of r28M-mediated T-cell activation and tumor cell killing is effective in preventing growth of tumor cells not only *in vitro* but also *in vivo*. To this end we inoculated U87MG cells into the striatum of nude mice. At day 7 after inoculation we injected a single dose of the bispecific single-chain antibody r28M antibody together with freshly isolated PBMC. This treatment resulted in a highly significant suppression of tumor growth. At day 50, survival was 100% in the r28M group and 0% in all control groups. Control animals injected with PBMC alone or with PBMC plus a mixture of Fab-fragments derived from parental anti-HMWG and anti-CD28 antibodies did not survive longer than mice injected with PBS (Grosse-Hovest et al. 2005).

Conclusion

In conclusion proteins with antigen-binding activity can be produced in abundant amounts in transgenic livestock when genes for the κ and γ chains of a monoclonal antibody are introduced into the germ-line. Purification of the antibodies from the serum revealed the presence of antibodies with two intact binding sites.

The gene farming approach underscores the principle that blood cells can be directed to produce high concentrations of fully active therapeutic molecules that are otherwise difficult to express. This can be achieved without compromising the animal's health, reproduction and development and without inputs from fermentation facilities and costly technical staff. Future applications of transgene expression in blood extend to producing large quantities of additional immunoglobulin fusion proteins for treatment of autoimmunity, cancer and infection or for enzymatic and diagnostic use.

Our results encouraged us to initiate clinical pilot studies in which the r28M protein is used for the treatment of patients with metastatic malignant melanoma and glioblastoma.

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National Statistical Data of Bovine Embryo Transfer Activity in Europe in 2007

TABLE 1: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **AUSTRIA** ***A.E.T.E 2008***

Data collected by
Dr. Lukas Kalcher

Total number of approved E.T. teams in the country	7
Number of teams providing data	7

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	170	B/A=	10.9
	Embryos collected	**B**	1848	C/A=	6.4
	Embryos transferable	**C**	1084	C/B=	58.7%
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**	1084	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	326		
In vivo	Frozen	**I**	531		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	857	H+I+J+K=	
Number of frozen stored embryos		**M**	552		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	62.0%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	164
Number of calves born from *in vitro* embryos	
Total	164

TABLE 2: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **BELGIUM** ***A.E.T.E 2008***

Data collected by
Dr. Peter Vercauteren

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	232	B/A=	9.2
	Embryos collected	**B**	2123	C/A=	4.7
	Embryos transferable	**C**	1100	C/B=	51.8%
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**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**		=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	312		
In vivo	Frozen	**I**	1047		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1359	H+I+J+K=	
Number of frozen stored embryos		**M**	788		
% of *in vitro* embryos transferred		**N**	%	(J+K)/L=	
% of frozen embryos transferred		**O**	77.0%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 3: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **CROATIA** ***A.E.T.E 2008***

Data collected by
Dr. Martina Karadjole

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

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		10	
	Nb of OPU sessions		36	
	Nb of transferable embryos	**D**	91	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	91	=(D+E)
Total number of transferable embryos		**G**		=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	77	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	77	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**	0%	(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2007)

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

TABLE 4: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **CZECH REPUBLIC** ***A.E.T.E 2008***

Data collected by
Dr. Jirina Peteliková

Total number of approved E.T. teams in the country	7
Number of teams providing data	8

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1002	B/A=	10.6
	Embryos collected	**B**	10589	C/A=	5.4
	Embryos transferable	**C**	5451	C/B=	51.5%
In vitro *(OPU)*	Nb of oocyte donors		1		
	Nb of OPU sessions		1		
	Nb of transferable embryos	**D**	3		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	91		
Total i*n vitro* embryos		**F**	94	=(D+E)	
Total number of transferable embryos		**G**	5545	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3361		
In vivo	Frozen	**I**	2467	42.3% frozen	
In vitro	Fresh	**J**	12		
In vitro	Frozen	**K**	40	76.9% frozen	
Total embryos transferred		**L**	5880	H+I+J+K=	
Number of frozen stored embryos		**M**	2141		
% of *in vitro* embryos transferred		**N**	0.9%	(J+K)/L=	
% of frozen embryos transferred		**O**	42.6%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 5: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **DENMARK** ***A.E.T.E 2008***

Data collected by
Dr. Henrik Callesen

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	548	B/A=	10.7
	Embryos collected	**B**	5873	C/A=	7.2
	Embryos transferable	**C**	3963	C/B=	67.5%
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**	3963	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1899		
In vivo	Frozen	**I**	1548		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3447	H+I+J+K=	
Number of frozen stored embryos		**M**	2012		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	44.9%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	1967
Number of calves born from *in vitro* embryos	-
Total	1967

TABLE 6: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **ENGLAND** ***A.E.T.E 2008***

Data collected by
Dr. Anita Meacock

Total number of approved E.T. teams in the country	31
Number of teams providing data	

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**	8480	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**	8480	=(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**	5717	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 (2007)

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

TABLE 7: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **ESTONIA** ***A.E.T.E 2008***

Data collected by
Dr. Ulle Jaakma

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

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 (2007)

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

TABLE 8: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **FINLAND** ***A.E.T.E 2008***

Data collected by
Dr. Marja Mikkola

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	430	B/A=	11.8
	Embryos collected	**B**	5081	C/A=	7.5
	Embryos transferable	**C**	3217	C/B=	63.3%
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**	23		
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	3240	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	851		
In vivo	Frozen	**I**	1999	70.1% frozen	
In vitro	Fresh	**J**	23		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2873	H+I+J+K=	
Number of frozen stored embryos		**M**	2050		
% of *in vitro* embryos transferred		**N**	0.8%	(J+K)/L=	
% of frozen embryos transferred		**O**	69.6%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	1168
Number of calves born from *in vitro* embryos	
Total	1168

TABLE 9: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **FRANCE** ***A.E.T.E 2008***

Data collected by
Dr. Bernard Guérin

Total number of approved E.T. teams in the country	28
Number of teams providing data	24

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5753	B/A=	9.5
	Embryos collected	**B**	54733	C/A=	5.4
	Embryos transferable	**C**	31282	C/B=	57.2%
In vitro	Nb of oocyte donors		52		
(OPU)	Nb of OPU sessions		83		
	Nb of transferable embryos	**D**	198		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	31480	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	21033		
In vivo	Frozen	**I**	12654	37.6% frozen	
In vitro	Fresh	**J**	140		
In vitro	Frozen	**K**	0	% frozen	
Total embryos transferred		**L**	33827	H+I+J+K=	
Number of frozen stored embryos		**M**	10723		
% of *in vitro* embryos transferred		**N**	0.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	37.4%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	2480
Number of calves born from *in vitro* embryos	29
Total	2509

TABLE 10: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **GERMANY** ***A.E.T.E 2008***

Data collected by
Dr. Hubert Cramer

Total number of approved E.T. teams in the country	45
Number of teams providing data	21

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2430	B/A=	11.5
	Embryos collected	**B**	28012	C/A=	6.7
	Embryos transferable	**C**	16305	C/B=	58.2%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions		1488		
	Nb of transferable embryos	**D**	3076		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	3076	=(D+E)	
Total number of transferable embryos		**G**	19381	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5145		
In vivo	Frozen	**I**	8784	63.1% frozen	
In vitro	Fresh	**J**	1540		
In vitro	Frozen	**K**	?		
Total embryos transferred		**L**	15469	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	10.0%	(J+K)/L=	
% of frozen embryos transferred		**O**	%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 11: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **GREECE** ***A.E.T.E 2008***

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**	18	B/A=	5.3
	Embryos collected	**B**	95	C/A=	4.7
	Embryos transferable	**C**	85	C/B=	89.5%
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**	85	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**			
In vivo	Frozen	**I**			
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**		H+I+J+K=	
Number of frozen stored embryos		**M**	85		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 12: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **HUNGARY** ***A.E.T.E 2008***

Data collected by
Dr. Laszlo Solti

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	48	B/A=	10.0
	Embryos collected	**B**	478	C/A=	5.4
	Embryos transferable	**C**	259	C/B=	54.2%
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**		=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	86		
In vivo	Frozen	**I**	256		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	342	H+I+J+K=	
Number of frozen stored embryos		**M**	350		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	74.6%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	196
Number of calves born from *in vitro* embryos	
Total	196

TABLE 13: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **IRELAND** ***A.E.T.E 2008***

Data collected by
Dr. Pat Lonergan

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	391	B/A=	8.0
	Embryos collected	**B**	3133	C/A=	5.3
	Embryos transferable	**C**	2074	C/B=	66.2%
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**	2074	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1058		
In vivo	Frozen	**I**	914		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1972	H+I+J+K=	
Number of frozen stored embryos		**M**	1160		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	78.8%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 14: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **ISRAEL** ***A.E.T.E 2008***

Data collected by
Dr. Yoel Zeron

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	85	B/A=	5.8
	Embryos collected	**B**	491	C/A=	5.0
	Embryos transferable	**C**	423	C/B=	86.0%
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**	423		
In vivo	Frozen	**I**	86		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	509	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	16.9%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	123
Number of calves born from *in vitro* embryos	-
Total	123

TABLE 15: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **ITALY** ***A.E.T.E 2008***

Data collected by
Dr. Giovanna Lazzari

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2350	B/A=	10.5
	Embryos collected	**B**	24660	C/A=	5.6
	Embryos transferable	**C**	13144	C/B=	53.3%
In vitro *(OPU)*	Nb of oocyte donors		151		
	Nb of OPU sessions		312		
	Nb of transferable embryos	**D**	1021		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	2289		
Total i*n vitro* embryos		**F**	3310	=(D+E)	
Total number of transferable embryos		**G**	16454	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	9863		
In vivo	Frozen	**I**	2290	18.8% frozen	
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	2228	95.7% frozen	
Total embryos transferred		**L**	14481	H+I+J+K=	
Number of frozen stored embryos		**M**	7194		
% of *in vitro* embryos transferred		**N**	16.1%	(J+K)/L=	
% of frozen embryos transferred		**O**	31.2%	(I+K)/L=	%

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	7413
Number of calves born from *in vitro* embryos	
Total	7413

TABLE 16: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **THE NETHERLANDS** ***A.E.T.E 2008***

Data collected by
Dr. Sybrand Merton

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	3470	B/A=	10.8
	Embryos collected	**B**	37444	C/A=	6.4
	Embryos transferable	**C**	22097	C/B=	59.0%
In vitro *(OPU)*	Nb of oocyte donors		226		
	Nb of OPU sessions		1535		
	Nb of transferable embryos	**D**	2173		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	223		
Total i*n vitro* embryos		**F**	2396	=(D+E)	
Total number of transferable embryos		**G**	24493	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	4499		
In vivo	Frozen	**I**	10851	70.7% frozen	
In vitro	Fresh	**J**	583		
In vitro	Frozen	**K**	1152	66.4% frozen	
Total embryos transferred		**L**	17085	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	10.2%	(J+K)/L=	
% of frozen embryos transferred		**O**	70.3%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 17: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **NORWAY** ***A.E.T.E 2008***

Data collected by
Dr. Eiliv Kummen

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	9	B/A=	8.9
	Embryos collected	**B**	80	C/A=	5.7
	Embryos transferable	**C**	51	C/B=	63.8%
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**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**		=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	12		
In vivo	Frozen	**I**	150		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	162	H+I+J+K=	
Number of frozen stored embryos		**M**	39		
% of *in vitro* embryos transferred		**N**	%	(J+K)/L=	
% of frozen embryos transferred		**O**	92.6%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	80
Number of calves born from *in vitro* embryos	
Total	80

TABLE 18: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **POLAND** ***A.E.T.E 2008***

Data collected by
Dr. Jedrzej Jaskowski

Total number of approved E.T. teams in the country	4
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	16	B/A=	6.0
	Embryos collected	**B**	97	C/A=	4.5
	Embryos transferable	**C**	72	C/B=	74.2%
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**	72	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	72		
In vivo	Frozen	**I**	181		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	253	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	71.5%	(I+K)/L=	

Number of E.T. calves born (2007)

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

TABLE 19: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **ROMANIA** ***A.E.T.E 2008***

Data collected by
Dr. Stela Zamfirescu

Total number of approved E.T. teams in the country	3
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	25	B/A=	9.4
	Embryos collected	**B**	235	C/A=	4.8
	Embryos transferable	**C**	119	C/B=	50.6%
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**		=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	28		
In vivo	Frozen	**I**	18		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	46	H+I+J+K=	
Number of frozen stored embryos		**M**	78		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	39.1%	(I+K)/L=	%

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	19
Number of calves born from *in vitro* embryos	
Total	19

TABLE 20: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **SPAIN** ***A.E.T.E 2008***

Data collected by
Dr. Julio De la Fuente

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	467	B/A=	9.6
	Embryos collected	**B**	4484	C/A=	4.3
	Embryos transferable	**C**	2014	C/B=	45.0%
In vitro *(OPU)*	Nb of oocyte donors		3		
	Nb of OPU sessions		16		
	Nb of transferable embryos	**D**	7		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	7	=(D+E)	
Total number of transferable embryos		**G**	2021	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	456		
In vivo	Frozen	**I**	1437	75.9% frozen	
In vitro	Fresh	**J**	6		
In vitro	Frozen	**K**	1	16.7% frozen	
Total embryos transferred		**L**	1900	H+I+J+K=	
Number of frozen stored embryos		**M**	1814		
% of *in vitro* embryos transferred		**N**	0.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	75.7%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	690
Number of calves born from *in vitro* embryos	2
Total	692

TABLE 21: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **SWITZERLAND** ***A.E.T.E 2008***

Data collected by
Dr. Rainer Saner

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	413	B/A=	12.3
	Embryos collected	**B**	5061	C/A=	8.0
	Embryos transferable	**C**	3324	C/B=	65.7%
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**	3324	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	683		
In vivo	Frozen	**I**	1869	73.2% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	7		
Total embryos transferred		**L**	2559	H+I+J+K=	
Number of frozen stored embryos		**M**	2633		
% of *in vitro* embryos transferred		**N**	0.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	73.0%	(I+K)/L=	

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	569
Number of calves born from *in vitro* embryos	3
Total	572

TABLE 22: EMBRYO TRANSFER ACTIVITY IN 2007

COUNTRY: **TURKEY** ***A.E.T.E 2008***

Data collected by
Prof. Ebru Emsen

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

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**		
In vivo	Frozen	**I**	26	% frozen
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	26	H+I+J+K=
Number of frozen stored embryos		**M**	26	
% of *in vitro* embryos transferred		**N**	%	(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2007)

Number of calves born from superovulated embryos	16
Number of calves born from *in vitro* embryos	
Total	16

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2007

I. EMBRYO PRODUCTION

(Data collected from 22 countries)

In vivo* produced embryos (superovulation)	
- number of flushed donors	18,287
- number of transferable embryos	106,064
- mean number per flushed donor	5.79
***In vitro* produced embryos:**	
From OPU	
- number of OPU sessions	3,471
- number of transferable embryos	6,569
- mean number per session	1.89
From slaughterhouse collected ovaries	
- number of transferable embryos	2626
Total *in vitro*	9,195
Total number of transferable embryos	123,739

* Specified data from one country not available and not included.

(S. Merton, AETE, Pau, France 2008)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2007

II. EMBRYO TRANSFERS

(Data collected from 22 countries)

***In* vivo produced embryos ***	**Number of embryos transferred** 97,292 (49,963 fresh / 47,329 frozen)
***In vitro* produced embryos**	5,832 (2,404 fresh / 3,428 frozen)
Total number of embryos transferred	108,841
Proportion of IVF embryos transferred	5.4%
Proportion of frozen embryos transferred	49.2%

* Specified data from one country not available and not included.

(S. Merton, AETE, Pau, France 2008)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2007

Species	Embryo Production	Embryo Transfers	Countries
Sheep	238	212	Denmark Greece Romania Turkey
Swine	621	32	Czech Republic
Goat	134	128	Denmark Romania
Horse	764 (incl. 32 IVP)	622 (incl. 32 IVP)	Austria Czech Republic Finland France Hungary Italy Netherlands

(S. Merton, AETE, Pau, France 2008)

INVITED LECTURES

EARLY EMBRYO ENVIRONMENT AND LONG TERM OUTCOMES: EFFECTS OF MATERNAL NUTRITION AND IN VITRO PRODUCTION PROCEDURES

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Abstract

The Developmental Origins of Human Adult Diseases (DOHAD) has initially emphasised the effects of maternal undernutrition during foetal development on long term outcomes in the adult offspring. More recent work has provided evidence that pre-conceptional nutritional conditions and periconceptional environment also play a major role in programming the offspring susceptibility to disease. Epigenetic mechanisms, which may be mediated by macro- and micro-nutriments, endocrine status and oxidative stress, are the focus of the mechanistic studies aimed at understanding the processes involved in these effects.

Introduction

Epidemiological studies in humans have demonstrated that the incidence of metabolic diseases in adults such as obesity, hypertension, insulin resistance and the metabolic syndrome is markedly increased when maternal nutrition is altered at critical periods of foetal development [1, 2]. This phenomenon with a high incidence on human health is now referred to as developmental programming. Animal models are being used to analyze the underlying molecular mechanisms where long lasting effect of perturbations brought to the environment of early developing organisms are suspected to induce a "predictive adaptive response" of genes that control the genetic program of development [3]. If maternal nutrition during pregnancy has been shown by many to affect long term development in the offspring, periconceptional nutrional restriction has also been shown to affect offspring outcome in terms of development of the hypothalamo-pituitary-adrenal axis, cardiac parameters, and many other parameters, therefore extending the range of key gestation periods where metabolic programming takes place [4-7].

In both periconceptional and foetal developmental programming, the mechanisms involved appear to be mediated by the alteration of the expression of key genes involved in the regulation of many physiological functions, amongst which carbohydrate metabolism, adipogenesis and adipocyte response to corticosteroids and adrenal function. Epigenetic mechanisms are most certainly involved and some genes may be more susceptible to these environmental changes compared to others as elegantly demonstrated in the mice with the use of maternal folate supplementation to modify the expression of epigenetically sensitive alleles in the offspring [8-10]. Since essential epigenetic events occur during the time of embryonic development, it seems logical that alterations of the embryo environment through maternal nutrition or embryo culture may permanently affect gene expression in the offspring.

Numerous reviews have been published lately on the influence of maternal nutrition at all stages of pregnancy on health of the offspring [4, 11-15]. This paper focuses on evidence from early embryonic programming in animals through the manipulation of maternal nutrition with regard to in vivo or in vitro fertilization, trying to use examples both from epidemiological work in humans and from laboratory and domestic animal studies.

The supply line concept

The supply of substrates is essential for growth and development of the embryo and foetus (Fig. 1). The fetal supply line, as first described by J. Harding [16], is dependent on the availability of nutriments coming from the maternal side. However, maternal nutrition can influence development, including that of the fetal reproductive system at all stages of development, with the involvement of a wide range of mechanisms. Indeed, maternal body condition and body reserves prior to conception influence both oocyte maturation, oviductal environment and maternal endocrine and metabolic responses to pregnancy, which in turn will affect embryonic environment and embryo development. Some of these effects may even stem from the mother's own foetal conditions, when oogenesis takes place, which may be determined by their mother's nutritional status, thus leading to trangenerational effects from

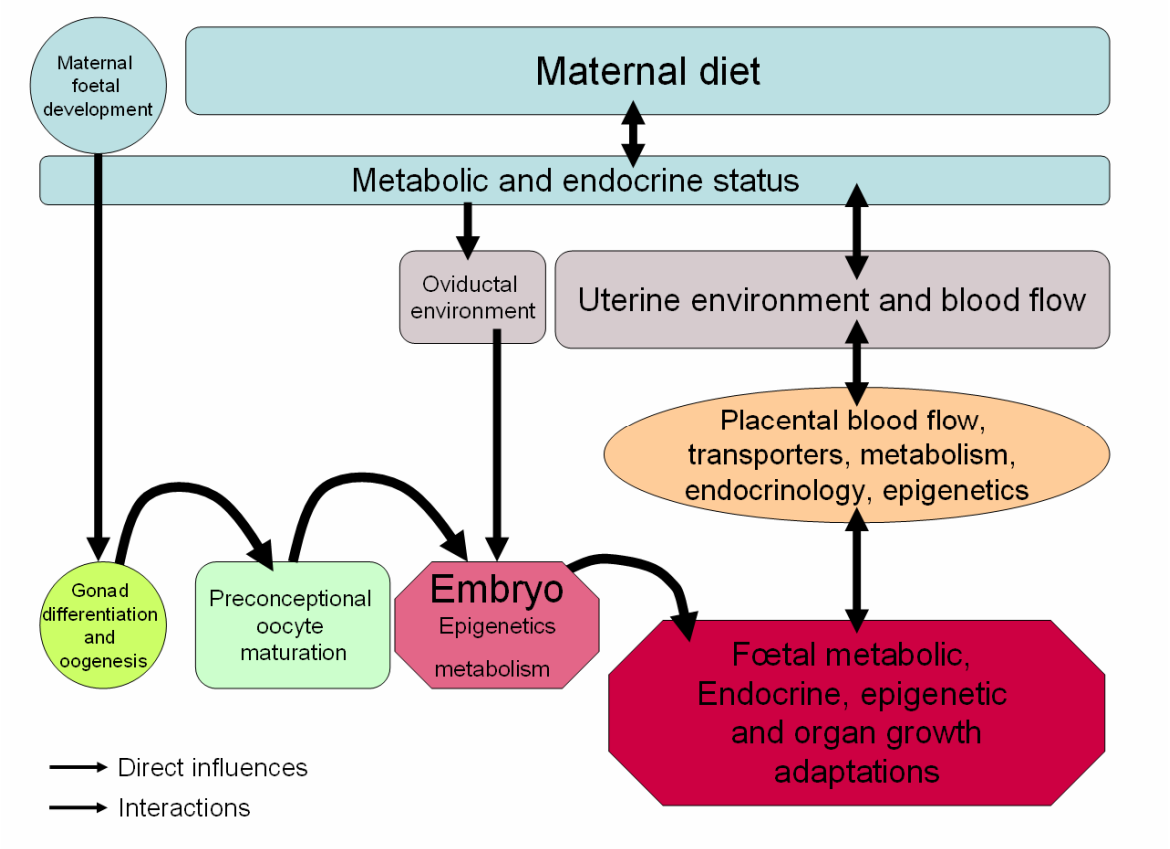

Figure 1: The embryo and foetal supply line, and how maternal conditions may influence offspring development (extended from the initial graph from [16])

the grandmother to the grand-children. Following implantation, the placenta plays a key role in mediating a maternal/foetal duet where nutriment transport is affected by endocrine signals and metabolic status from both sides. Implantation conditions and placental development may partly depend, however, on relative numbers of inner cell mass and trophoblast in the blastocyst and proper elongation of the ruminant trophoblastic membranes. Moreover, neonatal conditions will increase the effects of programming in case of mismatch between the foetal and the neonatal nutritional status. Finally, nutritional and endocrine influences during fetal or neonatal development, potentially can influence reproductive performance in the adult offspring but there is also evidence that effects can be exerted in subsequent generations, on fetal growth, fertility and endocrine status.

Maternal pregnancy conditions and offspring reproductive abilities

Maternal undernutrition and, under certain circumstances overnutrition, before or during pregnancy or during early postnatal life can alter reproductive function of the offspring, possibly through affecting the expression of genes implicated in ovarian differentiation (Fig. 2) [17-19].

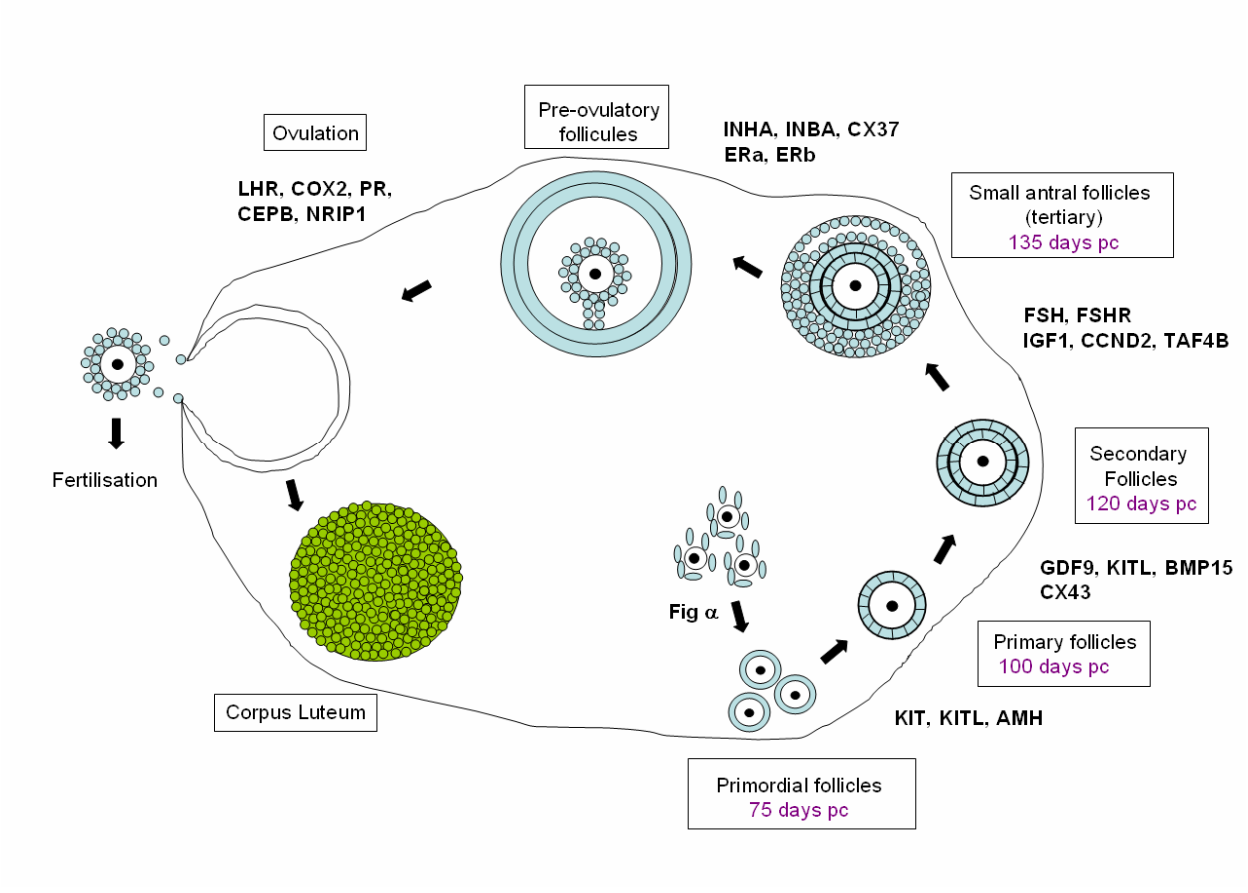

Figure 2: Genes involved in ovarian development with the timing of events in sheep in days post-conception (courtesy of Béatrice Mandon-Pépin). The gonadal sex differentiation occurs 30-32 days post-conception (pc) in sheep, with female meiosis starting at 55 days. Primordial follicles appear at 75 days, primary follicles at 100 days, secondary follicles at 120 days and tertiary follicles at 135 days of pregnancy.

Reduced maternal nutrition

Delayed puberty and reduced ovulation rates or numbers of offspring produced by adult animals have been reported in females that had been undernourished during their foetal life in several mammalian species [18, 20-23]. In contrast, in cattle, recent data have shown that high maternal Body Condition Score (BCS) during gestation has a favorable effect on daughter BCS, non-return rate and number of inseminations per conception [24]. Although some of these effects are relatively small, they can affect the productive life of the animals and may have economical consequences in animal production. For humans, the clinical consequences may be relevant in terms of fertility.

This effect of maternal undernutrition on the timing of puberty and on adult fertility is apparently accompanied with delayed foetal ovarian development [25]. Food restricted in ewes during the periconceptional period induces differences in fetal ovarian development at 110 days of gestation [26]. At the time of the food restriction, the ovarian structures were not present and therefore could not have been directly affected, although precursor tissues could have been. Indeed, maternal undernutrition alters cell proliferation and the expression of apoptosis-regulating genes in the developing foetal ovary, with the precise mechanism depending on the window of maternal food restriction [27, 28]. Maternal undernutrition during pregnancy is also associated with an increased incidence of DNA damage in fetal ovaries, associated with up-regulation of tumour suppressing protein p53, the antiapoptotic factor Bcl-2 and base-excision repair polymerase [29]. These observed changes represent potential mechanisms through which nutritional influences may affect early development and subsequent function. The hypothalamic–pituitary–gonadal neuro-endocrine system appears in some circumstances to be also affected by fetal nutritional influences, with apparently less effects observed in males compared to females [25, 30-32]. Although less striking than effects observed in females, variations in the timing of the onset of puberty have been reported in male sheep [30] with, for example, a 5- week delay in the onset of puberty in intrauterine growth retarded male lambs (2.8 kg at birth) compared with controls (5.2 kg) [33].

Excess maternal nutrition

Excess maternal nutrition may also affect the reproductive success of offspring. Indeed, in the obese adolescent ewe model developed by Wallace and collaborators at the Rowett institute, maternal nutritional excess results in reduced birth weights in the offspring [34], with underdeveloped ovaries in females [31]. Moreover, there is a higher pituitary LH expression in intra-uterine growth retarded foetuses from overfed ewes compared to normal-sized fetuses from ewes that were fed normally [35]. Although no differences were found for FSH expression, there were fewer follicles in the ovaries of foetuses from the overfed dams, regardless of foetal size. Interestingly, the total number of follicles was positively associated with placental mass. Although excess nutrition is relatively rare in animal production, these data are very relevant to human populations [36-38] with the prevalence of obesity rapidly increasing in Europe (x3 in the last two decades), and an estimation of 150 million obese adults (20% of the population) and 15 million obese children and adolescents (10% of the population) in the WHO European Region by 2010 (http://www.euro.who.int/obesity).

Transgenerational effects

Transgenerational effects on birth weight have been demonstrated in humans in studies on the consequences of the Dutch famine during World War II, where the German embargo on food transport in 1944 was followed by a harsh winter inducing a severe, well documented famine that ended in the spring of 1945 [39]. Women, whose mothers were exposed to famine

during their 1st and 2nd trimester in utero, subsequently had offspring with lower birth weights than women whose mothers were not exposed to the famine. The decrease in birth weight was in part due to slower fetal growth rate, and also partly to a shorter gestation. In contrast, the birth weights of the grandchildren of women exposed in their 3rd trimester in utero were not reduced [40].

Periconceptional nutritional influences

If maternal nutrition during the foetal period has been shown by many to affect offspring long term development, periconceptional nutrional restriction has also been shown to affect offspring outcome in terms of weight gain, development of the hypothalamo-pituitary-adrenal axis, cardiac parameters, and many other parameters, therefore extending the range of key gestation periods where metabolic programming takes place [4, 13].

Until relatively recently, most efforts to manipulate nutritional status, either for research or production purposes, centred on the days and weeks before mating in sheep and on the postpartum/pre-mating period in cattle because a moderately high level of nutrition at these times is known to maximise the rate or incidence of ovulation and therefore reproductive performance [41, 42]. It is now known that maternal nutritional during the periconceptional period significantly affects embryo development with long term outcomes on the health of the offspring. Such effects are exerted during the processes of oocyte maturation and embryo differentiation and development.

Oocyte maturation period

Essential fatty acids are classified in two families, i.e. n-3 Polyunsaturated fatty acids (n-3 PUFA) and n-6 PUFA. These fatty acids have metabolic properties as they are the source of long chain fatty acids present in the cell membrane structure and the are also metabolised to bioactive lipids like ecosanoids and docosanoids. They also regulate multiple metabolic pathways through intra-cellular processes or via the direct activation of transcription factors, and some omega 3, like EPA (eicosapentaenoic acid), have direct effects on immune function and inflammatory processes.

In cattle, a high fat supplement can be used in the periconceptional period to increase the energetic contents of the diet. The high-fat diet has been shown to reduce numbers of small and medium follicles without effects on the quality of oocytes (grades 1–4) nor on cleavage rate. The blastocyst production from matured and cleaved oocytes, however, was significantly improved with embryos having significantly more total, inner cell mass and trophectoderm cells than the low-fat group. Moreoover, the negative effects of milk yield, dry matter intake, metabolizable energy intake, and starch intake on blastocyst production observed in the low-fat group were not observed in the high-fat group [43], confirming other studies showing that the effect of diet is dependent on body condition [44]. In another study, the fatty acid supplementation of heifers with 6% calcium soaps of palm oil significantly increased the fat content of the Cumulus Oocyte Complexes (COCs) but the fatty acid composition is not altered by the PUFA content of the diet due to selective uptake of saturated fatty acids by the oocyte [45, 46].

Work performed in our lab on rabbits showed that fatty acid (soy oil, 8% lipids total) and cholesterol (0.2%) supplementation to female rabbits from before puberty through to lactation significantly affected in vivo embryo development with intra-uterine growth retardation observed as early as 9 days post fertilisation, i.e. shortly after implantation, with offspring becoming significantly fatter and heavier as adults, whereas the supplementation administered from the time of breeding had not adverse effects [47, 48].

Very recent data in the mouse show that exposure to a diet rich in N-3 PUFA for 4 weeks prior to fertilisation significantly affected mitochondrial distribution and calcium levels in the oocyte and increased the production of reactive oxygen species [49]. Moreover, even though the number of zygotes collected after in vivo fertilisation tended to be increased in the treated group, this was mainly due to an increase in low quality zygotes, and embryo development up to the blastocyst was significantly delayed. In contrast, these effects were not observed after in vitro fertilisation, suggesting that removal of the oocyte from the high n-3 PUFA environment rescued its developmental potential.

Low protein diets have also been shown to induce long term effects in the offspring as shown by Watkins et al. in the mice. Indeed, a low protein diet (9% casein) administered during only to one ovulatory cycle, 3.5 days prior to mating, did not affect gestation length, litter size, sex ratio or postnatal growth but induced anxiety behaviour and affected cardiovascular function and kidney nephron numbers with marked sex differences in the adult animals [50].

Pre-implantation period

Long term effects of the pre-implantation environment have been demonstrated in laboratory species and in sheep. In sheep, maternal nutritional restriction (70% of requirements) from 60 days before to 7 days after conception induced an increase in foetal arterial blood pressure in late gestation in twins but not in singletons, independent of the renin-angiotensin system activation, and had significant effects on the hypothalamic-pituitary-adrenal (HPA) axis activity depending on offspring number and sex [51]. Periconceptual undernutrition has been associated with altered endocrine profiles, specifically the pattern of ovine fetal plasma IGF-1 and IGFBP-3 concentrations, at later stages of fetal development, with potential consequences for reproductive organ development [52]. Preterm delivery and reduced foetal growth rate have even been reported after extreme periconceptional undernutrition (28% of maintenance levels), due to increased activity of the HPA axis [6, 53], with reduced adrenal weight and adrenal expression of key genes of the IGF family [54]. Work with a less severe undernutrition regimen (50% of requirements, from 15 days before to 30 days after) does not indicate any effect on gestation length (Chavatte-Palmer et al., unpublished data). In any case, periconceptional effects are observed until late in the life of the offspring, with, for example, changes in the cardiovascular function still observed at 1 year of age in sheep after maternal undernutrition from 0 to 30 days of pregnancy [55].

In rats and mice, the administration of a low protein diet during the post-fertilization period reduces cell numbers in preimplantation embryos, apparently due to a slower rate of cellular proliferation, alters birth weight with sex and species differences and induces higher body weight together with sustained hypertension and abnormal anxiety-related behavior, especially in females [5, 56]. Physiological adaptative mechanisms to the maternal nutritional environment are observed already at the blastocyst stage through increased endocytosis in the embryo derived visceral yolk sac endoderm [56]. This response is thought to activate physiological mechanisms of developmental plasticity in order to stabilize conceptus growth and enhance postnatal fitness. However, activation of such responses may also lead to adult excess growth and cardiovascular and behavioural diseases.

Influence of oviductal conditions

The periconceptional and preimplantation effects are largely mediated by oviductal conditions, and methods to analyse those have been elegantly reviewed [57]. Maternal metabolic status and nutrition prior to conception as well as during the preimplantation period are key factors in determining the endocrine, paracrine and metabolic conditions of the

embryo in the oviduct as previously reviewed [45]. Further work is needed to explore the relationship between oviductal environment and long term programming of the offspring. Moreover, although much is known on the carbohydrate and protein contents of oviductal fluids, less data are available on lipids, although they are also important for oocyte and embryo development [58].

Oxidative stress, antioxidants and pregnancy outcomes

Reactive oxygen species (ROS) are formed as by-products of normal aerobic metabolism. Under normal conditions, there is equilibrium between ROS production and the intracellular levels of antioxidant enzymes. This balance is essential for the survival of organisms and their health. An increase in the generation of ROS beyond the ability of the antioxidant systems is called oxidative stress potentially damage lipids, proteins and nucleic acids and ultimately lead to cell death by apoptosis. Cells are protected from ROS-induced oxidative damage by a highly integrated enzymatic antioxidant systems, as well as non enzymatic antioxidant nutrients. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR) and glutathione transferase (GST) which are the first line of defence against ROS-induced oxidative stress. Non-enzymatic antioxidants are mainly represented by glutathione (GSH) and dietary antioxidants, including ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), carotenoids, polyphenols and flavonoids. In addition, dietary essential micronutrients, such as copper, manganese, zinc and selenium, are vitally important because they form part of the active site necessary for the antioxidant enzyme function or act as cofactors in the regulation of antioxidant enzymes.

ROS function as double-edged swords: excessive ROS production damages DNA, whereas low levels of ROS affect cell signalling in physiological processes [59]. A great deal of evidence suggests that ROS affect physiological processes such oocyte maturation, ovulation, follicular and luteal steroidogenesis, implantation and early embryo development [60] and excessive production of ROS during pregnancy plays an important role in the pathophysiology of low birth weight [61, 62]. Oxidative stress has been implicated in the pathology and complications of diabetic pregnancy, and depletion of GSH and impaired responsiveness of GSH-dependent enzymes to oxidative stress during organogenesis have important role in the development of embryonic malformations in diabetes [63]. Close to the implantation period, oxidative damage to the conceptus induced by onset of the maternal placental circulation has been suggested to be a key factor in human early pregnancy loss and there is also evidence that placental oxidative stress is recognized to be associated with human pregnancy-related disorders, such as preeclampsia, embryonic resorption, spontaneous abortion and intra-uterine growth restriction [64, 65].

In animal models, recent results from our laboratory have shown that the activities of GSH-related enzymes increased markedly in sheep placenta during early pregnancy, suggesting that these antioxidant enzymes play a protective role against ROS-induced oxidative stress and apoptosis during early sheep feto-placental growth and development [66]. The biologically active components of nutrition, including trace elements and antioxidant nutrients, have an influence in every single cell at all levels. Data from animal and human studies show that antioxidant vitamins, alone or combined with other supplements, decrease embryonic mortality and improves foetal and birth outcome [67, 68], although it is worthy to note that the association between antioxidant vitamin levels and birth outcome is not conclusive in human and animal [69], maybe due to imbalanced administration of antioxidant vitamins. Dietary supplementation with pharmacological doses of antioxidant vitamin C and

vitamin E has been shown to have a negative effect on litter size and total number of offspring born in mice [70].

Recently, a nutraceutical product extracted from a strain of cantaloupe melon (Cucumis melo LC) chemically combined to wheat gliadin (GliSODin® or Oxykine®) for efficacy after oral administration has received considerable attention as an oral dietary antioxidant supplement. This extract contains antioxidant enzymes, non-enzymatic antioxidant quenching molecules and a variety of antioxidant vitamins. When this component is added to a nutritional formula specifically produced for animal feed, reproductive performance is improved in the immune-mediated abortion-prone, CBA/J-mated DBA/2J mouse model (Al-Gubory et al., unpublished data).

In vitro production

Choice of the oocyte donor

Oocyte donors in case of in vitro production of embryos are generally chosen for their genetic background in terms of animal production. In humans, IVF and especially ICSI is a common response to male infertility. As discussed before, however, nutrition during the period when ovarian follicles emerge from the primordial pool (approximately 6 months before they ovulate in ewes and 3–4 months in cows) can influence ovulation rate in ewes and oocyte quality in cattle, as well as offspring outcome. The quality of the oocyte production can be improved by flushing with different regimen according to the species and stage of production [45]. Effects appear to be mediated to some extent by the cytoplasm contents, as the use of oocytes from well-nourished ewes improved the cloning efficiency in sheep compared to oocytes originating from underfed animals [71].

Immature animals may be used for in vitro production of embryos but there are heavy early embryonic losses is high after transfer, due to poor oocyte quality. In sheep, the quality of the oocytes harvested from 9 week old lambs for IVP was improved by feeding their dams with a high energy diet (x1.5 maintenance levels) during late pregnancy (between 71 and 110 and/or 101–126 days of pregnancy) [71]

Unlike spontaneously ovulating sheep and cattle for which high-plane feeding is beneficial to oocyte quality the opposite is the case in superovulated animals and those donating oocytes for in vitro embryo production. The adverse effect is accentuated in animals in good body condition and those given large amounts of high-starch concentrates that are rapidly fermented in the rumen [45].

Embryo culture

Various factors including the length of gamete interaction and embryo culture conditions are known to influence the rate of development and sex ratio of mammalian embryos produced in vitro [72, 73]. In cattle, cytogenetic evaluation of IVP embryos revealed that the proportion of males and females vary under some laboratory conditions [74]. Furthermore, males tend to develop faster than females and to reach a more advanced stage of development during the first 8 days of in vitro culture [75].

Embryo culture conditions and especially medium supplementation with different batches of serum during a short in vitro culture period have proved to result in long term effects with an incidence on birth weight and perinatal losses in cattle [76, 77]. In a field study including 2288 calves born after in vitro production of embryos, a significantly higher frequency of abnormal offspring was observed (3.2%) compared to 0.7% in calves born from AI [76]. The frequency of large offspring syndrome is further increased when in vitro culture of the early is associated with nuclear transfer [78]. Lazzari et al, also reported significantly

increased birthweight of calves after a 5 days in vitro culture in SOF-BSA or SOF-Serum compared to similar in vitro produced zygotes that were cultured for 5 days in sheep oviduct [79]. The analysis of the transcripts of genes developmentally important in day 12 embryos (glucose transporters, SOD, IGF-Ir..) showed that out of the 9 genes studied, 5 had a higher level of transcription in in vitro cultured embryos than after early development in sheep oviduct. Differences in gene expression in bovine embryos following in vitro culture and nuclear transfer have been described, with genes that will play a role in foetal development being affected (silenced or overexpressed) [80]. Curiously, however, nuclear transfer appears to affect a smaller number of genes expressed at the blastocyst stage than IVF, compared to in vivo control embryos [81]. The situation is reverse, however, when placental gene expression was analysed at term [82].

Putative mechanisms

Epigenetics

Mechanisms involved in the effects described above appear to be mediated by the alteration of the expression of key genes involved in the regulation of many physiological functions, amongst which carbohydrate metabolism, adipogenesis and adipocyte response to corticosteroids and adrenal function. Epigenetic mechanisms are most certainly involved and some genes may be more susceptible to these environmental changes compared to others as elegantly demonstrated in the mice with the use of maternal folate supplementation to modify the expression of epigenetically sensitive alleles in the offspring [8-10]. Since essential epigenetic events occur during the time of embryonic development [83-85], it seems logical that alterations of the embryo environment through maternal nutrition may permanently affect gene expression in the offspring.

Indeed, the administration of a methyl-deficient diet (through vitamin B and folate deficiency) to ewes in the week before and up to 6 days after fertilisation, when embryos were transferred to control recipients, led to adult offspring that were both heavier and fatter, had altered immune response to rotavirus vaccination at 1 year of age and elevated blood pressure with males more affected than females. Methylation status was altered in 4% of the CpG islands examined in the liver, with the majority of the affected loci that were specific to males [86]. DNA methylation, however, is not the only candidate as DNA methylation was not responsible for the transgenerational effects in the Agouti viable yellow mice model where the agouti gene is epigenetically sensitive to methylation [8].

Cell differentiation

The structural and functional differences associated with different nutritional regimes may reflect short- and long-term regulation of expression of multiple genes. At least some of the effects reported may be attributable to nutritional or endocrine influences on endoderm, mesoderm and ectoderm tissues in the early embryo, resulting in differential gene expression and therefore organ structure and function. Some of the effects observed at later stages of development may reflect early, permanent alterations to the cells from which tissues are subsequently derived, resulting in modified responses to endocrine or metabolic signals in these tissues at the later times.

Conclusion

The data presented here show evidence from a large amount of scientific literature that pre-conceptional and periconceptional environment play a large role in programming foetal

and post-natal development. Oocyte maturation and embryo culture media may therefore affect offspring development to a much larger extent than we believed so far. In any case, with respect to these results, the evaluation of long term effects of human embryo culture media should be performed with preferably several animal models before full approval for human use.

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STATUS OF SPERM SEXING TECHNOLOGIES

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Among a number of biotechnological procedures, separation of spermatozoa into X- and Y-chromosome bearing populations is nowadays one of the most discussed and wanted biotechnologies that has found its way into practical application to fasten the genetic selection progress and to increase productivity. The demand comes mainly from cattle industry but also in pigs and other domestic animals its usefulness is foreseeable for example to improve animal welfare by reducing the incidence of dystocia in cattle, avoiding castration of male pigs, and producing less environmental impact due to elimination of animals with the unwanted sex.

In mammals the fetus develops into a female when an X-chromosome bearing sperm fertilizes the oocyte. This mechanism is overwritten by the sex-determining region (SRY) that has been mapped on the short arm of the Y-chromosome (Koopman et al. 1991). SRY induces the formation of the primary testicular tissue in the genital ridge with the further development to male reproductive organs being primarily under hormonal control. A very recent publication however provides evidence that the testosterone content of the follicular fluid may also have an impact on sex determination (Grant et al. 2008), a fact that would require re-thinking the whole mechanism.

Many techniques have been investigated to chance the sex ratio of offspring from mammalian livestock species. Among these only the technique that determines the difference in the relative amount of DNA between X- and Y- chromosome bearing sperm has been successful and highly repeatable. The difference in DNA content that ranges from 3.5% to 4.2% between X- and Y-bearing sperm can be exploited to effectively separate populations of X- and Y- sperm in mammals employing high-speed flow cytometry (Johnson et al. 1989). The technique has been described in detail elsewhere (Johnson 1992) and provides high purity of the sorted samples. However, due to the stress caused during the processing and sorting and the fact that sperm have to be identified individually its efficiency as well as the post sort sperm quality limit its application to a few species and individual semen donors. Nevertheless the technique has found its way into commercial cattle AI.

For this species the technology was first applied commercially in the UK in 2000 and recently in other countries like USA, Canada, Brazil, Argentina, The Netherlands and Denmark, with others on the list to come. Approximately two million calves have been produced from insemination with sexed sperm since then. Although the sexing technology has become more efficient over the past years, the number of sperm needed for regular inseminations (~20 million sperm in cattle) can still not be flow sorted in an adequate time. In

consequence the sperm number per AI has to be reduced to about a tenth (Seidel et al. 1997) are needed for commercial application of sex separated bovine sperm. Two million sperm may be too low to obtain satisfactory pregnancy rates especially in light of the reduced post-thaw quality of sorted sperm. Some authors found no decline of fertility and others reported a reduced fertility after insemination with frozen sex-sorted spermatozoa (Seidel 1999; McNutt & Johnson, 1996; Cran et al. 1994; Lu et al. 1999, Merton et al. 1997; Beyhan et al. 1999). This may only partly be related to the sorting process itself. For non-sorted semen it has been shown that in some bulls the non return rates decrease with high dilution of the ejaculates whereas in other bulls dilution has no significant effect (Den Daas 1998).

In addition to these individual sperm characteristics physical and/or chemical stress during sorting can diminish sperm lifespan. High laser intensity may cause more damage than lower laser intensity as shown for rabbit (Johnson et al. 1996) and bull spermatozoa (Schenk & Seidel, 2007). No changes were found in the frequency of endogenous DNA nicks after staining and UV light exposure, indicating that the dye is not genotoxic (Catt et al. 1997; Parrilla et al. 2004). The hydrodynamic pressure could also exert detrimental stress on the sorted sperm. Lowering the pressure from 2.59 to 2.07 mm Hg increased developmental rates of bovine IVF embryos produced from sex-sorted semen (Campos-Chillon & de la Torre 2003; Suh et al. 2005).

Recently, we demonstrated that bovine IVF-blastocysts derived from sex-sorted spermatozoa may show an aberrant mRNA expression pattern of developmentally important genes such as glucose transporter 3 (*SLC2A3*) and glucose-6-phosphate dehydrogenase (*G6PDX*), indicating a long lasting sorting effect on pre-implantation development (Morton et al. 2007). Moreover, cleavage and blastocyst rates after IVF with sex-sorted spermatozoa were significantly lower than in embryos produced with unsorted spermatozoa of the same ejaculate (Bermejo-Alvarez et al. 2008). Earlier reports had indicated that the number of cell cycles is reduced (Beyhan et al. 1999) and timing of embryo development may be disturbed after insemination with sorted semen (Cran et al. 1993; Lu et al. 1999; Morton et al. 2005).

In order to minimize the costs of sorted semen one approach to improve field results would be to use only those sorted sperm populations that fulfill specific minimal requirements for insemination. Beside others an important indicator to predict the fertile lifespan of sperm in the female genital tract is a 6-hour thermo tolerance test. In our opinion a reduced survival period after thawing of sorted semen is one of the main factors for the highly variable AI results, as it requires a non-physiologically late insemination time point in relation to ovulation. (Klinc et al. 2007; Klinc & Rath 2007). The use of a novel sperm preservation protocol (Sexcess®), significantly increased lifespan and motility of bull spermatozoa were obtained. Calving rates of heifers inseminated with sex separated semen treated with Sexcess® reached levels as for animals inseminated with non-sex separated semen. For yet unknown

reasons cows have still too low conception rates after insemination with sex sorted semen. In conclusion, flow cytometry based separation of sperm into X- and Y-chromosome bearing populations meets already the requirements for field application of bovine semen when heifers are used and semen from preselected bulls is processed using the most advanced protocols.

In other domestic farm animals more research is underway to improve AI with sex-sorted semen by a better preselection and protection of spermatozoa during sorting and cryopreservation (Maxwell et al. 2007; pig: Grossfeld et al. 2005; Bathgate et al. 2008; Cuello et al. 2005; horse: Clulow et al. 2007; Heer 2007). In the pig it was shown that media supplemented with PSP I/II heterodimers (Garcia et al. 2007), which increases motility and mitochondrial activity (Centurion et al. 2003) raised the proportion of viable sorted boar spermatozoa. In vitro fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) and intra-tubal insemination are promising approaches to adjust the sorting technology in pigs and horses, where the high number viable spermatozoa needed for regular fertilization cannot be produced, even for deep intrauterine insemination of sorted spermatozoa (Rath et al. 1993, 1997, 1999; Abeydeera et al. 1998; Probst & Rath 2003; Garcia et al. 2007). However, more research is needed to allow commercial application of sex sorted semen for other livestock species.

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SANITARY CONTROL IN BOVINE EMBRYO TRANSFER: WHERE PRACTICE MEETS SCIENCE

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INTRODUCTION

Embryo transfer in cattle is widely used in veterinary practice in order to get more offspring from a genetically valuable donor cow. Although transfer of bovine embryos is much less likely to result in disease trans than transport of live animals, the sanitary risk associated with bovine embryo transfer remains the subject of both scientific investigations (Givens and Marley, 2008) and adaptations of national and international legislation : these directives have therefore important consequences for the veterinary practitioner.

Scientific reflections on the sanitary risks associated with embryo transfer are focusing on the probability that embryos can be contaminated either via the oocyte, the semen or the zona (Van Soom et al., 2008). Since the risk of contamination is low but not negligible, detailed practical instructions to enable veterinarians to prevent any disease transmission by means of embryo transfer have been published in the manual of the International Embryo Transfer Society (Stringfellow and Seidel, 1998). In addition to these worldwide accepted guidelines EU legislation prescribes the sanitary conditions to which embryo collection and transfer should adhere (European Council, 1989, 1990, 1993, 2003; European Commission, 1994). This legislation is transcribed in national legislation, also in Belgium (Ministry of Small Enterprises, Trade and Agriculture, 1992).

It is the purpose of this manuscript to review the current scientific knowledge on infections taking place during embryo transfer in cattle from a practical point of view. This manuscript is an update of a more elaborate manuscript which has been published recently (Van Soom et al., 2007).

RISK OF DISEASE TRANSMISSION VIA EMBRYO TRANSFER OF A SPECIFIC PATHOGEN ORIGINATING FROM THE DONOR

Since the fifties, the scientific knowledge about bovine embryo transfer and the technical possibilities have improved a great deal. Superovulation has increased the number of embryos considerably to a mean of 5- 6 transferable embryos per donor, the high embryo survival rates after cryopreservation have made donor- recipient synchronization virtually redundant and both flushings and transfers are currently entirely being performed under farm conditions. The economic advantages of embryo transfer for cattle breeders are mainly situated in the possibility to increase the offspring of a valuable dam ten to fifty fold, to store valuable embryos frozen for future use and to commercialise the genetic potential of their herd, since exporting embryos is much cheaper and more considerate for animal welfare than exporting live animals. This is reflected by the fact that in 2006, over 80 000 *in vivo*-derived embryos were transferred in Europe, and almost 7000 *in vitro*-produced embryos. More than half of the embryos were transferred frozen (Merton, 2007).

In addition to economical benefits, embryo transfer can be considered as a particularly safe and effective means of preventing the spread of many pathogens in the international movement of genetic material provided that proper sanitary collection, handling and transfer techniques are used, and uniform procedures for embryo identification and record keeping are followed to ensure that health certification corresponds to the appropriate embryos (Stringfellow and Seidel, 1998; Thibier, 2005, 2006).

In what follows, an overview is given of the current scientific knowledge on bacteria, both originating from the donor animal as from the environment, viruses, parasites and prions that may interact with the oocyte or the early embryo and therefore mean a risk for its development. By the word "embryo" we mean a zona-intact 6- to 8-day old embryo, frozen-thawed or fresh, which is between the compacted morula to expanded blastocyst stage. At this stage, the embryo is ready for transfer to the uterus, and is still protected by the zona

pellucida, an extracellular coat, which is surrounding the oocyte and early embryo until the process of hatching, and which is composed of a few highly modified glycoproteins (Figure 1-3).

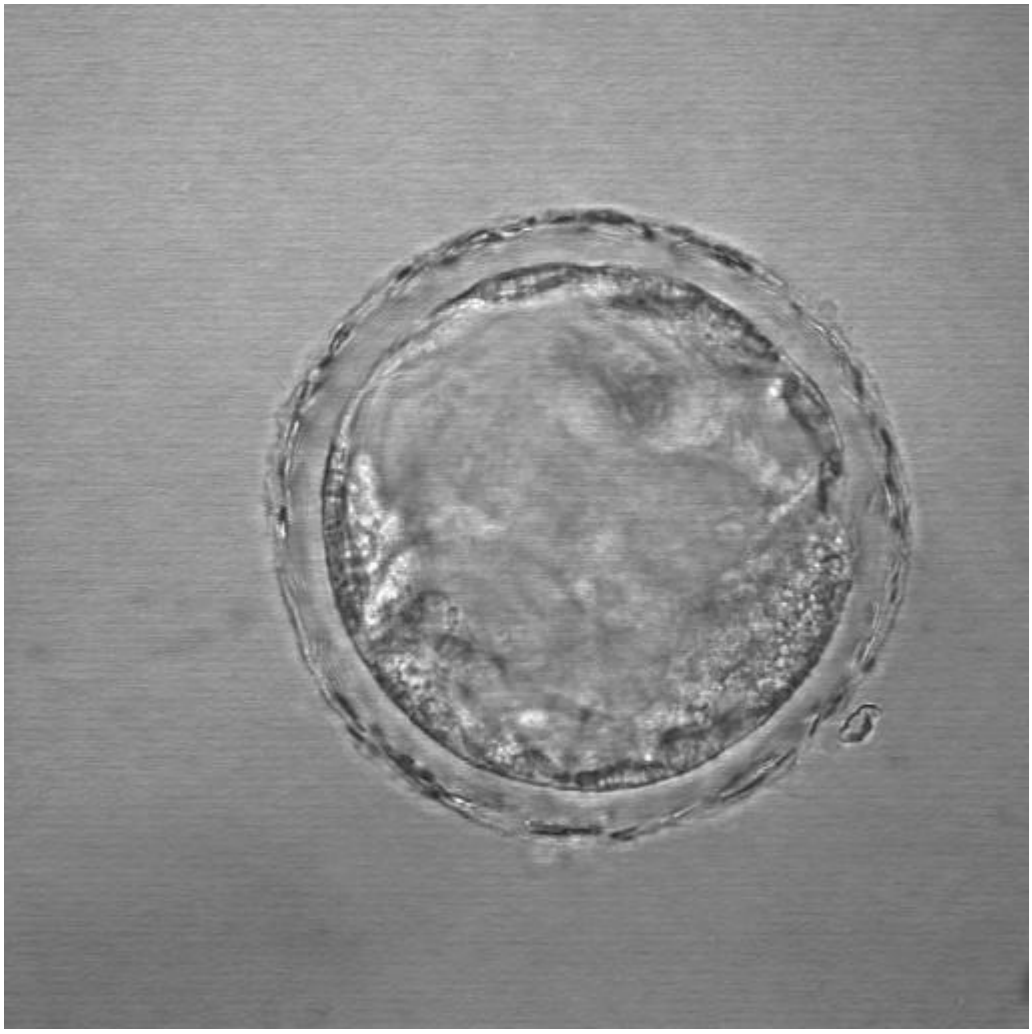

Figure 1: *In vivo* produced pig embryo at the blastocyst stage. Note that the entire embryo is being surrounded by the zona pellucida. Typical for pig embryos is the presence of multiple sperm cells in the zona pellucida. In the same way some pathogens, like *Leptospira* or *Histophilus*, may adhere and even partially penetrate the zona pellucida.

2.1. Bacteria

Obviously, in a normal, healthy animal, the embryo develops without the presence of any micro-organism. However, in septicemic animals with bacteria that have a tropism for the genital tract, embryonic infection may occur. Also while manipulating the embryo during transfer, bacteria may come in contact with the zona pellucida or with the solutions used. For instance, *Leptospira spp*. may cross the channels in the zona pellucida, thus reaching the embryo. In addition, *Leptospira*, *Brucella*, *M. paratuberculosis*, *Mycoplasma* and *Streptococcus* spp. may adhere tightly to the zona pellucida in such a manner that intensive

Table 1. Zona-intact bovine embryos were exposed to bacteria, washed and then assayed (adapted from Stringfellow and Givens, 2000)

Pathogen	No. of embryos exposed	No. of embryos positive for pathogen (95% CI * expressed in percent)	References
Brucella abortus	96	0 (0 – 3)	Mallek et al., 1984, Stringfellow et al., 1984
Haemophilus somnus	38	10 (13 – 43)	Thomson et al., 1988
Mycoplasma bovis	111	111 (97 – 100)	Bielanski et al., 1989, Riddell et al., 1989
Mycoplasma bovigenitalium	49	49 (94 – 100)	Riddell et al., 1989
Mycobacterium paratuberculosis	20	6 (12 – 54)	Rohde et al., 1990
Ureaplasma diversum	26	26 (89 – 100)	Britton et al., 1988

* 95% confidence interval (binomial exact).

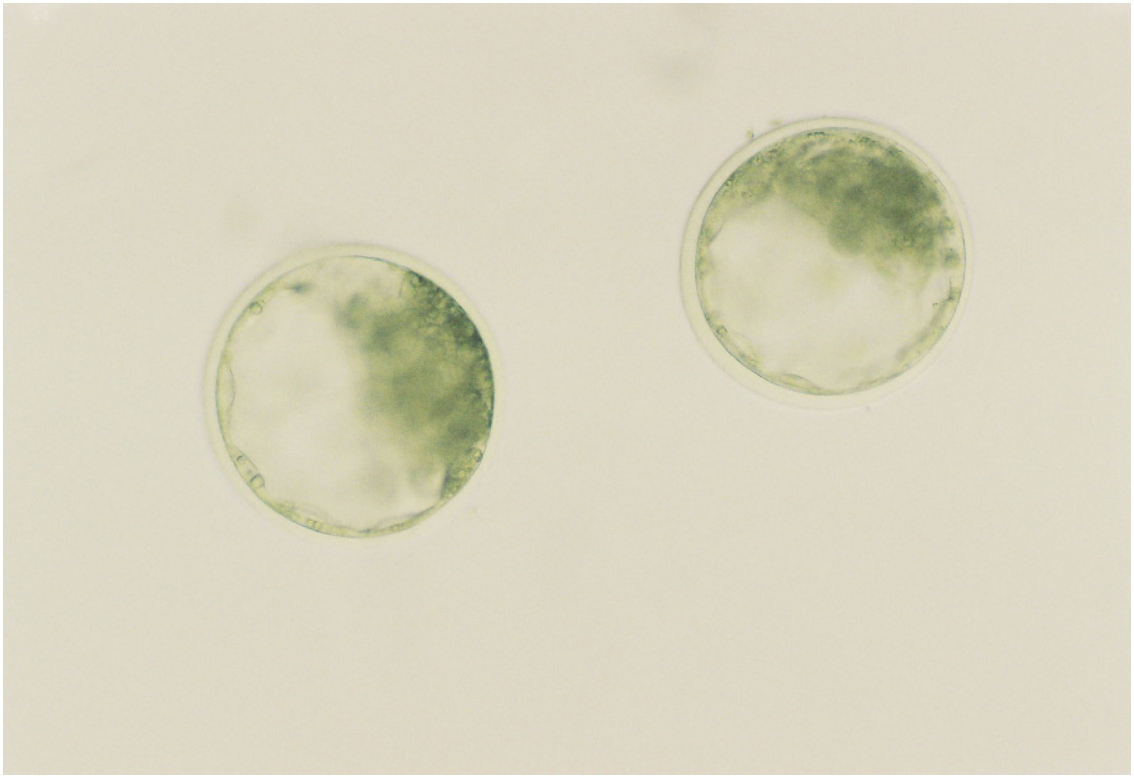

Figure 2: Two zona- intact in vitro produced bovine blastocysts without any adhering cells. Such embryos can be selected for transfer.

washing cannot remove them. Some of these tight associations have only been demonstrated *in vitro* (Table 1), and the probability that this occurs *in vivo* is not known (e.g. *E. coli* O9:K99 and *Leptospira*). Commercial media used for recovering of embryos therefore contain antimicrobials (Givens et al., 2008).

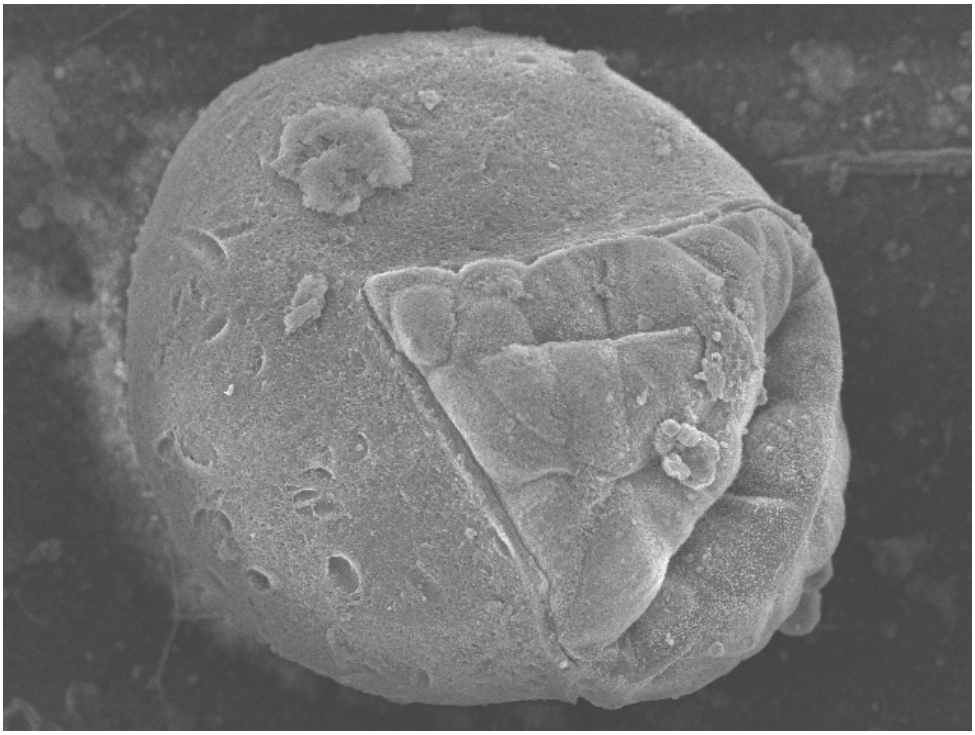

Figure 3: SEM-micrograph of bovine *in vitro* produced embryo at the hatching blastocyst stage. Note the triangular demarcation of the hole in the zona pellucida, through which the embryonic cells are protruding. The use of zona-damaged or hatching embryos therefore represents a danger for pathogen transmission and such embryos must not be selected for transfer.

Pathogens originating from the donor

To estimate the risk that the bacterial agents listed in Table 2 may present for the Belgian sector, information on the occurrence (exposure), insight in the pathogenesis and incubation times of the infectious agents and the available tests are needed. In what follows, a brief description of the available information is given.

- Brucellosis is characterised by contagiousness linked to the presence of large numbers of organisms in reproductive fluids and milk (Saegerman et al., in press). However it was observed that *Brucella abortus* were not adsorbed on the zona pellucida after experimental contamination (Mallek et al., 1984). In Belgium, until the end of the eighties, *Brucella abortus* was a main cause of abortion in cattle. However, Belgium is officially free from bovine brucellosis since 25 June 2003 according to the decision

2003/467/EC (European Commission, 2003). Therefore, the risk to transfer bovine brucellosis through embryo transfer is negligible in Belgium.

- *Mycobacterium bovis* causes nodular granulomas (tubercles). Although lesions are most commonly found in the lymph nodes of the head and thorax, lungs, intestines, liver, spleen, pleura, and peritoneum, lesions may also occur in the ovaries. Mostly, the course of the disease is chronic. In 2002 to 2005, less than 10 cattle herds were found infected by *Mycobacterium bovis* (Federal Agency for the Safety of the Food Chain, 2005). Therefore, the risk to transfer bovine tuberculosis through embryo transfer must be considered as negligible in Belgium.

- *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) causes an intestinal granulomatous infection, initially located in the small intestine and its lymph nodes. At a later phase, lesions also occur in the large bowel and *M. paratuberculosis* may finally be found throughout the body. Cattle may be infected by ingestion of *M. paratuberculosis* from the contaminated environment, and infection can spread vertically to the foetus and through semen. In 2000, the true herd prevalence of *M. paratuberculosis* infection in Belgium was estimated at 6%, but due to the relatively low test specificity, this figure must be considered with caution (Boelaert et al., 2000). Cows with subclinical Johne's disease however do not seem to transmit the disease by embryo transfer when the embryos have been washed as recommended by the IETS (Bielanski et al., 2006, Perry et al., 2006)

- *Campylobacter fetus* subspecies *venerealis* infection causes infertility, early embryonic mortality, and abortion. Healthy carrier bulls with contaminated prepuces represent the natural reservoir. During natural mating, but also during artificial insemination, the bacterium may pass on to the cow. *Campylobacter fetus* subsp. *fetus* is less pathogenic than *C. fetus* subsp. *venerealis*, but may be isolated from aborted foetuses as well. In Belgium, *Campylobacter fetus* has not been isolated during the last 5 years.

- Various *Leptospira* serovars (e.g. pomona, grippotyphosa, hardjo) may infect cattle and reside in liver and kidneys. These animals excrete *Leptospira* with their urine. Chronic infections with *Leptospira* spirochetes may cause abortion, stillbirth or birth of weak offspring. Species involved are, amongst others, *L. borgpetersenii* serovar *hardjo* (hardjobovis) and *Leptospira interrogans* serovar *hardjo* (type hardjoprajitno). Both a serological test (agglutination with living *Leptospira* cultures for the detection of specific antibodies) and an antigen detection (immunofluorescence) test are available. However, only old data on the prevalence of *Leptospira* infections in Belgian cattle is accessible. One study from 1989 on dairy herds distributed over the country showed 9.2% of herds seropositive for serovar hardjo (Dom et al., 1991). The presence of *L. borgpetersenii* serovar *hardjo* has been examined in *in vivo* derived bovine embryos after experimental infection of heifers: the pathogen was present in uterine fluids and its DNA was associated with some of the embryos, but no seroconversion could be established after inoculating embryos into seronegative heifers (Bielanski et al., 1998). For *in vitro* produced bovine embryos, *Leptospirae* were able to penetrate the zona and damage the embryonic cells (Bielanski and Surujballi, 1998). Several washings were not able to remove the pathogens from the embryo, and accordingly this pathogen, which is prevalent in Belgium, is still in category 4, and provides a risk during embryo transfer.

- *Hemophilus somnus* (current name: *Histophilus somni*) is less frequently encountered in cattle (mainly young bovines). Generally, respiratory disorders (e.g. coughing, nasal

discharge, salivation) are present in cattle before neurological disorders (thrombo-embolic meningo-encephalitis) and not necessarily in the same animals (Saegerman et al., 2003). Embryos from *H. somnus*-infected heifers survived in culture media for a significantly shorter time than embryos from control heifers (Kaneene et al., 1986). *Hemophilus somnus* may have effect on ovarian activity (Kaneene et al., 1987) and may adhere to zona pellucida-intact embryos (Thomson et al., 1988, Figure 1, Table 1).

- The intracellular parasite *Chlamydophila abortus* (formerly called *Chlamydia psittaci* serovar 1) infects cattle via the oral route, via inhalation or via direct inoculation of the urogenital tract during mating or artificial insemination. Subclinical infection as well as abortion may occur. *C. abortus* has been demonstrated in the bull's semen and has been associated with reduced fertility (DeGraves et al., 2004). Several studies report high levels of anti-Chlamydophila antibodies in cattle (Kaltenboeck et al. 1997), as well as the detection of *C. abortus* in the vaginal tract (De Graves et al., 2003). Culture of *Chlamydophila* is labour-intensive and requires inoculation of embryonated chicken eggs or cell lines. Its occurrence in Belgium is not known.
- *Escherichia coli* O9:K99 are enterotoxigenic germs, causing diarrhoea mainly in young calves, but may be found in older animals also. It is known that K99 positive *E. coli* may firmly adhere to the zona pellucida of embryos (Otoi et al., 1993). A contamination of embryos with K99 positive *E. coli* during prelevation and washing is possible. In Belgium, approximately 150 to 300 *E. coli* strains isolated from diseased cattle are tested annually for virulence sequences by means of PCR. About 5% of the strains are K99 positive (non published results).
- *Mycoplasma* species have been linked to respiratory disease, mastitis and infertility (Appleby and Gourlay, 1980; Saed and Al-Aubaidi, 1983, Nicholas and Ayling, 2003). *Mycoplasma bovigenitalium* has been isolated from the reproductive tract of cows with vulvovaginitis and infertility (Ayling et al., 2004). However, in this survey conducted in Great Britain between 1990 and 2000, only 8 isolates of *M. bovigenitalium* were found on a total of 1413 isolates, whereas *M. bovis*, which is more frequently associated with respiratory disease, accounted for 735 of the cases (52 %) (Ayling et al, 2004). In general, *Mycoplasma* is not considered to be a major pathogen. Moreover, their culture is laborious and expensive, which may explain why veterinarians may not easily send samples to the laboratory for isolation. In Belgium, the only data which are available on the prevalence of *Mycoplasma* describe the isolation of six *Mycoplasma* species in healthy cattle (16 % positive) and cattle with respiratory disease (65-78 % positive) (Thomas et al., 2002), but there are no recent data on the prevalence of *Mycoplasma* and *Ureaplasma* in cattle with infertility problems.

Environmental pathogens

It is well recognized that semen and embryo collection procedures as well as semen and embryo processing and cryopreservation are not sterile techniques. Both semen and embryos can be associated with potentially pathogenic bacterial agents. It has been shown that microbial contamination may be present in liquid nitrogen, frozen semen and embryos (Bielanski et al., 2003). Thirty two bacterial and one fungal species have been identified in the latter study (among them *Enterobacteriaceae* and *Stenotrophomonas* species), but the source of the bacterial infection was not identified and may probably have resulted from laboratory contamination rather than genuinely being present within the sample (i.e. resulting from the

donor). *Stenotrophomonas maltophilia* has been reported to be detrimental for embryo development *in vitro* (Stringfellow et al., 1997) but data on *in vivo* effects are lacking.

Samples from flushing and washing media and degenerated embryos and unfertilized oocytes which have been analysed by the Veterinary and Agrochemical Research Centre for bacterial contamination in the period from 2000 to 2004 are represented in Table 3. Because of the low number of reports and the change in Agar media used since 2003, which increased the chance of pathogen isolation, the interpretation of these results is difficult.

Table 2. Diseases or infectious agents in cattle listed according to the risk for their transmission via *in vivo* derived embryos (update International Embryo Transfer Society, 1998) and their occurrence in Belgium

Disease category [(a)]	Disease agent	Occurrence in Belgium
Category 1: Diseases or disease agents for which sufficient evidence has accrued to show that the risk of disease transmission is negligible provided that the embryos are properly handled between collection and transfer	Bovine leukemia virus (BLV)	Officially free
	Foot-and-mouth disease virus (cattle)	-
	Bluetongue virus (BTV)	Outbreaks since 2007
	Brucella abortus	Officially free
	Bovine herpesvirus 1 (BoHV-1)(trypsin required)	++++
	Bovine spongiform encephalopathy (BSE) agent	+
Category 2: Identical to category 1 but for which additional transfers are required to verify existing data	None	
Category 3: Diseases or disease agents for which preliminary evidence indicates that the risk of disease transmission is negligible provided that the embryos are properly handled between collection and transfer, but for which additional *in vivo* and *in vitro* experimental data are required to substantiate the preliminary findings	Bovine immunodeficiency virus (BIV)	-
	Rinderpest virus (RV)	-
	Bovine viral diarrhea virus (BVDV)	++++
	*Histophilus somnus (*formerly, *Haemophilus somnus)*	+++
		++++
	Mycobacterium paratuberculosis	++++
	Neospora caninum	+
	Campylobacter fetus (subspecies venerealis)	
Category 4: Diseases or disease agents on which preliminary work has been conducted or is in progress, that indicate: 1. that no conclusions are yet possible with regards to the level of transmission risk; or 2. the risk of transmission via embryo transfer may not be negligible even if the embryos are properly handled according to the IETS-manual between collection and transfer.	Akabane virus (AV)	-
	Bovine anaplasmosis	-
	Bovine enterovirus (BEV)	?
	Bovine herpesvirus-4 (BHV-4)	+
	Chlamydophila psittaci	?
	Escherichia coli 09:K99	+++
	Leptospira borgpetersenii serovar *hardjobovis*	+
		Officially free
	Mycobacterium bovis	++++
	Parainfluenza-3 virus	?
	Trichomonas foetus	+++
	Ureaplasma-Mycoplasma species	-
	Vesicular stomatitis virus (VSV)	

The current legislation on quality control in Belgium is presented in Annex 1. Samples in which bacteria were identified may be indicative for the infection status of the animal.

Since there is no indication that the corresponding animals showed clinical signs, the results suggest that the farm and handling conditions (circumstances in which the embryos were processed) were not optimal. However, these laboratory results are not conclusive to estimate the risk for disease transmission. Even if the bacteria are originating from the reproductive tract of the cow, their pathogenic significance is probably limited. In literature, mostly older data are available on the correlation between vaginal microflora and infertility in cattle, but it has been difficult to demonstrate a causal relationship. In repeat breeders, the same micro-organisms have been isolated from vaginal samples as in normal cows, but organisms of the family Enterobacteriaceae and the genus Corynebacterium occurred in significantly higher numbers in the repeat breeder group (Panangala et al., 1978).

Table 3. Bacteriological results of the examination of the flushing and washing media and degenerated embryos and unfertilized oocytes which have been analysed in Belgium (years 2000-2006)

Year	Number of reports	Number of analysed samples	Reports with favourable results [$]	
			Number	Percent (95% CI [#])
2000	5	116	4	80 (28 – 99)
2001	17	247	14	82 (57 – 96)
2002	19	155	13	68 (43 – 87)
2003[*]	24	259	3	12.5 (3 – 32)
2004	19	116	6	32 (13 – 57)
2005	16	105	7	44 (20 – 70)
2006	13	64	8	62 (32 – 86)
Total	84	893	40	48 (37 – 59)

[$] A favourable result is considered when the bacteriological examination gives a result equal or less than 5 colony forming units. If the quality control for bacteriological official procedure (cf. Annex I) is not respected a new batch of samples must be sent by the considered embryo transfer team to the authorized laboratory for re-analysis.
[#] 95% confidence interval (binomial exact).

2.2. Viruses

From the data presented in table 2 it is clear that sufficient evidence exists that the risk for disease transmission of the following viruses is negligible provided that the embryos are properly treated: enzootic bovine leukosis (caused by bovine leukemia virus), foot-and-mouth disease, bluetongue and infectious bovine rhinotracheitis (IBR) (caused by bovine herpesvirus-1). Bovine viral diarrhoea virus (BVDV) however requires further investigation. BVDV is an economically significant pathogen with a worldwide distribution among cattle populations. It is known to be associated with semen, ovaries and serum of infected cattle. Despite early studies indicating that it was safe to transfer embryos from BVDV infected cattle, some doubt has risen on the safety related to embryo transfer with BVDV infected cattle. It has been shown that BVDV is present in oocytes of persistently infected cattle (Fray et al, 1998) and in bovine oocytes after acute infection (Grooms et al., 1998). However, despite the fact the BVDV antigen has been detected in developing oocytes, no report so far has been able to show BVDV associated with zona-pellucida intact embryos that have been

washed according to the IETS protocol (Stringfellow and Givens, 2000). Moreover, the few calves that have been produced from infected cows have been BVDV free (Stringfellow and Givens, 2000). It is not clear whether the oocytes from infected cows indeed are developmentally competent. If this is not the case, the risk for disease transmission should be negligible. Further research should address the oocyte quality and also the efficiency of the IETS washing procedure after *in vitro* exposure of embryos to representative field isolates of type I and type II non-cytopathic BVDV (Stringfellow and Givens, 2000). Some high-affinity isolates cannot be removed using standard IETS procedures (Waldrop et al., 2004b) and when such embryos which were previously infected with the high-affinity noncytopathic BVDV strain SD-1 were coincubated with uterine cells in vitro, the quantity of virus associated with a proportion of individual embryos (both washed and trypsin treated) was sufficient to infect the uterine cells (Waldrop et al., 2004a), and was also infective in vivo (Waldrop et al., 2006). A compilation of the measures which can be taken to prevent BVDV transmission by semen and embryos has been published recently (Gard et al., 2007).

2.3. Parasites

It is generally accepted that adverse effects on embryos are indirect such as placental lesions or late foetal lesions (e.g. *Tritrichomonas fetus*, *Neospora caninum*, *Aspergillus* sp., *Toxoplasma gondii*) (Hill et al., 1971; Rhyan et al., 1995; Hemphill et al., 2000).

2.4. Prions

The great majority of cattle in the British BSE epidemic acquired infection by consumption of contaminated feed (Wilesmith, 1998). Despite this, the maternal transmission of BSE was demonstrated in an experimental model using transgenic mice (Castilla et al., 2005). Whether the latter takes place vertically (i.e. via the embryo or across the placenta before parturition), or horizontally after parturition is unknown. The results of several field epidemiological studies in cattle in the UK suggest a rate of maternal risk enhancement of approximately 10 % in the offspring of dams within 12 months of the onset of clinical signs of BSE (e.g. Wilesmith et *al.*, 1988; Donnelly, 1998). This finding was not confirmed according to the Belgian data (e.g. Saegerman et al., 2001). A recent model study that took into account all data on the epidemic curve in the UK has estimated the current risk to be around 2% with a 95% confidence interval that includes the zero value (J.W. Wilesmith, personal communication). One explanation may be that the maternal transmission only played a role when the exposure to BSE risk was very high (Prince et al., 2003).

If the time lapse between parturition and the onset of clinical symptoms is longer than 12 months, the rate of maternal transmission is reduced. Whether infectivity is transferred directly before birth or after birth by a variety of mechanisms (e.g. calf infection by contaminated material, environmental contaminated with blood, faeces, infected feed, etc.) is uncertain and should be further investigated (European Commission, 2002). There is no scientific data to support the hypothesis that infected calves are unduly sensitive to infection on a genetic basis. It appears that there is no enhanced risk of the development of BSE in the offspring of sires that developed BSE. It is therefore unlikely that semen constitutes a risk factor for BSE transmission (European Commission, 2002).

Preliminary results suggest an extremely low risk of transmission (95% confidence interval: 0-1.5%) of BSE using embryo transfer. These results are consistent with maternal transmission being mediated later in the pregnant period either during or following birth of the animal. Recent studies published by Wrathall and coworkers (2002), indicate that embryos are unlikely to carry BSE infectivity even if they have been collected at the end-stage of the disease, when the risk of maternal transmission is believed to be highest. Transmission of

BSE by artificial insemination is unlikely for semen derived from BSE-affected bulls early in their incubation period. For these reasons, transmission via embryos is unlikely provided IETS protocols are followed (European Commission, 2002).

3. RISK OF DISEASE TRANSMISSION VIA EMBRYO TRANSFER OF A SPECIFIC PATHOGEN ORIGINATING FROM THE MATERIALS OF ANIMAL ORIGIN

It is widely recognized that the use of material of animal origin carries a risk to introduce infectious agents into the recipient of the contaminated material (Brock, 1998). It is essential to find alternatives to the use of materials of animal origin, but until satisfactory replacements have been developed it is necessary that the used materials are safe and free of infectious agents. An overview of how to validate the efficiency of virus clearance in biotechnological products such as serum and hormones has been published more than ten years ago. The authors emphasized that evidence of viral clearance must be obtained in all stages of purification and adequate virus removal and/or inactivation must be proven (Trijzelaar, 1993).

3.1. Bovine foetal serum

Commercial batches of bovine serum are often contaminated with various bacteria and viruses, such as BVDV, BoHV-1 and bovine parainfluenzavirus-3 (PI-3). Probably, the assumption has to be made that all commercial lots of foetal bovine serum are potentially contaminated with at least BVDV (Brock, 1998). Even a certificate stating that a batch is free from BVDV is not reliable, since the performed analysis does not allow identifying small amounts of virus by direct infection of cells. Serum can be made virus free by chemical inactivation by means of binary ethylenimine and by gamma irradiation (30 kiloGray) (European Agency for the Evaluation of Medicinal products, 2005). Such methods have been shown to be effective in terms of virus inactivation, but may have some effects on hormone activities (Kyvsgaard et al., 1997) or on other proteins present in the serum. Virus inactivation with beta-propiolactone may not be equally efficient in all blood products and must hence be tested independently in individual conditions (Scheidler et al., 1998). Serial ultrafiltration through 40 nm porosity filters has been advocated to reduce viral contamination to a minimum level but it may be necessary to ask for additional treatment to make the sample safe. Recently, EU regulators have formalised their position on issuing guidelines on the use of bovine serum during manufacture (Robertson, 2006).

3.2. Bovine serum albumin

The risk of infectious agents in BSA is less than in whole foetal bovine serum. Nevertheless, these products should be equally tested and declared free of pathogens. Alternatives which may replace BSA during bovine embryo culture and freezing, can either be macromolecules of non-animal origin such as vegetal peptones (George et al., 2006) or chemicals with surfactant properties (Palasz et al., 1995).

3.3. Hormones

Embryo transfer requires different natural and synthetic hormones for the synchronisation or induction of oestrus and for superovulation of the donor females. Prostaglandins are the most frequently used hormones to synchronise females presenting a cyclic activity. Numerous molecules are presently synthesized by the pharmaceutical industry. Synthetic progestagens as implants and the progesterone included in the silastic, the inert polymer of the vaginal spiral, can also be used for the synchronisation or induction of oestrus. One injection of oestrogen ester is frequently combined with these treatments in order to

stimulate the ovary. The treatments to induce a superovulation are aimed to increase the number of follicles on the ovaries at around superovulation. At first, Pregnant Mare Serum Gonadotrophin (PMSG) (current name – equine chorionic gonadotrophin (eCG)) extracted from mare serum was used. The most important disadvantage of this gonadotrophin lies in its long half life and in its bifunctional activity, leading to unreliable superovulatory responses. More recently, pituitary extracts, with predominantly FSH-activity, have been used for superovulation with a significant increase of the results in term of the number and the quality of the embryos. Presently, all these substances are commercially available with official registrations at the national commissions of drugs. These registrations permit to reduce the risk of contamination by a biological agent. Moreover, the inocuity of batches of those preparations in terms of prion transmission can be verified with an appropriate testing procedure (Degand et al., 2004).

3.4. Enzymes

Trypsin should be tested and declared free of *Mycoplasma* and viruses. We have found no data on the possible contamination of trypsin with pathogens. As for serum and BSA, commercial alternatives for animal-derived trypsin are being tested for use in bovine embryo manipulation, such as recombinant trypsin-like proteases including TrypLE (Marley et al., 2008) and Trypzean (Seidel et al., 2007). Although some results were promising, more data are needed before any conclusion can be drawn about the safety and efficacy of these products.

3.5. Antiviral compounds

Pretreatment of embryos with antiviral compounds has been investigated as a possible approach to prevent virus transmission via embryo transfer. Adding an antiviral agent to culture medium could provide an integral safeguard by preventing the replication of cytopathic and noncytopathic, type one and two BVDV that could have been introduced accidentally (Givens et al., 2006). Several antiviral compounds have been shown to be effective against BVDV and non-toxic for bovine embryos in vitro, such as DB606 (Givens et al., 2005) and BPIP (Mestach et al., 2007). Transfer of DB606-treated embryos led to the birth of normal calves (Givens et al., 2006) and the female calves developed to heifers with a normal reproductive capacity (Givens et al., 2007).

3.6. Media and antibiotics

The exact composition of most commercial media such as ViGro Holding Plus is not made available to the general user. The manufacturer only states that it is a HEPES-buffered complex salt solution, with a protein content provided by 0.4% certified and export cleared BSA. The solution also contains growth factors, amino acids, co-factors, vitamins and antibiotics (penicillin, streptomycin, kanamycin and gentamicin).

CURRENT TESTS

Current testing, as imposed by the Belgian legislation, is focusing on the flushing and washing fluids and media. Obviously, it is beneficial for sanitary, economic and practical reasons to consider the sanitary conditions of the donor, or even the population to which the donor animal belongs, in order to evaluate the risk of transmission of infectious agents.

As an example, for the viral infections BoHV-1 and BVDV, it is helpful to direct the efforts on the individual status of the donor cow and on the status of its herd. Indeed, the virological tests on embryos are very time consuming and should be done systematically in order to be efficient. Furthermore, their sensitivity is probably quite low. Therefore, it is

recommended to select only BoHV-1 seronegative donor cows or cows that have tested negative against glycoprotein gE. Alternatively, the donor cow should originate from a BoHV-1 free herd or a herd controlled for BoHV-1 infection and which has an approved IBR control programme. Concerning BVDV, the donor should have been tested individually for the absence of persistent infection, by a validated RT-PCR or antigenic test.

LEGAL AND RECOMMENDED SANITARY MEASURES

In 1920, the transit of zebus through Belgium unintentionally stood at the origin of a new international sanitary legislation. The animals originated from India and were shipped for Brazil via Antwerp's harbour. These animals caused multiple outbreaks in Belgium of rinderpest by infecting cattle that were housed in the same quarantine stables and that were later sold at the cattle market places of Ghent and Brussels. This economical and sanitary catastrophe had an important consequence at international level. Twenty-eight countries or territories agreed in 1924 to create the "*Office International des Epizooties*" in Paris, which recently has changed its name to World Organisation for Animal Health (WOAH) and which is until our days managing the world animal health information system. To prevent the occurrence of future epidemics, Member Countries were asked to notify to the WOAH the main animal diseases. The list of notifiable diseases, which includes zoonoses, can be consulted at its website (http://www.oie.int/eng/maladies/en_classification.htm).

This historical example clearly illustrates the importance of certification of the sanitary status, as organised under the auspices of the WOAH.

International recommendations

The official sanitary control of *in vivo* derived embryos destined for international transport is covered by the Terrestrial Animal Health Code of the World Organisation of Animal Health (http://www.oie.int/eng/normes/mcode/code2006_back/en_chapitre_3.3.1.htm). The aim of this control is to make sure that a number of organisms, which could be pathogenic for embryos, are controlled and that transmission of infection to recipient animals and their offspring is avoided. The recommendations described in the WOAH code are based on the International Embryo Transfer Society (IETS; http://www.iets.org/) handbook published in 1998.

According to the available knowledge, the Research Subcommittee of the IETS has categorized a number of microbial agents in order to estimate the risk for their transmission via *in vivo* collected and properly treated embryos. The categories with agents infectious for cattle are listed in Table 2. Infections of which most evidence is available that their risk of transmission may be neglected under the condition that embryos are handled following the instructions as described in the IETS handbook, belong to category 1. The list should be used as guidance when evaluating the risk for disease transmission via embryo transfer.

The transfer of *in vivo* obtained embryos is a method for transplantation of animal genetic material that, if correctly done, is only associated with a limited risk for transmission of microbial agents. Concerning risk assessment, and independently of the animal species, the WOAH sanitary code (Article 3.3.1.5: http://www.oie.int/eng/normes/mcode/code2006_back/en_chapitre_3.3.5.htm) identifies three levels that determine the risk associated with the technique of embryo transfer:

- The first concerns infections not listed in the IETS category 1 and deals with the probability an embryo gets infected. This probability depends on *(i)* the zoosanitary situation of the country or region, *(ii)* the sanitary situation of the herd and of the donor

animals from which the embryos are taken, and *(iii)* the virulence of the microbial agent.

- The second level concerns the application of the sanitary measures as described in the IETS handbook. These procedures concern the washing, treatment and analysis of embryos (separate rinsing of embryos that originate from different donor animals, sufficient number of washings, use of single-use material, eventual use of trypsin in order to inactivate certain viruses, examination of the zona pellucida of each embryo (Figure 1-2).
- The third level also concerns infections not listed in the IETS category 1 and concerns the following measures: (i) surveillance of the donor animals and herds from which they originate as for the outbreak of infectious diseases, taking into account their incubation periods, and (ii) examination of embryo-collection (flushing) fluids and non-viable embryos, or other samples such as blood, for presence of specified disease agents.

As for embryo transfer, Stringfellow (1998) argued that certification on the health of transferred embryos relies on specific tests conducted on the donor animal, appropriate washing or trypsin treatment of the embryos (Table 4), and testing of the samples associated with the embryo collection procedure.

Justification for testing samples of collection fluid and washes lies in the fact that it gives some indication of the pathogens to which embryos have been exposed in the uterus and to the efficacy of the washing procedure (Stringfellow, 1998). It may be worthwhile to test embryo samples when the donor is seropositive for a particular disease, but it would be unnecessary to test these samples if donor males and females were shown to be free of disease (Stringfellow, 1998).

It may be worthwhile to focus on serological or other testing of the donor animals instead of collecting fluids from all flushes for future analysis, because this approach may lead to more consistent results than sample testing for various reasons.

The description of the general guidelines on the collection and processing of embryos is available (http://www.oie.int/eng/normes/mcode/en_titre_3.3.htm). Working with disease and pathogen free donor animals and pathogen free media and with sterile plastic or glass recipients is certainly the best approach to prevent inadvertent disease transmission via assisted reproductive technology.

Belgian legislation

The continuous introduction of new technologies requires new legislation: the development of embryo transfer techniques during the eighties prompted the Belgian government in 1992 to enact a regulation concerning the sanitary control for bovine embryo collection and transfer teams (Royal Decree of January 23, 1992 and its modifications; http://www.juridat.be/cgi_loi/legislation.pl). This legislation lists measures that are necessary in view of Good Veterinary Practice such as conditions for approval of an embryo collection and transfer team and official measures concerning quality control during embryo collection and transfer (Annex 1), conditions relating to the collection, processing, storage and transport of embryos by the approved embryo collection and transfer teams, conditions applying to donor animals, identification of consignment, identification and registration of embryo collection and transfer teams, conditions of animal health certification.

Table 4. Essential requirements for proper washing and trypsin treatment of embryos (adapted from the International Embryo Transfer Society, 1998)

Requirements for proper washing of embryos
Only embryos from a single donor washed together
Ten or fewer embryos washed at a time
Only zona-intact embryos washed
Only embryos free of adherent material washed
Minimum of ten washes
Use a new sterile micropipette each time embryos are moved from one wash to the next
Regulate volumes so that each wash is at least 100-fold dilution of previous wash
Requirements for trypsin-treatment
Transfer embryos through five washes in PBS with 0.4 % BSA and antibiotics
Expose to sterile trypsin [1:250] at a concentration of 0.25 % for 60 to 90 s
Transfer embryos through five additional washes in PBS with 2 % serum and antibiotics

Meticulous and strict implementation of the above regulation, although susceptible to improvement, is time-consuming and costly but aims at the early detection of viral or bacterial contaminants, and therefore at an embryo transfer where the risk for infection is maximally reduced.

The Royal Decree specifies that donor animals have to be derived from herds that are officially free from brucellosis, tuberculosis and enzootic bovine leukosis, when the embryos are subject to intra-community trade. No specifications of other important bovine pathogens such as BoHV-1 and BVDV are mentioned. However, these viruses may cause persistent infections in cattle and therefore may escape the attention of the herd owner or the veterinarian.

Taking into account these WOAH recommendations, it is reasonable to consider the following levels of qualification:

- Qualification of the country or region (considering the existing surveillance programmes);
- Qualification of the herds origin where donor animals originate from (considering the existing surveillance programmes);
- Qualification of the donor animals based on the absence of clinical symptoms and / or favourable analytical results;
- Qualification of the embryos on the basis of laboratory analysis of flushing and washing fluids, and of non-viable and degenerated embryos. This examination also serves as quality control for the manipulations as described in the IETS handbook: a bacterial contamination of the rinsing liquids, but absence of contamination of the embryos themselves, is indicative for non-septic work.

The Scientific Committee of the Federal Agency for the Safety of the Food Chain advised to adapt the existing Belgian legislation according to these considerations.

GENERAL CONCLUSIONS

The *in vivo* recovery of embryos from cattle may represent a risk in terms of infection with pathogenic bacteria, viruses, parasites or prions. Obviously, these pathogens may originate from the donor animal, the fluids and media used for recovery of the embryos, and from the environment.

Due to well established, globally accepted sanitary measures during handling, the risk of pathogen transmission may be significantly reduced. However, in order to improve the confidence and quality of the manipulations and to further reduce the possible contamination with pathogens, the donor should be selected from countries, regions or herds that have been shown to be free of specific pathogens. If this information is not available, the donor should be tested individually. Officially accepted tests that appear in the manual of diagnostic tests and vaccines for terrestrial animals (http://www.oie.int/eng/normes/mmanual/A_summry.htm) maximally guarantee the absence of specific pathogens. Also fluids, media and other material should be certified free of pathogens. The use of antibiotics is also a generally accepted procedure in this context. Finally, specific effort should go to the aseptic environment and clean handling during *in vivo* recovery.

It should be noted that professional workers (farmers, veterinarians and officials of the veterinary services) should adhere to a professional scientific awareness about the veterinary public health aspects of the bovine embryo transfer.

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Annex 1. Quality control which is required for the official recognition of the embryo transfer team (Ministry of Small Enterprises, Trade and Agriculture, 1992)

Chapter IV : Quality control

Article 6:
Samples of flushing media [A], of washing media [B] and of degenerated and unfertilized oocytes [C] which have been obtained while performing embryo transfer activities will be transferred on a yearly basis by the team to a certified diagnostic laboratory for investigation according to stipulations in addendum III. Costs will be charged to the team.

Addendum III:
1. Sampling
- Flushing fluids : must be kept in a sterile recipient
- Washing fluids : media of three last washes of embryos from a single donor must be mixed and preserved
- Degenerate embryos and unfertilized oocytes of a single donor animal must be put together, washed ten times and preserved

2. Preservation of samples
All aforementioned samples must be kept in liquid nitrogen at a temperature of -196°C
3. Organisation of quality control
- Each year
- By an authorized laboratory
- Random sampling among all embryo harvests

4. Interpretation of the results
- Quantitative bacteriological analysis for [A] and [B]:
  Less than 5 CFU (colony forming units) : 0
  Between 5-100 CFU : +
  Between 100-1000 CFU : ++
  Between 1000-10 000 CFU : +++
  More than 10 000 CFU : ++++
- Qualitative bacteriological analysis for [A] and [B]
- Qualitative analysis for *Mycoplasma* for [A] and [B]
- Virological analysis for BoHV1 and BVDV for [B] and [C]
- Results of the analysis *
  Favourable result :
  mixture of three washes [B] has to be sterile
  no virus may be present in washing fluid [B] and embryos [C]
  some contamination in the flushing fluid [A] is acceptable but not systematically
  All other results are unfavourable and will require a second analysis, which will lead in case of confirmation of the unfavourable results, to suspension of the licence of the collection and transfer team

* On the basis of above mentioned bacteriological and virological criteria.

SHORT COMMUNICATIONS

COW IN VITRO OOCYTE MATURATION

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The oocyte maturation in vitro is a critical step in the production of in vitro embryos. It is the third biotechnology generation, widely spread in the word but remains relatively unknown in Algeria.

This work aims to introduce this technique for the first time in Algeria and will lead us to master the in vitro fertilization technique in cattle.

Our goal is to study the oocytes maturation variation rate according to the culture media composition, including the addition of hCG effect in a basic media supplemented with foetal calf serum (SVF), glutamine and antibiotic.

The ovaries are taken from uteri cows collected at the slaughterhouse and transported in an insulated bag maintained at a temperature of 30 to 35 ° C. They were then delivered within two hours after slaughter at the molecular biology laboratory of the Pastor Institute of Algeria where they were picked up with a needle G18 mounted on a syringe. After being classified, the harvested oocytes were put in a media in an oven at 5% CO2.
Three medium were used, the first containing: M199 + SVF + glutamine + antibiotic, the second: MEM + SVF + glutamine + antibiotic, the third one: first medium + hCG. All manipulations were made in a laminar flow hood.

The overall maturation rate was 57.02%, enriched with hCG to reach a maximum of 63.64%.
According of the type of media used, the maturation rate is for:
Media 1 (TCM 199 + SVF): 54.92%; Media 2 (MEM + SVF): 50%; Media 3 (Media 1 + hCG): 60%.

In 2002 Bryuere obtained a rate of 67.81%; This difference can be explained by the absence of certain enrichment factors as growth factors which increase the growth of cumulus (Neufeld et al. 1987), or gonadotropins, which induce maturation cytoplasmic (Calder et al. 2003).

Keywords: mature oocyte, in vitro, cow, culture media, embryos.

Notes

EFFECT OF PARITY ON PREGNANCY ASSOCIATED GLYCOPROTEIN (PAGS) LEVELS MEASUREMENT BY RADIOIMMUNOASSAY IN BOVINE. PRELIMINARY RESULTS

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Pregnancy-associated glycoproteins constitute a large family of molecules specifically expressed in the outer epithelial cell layer of the placenta in eutherian species. The detection of these placenta-secreted proteins in maternal peripheral blood can be used for early pregnancy diagnosis in cattle from Day 28 of gestation onwards (Zoli *et al.*, Biology of Reproduction, 1992, 46, 83-92). The present study was undertaken to explore the effect of parity on PAG concentrations during the first trimester of pregnancy by use of homologous and heterologous RIA systems.

Thirty-seven Holstein Friesian females (4 nulliparous, 12 primiparous and 21 multiparous) with mixed age were followed at different periods after artificial insemination: Days 21, 30, 45, 60 and 80. The day of AI was considered as Day 0 for calculation of pregnancy day. Blood samples were removed from the coccygeal vein into tube containing EDTA. The plasma was obtained by centrifugation (1,500 x *g*, 15 min) immediately after collection and stored at -20 °C until assay. In all RIA systems, 67 kDa PAG preparation was used as tracer (labeled with ^{125}I according to the Chloramine T method) and as standard. Five antisera were raised in rabbits against different PAG preparations according to the technique of Vaitukaitis. Plasmatic PAG concentration was measured by radioimmunoassay technique with some modifications (Ayad *et al*., Reproduction in Domestic Animals, 2007, 42, 433-440). PAG concentrations tended to be higher in primiparous than in nulliparous females. On the other side, PAG concentrations were equal or higher in primiparous than in multiparous cows (excepting at Day 80 by using RIA-809). However, due to the small number of pregnant females in the three groups, differences could not be considered as significant.

In conclusion, parity had no significant effect in plasma PAG level. Further investigations are to be carried out in order to better understand PAG secretory profiles according to parity status.

Notes

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QUANTITATIVE ASSESSMENT OF TRANSCRIPTS ENCODING PROTEINS CONTRIBUTING TO MATURATION ABILITY OF OOCYTES IN MORPHOLOGICALLY VARIED GROUPS OF PORCINE CUMMULUS OOCYTE COMPLEXES

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Gap junctions are collections of intracellular membrane channels and are composed of connexins. The connexins are members of a large family of more than 20 proteins, which participate in communication between oocytes and the complex of cummulus cells. Therefore, connexins play a significant role in process of oocyte maturation. The cyclin-dependent kinases (CDKs) are involved in cell cycle progression. CDKs are associated with activating molecules called cyclins. The activation of CDKs and the subsequent phosphorylation of their substrates regulate the cell cycle.

We decide to evaluate the transcript levels of connexins 43 and 45, CDK 5, CDK 5-regulatory subunit 1 (p35), and CDK inhibitors 1, 2B and 3 (CDKN2B, CDKN1 and CDKN3) in morphologically different groups of porcine oocytes isolated from prepuberal gilts.

Total RNA was isolated from porcine oocytes (n=20) using an RNeasy mini column Qiagen GmbH (Hilden, Germany), treated by DNase I, and reverse-transcribed into cDNA. The quantity of mRNA product in each sample was adjusted to the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA or calculated per 20 oocytes. The classification of cummulus oocyte complex (COCs) morphology was based on the four grade scale proposed by Pujol M *et al*. (2004).

We found an increased level of CDKN 1 transcript in oocytes graded I and II as compared to other groups ($P<0.05$). Furthermore, we observed a higher level of connexin 43 and 45, CDK 5, and p35 mRNAs in oocytes graded I compared to cells graded II, III, IV ($P<0.05$). However, we did not detect any differences in CDKN3 and CDKN2B mRNA contents in these analyzed groups of COCs.

Our results demonstrate that connexins, CDKN 1, CDK 5 and p35 transcript contents may be associated with COCs morphology.

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Notes

EFFECT OF EXOGENOUS LEPTIN ON IN VITRO DEVELOPMENT OF BOVINE EMBRYOS

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Plasma leptin concentration is related to body fatness and level of feeding and plays an important role in reproduction through oocyte maturation and early embryo development. The objective of this study was to analyse the influence of leptin supplementation during *in vitro* maturation (IVM) and *in vitro* embryo culture (IVC) on bovine embryo development. A total of 1343 cumulus-oocyte complexes (COC) were matured in TCM 199 supplemented with 10% FCS, 0, 10 or 100 ng/ml leptin for 24 h. After IVM, COC were fertilized *in vitro*. Groups of 25–30 presumptive zygotes were cultured in 25 µl drops under mineral oil in SOF medium with 3 mg/ml BSA and supplemented with 10% FCS, 0, 10 or 100 ng/ml leptin. Cleavage rate was recorded at 48 h post-insemination and blastocyst yield on Days 6, 7, 8 and 9. Statistical analysis was performed by one-way ANOVA. In embryos cultured without leptin, cleavage rate was significantly lower when COC were *in vitro* matured with 100 ng/ml leptin compared to 0, 10 ng/ml leptin and FCS treatments (P< 0.001) (Table 1). The lowest blastocyst yield was also obtained using 100 ng/ml leptin during IVM (P= 0.055). However, when testing different concentrations of leptin during IVC (IVM 0 ng/ml leptin) no significant effect on blastocyst development was found. Finally, the presence of 10 ng/ml leptin in both IVM and IVC showed similar blastocyst yield than FCS/FCS group and higher than 100/100 ng/ml leptin group (P< 0.002). Furthermore, 10/10 ng/ml leptin and FCS/FCS groups, significantly increased Day 6 blastocyst yield over that obtained with other treatments (P< 0.001). In conclusion, the present study suggests that a high concentration of leptin (100 ng/ml) in both IVM and IVC media is detrimental for the development of bovine preimplantation embryos *in vitro;* however, a physiological concentration (10 ng/ml) during both IVM and IVC had a positive effect on blastocyst formation leading to similar percentages as those obtained with serum.

Table 1. Effect of leptin during IVM/ IVC period on development of bovine embryos *in vitro*

Leptin (ng/ml) IVM/IVC	N	Cleavage Rate n (%)	Blastocyst yield per day n (%)				Total Blastocysts n (%)
			Day 6	Day 7	Day 8	Day 9	
0/0	281	215 (76.5)$_{a, b}$	10 (3.5)$_{c, d}$	34 (12.1)	32 (11.3)	4 (1.4)	80 (28.4)$_{d, e, f}$
10/0	123	100 (81.3)$_{a}$	8 (6.5)$_{c}$	15 (12.2)	10 (8.1)	1 (0.8)	34 (27.6)$_{d, e, f}$
100/0	180	115 (63.8)$_{c}$	3 (1.6)$_{c, d}$	11 (6.1)	14 (7.7)	4 (2.2)	32 (17.7)$_{f}$
0/10	147	120 (81.6)$_{a}$	1 (0.6)$_{d}$	20 (13.6)	11 (7.4)	5 (3.4)	37 (25.1)$_{d, e, f}$
0/100	116	79 (68.1)$_{b, c}$	3 (2.5)$_{c, d}$	12 (10.3)	14 (12.0)	5 (4.3)	34 (29.3)$_{d, e}$
10/10	156	127 (81.4)$_{a}$	19 (12.1)$_{b}$	22 (14.1)	13 (8.3)	2 (1.2)	56 (35.9)$_{d}$
100/100	202	131 (64.8)$_{c}$	1 (0.5)$_{d}$	14 (6.9)	17 (8.4)	8 (3.9)	40 (19.8)$_{e, f}$
FCS/FCS	138	109 (78.9)$_{a}$	24 (17.3)$_{a}$	17 (12.3)	7 (5.0)	1 (0.7)	49 (35.5)$_{d}$

N: Total number of oocytes for IVM/F/C. a, b, c, P< 0.001; d, e, f, P< 0.002.

Notes

THE EFFECT OF METHOD OF CULTURE MEDIA REPLACMENT IN SEQUENTIAL EMBRYO CULTURE ON IN VITRO DEVELOPMENT OF BOVINE BLASTOCYSTS

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In order to recognize differences in the oviduct and the uterus environment and to closer imitate in vivo conditions sequential culture media for mammalian embryos were introduced. However the benefit of the use of such system showed to be conflicting between different studies (Lane M et al, 2003. Theriogenology 60;407-419; Amorim et al, 2008, Reprod Fert and Dev 29;141; Block et al, 2008 Reprod Fert and Dev 29;142). One of the reasons of such inconsistency could be the difference in methods used of fresh media adding or replacement. The objective of this study was to compare continues embryo culture in the same drops of media from Day 2 until Day 8 with 2 methods of fresh culture medium replacement on developmental capacity of bovine embryos. A total of 1164 oocytes (7 replicates) were matured in TCM-199 medium containing 10% FCS and 10 ng/ml of epidermal growth factor and inseminated in Fert-Talp supplemented with 25 mM bicarbonate, 22mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA-FAF and 10 µg/ml heparin with $1x10^6$ ml spermatozoa in group of 50 in 250µl at 39°C. After 24h of oocytes-sperm co-incubation presumptive zygotes were cultured in SOFaa culture medium supplemented with 5% FCS in 30 µl drops in the following groups: Group 1 (control), embryo culture in the same drops (no medium change or added until end of the culture), Group 2, embryos were transferred to fresh culture drops after 96 post insemination (pi) and Group 3, half of the culture drops volume was replaced by fresh media after 96 hr pi. Embryos were culture under paraffin oil at 39 ^{0}C, 5% CO_2 and 5%O_2 in humidified air. Cleavage rates were recorded on Day 2 and on Day 4 (96 hr pi) embryos from group 2 were transferred to fresh culture drops and in Group 3, 15 µl of medium was aspirated from each culture drop and replaced by the same amount of fresh media. The embryos from control groups remained intact and were culture continuously. The numbers of blastocysts were recorded on Days 7 and 8. Embryos development was analyzed by Chi square analysis. The results are shown in Table 1.

Table 1. Effect of the methods of culture media replacement on bovine embryo development.

Treatment groups	Number of oocytes	Cleavage rates Day 2	Total number of blastocysts Day 8
Group 1. Static culture	370	278 (75.1%)	102 (36.7%)[a]
Group 2. Fresh culture drops	404	286 (70.1%)	77 (26.9%)[b]
Group 3. Fresh media added	390	285 (73.1%)	106 (37.2%)[a]

ab - percentages within a rows with values not in common differ (P< 0.05)

It can be concluded that continuous embryo culture from Day 2 until Day 8 of culture or replacement of the half of the volume of culture drops by fresh media at Day 4 produced comparable numbers of blastocysts that were both higher than number of blastocysts developed in fresh culture drops after Day 4 of culture. However, blastocysts in Group 3 were significantly more developmentally advanced than embryos in Group 1 and 2.

Notes

DEVELOPMENTAL KINETICS OF MALE AND FEMALE BOVINE EMBRYOS PRODUCED IN VITRO WITH SEX-SORTED SPERM

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Male embryos are known to develop faster than their female counterparts. Time of first cleavage has been related with developmental competence and differences in kinetics of cleavage between sires have been reported. The objective of this study was to examine the developmental kinetics of male and female bovine embryos produced in vitro using sex-sorted sperm. Bovine cumulus oocyte complexes (n=3342) were matured in vitro and fertilized with X- or Y-sorted sperm from 1 of 3 bulls (5 replicates for Bull 1 (n=X:618, Y:533) and 4 replicates for Bulls 2 (X:445, Y:411) and 3 (X:411, Y:408) or with unsorted semen from Bull 1 (n=516). The resulting zygotes were cultured in vitro and development was assessed at 24, 27, 30, 33, 36, 40, 44 and 48 hpi to establish the evolution of cleavage (Fig.1). Blastocyst development was recorded from Day 6 to Day 9 (Fig.2). Different letters indicate statistically significant differences (ANOVA Holm-Sidak, $p \leq 0.05$). Cumulative cleavage rate and blastocyst yield were significantly higher in the unsorted group when compared with the X or Y sorted group from the same bull. Differences also appeared between bulls at different cleavage time points (Fig.1). No statistically significant differences between bulls appeared at the blastocyst stage, but a tendency towards a relationship between higher blastocyst yield and earlier cleavage was noted. Finally, no differences in cleavage rate and blastocyst yield were observed between sexes when X and Y groups from the same bull were compared, except at 40 hpi for bull3. Nevertheless, a higher cleavage rate and blastocyst yield was obtained in all bulls when Y-sorted semen was used. In conclusion, no differences were observed when using X- and Y-sorted sperm from the same bull in IVF. The use of sorted semen in IVF significantly delayed the first cleavage when compared with unsorted. However, kinetics of cleavage differed significantly between bulls suggesting differences in susceptibility to the sorting procedure between bulls.

Fig.1

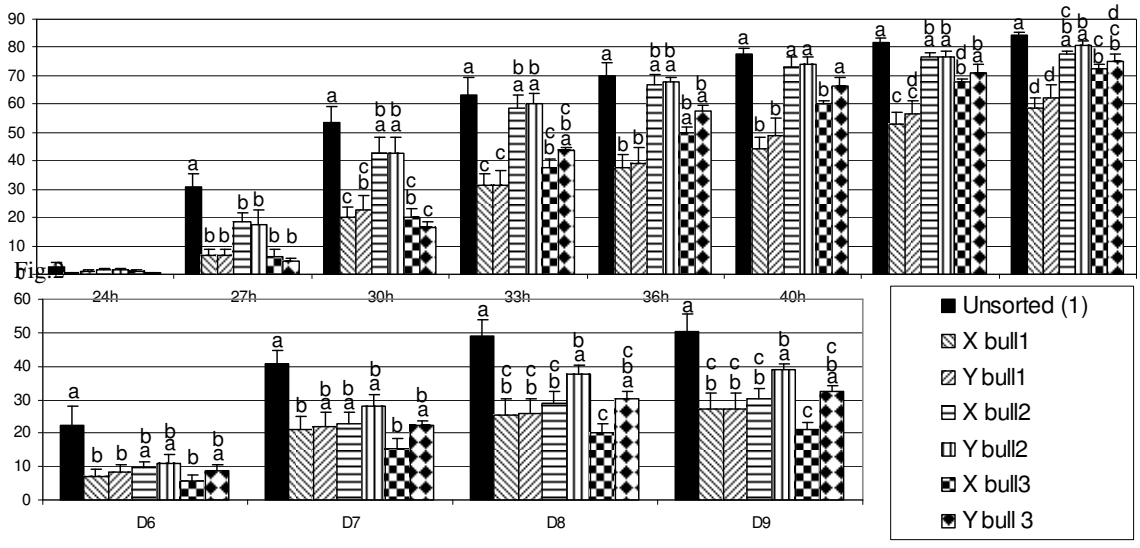

Fig.2

Notes

MEIOTIC SEGREGATION ANALYSIS IN COWS HETEROZYGOUS FOR THE T(1;29) ROBERTSONIAN TRANSLOCATION

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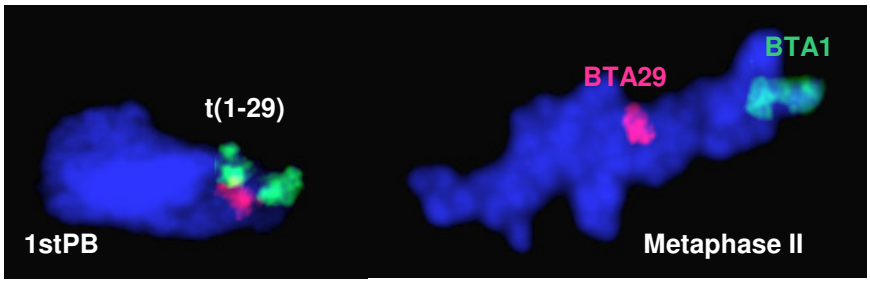
Heterozygotes for Robertsonian translocations have usually a normal phenotype but show variable decrease in fertility. In humans, meiotic segregation studies carried out in male carriers reveal a majority of balanced spermatozoa. The rate of unbalanced segregation products appears higher in females than in males, but the number of human oocytes analysed so far remains limited (about 100). The occurrence of the t(1;29) translocation in several cattle breeds makes bovines an interesting animal model to study the meiotic segregation in female carrier of Robertsonian translocations. The aim of our project is thus to assess the rates of unbalanced oocytes produced by four cows and to compare these values with those obtained in bulls carriers of the same translocation. The superstimulated cows were subjected to twenty ovum pick up (OPU) sessions. Dual colour whole chromosome painting probes for BTA1 and 29 were hybridized on oocytes preparations (figure below).



A total of 817 oocytes have been punctured and 770 matured *in vitro*. Among the 597 oocytes successfully spread and used for the FISH procedure, 394 (66%) produced FISH results. Out of these 394 oocytes, 292 (74.11%) were balanced including 152 “translocated” and 140 “normal”, 86 (21.82%) were diploid and 16 (4.06%) unbalanced. There are little more “translocated” than “normal” oocytes but this difference is not statistically significant. There is neither significant departure from 1:1 ratios for the adjacent categories (nullisomy 29 vs disomy 29 or nullisomy 1 vs disomy 1). The average diploidy rate is higher than expected. Indeed, one cow mainly contributes to this value with 45% of diploid oocytes while the diploidy rates of the other cows ranged from 7.7 to 17% which is more consistent with the 10.7% of diploidy described in the literature. As already described in several humans studies, we found cases of premature separation of sister chromatids (PSSC) which lead to unbalanced oocyte with one extra chromatid.

The percentage of balanced gametes (74.11%) in females is lower than the proportion of balanced spermatozoa (97.21%) observed for two heterozygous bulls. This difference is primarily due to the higher rate of diploid gametes in females (21.83%) compared to males (0.04%). Human data show also that female meiosis produce more diploid gametes (5.4 to 10.1%) than male meiosis (0.03 to 0.085%). If diploids are not taken into account, we obtain 94.8% of balanced oocytes. The rate of unbalanced gametes (5.19%) in females heterozygotes for the Robertsonian translocation t(1;29) is still significantly higher (p-value<0.05) than in males (2.75%). In the case of the human Robertsonian translocations t(13;14) and t(14;21), Munne et al. (2000) assessed a higher difference between males and females (33% vs 25% and 42% vs 12% respectively).

Finally, the percentage of unbalanced oocytes (5.19%) is lower than expected from previous studies of carrier cows. To our knowledge, this is the first time that such kind of study is carried out on females heterozygous for a Robertsonian translocation.

Notes

REPEATED OPU ON FOUR BLONDE D'AQUITAINE COWS: VARIATION IN THE STIMULATION RESPONSE AND IN THE RATE OF DIPLOID OOCYTES AFTER *IN VITRO* MATURATION.

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In order to assess the percentage of unbalanced oocytes produced by heterozygous cows for the Robertsonian translocation (1;29), 21 ovum pick up (OPU) sessions have been carried out after FSH ovarian stimulation (Stimulfol® -ULg FMV) on four donors in a every two weeks scheme. Punctured cumulus oocytes complexes (COC) from each cow were cultured *in vitro* separately (for details see Bonnet-Garnier et al., *Cytogenet Gen Research* 2008 120(1-2):91-96). A total of 923 follicles were punctured, 817 (89%) COC were recovered and 770 (94%) were *in vitro* matured. The average number of COC retrieved for each cow over the 21 OPU sessions ranged from 5.62 to 13.24. Among the matured oocytes, 394 were analysed by fluorescent *in situ* hybridization (FISH) to examine their genetic content (haploid vs. diploid) as described by Bonnet-Garnier et al (2008).

The follicular response as well as oocyte retrieval was very high in the four first sessions. The total numbers of punctured follicles and collected COC then decreased during further sessions. This tendency was also observed for individual donors (data not shown). We observed large differences in average number of oocytes retrieved and percentage of diploid oocytes between donor cows. Indeed the two older cows (n° 2 and 3) were low responders to gonadotrophin stimulation, as compared with the two others. Nevertheless, among these last two cows, Cow 4 displayed a high rate of diploid oocytes (45%) as compared with the three others (12% in average) and to the literature (about 11.5 %). This cow can be described as a high responder but a donor of low quality oocytes for *in vitro* embryo production. As the COC of these two cows have been matured in the same conditions, we can speculate that individual factors related to oocyte ability to achieve the meiotic process could explain such difference. In addition, this unexpected result could at least partly explain why for some individuals few embryo numbers are produced in vitro, even if (as this cow – data not shown) large numbers of grade 1 and 2 immature COC are collected.

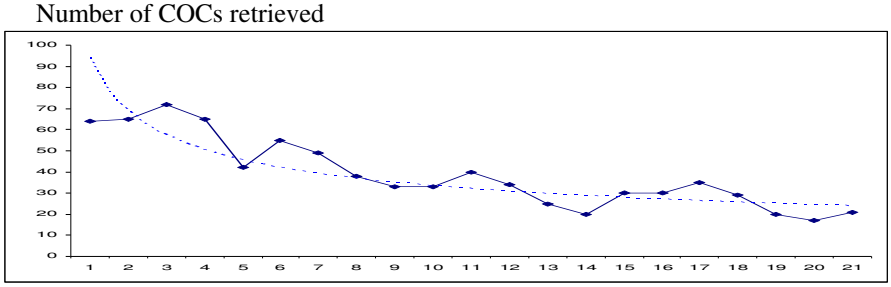

Figure 1: Evolution of the number of COCs retrieved toward the OPU sessions - $R^2 = 0,743$

Table 1: Numbers of follicles, COCs and oocytes obtained per cow.

Cows	Cow 1	Cow 2	Cow 3	Cow 4	Total
Age	*5*	*11*	*10*	*5*	
Follicles aspired	*295*	*185*	*142*	*301*	*923*
Collected COCs	278	160	118	261	817
Mean (+/- SD) number of COCs per session	13.24(+/- 4.01)	7.62 (+/- 5.45)	5.62 (+/- 4.06)	12.43 (+/-5.93)	38.90 (+/- 16.23)
Cultured COCs	264	155	113	238	770
Oocytes analysed by FISH	135	78	59	122	394
Diploid oocytes	15	6	10	55	86

Notes

ASSESSMENT OF OVIDUCT PERMEABILITY IN A SUPEROVULATED COW THAT FAILED TO PRODUCE EMBRYOS

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Superovulated cows are occasionally encountered that consistently fail to produce embryos, despite a superovulatory ovarian response.

A 6-year-old on-farm Prim'Holstein cow exhibited inexplicable repeat breeding. Because of her high genetic value, she was brought to the genetic station of Denguin (France) in August 2006 and was superovulated by pFSH treatment (Stimufol ®) three times during the year. No embryo was recovered at any time despite the superovulatory response.

We hypothesized that an obstruction of the oviducts might prevent transport of the embryos to the uterus. To confirm this hypothesis, we performed a phenol sulphonephthalein (PSP) test during the luteal phase to investigate fallopian tube patency.

This PSP test involves deposition of a dye solution into the uterine horn. The dye spreads from the uterus to the peritoneum through the normal oviduct and is excreted in the urine within 10 to 20 minutes. In the case of oviduct occlusion, the dye appears in the urine more than 20 minutes after PSP administration.

The bladder was catheterised and emptied of urine, and a PSP solution infused into the uterine lumen. Each oviduct was investigated separately. Urine was collected prior to dye instillation, then 8 to 40 minutes later and alkalinised. The permeability of the right oviduct and occlusion of the left oviduct were evidenced.

In conclusion, the repeat breeding and absence of embryo could be partly explained by the alteration of oviduct permeability.

The prevalence of an occluded uterine tube, based on post-mortem findings, can attain 5 % although approximately half the oviducts are macroscopically normal (JAVMA 1978, 172 :1308). The PSP test is a sound and practical method for diagnosing fallopian tube obstruction. It could also be used to restore permeability when oviduct blockage is due to accumulated debris or mucus rather than to permanent fibrous adhesions resulting from salpingitis.

Notes

RETROSPECTIVE STUDY OF FACTORS OF VARIATION OF THE SUPEROVULATION RESPONSE IN MIDATEST EMBRYO TRANSFER TEAM

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The average number of embryos produced in embryo transfer programs has changed little over the past two decades. The significance of experimental studies involving a small number of animals is hindered by high variability both within and between cows in response to superovulatory treatment.

Data compiled by the Midatest Embryo Transfer Team (Denguin, France) from 1984 to 2007 and including 6358 embryo collections were retrospectively analysed. The influence of five factors (breed, parity, reference estrus, association of progestagen with FSH, number of AI) and their interactions on embryo production were analysed using a general linear model (GLM) (Table 1). No significant interaction was obtained for the number of transferable embryos whereas threefold or twofold interactions between heat, parity and treatment were observed for total embryos. The factors included in the GLM explained only 2.5 % of the variability in embryo production.

Table 1: Influence of five factors on transferable embryos (mean ± SD) in donor cows.

Factor	Mean ± SD of transferable embryos (number of donors per group)	
Breed, p=0.02	Dairy cattle (n=3870) : 4.93 ± 4.78	Beef cattle (n=2488) : 6.02 ± 5.62
Parity, p<0.05	heifers (n=2453) : 4.74 ± 4.23	cows (n=3905) : 5.75 ± 5.62
Reference estrus, p=0.6	Natural (n=5143) : 5.32 ± 5.08	Induced (n=1215) : 5.51 ± 5.44
FSH treatment, p=0.02	alone (n=5174) : 5.51 ± 5.26	with progestagen (n=1184) : 4.72 ± 4.6
AI, p=0.7	1 AI (n=890) : 5.32 ± 5.48	2 IA (n=5468) : 5.37 ± 5.1

These results are in general agreement with those of most studies (Anim Reprod Sci 1997,48:137) which report enhanced superovulatory responses in beef cattle, compared to dairy cattle and in cows compared to heifers. Embryo production was unaffected by AI number or oestrus reference type. The relevance of the treatment effect should be interpreted with caution because poor donors were more frequently included in the FSH group associated with progestagen.

The influence of ablation of dominant was analyzed by a paired t-test in a subpopulation of 82 donors recovered at least twice within a year, and in which the dominant follicle was ablated or not, prior to FSH stimulation. For the paired data, a greater number of total embryos was recovered in the ablated group (mean ± SD, 9.8 ± 6.9 vs 7.7 ± 6.1, P<0.001), and the number of transferable embryos tended to be higher in the ablated group (5.0 ± 4.8 vs 4.1 ± 3.9, P=0.1), in accordance with previous results (Theriogenology, 2000, 53:1521).

These results highlight the interest and limits of field experiments performed with a large number of animals but under non-controlled conditions which precludes a consideration of environmental factors.

Notes

HISTONE H3 LYSINE 27 TRI-METHYLATED LINKED TO INACTIVE X-CHROMOSOME IN IN VITRO FERTILIZED AND CLONED BOVINE EMBRYOS

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To equalize the dose of X-linked genes in male and female, one of the two X-chromosome is transcriptionally silenced in mammalian female cells. The X-chromosome inactivation takes place during early embryonic development and consists in chromosome-wide establishment of facultative heterochromatin. In trophectoderm cells, the inactivation process is under a parental imprint: the paternally inherited X-chromosome is preferentially inactivated in these cells. Considering cloning procedure by nuclear transfer of an adult somatic nucleus the X chromosome status will be different. The parental inherited information is lost in clones and the donor nucleus has one active X chromosome and one inactive X chromosome. Surprisingly, even in the absence of parental imprint, there is still a bias of inactivation in trophectoderm cells of clones able to survive in mouse and bovine. More precisely, the inactive X-chromosome from the donor cell nucleus is the inactive X-chromosome in the trophectoderm cells. These results suggest that, through remodelling process, a mark is maintained to provide an epigenetic memory of the initial X-chromosome status in the donor cell nucleus. We choose to study the histone H3 lysine 27 tri-methylated (H3K27me) mark in embryos produced by in vitro fertilization (IVF) and cloning to determine whether or not it could be the remaining mark that leads to the bias of inactivation in the clones trophectoderm.

In vitro fertilized and cloned bovine embryos are produced in the laboratory. After fertilization and cloning, the embryos are cultured in vitro. We fixed embryos at different stages and then performed immunocytochemistry with H3K27me3 antibody. The H3K27me3 mark linked to the inactive X-chromosome is seen as a large domain in female nucleus. The embryos analysis is performed in confocal microscopy.

Our results show that H3K27me3 is associated to inactive X-chromosome in bovine and we can detect it in 30% of the donor cell population. In IVF embryos, H3K27me3 linked to inactive X-chromosome appears at blastocyst stage (Day 7). In cloned bovine embryos, the H3K27me3 mark seems to be present between one-cell stage and 8-cell stage and then the rate of blastomeres presenting the mark decreased. At blastocyst stage, the rate of blastomeres with the H3K27me3 mark increases and is similar to the rate observed in IVF blastocyst embryos.

Through remodelling process, epigenetic marks can be maintained. Decreased of the rate of blastomeres with the mark H3K27me3 in cloned embryos allow us to suggest that H3K27me3 disappeared in a stepwise manner through the remodelling process.

Notes

USE OF A RAPID LATERAL FLOW DEVICE (WITNESS® LH) TO DETECT BOVINE LUTEINIZING HORMONE

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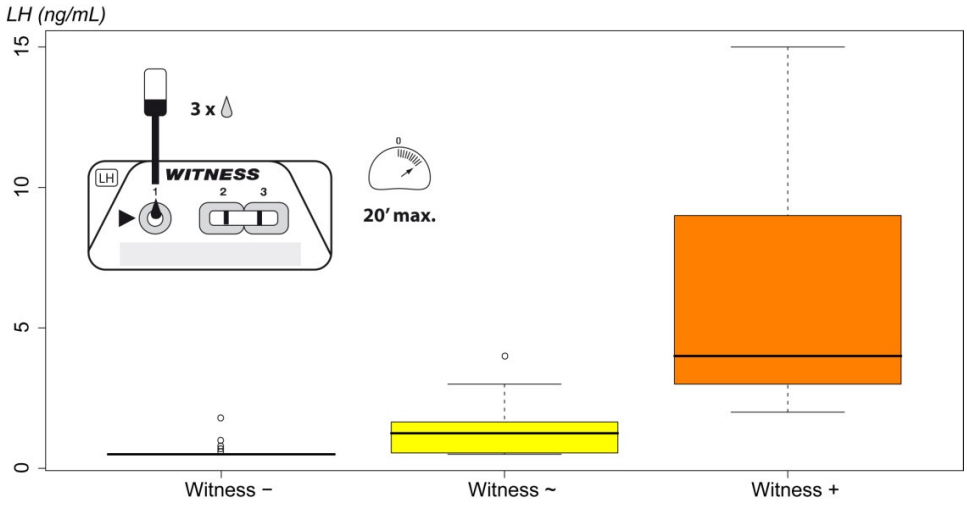
Estrus detection has always been a key factor for the fertility results. Advisors used to stress that watching the cows more carefully would improve the detection rate. However, in recent years it has become evident that cows do not express their estrous behavior as intense as reported in older studies. Intervals from estrus or LH surge to ovulation are now considered the best markers to predict ovulation time. But the onset of LH peak seems to be a more precise indicator. Until recently, prediction of ovulation by LH assay was not reliable on the field, due to time consuming techniques and requirement for expertise of the technician people. SYNBIOTICS did recently introduce an "in-house" kit (Witness® LH), originally designed to detect LH in dogs and cats. This lateral flow device allows rapid detection (20 min) of LH from serum samples and is simple to perform. The rationale for this study was first to confirm the feasibility of the test with bovine serum samples, second to estimate the correlation between Witness® LH results and reference quantitative assay.

Eight cyclic non-lactating cows were synchronized with a double injection of $PgF_{2\alpha}$ 11 days apart and checked for estrous behavior during 5 days. Ultrasound examinations were carried out daily to follow ovarian activity. Blood samples (10 mL) were collected on vacuum tubes every 6 hours starting the day after injection of $PgF_{2\alpha}$. Samples were allowed to clot, centrifuged (3000 x *g*, 10 min), and aliquots were stored at –20°C until assay. Plasma LH concentrations were determined by ELISA technique using LH Detect® (INRA, France).

Witness® LH results were classified as negative (no LH detected), intermediate (test line lighter or similar to control), or positive (test line darker than control). The figure shows a boxplot of LH concentrations pooled by Witness® LH results. The overall correlation coefficient (Spearman's rank) between LH concentration and Witness® LH result was ρ=0.738. A pairwise comparison using *t* test did confirm that Witness® LH results cannot be misinterpreted (p=0.019 for negative results compared to intermediate results, p<0.001 for positive results compared to any other class). The LH results (Witness® LH and LH concentrations) were in accordance to previous descriptions reported into the literature as well (*eg*. timing of follicular events, estrous behavior or ultrasonographic observations).

These results confirm Witness® LH can be used for rapid detection of LH peak in bovine serum samples. It may allow better determination of the timing of ovulation and optimize the timing of IA in bovine breeding programs.



Notes

ASSESSMENT OF SPERM APOPTOSIS USING FLOW CYTOMETRY IN DIFFERENT AGE GROUPS OF DOGS

BUKOWSKA D.[1], KEMPISTY B.[2], SIKORA J.[3], BUDNA J.[2], ANTOSIK P.[1], LIANERI M.[2], JAŚKOWSKI J.M.[1], WIKTOROWICZ K.[3], JAGODZIŃSKI P.P.[2]

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We evaluated the level of apoptosis and necrosis in swim-up purified spermatozoa isolated from dogs of various age and using varying times of spermatozoa incubation in Sperm-TALP (Tyrode's albumin lactate pyruvate) medium *in vitro*.

Dog semen samples were collected from three groups, each comprised of six animals, aged 0.5 – 1.5, 6 – 8, and 11 – 13 years, respectively. Spermatozoa were then purified by swim-up technique and incubated for 15, 30, and 45 min in Sperm-TALP medium. To evaluate apoptosis/necrosis levels in dog semen, the spermatozoa were stained with annexin V-fluorescein isothiocyanate (FITC)/propidium iodine (PI) and analyzed by flow cytometry and fluorescence microscopy assays.

We observed a significantly higher number of apoptotic/necrotic cells in swim-up purified spermatozoa from ejaculates of the oldest dogs as compared to the youngest animals ($P<0.05$). Moreover, we also found significantly higher numbers of apoptotic/necrotic spermatozoa after 30 and 45 minutes of incubation in the oldest dogs compared to the youngest animals.

Our observations suggest that dogs of advanced age exhibit an increased number of apoptotic/necrotic spermatozoa and an increased apoptotic susceptibility of sperm, which may reflect their reduced reproductive potential.

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Notes

EFFECT OF EXOGENOUS PROGESTERONE IN VITRO ON THE QUALITY OF BOVINE BLASTOCYSTS ON DAY 7 AND DAY 14

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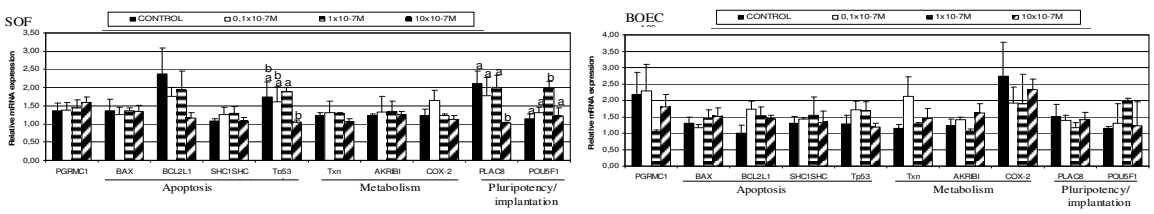
Progesterone (P_4) is the key hormone responsible for maintenance of pregnancy in mammals and circulating levels in the early post conception period have been associated with pregnancy success. It is not clear whether P_4 acts directly or indirectly on the embryo to alter gene expression and development. The aim of this study was to examine the effect of addition of P_4 to culture medium (i) on the relative abundance of genes related to quality in blastocysts and (ii) on the survival and morphological appearance of embryos on Day 14 following transfer to synchronized recipients. In Experiment 1, blastocysts were produced by IVM/IVF, and IVC either in SOF alone or in co-culture with bovine oviduct epithelial cells (BOEC) in the absence or presence of P_4 at one of three concentrations (0.1, 1 or 10 x 10^{-7} M). Day 7 blastocysts from each group were snap-frozen in LN_2 in groups of 10 for mRNA extraction. Quantification of transcripts for progesterone receptor membrane component 1 (PGRMC1), BCL2-associated X protein (BAX), BCL2-like 1 (BCL2L1), Src homology 2 domain containing transforming protein 1(SHC1SHC or $P66^{shc}$), tumor protein p53 (Tp53), genes related to apoptosis, thioredoxin (TXN), aldose reductase mRNA (AKRIBI), prostaglandin G/H synthase-2 (PGHS-2 – COX-2), genes related to metabolism, placenta-specific 8 (PLAC8), related to implantation and POU class 5 homeobox 1 (Oct3/4 – POU5F1) related to pluripotency was carried out by real time quantitative RT-PCR. In Experiment 2, blastocysts produced by IVM/IVF and IVC in SOF alone or in the presence of P_4 at a concentration of 10 x 10^{-7} M were transferred in groups of 20 to the ipsilateral uterine horn of synchronized recipients (n=6 recipients/group). On Day 14 (Day 0=day of fertilization/day of oestrus) animals were slaughtered and the embryos were recovered by flushing the uterus. The recovery rate and the dimensions of all embryos were recorded and embryos individually were snap-frozen in LN_2 for further studies. Data on differences in transcript abundance were analysed by ANOVA and embryo size on Day 14 by t-test. In experiment 1, the relative abundance of only 3 of the genes tested varied significantly between groups but these differences were not apparent when culture was with BOEC (see figure below). For experiment 2, the recovery rate of embryos on Day 14 was similar for both groups (SOF:65.0±2.2 % vs SOF+P4:65.0±9.3%). Embryos derived from culture in the presence of progesterone tended to be larger, on Day 14 ($P \leq 0.066$) (Length: 4.02±0.8 mm; Width: 0.78±0.07 mm) than embryos cultured in SOF alone (Length: 2.70±0.4 mm; Width: 0.58±0.04 mm). These results suggest that exogenous P4 during in vitro culture does not have a major effect on transcript abundance in Day 7 blastocysts but may influence post-hatching elongation.



Notes

NOCODAZOLE- AND DEMECOLCINE-ASSISTED ENUCLEATION IN MOUSE OOCYTES

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Chemically-assisted enucleation has been performed in porcine, bovine and, more recently, in goat to successfully produce cytoplasts for nuclear transfer (NT). In our study, we adapted this technique to enucleate mouse oocytes. The enucleation efficiency, amount of cytoplasmic volume eliminated and distribution of spindle associated γ-tubulin in the cytoplasts and karyoplasts produced were compared to the standard mechanical enucleation method. Moreover, spindle morphology after reconstruction of the cytoplasts by NT was also compared between both methods.

Outbred CD-1 and hybrid B6CBAF1 oocytes were collected at 13-14h post-hCG. In chemically-assisted enucleation experiments, oocytes were treated with either 0.4 µg/ml demecolcine (DEM) or 0.3 µg/ml nocodazole (NOC) in KSOM medium for 30 min. Induced protrusions were easily identified both in CD-1 (92.2%, n= 695) and B6CBAF1 (83.3%, n= 370) oocytes and were then aspirated with a micropipette attached to a piezo-drill actuator, in drops of H-KSOM containing 2.5 µg/ml cytochalasin B and 0.05 M sucrose. Enucleation efficiencies were higher than 90% in both strains (90.1%, n= 403 CD-1; 90.4%, n= 260 B6CBAF1), although they were significantly lower than the obtained by mechanical enucleation in non-treated CD-1 (98.4%; n= 126; P= 0.005) or B6CBAF1 (97.8%, n= 137; P= 0.011) oocytes. The cytoplasts prepared by chemically-assisted enucleation showed a lower reduction in cytoplasmic volume than those prepared by mechanical enucleation (2.1% and 3.9%, respectively; P<0.0001). Both enucleation protocols resulted in the elimination of spindle-associated γ-tubulin from the cytoplasts, even though chemically-assisted enucleation avoided the removal of the spindle microtubules, as confirmed by immunofluorescence analysis of γ-tubulin, microtubules and DNA in the cytoplasts and karyoplasts produced. The same staining protocol was used to analyse enucleated oocytes reconstructed by NT with a cumulus cell nucleus and fixed at 2 or 4h post-NT.

Reconstructed CD-1 cytoplasts displayed a metaphase spindle by 2h post-NT (55.9-79.5%). Most spindles were bipolar in the cytoplasts prepared by mechanical and NOC-assisted enucleation (78.9% and 81.8%, respectively), but monopolar in DEM-assisted enucleated oocytes (61.3%). In B6CBAF1, the percentage of reconstructed oocytes displaying a spindle was higher than in CD-1 oocytes when cytoplasts were prepared by mechanical or NOC-assisted enucleation (84.6-87.5%). As in the CD-1 strain, only a low percentage of the spindles in B6CBAF1 oocytes reconstructed from DEM-assisted enucleated cytoplasts were bipolar at 2h post-NT (29.1%). However, when the culture was extended to 4h post-NT, the proportion of bipolar spindles increased in comparison to equivalent groups fixed at 2h post-NT in both strains (55.6-60%) and was similar to the mechanically-enucleated group (81.8-90%).

In conclusion, chemically-assisted enucleation can be efficiently used in the mouse, allowing the removal of the oocyte chromosomes with a smaller amount of cytoplasm than when mechanical enucleation is performed. Cytoplasts produced by NOC or DEM-assisted enucleation are able to form a morphologically normal spindle after NT, just as those produced by standard mechanical enucleation.

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Notes

THE EFFECT OF SERUM OR CYSTEAMINE DURING IN VITRO MATURATION ON THE SENSITIVITY TO LIPID PEROXIDATION IN BOVINE CUMULUS OOCYTE COMPLEXES

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The production of reactive oxygen species (ROS) is a normal process during cell metabolism. ROS, however, cause lipid peroxidation, wich usually implicates cell damage and thus impaired oocyte developmental capacity. Cysteamine supplementation during *in vitro* maturation (IVM) significantly improves embryo development by stimulating glutathione (anti-oxidant) synthesis and therefore can be used to prevent ROS damage to the embryo (de Matos *et al*; 2000). It is also known that cumulus cells play an important role in oocyte glutathione synthesis during IVM (Cetica *et al*; 2001).

The aim of the present study was to evaluate the effect of adding serum or cysteamine to maturation medium on the sensitivity to lipid peroxidation of oocytes and cumulus cells.

After puncturing 2-8 mm sized follicles of slaughterhouse ovaries, only grade I cumulus oocyte complexes (COCs) were selected for IVM. In the first experiment the effect of adding serum to the maturation system was tested. The COCs were matured in maturation medium with 20% serum (n= 196) or in serum-free maturation medium supplemented with 20 ng/ml mEGF (epidermal grouth factor, n= 193). In a second experiment, 957 COCs were placed in maturation medium supplemented with 20 ng/ml mEGF with (n=492) or without (n=465) 0.1 mM cysteamine. In the third experiment 360 COCs were matured in serum containing medium with 20% serum with (n=184) or without (n=176) 0.1 mM cysteamine. After 24 hours of maturation, the COCs were stripped in 0.1% hyaluronidase. Oxidative stress was induced in the denuded oocytes and, separately, in the detached cumulus cells, by incubating both in the presence of 20 mM ascorbic acid and 4 mM ferrous sulfate for 1.5 hrs at 37°C (oocytes in groups of 80-95, cumulus cells per 1 million/ml). Subsequently, the concentration malondialdehyde (MDA, end product of the lipid peroxidation), was spectrophotometrically analysed through a detection of the levels of tiobarbituric acid reactive substances (TBARs). A pink chromogen was formed after the reaction of 1 molecule MDA and 2 molecules tiobarbituric acid (TBA). A negative control group without oxidative stress induction was included in the analyses. MDA concentrations were compared non-parametrically and are expressed in µg/l MDA produced per oocyte or per 1 million/ml living cumuluscells.

The incubation of oocytes or cumulus cells significantly increased (>53%) the MDA production. Oocytes matured in the presence of serum showed a similar sensitivity to oxidative stress induction compared to those cultured in serum-free medium. The latter cumulus cells, however, were prone to produce a higher amount of MDA compared to cells from serum containing medium ($P < 0.05$). Neither in a serum containing, nor in a serum-free maturation system, the addition of cysteamine changed the sensitivity of the oocyte nor cumulus cells to the induction of oxidative stress. Furthermore, MDA production in oocytes and cumulus cells was correlated ($r = 0.68$; $P< 0.05$).

These findings suggest that a serum-free maturation system or adding cysteamine in the maturation medium does not reduce lipid peroxidation in terms of MDA production in COCs. Only cumulus cells matured in a serum containing medium showed a reduced sensitivity to oxidative stress induction.

Notes

DONOR AND RECIPIENT EFFECTS ON SUCCESS OF FROZEN OVINE EMBRYO TRANSFER

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Pregnancy rates from embryo transfer (ET) vary widely. Variation in donor factors, recipient suitability and breed, the number of corpus luteum and number of transferred embryos per recipient are thought largely responsible for variation in observed success (Emsen and Gimenez Diaz 2006). Donor effects on pregnancy rates have been studied most and reviewed recently (Seidel 1984), but there is considerably less literature on recipient factors affecting ET pregnancy rates in sheep.

The fertility rate of fat-tailed ewes used as recipients ewes in frozen thawed embryo transfer program were evaluated by investigating factors intrinsic to donor and recipient. Estrus was synchronized by means of fluorogestone acetate (FGA) intravaginal sponges and PMSG (400 IU) was used. Recipients received two frozen thawed embryos via semi laparoscopy technique 6 days following the onset estrus. While pregnancy rate was found significantly ($P<0.0001$) higher (67%) in recipients showed estrus 60 h after sponge removal compare to those estrus occurred 36h (33%) and 48 h (31%) after sponge removal. The breed of recipients, the number of corpus luteum and the transfer number of embryos had no significant effect ($P>0.05$) on subsequent fertility rate. Pregnancy rate varied between 22 % to 78 % for eight embryo donors and pregnancy rates differed significantly ($P<0.001$) among donors. These preliminary results show that, the time to estrus following the synchronization program, donors have played an important role in fertility of recipient ewes.

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Notes

IN VITRO ASSESSMENT OF THE EFFECTIVENESS OF A COMMERCIALLY AVAILABLE POST-THAW BOVINE SEMEN SEXING KIT ON SEMEN QUALITY PARAMETERS, *IN VITRO* FERTILIZING ABILITY AND SEX-RATIO DEVIATION

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The use of sexed bovine semen is getting more and more popular among farmers. Up today, the flow cytometry sorting technology has been the most efficient and several suppliers are marketing bull sexed semen originating from different countries. Alternative processes, currently proposed to breeders, are presented as effective, reliable and often cheaper but are often not well enough documented. This study aimed to assess *in vitro* the effectiveness of the BullPlus, HeiferPlus processes (Emlab genetics ®) both in terms of semen quality, fertilising ability and sex-ratio deviation.

The semen of 3 bulls whose in vitro fertility was known was used in the experiment. For each bull, 3 straws of the same ejaculate were thawed and incubated for 20 mn at 35°C. Two of them were treated respectively according to HeiferPlus and BullPlus procedures and the third one was not treated and used as a control.

Semen quality was assessed by phase contrast microscopy (motility and % motile sperm), by flow cytometry (viability) and by CASA (Ivos, Hamilton Thorn). Fertility was estimated *in vitro* (3 replicates per bull). The sex-ratio deviation was measured on *in vitro* produced embryos by using the PCR method developed by UNCEIA. ANOVA (SAS proc GLM) and Chi square analyses were used for statistical analysis on quantitative variables and qualitative variables respectively.

Semen quality parameters except LAH and *in vitro* fertility results were significantly decreased by both treatments when compared to control (no interaction with the bull effect) (tables 1 and 2).

Table 1: Effect of treatment on the main semen quality parameters

Treatment	% live	% mot	score	VAP	VSL	VCL	LAH	%Prog
Control	37,9	47,0	2,5	124,2	113,1	206,9	8	21,9
Heifer +	14,8**	30,6*	1,6*	66,1**	54,0**	131,4**	7 NS	3,2*
Bull+	22,4*	33,5*	1,8*	77,1**	68,7**	134,8**	7,3 NS	11,2 NS

Treatment versus control: ** ($p<0,001$); * ($p<0,05$); NS: non significant

Table 2: Effect of treatment on fertilization parameters measured in vitro

	N° inseminated	% fertilized	% developed	% polyspermic
Control	647	77,7 (503)	66,5 (430)	4,8 (24)
Heifer+	655	44,7 (293)*	32,2 (211)*	10,2 (30)
Bull+	622	54,0 (336)*	40,2 (262)*	9,5 (32)

Treatment versus control:* ($p<0,05$)

A total of 757 embryos were sexed by PCR on Day 5 post IVF (table 3). Both treatments enhanced the percentage of female embryos ($p<0,005$).

Table 3: Effect of treatment on sex-ratio deviation in embryos

Control			Heifer+			Bull+		
% fem	% males	N	% fem	% males	N	% females	% males	N
50,7	49,0	351	69,0	27,6	145	59,2	39,9	238

These results show that the use of HeiferPlus induced a significant deviation of the % of female embryos produced in vitro but such a treatment may finally result in producing fewer daughters than the control due to impaired fertility.

Notes

EXPRESSION OF GENES INVOLVED IN COMPACTION, BLASTULATION AND METABOLISM IN BOVINE IVF EMBRYOS AND PARTHENOTES DEPENDS ON TIMING OF BLASTOCYST EXPANSION

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Sex and quality of in vitro fertilized (IVF) embryos has been associated to timing on development. Parthenogenesis is a process by which the oocyte develops without the male gamete. However, once transferred to surrogate mothers, parthenotes are unable to develop to term, although they can go through gastrulation and early stages of organogenesis.

The arrest in development of parthenotes is thought to be due to genomic imprinting. Maternal and paternal genes are reciprocally imprinted, such a way both genomes are needed for correct growth, being complementary but not equivalent. Indeed, monoparental duplication of gene expression could lead to embryonic lethality. Therefore, both imprinted and other non-imprinted genes could be altered at early embryonic stages.

In this work we analyzed mRNA transcription of genes related to metabolism, morula compaction and blastocyst formation in Day 7 and Day 8 parthenotes and IVF expanded blastocysts. In vitro matured slaughterhouse oocytes were fertilized with frozen-thawed, swim-up separated sperm or activated with ionomycin + 6-DMAP, and cultured for 8 days in synthetic oviduct fluid with 6g/L BSA. Gene expression was analyzed by RT-PCR starting from pools of 15 expanded Day-7 or Day-8 blastocysts within 4 replicates. Among metabolism related genes, SLC2A1 was downregulated in parthenotes ($p<0.05$), while AKR1B1, COX2 and TXN were upregulated. H6PD was downregulated in Day-7 embryos, and SLC2A5 decreased in Day-8 parthenotes. Among genes involved in compaction/blastocyst formation, GJA1 was downregulated in parthenotes, while no differences were detected within ATP1A1 and CDH1. Within parthenotes, expression of SLC2A1 and H6PD, and possibly AKR1B1, resembles patterns described in female embryos. Genes that differ between parthenotes and IVF embryos might be potential candidates to be imprinted, which opens doors to further research.

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Notes

PREGNANCY RATES AFTER SINGLE DIRECT TRANSFER OF BIOPSIED FROZEN-THAWED BOVINE EMBRYOS ACCORDING TO QUALITY

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Recently, a reliable method for whole genome amplification (WGA) has been implemented for DNA pre-amplification and subsequent genotyping from embryo biopsy containing 5 cells or more in cattle (Le Bourhis et *al*., Proc 23rd AETE Meeting, 2007). In France, these methods are now tested under field conditions. Several studies report pregnancy rates after direct transfer of biopsied frozen-thawed grade 1 embryos similar to that intact frozen ones. As grade 2 and 3 embryos represent 25.5% of the transferable embryos (AETE data 2007) and may limit the use of the above mentioned techniques if results are not satisfactory, the impact of the embryo quality on the pregnancy rates after single direct transfer of biopsied frozen-thawed embryos was investigated.

Embryos were collected on 12 donor cows after 15 sessions of superovulation treatment using FSH injected twice daily in decreasing doses over 4 days. Cows were inseminated at 12 and 24 hours after onset of estrus. Embryos were recovered 7 days post-insemination and evaluated according to IETS standards. Biopsies were performed on stage 4 to 7 grade 1 to 3 embryos with a steel blade attached to a micromanipulator. Embryonic cells from the biopsy were dry deposited in microtubes and frozen before WGA and multi-genotyping. Each biopsied embryo was equilibrated for 10 min in 1.5 M ethylene glycol and then loaded into straw containing two columns of F1 medium separated by a central column of 1.5 M EG with the embryo. The freezing sequence was: -7°C directly; seeding; held for 10 min; 0.5°C/min until -35°C before plunging into liquid nitrogen. Embryos were thawed (straws 10s in air and 20s in water at 20°C) and directly transferred into synchronized recipient heifers. Pregnancy was diagnosed by ultrasonography at 35 and 90 days. Effects of embryonic stage and quality on pregnancy rates were analysed by log linear models (Proc CATMOD, SAS Institute Inc).

A total of 58 embryos were micromanipulated. All G1 and 2 embryos were successfully biopsied and frozen and, 0.8% of G3 (2/25) were discarded due to their low quality after micromanipulation. No significant effect of embryo stage and quality on pregnancy rates was found after direct transfer (table 1).

Table 1. Pregnancy rates following single direct transfer of biopsied frozen-thawed G1 to 3 embryos

	Number of transfers	Pregnancy rates	
		D35	D90
Grade 1	24	62.5%	54.2%
Grade 2	7	57.1%	57.1%
Grade 3	23	52.2%	47.8%

These preliminary results suggest that high pregnancy rates can be achieved after direct transfer of biopsied frozen-thawed embryos of Grade 1, 2 and 3 allowing most of the embryos to be involved in the genotyping process.

This work has been performed through the programme TYPAGENAE (GENANIMAL 4-03) supported by FRT/ANR and Apis-Genes.

Notes

SINGLE CULTURE IN WELL OF THE WELL DOES NOT IMPROVE THE *IN VITRO* DEVELOPMENTAL CAPACITY OF BOVINE EMBRYOS CULTURED ON A CUMULUS CELL MONOLAYER

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The routine practice in bovine *in vitro* embryo production (IVP) is to handle oocytes and embryos in groups, although an individual IVP system has many advantages, allowing for following a single oocyte from retrieval through development to the blastocyst stage. Individual culture of zygotes on a cumulus cell monolayer was shown to increase blastocyst rates, compared to the absence of coculture (Goovaerts *et al*., 2007). Moreover, using a modified well of the well (mWOW) system (Vajta *et al*., 2000) gave promising results for individual culture in preliminary tests. Therefore, the aim of this study was to evaluate if the combination of a cumulus cell monolayer and a mWOW system, results in an improved developmental capacity of singly cultured bovine zygotes, compared to coculture treatment alone.

To do so, culture plates were prepared 6 days before zygote culture in order to allow monolayer formation. In 24-well plates, droplets of 15 µL SOF + 5% FCS were made under oil. For the mWOW treatment, a well (450 µm diameter) was pressed with a pen in the bottom of each well of the 24-well plates. In all plates for single culture, 5 µL of a cumulus suspension with 100 viable cumulus cells/µL was added to the 15 µL droplets. Four days later, grade I COCs (n = 557) were collected from slaughterhouse ovaries (4 replicates) and routinely matured and fertilized in groups of ± 100. For culture (90% N_2, 5% O_2, 5% CO_2), presumptive zygotes were ad random divided to 3 treatments: 1) group control: ± 25 zygotes in 50 µL droplets under oil; 2) single control: 1 zygote in 20 µL droplets under oil on a cumulus cell monolayer; and 3) single mWOW: 1 zygote in a mWOW in 20 µL droplets under oil on a cumulus cell monolayer. Cleavage, blastocyst and hatching rates were assessed 2, 8 and 10 days after fertilization, respectively. Possible effects of treatment were evaluated with binary logistic regression (SPSS 13.0).

No interactions between replicate and treatment could be found. Cleavage, blastocyst and hatching rates were not significantly different between the single treatments and the group control (Table 1) ($P > 0.05$). Moreover, between the two single treatments no significant difference in development could be found.

In conclusion, using a mWOW system in combination with a cumulus cell monolayer, does not enhance single embryo development compared to a coculture with cumulus cells only.

Table 1: Cleavage, blastocyst and hatching rates of bovine zygotes cultured in group and individual on a cumulus cell monolayer or a monolayer combined with a modified well of the well (mWOW)

Treatment	Nr of oocytes	Cleavage (%)	Blastocysts D8 (%)	% Hatched D10
Group control	207	141 (68.1)	58 (28.0)	67.2
Single control	182	137 (75.3)	51 (28.0)	60.7
Single mWOW	168	123 (73.2)	44 (26.2)	65.3

Notes

COMPARISON OF IN VITRO CULTURE OF BOVINE EMBRYOS USING DIFFERENT PROTEIN SUPPLEMENTATIONS VERSUS IN VIVO CULTURE

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Blastocyst quality is known to be affected by many factors, among which the post-fertilization culture conditions play a key role. During this period, several crucial developmental events take place, e.g. the embryonic genome activation, the compaction of the morula and blastocyst formation. Modifications of the culture conditions can affect any or all of these processes and thereby the quality of the blastocyst produced in vitro. The aim of the present study was to examine the effects of different protein supplementation versus in vivo culture conditions on embryo development and embryo kinetics.

A total of 4084 in vitro matured COCs were divided into six groups. In five groups, matured COCs were fertilized and subsequently cultured in vitro for 8 days in SOF medium supplemented with 3mg/ml (791) or 6 mg/ml BSA (795) or 1% (761), 5% (747), or 10% ECS (755), respectively. In the sixth group, 235 in vitro matured COCs were mixed with $2x10^6$/ml swim-up separated spermatozoa and then transferred into the bovine oviducts of synchronized heifers by transvaginal endoscopy. Seven days later, embryos were flushed from the oviduct and uterus and cultured in vitro for 24 hours (GIFT-group).

After transfer into the bovine oviducts for in vivo culture, 150 COCs or embryos were recovered (recovery rate 63.8%). The blastocyst development in groups 3 and 6 mg/ml BSA and 1%, 5% and 10% ECS and GIFT-group was 10.4%, 9.6%, 16.4%, 21%, 17% and 20.7% at day 7 and 21.2%, 21.1%, 25.1%, 30.7%, 26% and 26.7% at day 8, respectively. Significantly less blastocysts were present at day 7 in the presence of BSA than in the presence of ECS in SOF or cultured in vivo. However, on day 8 this difference was evident only between BSA groups and blastocysts produced in 5% ECS. The kinetics of blastocyst appearance was significantly affected by the presence of BSA or the level of ECS; significantly more blastocysts were present at day 7 in the presence of ECS compared with BSA. Within the ECS groups more blastocysts were observed at day 7 in the presence of 5% ECS and 10% ECS compared with 1% ECS. The highest rate of blastocysts being developed at day 7 was reached after homologous in vivo fertilization and culture in comparison to in vitro culture irrespective of the level of ECS and BSA, respectively.

In conclusion, the use of different concentration of BSA or ECS did not affect the total blastocyst production but the use of ECS accelerated the appearance of blastocysts at day 7. Nevertheless the highest kinetics of blastocyst development was reached after the long-term culture of bovine embryos in homologous oviducts.

Notes

THE EFFECT OF ANIMAL REGROUPING ON THE RESULTS OF A BOVINE IN VIVO EMBRYO PRODUCTION PROGRAM

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Every year, CRV flushes 240 Holstein yearling heifers within her nucleus program in order to produce first class embryo's of high genetic merit animals. These embryos are sold to farmers and a selection of the subsequently born calves will be incorporated in the breeding program for the next generation. Heifers are being flushed two to three times before insemination. During this period heifers are housed as much as possible in fixed groups of 15 animals each. However, regrouping sometimes occurs resulting in changes of group composition. Several factors are known for having an effect on embryo production. Regrouping of yearlings can result in antagonistic behavior between animals (Raussi et al., Appl. Anim. Behaviour Sci, 2005) and might also have an effect on also embryo production (Dobson et al. Reproduction, 2003). Therefore the goal of this study was to determine the effect of regrouping of yearlings on in vivo embryo production.

Data were used from 398 flushings of 218 heifers in the period from 2006 to 2007. Only first and second flushings were used. Number of ova and embryo's were Poisson distributed, therefore they were analyzed by log linear regression using Genstat. Effects included in the model were stress classification, hormones used, stimulation dose and days between insemination and moment of flush. Stress classification was based on the replacement of the yearling within two weeks before flushing. No replacement was defined as no stress, replacement as stress.

Mean embryo production per donor was 3.4 (SD 3.6 median 2) and ranged from 0 to 27 embryos. The mean number of flushed ova was 8.0 (SD 6). No differences were found between first and second flushings. Regrouping of the heifers in our barn did not affect the number of first class embryos or total number of flushed ova (see Table 1).

Table 1. Total number of flushed ova and the number of first class embryo's and for the not regrouped yearlings (no stress) and the regrouped yearlings (stress).

	No stress	Stress	Mean
Number of flushes	232	165	397
Number of flushed ova	7.6	8.6	8.0
Number of first class embryos	3.2	3.7	3.4

These results found in this study suggest that stress caused by regrouping of heifers on our barn during their embryo production period, does not affect the results of a super ovulation treatment for both the number of first class embryos and total number of ova flushed.

Notes

GENE EXPRESSION DIFFERENCES BETWEEN BOVINE EMBRYO BIOPSIES DERIVED FROM BLASTOCYSTS RESULTED IN DIFFERENT PREGNANCY OUTCOMES AFTER TRANSFER TO RECIPIENTS

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Bovine embryos which differ in their developmental potential are know to differ in relative abundance of developmentally important genes. However, the direct connection between transcript abundance and further developmental capacity has been a challenge for the last decade. Here we aimed to establish this connection through gene expression analysis of biopsies derived from blastocysts prior to transfer. For this in vivo derived bovine embryos were subjected to biopsying and reexpanded embryos were transferred to recipients and based on the pregnancy outcome, biopsies were pooled in three groups: those resulted in no pregnancy, those resulted in resorption and those resulted in calf delivery. Triplicate pools (each with 3 biopsies) representing the three groups were used for gene expression analysis using BlueChip (with ~2000 clones) cDNA array. Microarray data analysis revealed a total of 50 and 52 genes were differentially regulated among biopsies derived from blastocyts resulted in no pregnancy vs. calf delivery and resorption vs. calf delivery respectively. Biopsies from calf delivery group were found to be enriched with genes regulating nucleosome assembly (H2FAZ), translation (RPLP0), cell cycle (RGS2), and metabolic process (ELOVL1). Biopsies from no pregnancy and resorption group are enriched with transcripts involved in mitochondrial electron transport (FL405), response to stress (HSPD1), and cell cycle arrest (PA2G4 and 7030402D04Rik). The transcript abundance of six differentially regulated genes was confirmed by quantitative RT-PCR in single independent biopsies from the three groups.

BMP-15, KRT8, Plac8 and RGS2 were found to be downregulated in biopsies which had lead to no pregnancy and resorption compared with biopsies which had resulted in calf delivery, whereas Fl405 and S100A10 were found to be highly upregulated in biopsies which resulted in no pregnancy and resorption. Further functional analysis of these candidates in bovine embryogenesis will supplement the results of the present study.

Notes

CONTRIBUTION TO THE STUDY OF COWS EMBRYONIC TRANSFER IN ALGERIA

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The reproduction is a luxury function, and can be, for herds performances a limiting factor. Its control has always been sensitive. The biotechnology development related to reproduction in cattle, has promoted the technologies development for induction and synchronized estrus, artificial insemination, embryo transfer, sexing, in vitro fertilization, cloning and transgenesis.

This biotechnology plays a very important role in initiating programmes for breeding cattle. These types of programs should mobilize several actors (governments, researchers, technicians) in the development of milk and meat production, where two main actions could be carried out:

- improving the system of reproduction conduct
- improving the animal genetic potential

This work is part of a project in collaboration with the National Centre of Artificial Insemination and Genetic Improvement (CNIAAG) which consist on producing select bulls to be tested and indexed.

After selecting and synchronising (heat) the donors and recipients, treatment was used for super-ovulation (FSHp.LHp "STIMUFOL 40%")prior to insemination.
The quality response to the super-ovulation treatment, embryo: extraction classification, transfer and freezing were evaluated.

The results were satisfactory and encouraging. The Class II embryos were transferred and those of class I have been frozen successfully.

Keywords: embryo transfer, cow, superovulation, donors and recipients

Notes

TRANSCRIPTIONAL ANALYSIS OF BUFFALO (*Bubalus bubalis*) OOCYTES DURING IN VITRO MATURATION USING BOVINE cDNA MICROARRAY

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The need for improving in vitro production of buffalo embryos necessitates a better understanding of the molecular mechanisms regulating early development including oocyte maturation. The aim of the present study is to investigate the gene expression profile of buffalo oocytes before and after in vitro maturation using bovine specific cDNA platform.

For this, a total of six pools each contain 50 immature or in vitro matured buffalo oocytes were used for mRNA isolation and subsequent cDNA synthesis. Ten cycles of amplification was used to amplify cDNA of all samples as established in our laboratory that has no effect on the representation of original mRNA population. The BlueChip bovine cDNA microarray (with ~2000 clones) was used to analyse gene expression profiles between immature and matured oocytes in triplicate target and corresponding dye-swap hybridisations.

Significance analysis of microarray (SAM) data revealed a total of 104 transcripts to be differentially expressed between the two oocyte groups. Out of these, 69 transcripts were up-regulated, while 35 were down-regulated in immature compared to matured oocytes. Gene Ontology Consortium classification (http://www.geneontology.org) was used to assess the possible biological themes in which up- or down-regulated genes are involved. Based on this, transcription factors (ZFP91), M-phase mitotic cell cycle (MPHOSPH9), growth factor (BMP15) and DNA binding (HMGN2) were found to be up-regulated in immature oocytes. Similarly, matured oocytes were found to be enriched with genes involved in cytoskeleton (ACTB), hydrogen ion transporting (ATP6V1C2), Calcium ion binding (ANXA2) and structural constituent of ribosome (RPS27A). Quantitative real-time PCR validated the expression profile of some selected transcripts during array analysis.

In conclusion, to our knowledge this is the first large-scale expression study to identify differentially abundant candidate genes that have potential roles during buffalo oocyte maturation.

Notes

COMPARISON OF BOVINE OOCYTE RECOVERY AND *IN VITRO* EMBRYO DEVELOPMENT AFTER OVUM PICK UP IN SIMMENTAL, CHAROLAIS AND HOLSTEIN FRIESIAN COWS

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The aim of the present study was to investigate the effect of three cattle breeds on number of aspirated follicles, retrieved oocytes, oocyte recovery rate, oocyte quality and subsequent embryo development after transvaginal Ovum Pick-Up. Transvaginal ultrasound guided (Pie Medical, the Netherlands) oocyte collection was performed in 12 nonlactating donor cows (4 Simmental, 4 Charolais and 4 Holstein-Friesian). Cows were synchronized with $PGF_2\alpha$ and stimulated with pFSH, twice a day during two days (Folltropin®, Bioniche, a total dose: 200 mg NIH-FSH-P1). OPU was performed 24 hours after the last FSH injection and procedure was repeated every second week for eight consecutive weeks. The number of aspirated follicles, the number of retrieved oocytes and oocyte recovery rate were recorded. The recovered oocytes were morphologically classified into four (1-4) quality categories. Grade 1 and 2 oocytes were matured, fertilized and cultured *in vitro*. The culture medium was composed of synthetic oviductal fluid (SOF) medium with aminoacids and 8 mg/mL BSA, and the embryos were cultured for 9 days at 38.5°C in 5% CO_2, 5% O_2, 90% N_2 atmosphere with maximum humidity. 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. All results were analyzed with ANOVA (StatSoft, Statistic, version 7.1.) and with Tukey's tests post-hoc analysis. The OPU results are presented in Table 1.

Table 1. Number of aspirated follicles, oocytes recovered and oocyte morphology in Simmental, Charolais and Holstein-Friesian cows (mean ± S.E.M.)

Animal breed	Number of follicles punctured per animal	Number of recovered oocytes per animal	Oocyte recovery rate per animal	Number of grade 1-2 oocytes per animal	Number of grade 3-4 oocytes per animal
Simmental	14.1±1.1[a]	9.7±1.1[a]	68.8±0.08	6.2±0.8[a]	1.4±0.3
Charolais	13.1±1.4[a]	9.1±1.4[a]	69.4±0.05	6. 9±1.2[a]	1.6±0.2
Holstein-Friesian	8.6±0.5[b]	4.9±0.5[b]	56.9±0.09	3.5±0.5[b]	1.2±0.2

[ab]Values with different superscripts differ between columns ($P<0.05$)

There was no differences in the proportion of good quality (grade 1-2) oocytes undergoing *in vitro* fertilization and subsequent development to the blastocyst stage between Simmental, Charolais and Holstein-Friesian breed. In conclusion, we reported that Simmental and Charolais donor cows showed significantly better results in number of follicles punctured, total number of oocytes and number of grade 1 and 2 oocytes recovered by OPU, but there was no differences in developmental competence of retreived oocytes of similar quality between three donor breeds.

Notes

THE MORPHOLOGY OF PREPUBERAL GILT OOCYTES IS ASSOCIATED WITH ZONA PELLUCIDA GLYCOPROTEIN TRANSCRIPT CONTENTS

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The mammalian zona pellucida contains 3 major zona pellucida glycoproteins, ZP1, ZP2, and ZP3, which function as essential structural components of the zona pellucida. These molecules are also significant contributors in the sperm's interaction with the oocyte during fertilization. The porcine *ZPs* genes encode pZP1, pZP2, pZP3 and pZP3α (pZP4) glycoproteins, where pZP3 and pZP2 bind to the sperm cell membrane proteins before and after the acrosome reaction, respectively.

Total RNA was isolated from porcine oocytes (n=20) using an RNeasy mini column Qiagen GmbH (Hilden, Germany), treated by DNase I, and reverse-transcribed into cDNA**.** Quantitative analysis of pZP1, pZP2, pZP3, and pZP3α 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 quantity of mRNA product in each sample was adjusted to the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA or calculated per 20 oocytes. The classification of cummulus-oocyte complex (COCs) morphology was based on a four grade scale proposed by Pujol M *et al*. (2004).

We found a 2-4 fold increase in the level of pZP1 transcript in oocytes graded as I as compared to oocytes graded as II, III, and IV, respectively. We also observed higher levels of pZP2 and pZP3 mRNA in oocytes graded as I as compared to others ($P<0.05$). The pZP3α transcript contents in oocytes graded as I was 2-fold higher compared to the oocytes graded as II, III, and IV.

Our observations suggest that porcine oocyte morphology is correlated to pZPs transcript contents, which may be associated with increased fertilization ability of oocytes. This knowledge may be useful in the primary selection of COCs before IVM and IVF in pigs.

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Notes

FOLLICULAR SIZE IS ASSOCIATED WITH QUANTITY OF TRANSCRIPTS ENCODING SELECTED MOLECULES CONTRIBUTING TO FERTILIZATION ABILITY OF OOCYTES FROM PREPUBERAL GILTS

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The quality and developmental competence of an oocyte is accomplished during folliculogenesis. It has been suggested that follicular size may correspond to transcript and protein stores, which reflect oocyte competence. However, it is still unclear whether follicular size is associated with the levels of transcripts that encode proteins contributing to the fertilization ability of porcine oocytes.

We evaluated the quantity of transcripts encoding porcine zona pellucida glycoproteins pZPs (pZP1, pZP2, pZP3 and pZP3α) and integrin β1 in oocytes isolated from prepuberal gilt follicles of different sizes.

A total of 300 follicles were collected from slaughterhouse gilt ovaries and classified as small (< 3 mm, n=100), medium (3-5 mm, n=100), or large (>5 mm, n=100). Only cumulus oocyte complexes (COCs) with more than two layers of cumulus and homogenous dark cytoplasm were used. Total RNA was isolated by Chomczyński method and immediately used for reverse transcription. Employing Real-time quantitative PCR reaction methods we determined the transcript levels of pZP1, pZP2, pZP3, pZP3α and integrin β1 in oocytes isolated from prepuberal gilt ovaries.

We observed a significantly higher level of transcripts of pZP1, pZP3, pZP3α and integrin β1 in oocytes collected from medium follicles as compared to small follicles ($P \leq 0.001$). Moreover, we found a higher content of pZP3α and integrin β1 mRNAs in large follicles as compared to small follicles ($P \leq 0.001$). However, we did not observe differences in pZP2 transcript levels in oocytes from these three groups of follicle size.

Our results suggest that the level of transcripts encoding selected molecules contributing to the fertilization ability of oocytes is related to follicular size in prepuberal gilts.

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Notes

EMBRYONIC DEATH RATES IN EWES ARTIFICIALLY INSEMINATED IN SYNCHRONIZED ESTRUS

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Embryonic death is an important problem in sheep breeding. Conception, gestation and giving birth to live foetus is a very complex process involving the embryo, the uterus, the mother's organism and the environment. Synchronization of estrus can save labor and is a key component in artificial insemination (AI) programs. It was reported that synchronized ewes exhibited markedly higher total reproductive losses, in both season (49%) and out of season (58%) (Lunstra and Christenson, 1981). The object of this study was to determine embryonic death in ewes artificially inseminated at their synchronized estrus. Fat tailed ewes (n= 68) were treated with vaginal sponge containing 30 mg fluorgestone acetate (FGA; Chrono-gest, Intervet, Boxmeer, The Netherlands) for 12 d. Immediately following sponge removal, ewes received an injection of 600 IU, i.m. eCG. Animals that showed any mating marks by 60 h were inseminated 54 to 56 h after sponge removal with freshly diluted semen containing 100 x 10^6 motile spermatozoa. Pregnancy was determined by serum progesterone concentration 28 days after insemination and transrectal ultrasound. Hormone concentration was determined by chemiluminescent assay. The discriminatory level of P4 for early pregnancy diagnosis in ewes was 1.25 ng/ml. In the present study, the percentage of ewes which became pregnant after insemination following synchronized estrus was 48% as it is detected by transrectal ultrasonography 28 days after AI. Regarding the progesterone test, fifteen non-pregnant ewes had progesterone levels higher than 1.25 ng/ml (false positive diagnoses) at the 28 day of insemination. Animals were detected with 14% embryonic loss when they were scanned on 80 days of insemination. Lambing rates was 30% and total reproductive losses was 18%. It was concluded that exogenous hormones is not the main reason for the incidence of embryonic loss but could be genetic factors, stress and animal health.

Notes

EFFECT OF SOMATOTROPIN ON THE DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTE FROM FOLLICLES OF DIFFERENT DIAMETERS

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In previous studies we demonstrated that recombinant bovine somatotropin (rbST, "Monsanto") supplementation remarkably enhanced the proportion of the late morulae and blastocysts obtained from oocytes aspirated from follicles of 3-5 mm diameter and matured in vitro (Kuzmina et al., 2003). We have shown that rbST in interaction with granulosa cells stimulates the oxidative activity of ooplasmic mitochondria and decreases the content of membrane bound [$Ca^{(2+)}$] in oocytes [Kuzmina et al., J Reprod Develop 53 (2): 309-316, 2007]. The aim of the present study was to evaluate the developmental competence of bovine oocytes aspirated from follicles of different diameters after maturation in culture medium with 10 ng/ml rbSt.

Bovine cumulus-oocyte complexes (COCs) were collected from antral follicles and graded into three categories depending on the diameter of follicles: small (<3mm); middle (3-5 mm); large (5-8 mm). Only those oocytes having a compact cumulus investment were used in the experiments. COCs were incubated in TCM 199 containing 10% (v/v) heat-treated FCS (Sigma) and 10^6/ml granulosa cells (control group). The culture medium of the treatment groups was modified by supplementation of the control medium with 10 ng/ml rbST (experimental groups). Oocytes were fertilized and embryos were cultured by standard protocols. We have estimated oocyte maturation, cumulus morphology and quality of early embryos including status of chromatin (cytogenetic analysis by Tarkowsky, 1966). Data were analyzed by Chi^2 – test.

Cumulus of 583 oocytes was evaluated after 24 hours of cultivation. All oocytes from 5-8 mm diameter follicles had cumulus with high level of expansion. rbST affected on the expansion of cumulus in experimental groups. The level of oocytes with high expanded cumulus was higher in treatment groups of oocytes from small follicles [45% (30/66) vs. 27 % (20/73), $P<0.05$] and of middle [89% (117/131) vs. 68% (102/151), $P<0.001$]. No significant differences were obtained in oocytes reaching metaphase II within control and experiments groups. But the cleavage rate on day 2 after fertilization was significantly higher in oocytes matured in the presence of rbST from middle follicles [49% (48/98) vs. 69% (66/95), $P<0.05$] and from large follicles [50% (50/101) vs. 71% (70/98), $P<0.05$]. The highest percentage of blastocysts was obtained in group of oocytes from follicles 3 - 5 mm in diameter cultured with rbST: 40% (38/95) vs. 26% (25/98). Only 7% (4/60) of oocytes from follicles < 3mm in diameter have reached the blastocysts stage, in the same time 15% (8/55) of oocytes from small follicles matured with rbST have developed to blastocysts. Analysis of morphology and chromatin abnormalities in 2 to 16 cell embryos have shown decreasing percentage of degenerative embryos obtained from the oocytes matured in the presence of rbST independent of follicle size: small - 77% (33/43) vs. 53% (27/51), $P< 0, 05$; middle - 48% (35/73) vs. 29% (28/98), $P<0.02$; large - 68% (49/72) vs. 48% (46/96), $P<0.01$. Based on the results of the present study we concluded that cultivation of bovine oocytes from follicles in different diameter in the presence of rbST and granulose cells improves quality of early preimplantation embryos in vitro.

Notes

USE OF EMBRYO SEXING AND MOET IN AUBRAC COWS TO SAVE THE DAIRY GENETIC TYPE

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Since 1961, the Laguiole cheese has taken benefit from a quality label ("Appellation d'Origine Contrôlée" (A.O.C.)). Strict specifications related to the geographic area of production (Aubrac region) and to the use of milk originating from Aubrac cows have to be respected to keep the A.O.C. label. However, the number of milked Aubrac cows (with a dairy type) has declined in a drastic way since 1950, impairing cheese production and threatening the breed. Since 1990, different programs have been implemented to enhance the genetic evaluation of milk performances in the Aubrac breed. In 2005, use of embryo transfer associated with embryo sexing was initiated to accelerate production of 250 supplementary dairy cows. Donor cows (n=45) were selected from 274 Aubrac dairy cows satisfying the following inclusion criteria: calving to calving interval ≤ 380 days, 5 previous calving at least and sufficient genetic indexes (IBOVAL Milk > 106, IVMAT > 97).

A total of 978 Day 7 bovine embryos were biopsied and sexed according to Lacaze et al. (AETE 2007). They were frozen using ethylene glycol (1.5 M) plus sucrose as cryoprotectants. Frozen biopsied embryos were collected from 226 sessions by conventional techniques (donor cows inseminated twice on observed oestrus following a standard superovulatory treatment and collected on Day 7, Table 1). Female embryos (n=213) were thawed (straws in air for 5-10 s and in a water bath for 30 s) and directly transferred to heifer recipients.

Table 1: Use of embryo sexing and MOET in Aubrac donor cows since 2005(3 years)

Numbers of	April2005	2006	2007	2008 (Jan – May)	TOTAL
Flushes	26	81	76	43	226
Collected embryos	381	1107	723	533	2744
Biopsied embryos	85	365	322	206	978
Embryos with undetermined sex	5	11	8	2	15
female embryos (frozen)	34	179	134	91	438
female embryos (transferred)	17	43	112	41	213
pregnancies	10	19	44+/72		73+/132

On average, 4.3 embryos per flush were biopsied and sexed. Female embryos represented 44.8 % of the biopsied embryos. Cases in which the sex remained undetermined represented 1.5 % of the biopsies. A total of 73 female pregnancies (55.3%) were obtained up to day following transfer of 132 female frozen thawed embryos .If 100 female embryos are transferred each year (because of limited numbers of recipients), the target of 250 females born will be reached in 2010.

Altogether, these preliminary results confirm that use of embryo sexing associated with MOET is a useful tool to promote and preserve endangered breeds contributing to maintain biodiversity. With MOET, high numbers of embryos and calves can be rapidly produced. The additional use of sexing is very useful to accelerate the process, especially when a limited number of recipients is available.

Notes

A POTENTIAL CONTAMINATION OF THE BIOPSY BY ZONA PELLUCIDA ASSOCIATED Y DNA DOES NOT AFFECT BOVINE EMBRYO SEXING RESULTS

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The biopsy used for embryo sexing can be prepared either by aspiration or cutting the embryo with a micro blade. In the later case, part of the Zona Pellucida (ZP) can remain attached to embryonic cells even after several washings. It can be hypothesised that sperm heads found attached to ZP fragments may influence the results of PCR embryo sexing due to the presence of Y DNA together with the biopsy. In this study, the influence of ZP fragments on the results of embryo sexing was investigated from field data collected by MIDATEST ET team from 2000 to 2007.

Bovine embryos were micro manipulated by a single operator and biopsed with a steel blade. Biopsies (between 3 and 10 cells) were rinsed 2 times consecutively in KCL medium, then put in a microtube for sex determination. For each biopsy the presence or the absence of ZP fragments following rising has been noted.

The UNCEIA R&D sexing kit and procedures developed for farm use from the initial PCR method based on a specific Y DNA sequence (INRA patent 1987) were used. The present protocol allows to identify the sex of the embryos within 2hours and 30 min.

The percentages of embryos from each sex were determined from 1358 micro manipulated and sexed days 7 embryos. From those, 45.8% were females, 49.9% were males and in 4.2% of the cases, sex was not determined due to a low amplification of the autosomal sequence and or/ lost biopsy. A total of 129 (9.5%) biopsies were associated to ZP fragments. As shown in table 1, the presence of DNA fragments did not increase the percentage of undetermined results nor the percentage of biopsies diagnosed as originating from a male embryo.

Table 1: Sex ratio from embryos for which the biopsy was associated or not to ZP fragments

	Biopsies	Undetermined N		Female		Male	
ZP fragments	Total	%		N	%	N	%
NO	1229	51	4.1	557	45	621	50.5
YES	129	7	5.4	65	50	57	44.2
Total	1358	58	4.7	622	45.8	678	49.9

A subset of 464 embryos diagnosed as females were transferred either fresh or frozen. This included 42 embryos for which ZP fragments were associated to the biopsy used for sex determination. The sex of born calves (n=237) have been subsequently registered. Only one error (birth of a male instead of a female) has been noted from the group with no ZP fragment (1/215) whereas in the group with ZP fragments all diagnosed females embryos gave birth to a female calf (21/21).

Taken together the results of this retrospective study show that, when using the above described PCR based sexing kit, a potential contamination of the biopsy by ZP associated Y DNA, if existing, does not affect sex determination and its accuracy.

Notes

BOVINE EMBRYOS EXPOSED TO TNFα DURING PREIMPLANTATION CULTURE UNDERGO NORMAL DEVELOPMENT

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The reduction of fertility in dairy herds is a growing problem in cattle breeding. Several factors are reported to be involved including nutrition, management, milk production, inbreeding and also uterine diseases. The latter, especially in the form of subclinical endometritis and inflammation, are considered important players in this context. In order to evaluate the consequences of uterine inflammation on embryo development we have investigated the effect TNFα supplementation to culture media of bovine embryos. The embryos were produced by in vitro maturation and fertilization of abattoir oocytes and were cultured in vitro up to day 8 in SOF-based media with or w/o 100ng/ml of TNFα. The morula compaction and blastocyst rate were evaluated on day 6, 7 and 8. In addition, the blastocysts obtained on day 7 were analysed for the expression of iNOS, Hsp70, G6PDH and Bax by semi-quantitative RT-PCR. Considering that in cattle most embryonic losses occur in the second week of development we investigated also the ability of TNFα-exposed embryos to undergo elongation as a measure of viability/ability to establish pregnancies. For this purpose day 7 blastocysts developed in vitro with and w/o TNFα were transferred in temporary recipients up to day 12 when the embryos were recovered and their number and size were recorded.

We found that TNFα does not affect embryo development rate up to day 7-8 in vitro (table 1). In addition we found no changes in the relative expression level of the genes analysed by RT-PCR. Finally, we found no difference in the size of the embryos recovered on day 12 although the embryos in the TNFα group tended to be larger (table 2).

Table 1. Embryo development up to blastocyst with and w/o TNFα

	N° Oocytes	Cleaved (%)	Compacted morulae d+5 (%)	Compacted morulae d+6 (%)	Blastocysts d+7 (%)	Blastocysts d+8 (%)
CTR	1031	795 (77.11)	120 (15.09)	180 (22.64)[a]	179 (22.52)[a]	251 (31.57)[a]
100ng TNFα	1023	802 (78.40)	95 (11.85)	157 (19.58)[a]	170 (21.20)[a]	252 (31.42)[a]

Table 2. Development and size of IVM-IVF bovine embryos grown with or w/o TNFα up to day 7 and then transferred in temporary recipients and finally collected on day 12

	Transferred (day 7)	Total recovered (day 12)	Recovery rate	N° of ovoid embryos	N° of spherical embryos	Mean length	Mean width
CTR	45	30	66.67	1	29	0.38	0.38
100ng TNFα	49	28	57.14	4	24	0.46	0.43

In conclusion TNFα exposure during embryo culture is not detrimental to development.

This work has been supported by Regione Lombardia (Uterofert).

Notes

EMBRYO GENOTYPING FROM IN VIVO BIOPSED BOVINE EMBRYOS

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Genomic tools are now available for most livestock species and used routinely for marker assisted selection (MAS) in cattle. One major challenge to facilitate multiple character selection would be to detect multiple markers from biopsies of pre-implantation stage embryos and then transfer only the selected embryos with success. This strategy provides also the opportunity to select embryos with traits of particular interest together with the absence of know abnormalities . This work aims to test the reliability of the MAS method for 45 micro satellites (MS) and 13 single nucleotide polymorphisms (SNP) from bovine embryo biopsies under field conditions.

After superovulation (10 cows), bovine embryos were *in vivo* produced and collected at day 6 or day 7 of pregnancy. Only grade 1 embryos were washed and biopsied using a microblade under a stereomicroscope. Biopsied embryo were either frozen or transferred back to synchronized recipients. Individual biopsies were transferred as dry samples to the laboratory. Genomic DNA was amplified using a whole genome amplification kit according to the manufacturer instructions (WGA; QIAGEN REPLI-g Mini Kit). Embryos were genotyped using GeneMapper® software (Applied Biosystems Europe). The sex of embryos was also determined by using the PCR method developed by UNCEIA and based on a specific Y DNA sequence.

From 123 collected embryos, 79 were classified as grade I or II transferable embryos (64.2%) and 57 were biopsied (34 were classified as stage 4-5 and 23 as stage 5-6, according to the IETS criteria). Using the stereomicroscopic analysis , 44 biopsies had a number of cells ranging from 4 to 7 (5.6±1.4) and 13 biopsies from 8 to 10 (8.4±1.6) . Overall, at least 95% of markers (MS+SNP) were detected in 49.1% of biopsies (28/57). The total detection rate for SNP was significantly higher than for MS; 70.2% (40/57) *vs* 31.6% (18/57) respectively, ($P<0.01$). Among the analysed biopsies, 45.6% were females (26/57), 52.6% (30/57) were males and for 1.8% (1/57) the sex could not be determined. The detection rate of the markers was not significantly affected by the embryo stage, the biopsy size or the sex of the embryo.

Our results confirm that genotyping a large number of markers from biopsy samples after whole genome amplification is possible under field conditions. A larger number of biopsies is required to assess the reliability of this method that may allow the development of MAS from early embryos .

This work has been performed through the programme TYPAGENAE (GENANIMAL 4-03) with the financial support of FRT/ANR and Apis-Genes.

Notes

DONOR AGE EFFECT ON SUPEROVULATORY RESPONSE OF BOER GOAT DOES

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This study was conducted to evaluate the effect of the age of the donor on the superovulatory response and embryo recovery rate in Boer goats. Seven maiden does (young, 1-2 years) and 9 multiparous does (adult, 3-4 years) 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 pFSH (Folltropin®-Vetrepharm) during the natural breeding season. The superovulation treatment was administered as a total dose of 200 mg pFSH/doe given i.m. in 7 dosages (at 12h intervals) - starting 48h prior to CIDR removal. Does were observed for oestrous behaviour 3 times daily at 8h intervals following CIDR withdrawal. Cervical inseminations (0.1ml fresh undiluted semen) were performed 36h and 48h following CIDR removal and the embryos surgically flushed 6 days following the second AI. All does showed overt signs of oestrus. The time interval from CIDR removal to the onset of oestrus in adult does (24.0±4.0h) was significantly ($P<0.01$) shorter than that recorded in the young does (32.0±4.6h). Age of donor did not have any effect on the duration of the induced oestrous period. The mean number of CL's, structures and embryos recovered were significantly ($P<0.01$) higher in the adult does (19.8 ± 4.8, 21.3 ± 3.9 and 20.9 ± 4.5, respectively), compared to the young does (13.7 ± 3.8, 11.7 ± 5.0 and 11.7 ± 5.0, respectively). The fertilisation rate, the mean number of unfertilised ova and the mean number of degenerate embryos did not differ between the young and adult does. The mean number of transferable embryos in the adult does (15.8 ± 6.4) was however significantly ($P<0.01$) higher than in the young does (9.5 ± 3.7). The longer reaction time taken to the onset of oestrus did no influence the fertilisation rate in young does. Although the young does recorded an acceptable fertilisation rate, the number of transferable embryos was however lower due to lower total number of embryos produced by the young donor does.

Notes

PRODUCTION OF PORCINE CHIMERIC EMBRYOS WITH AGGREGATION OF EMBRYOS OR BLASTOMERES

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Aggregation of different embryos or blastomeres may complement the possible handicaps of cloned embryos and consequently improve development of chimeric embryos. The ability to form aggregate blastocysts can even cross species boundaries to produce individual offspring (Fehilly et al., Nature, 1984; Williams et al., Reprod. Fertil. Dev. 1990). In the present study, chimeras were produced by aggregation of embryos or blastomeres to test their potential to develop to blastocyst *in vitro*. Porcine zona-free embryos were created by parthenogenetic activation or handmade cloning (HMC) using two different fluorescent transgenic cells: green (HMCg) or red (HMCr). All embryos were cultured in WOWs (Vajta et al., Mol. Reprod Dev., 2000) in PZM-3 medium. On Day 1 or 2 of culture, cleaved HMCg or PA embryos were incubated in 0.25% trypsin-0.01% EDTA to separate individual blastomeres. Subsequently, a single HMC embryo and single PA blastomeres from 2-cell stage (1/2 HMCg-PA) or 4-cell stage (1/4 HMCg-PA) embryos were cultured in one WOW for aggregation. Intact single HMCg and HMCr morulae were also aggregated with each other using the same method on Day 4. Results shown in Table 1 indicate that the highest blastocyst development was achieved with aggregation of single blastomeres of 2-cell stage embryos, while aggregation of morulae resulted in the highest cell number per blastocyst. For visual allocation of specific blastomeres in the inner cell mass vs. trophectoderm a simple fluorescence microscopic study proved inefficient. The task may require confocal laser examination. Formation of chimeras with contribution of handmade cloned embryos is technically simple, may improve qualitative and quantitative efficiency, and may also be used as a new way to study cell-cell interactions and cell allocations where the genotype or genome status differ.

Table 1. The developmental competence of different aggregated embryos

Developmental stage	Original embryos	Number of blastomeres or embryos	Aggregated blastocysts ratio %	Cell number per blastocyst
2-cell stage	HMCg	1 blastomere	62.1±0.7[a]	45.5±3.0[d]
	PAZF	1 blastomere		
4-cell stage	HMCg	1 blastomere	31.5%±0.9[b]	24.3±2.8[e]
	PZAF	1 or 2 blastomere		
Morula stage	HMCg	1 embryo	52.2%±1.1[c]	142.3±22.4[f]
	HMCr	1 embryo		

Values with different superscripts in the same column are statistically different (P<0.05)

Notes

OSMOTIC PRE-TREATMENT OF PORCINE OOCYTES IMPROVES THEIR CRYOTOLERANCE AND DEVELOPMENTAL COMPETENCE

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Cryopreservation of porcine oocytes is a demanding task because of their extreme sensitivity to chilling and other cooling-related injuries. The purpose of our work was to find the optimal way to improve cryotolerance by using osmotic pre-treatment with 3 different chemicals. In 6 replicates, a total of 1200 slaughterhouse-derived porcine oocytes were matured for 41-42 hr. COCs were treated with either NaCl, sucrose or trehalose (710 mOsmol for each in TCM199 and 2% cattle serum) at 38.5ºC for 1 hour. After an additional 1 hour recovery in IVM medium cumulus cells were removed with 1mg/ml hyaluronidase. Denuded oocytes were vitrified with the Cryotop method, activated with a single electric impluse of 63 V/cm plus incubation in 5 µg/ml cytochalasin B and 10 µg/ml cycloheximide for 4 h (Du et al., 2007, Cryobiology 54: 181-7). Embryo culture was performed in PZM-3 medium (Yoshioka et al., 2002, Biol. Reprod. 66:112-9) in 5% CO_2, 5% O_2 and 90% N_2 and maximum humidity. Cleavage and blastocyst rates were checked at 48 and 168 h after activation, respectively. Blastocysts were stained with Hoechst 33342 and cell numbers were counted under a Diaphot 200 inverted microscope with epifluorescent attachment and UV-2A filter (Nikon, Tokyo, Japan). SPSS 11.0 program (SPSS, Chicago, USA) was used for statistical calculations, differences with $P<0.05$ were regarded as significant. Results are shown in Table 1. In conclusion, osmotic pre-treatment with all the three tested chemicals improved in vitro developmental competence of vitrified and activated porcine oocytes. Further studies after vitrification and in vitro fertilization or somatic cell nuclear transfer are required to determine the effect of the osmotic pre-treatment on pregnancy, birth and post partum development.

Table 1: Developmental competence of porcine oocytes after osmotic pre-treatment with different chemicals, vitrification and parthenogenetic activation. Values with different superscript in the same column mean significant difference ($P<0.05$).

Group	No. of embryos cultured	No. of cleavage (%)	No. of total blastocysts (%)	No. of expanded blastocysts (%)
control	240	68 (28%) [a]	6 (3%) [a]	2 (1%) [a]
NaCl	233	105 (45%) [b]	21 (9%) [b]	13 (6%) [b]
Sucrose	221	97 (44%) [b]	17 (8%) [a, b]	13 (6%) [b]
Trehalose	224	105 (47%) [b]	24 (11%) [b]	16 (7%) [b]

Notes

INFLUENCE OF AN EMBRYO OR THE RECIPIENT ON PREGNANCY RATE AFTER EMBRYO TRANSFER IN DAIRY CATTLE

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A field MOET called "The Best cow –project II" in the Southern Savo, Eastern Finland during 2006-2007 aimed at better pregnancy rates after embryo transfer by using body condition score or milk progesterone concentration on Day 4 or on Day 7 (Day 0 = day of oestrus) for qualification of recipients. The aim of this paper was to study the influence of a transferred embryo or the recipient on the pregnancy rate.

Qualification of the recipients was performed before 188 embryo transfers (ET) on 48 farms (104 heifers, 84 cows). Transferred embryos originated from 99 embryo flushings in Finland and abroad. Before ET, an AI technician (nine technicians) performed body condition scoring and measurement of corpus luteum (CL). Farmers conducted heat detection and on Days 0 (oestrus), 4 and 7 after morning or evening milking, complete whole milk sample series were collected from 37 recipients for later analysis of progesterone concentration at Promilk Oy, Lapinlahti, Finland. Logistic mixed model was used to assess whether there is a relation between pregnancy rate and quality of embryo (fresh/frozen, intact/biopsied) or recipient (heifer/cow, body condition score (scale from 1 to 5), size (estimated diameter in cm during rectal palpation) and cavity of CL (existing or not), day of oestrous cycle at transfer (from Day 5 to Day 9), heat natural or synchronized, calving rate (from 0 to 8), rest period (time in postpartum days), progesterone level on Day 4 and on Day 7). In this model, the possible correlations between observations due to the farm and/or flushing can be taken into account putting those variables as random factors into the model.

Total pregnancy rate was 52 % (97/188). The pregnancy rate for heifers was 53 % and for cows 50 % (p=0.83 for the difference in rates between heifers and cows). Biopsied embryos had a lower pregnancy rate compared to non-biopsied embryos (42 vs. 54 %) though the difference was not statistically significant (p=0.19). Fresh embryos had a lower pregnancy rate than frozen-thawed embryos (47 vs. 53 %, p=0.45). The greater the body condition scores, the higher the pregnancy rates which were 24, 54 and 59 % for the classes of scores <3.0, 3.0-3.5, >3.5, respectively (p=0.04 for the difference in rates between classes). Similar, but statistically non-significant, trend was visible in both the size of the CL (p=0.42) and the day of oestrous cycle at transfer (p=0.84). The pregnancy rates were 47, 50 and 61 % for the size of the CL classes <2.00, 2.00-2.75, >2.75 and 42, 52 and 54 % for the day of oestrous cycle at transfer classes 5/6, 7 and 8/9, respectively. The pregnancy rates for milk progesterone concentrations on days 4 and 7 were 53, 29 % and 59, 50 % for the classes below and above the median values 6.8 nmol/l (p=0.15) and 28.3 nmol/l (p=0.60), respectively. The pregnancy rates for the length of rest period were 37 and 65 % for the classes below and above the median value 46 days (p=0.10).

In summary, results of this relatively small material reveal that recipients with body condition score below 3.0 on the day of embryo transfer will not make useful recipients in terms of pregnancy rate. In Finland, embryo transfer veterinarians recommend body condition score 3 to 4 for dairy recipients. Two fifths of fresh embryos were of quality grade II or III and biopsied, thus lowering the pregnancy rate when compared to frozen-thawed embryos (mainly grade I and non-biopsied). Pregnancy rates concerning milk progesterone concentrations on Days 4 and 7 warrant further studies.

Notes

PERMEABILIZATION OF PORCINE EAR FIBROBLASTS BY STREPTOLYSIN O

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Permeabilization is the first step in treatment of cells by oocyte extracts, and is useful for reprogramming of somatic cells. Streptolysin O (SLO) is the archetype of a cholesterol-binding bacterial cytolysin that forms large pores in the plasma membrane of mammalian cells (Walev et al., Infect. Immun., 1995; 63:1188-1194). The aim of the present work was to find the proper conditions for SLO permeabilization (according to Håkelien et al., Nat. Biotech., 2002; 20:460-466) of porcine ear fibroblasts.

Porcine ear fibroblasts were plated on poly-L-lysine coated coverslips in 4-well dishes. After growing to 50-70% confluence, cells were washed in ice cold PBS and in ice cold Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). For visualization of permeabilization, cold HBSS, Texas Red-conjugated dextran (70,000-Mr, 50 µg/ml; Invitrogen) and SLO were added. The tested concentrations of SLO were 100, 200, 250, 300, 500 and 1000 ng/ml. The cells were permeabilized at 37°C in atmospheric air for 30 min and subsequently sealed in DMEM with 2mM $CaCl_2$. The permeabilization rate was evaluated by the amount of Texas Red positive cells using fluorescence microscopy. SLO could permeabilize porcine ear fibroblasts in all concentrations used. The highest efficiency was obtained with concentration of 300 ng/ml, where 80% of the cells were permeabilized (Figure 1).

The optimal concentration of SLO was determined for permeabilization of porcine ear fibroblasts. Further work is on-going with treatment of cells by Xenopus egg extracts, followed by use of treated cells for porcine somatic cell nuclear transfer.

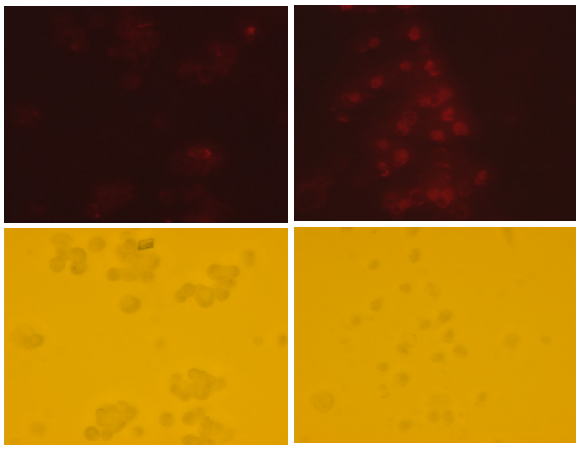

Figure 1. Permeabilization of porcine ear fibroblasts by SLO with concentrations of 0 or 300 ng/ml and observed by fluorescence microscopy (200×).

Notes

EFFICIENCY OF OOCYTE COLLECTION AND EMBRYO PRODUCTION FROM GENETICALLY VALUABLE COWS WITH AND WITHOUT ESTROUS CYCLE SYNCHRONIZATION

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In our preliminary study with slaughtered cows we found that oocytes recovered in the growth phase of the first follicular wave and fertilized by spermatozoa of a standard bull produced more and faster developed embryos than did oocytes recovered in the other stages of follicular development (Machatkova et al., 2006). In the present, field study, embryos were derived from 52 of genetically highly valuable cows excluded from breeding for reproductive disorders. Cows, aged 5 to 10 years, of Czech Siemmental, Holstein Dairy and Beef Cattle breeds were slaughtered, and used as oocyte donors. Before oocyte collection, donor estrous cycle was or was not synchronized (n = 31 or n = 21). Oocytes were obtained either in the growth phase of the first follicular wave (Days 3 - 4 after estrus) or in any other phases of follicular development (undetermined Days). The frozen-thawed sperm of 21 elite bulls of the same breeds were used for oocyte fertilization. The embryos were prepared by a standard protocol. The mean number of usable oocytes, transferable and freezable embryos per donor, the mean percent of usable, transferable and freezable embryos were assessed. The results were analyzed by Student's -t and Chi-square tests. Irrespective of the breed, the mean numbers of usable oocytes, and transferable and freezable embryos collected per donor were significantly higher ($P < 0.01$) for the synchronized than the nonsynchronized donors (20.4 vs 11.7, 4.3 vs 1.0 and 3.2 vs 0.8 respectively). Similarly, the mean percentages of usable oocytes, transferable and freezable embryos were significantly higher ($P < 0.01$) for the synchronized than the nonsynchronized donors (28.5% vs 20.5%, 20.9% vs 9.0% and 15.8% vs 6.5%, respectively). On comparison of the synchronized and nonsynchronized donors of each breed, the difference in the mean percent of usable oocytes was significant ($P < 0.01$) in cows of all three breeds, the difference in the mean percent of transferable embryos was significant in Czech Siemmental and Holstein Dairy cows ($P < 0.01$) and the difference in the mean percent of freezable embryos was significant only in Holstein Dairy cows ($P < 0.01$). In order to confirm viability of the embryos, some of them were transferred to recipients on Day 7 after estrus. After transfer of 41 frozen-thawed embryos and 43 fresh embryos, 20 heifers and 24 heifers became pregnant, respectively. In conclusion: a) higher number of oocytes from infertile, genetically valuable cows was recovered in the growth phase as compared with the other phases of follicular development; b) greater development of these oocytes resulted in more embryos for transfer and cryopreservation; c) transfer of frozen-thawed and fresh embryos gave a 48.8% and 55.8% pregnancy rate, respectively.

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Notes

VIABILITY OF RABBIT GENE-MICROINJECTED EMBRYOS CRYO-PRESERVED USING TWO VITRIFICATION SCHEMES

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The aim of this study was to compare two vitrification schemes (VS), using either ethylene glycol (EG) in combination with dimethylsulfoxide (DMSO, VSI) or Ficoll 70 (VSII), for rabbit embryo cryopreservation based on their post-thaw survival, cell death and actin cytoskeleton. The pronuclear stage eggs were flushed from the oviducts of the slaughtered New Zealand White rabbit does 19 to 20 hr *post coitum* (hpc) and randomly divided into 2 groups: intact (control) and gene-microinjected (Mi). Mi embryos or control embryos were cultured for up to 72 hpc (morula stage), and then vitrified using either VSI (VSI+Mi, VSI) or VSII (VSII+Mi, VSII). After 2-3 days at -196 ºC, the embryos were thawed and cultured until 96-100 hpc to assess their development to blastocyst, apoptotic rate (TUNEL assay) and state of actin cytoskeleton (phalloidine-TRITC).

Mi procedure reduced blastocyst yield ($p< 0.001$, versus intact embryos), but it was higher than in either vitrified (VSI, $p<0.05$) or Mi vitrified (VSI+Mi, $p<0.001$) embryos. VSI compromised, whereas VSII did not significantly affect blastocyst development compared to intact embryos ($p< 0.001$). Mi and VS both affected the embryo quality increasing TUNEL-index and decreasing the ratio of embryos with high quality actin cytoskeleton compared to control. A higher apoptotic index was recorded in VSI group. A combination of Mi and VS induced an increase in apoptotic rate (10.35% and 7.54% for VSI+Mi and VSII+Mi, resp.) as compared to Mi alone (5.7%). Ratio of embryos belonging to best actin quality (grade I) was different among groups and most of the embryos with grade I actin were in intact (84%), Mi (71%) or VSII (70%) groups. A significantly lower number of embryos with grade I actin quality was observed in VSI (58%), VSI + Mi (54%) or VSII + Mi (66%).

These observations indicate that of the vitrification schemes tested, the VSII using EG and ficoll 70 as cryoprotectants, was less harmful than VSI (EG combined with DMSO in vitrification medium).

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Notes

SEASONALITY OF SHEEP IN *IN VITRO* EMBRYO PRODUCTION IN SARDINIA

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It has been reported that the recovery and the quality of embryo production is related to season. Indeed, even where donor animals are identified and carefully managed, seasonal effects on fertility cause variation in embryos yields. The aim of this work was to assess the effect of season (breeding and anestrous) and annual variations on the cleavage and blastocyst rates of *in vitro* produced ovine embryos during three years of data collection (from 2005 to 2007).

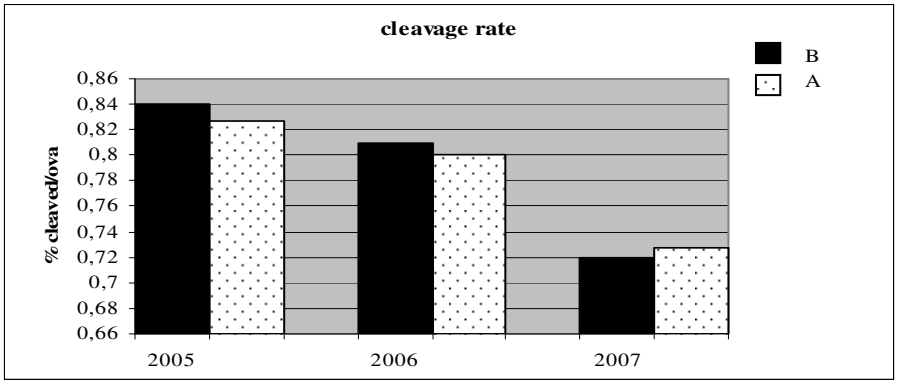
Ovaries of Sarda sheep were collected from the slaughterhouse. A total of 4404 oocytes were matured in TCM-199 supplemented with 4 mg-/ml BSA, 100 □M cysteamine, 0.3 mM Na Pyruvate, 0.1 UI/ml r-FSH, 0.1 UI/ml r-LH, 1 □g/ml estradiol-17β. Matured oocytes were fertilized with fresh semen in synthetic oviductal fluid (SOF) with 20% of heat inactivated estrous sheep serum. The presumptive zygotes were cultured for 6-7 days until blastocyst stage in medium consisting of SOF supplemented with 1% BME, 1% MEM, 1mM glutamine and 8 mg/ml fatty acid-free BSA.

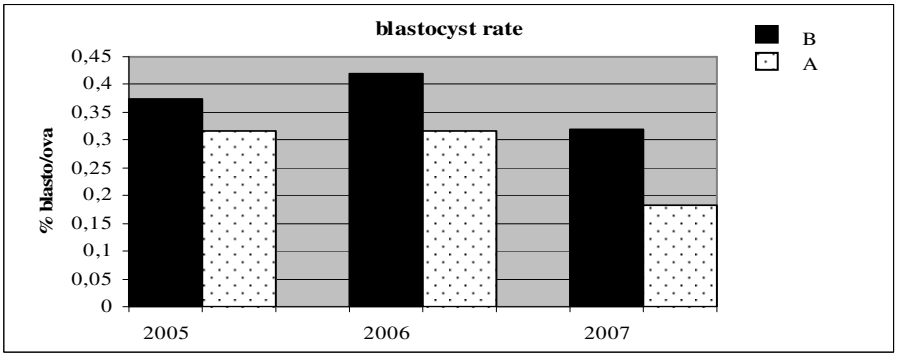
The cleavage rate did not show significant differences between seasons within the years (84% vs 83%, 81% vs 80 % and 72% vs 73%, respectively). However, when data among years were compared, the 2007 showed the lowest values, with a significant difference ($P<0.001$) in respect to the other periods.

The blastocyst rate showed a significant difference between seasons within the years 2006 and 2007 ($P<0.001$), whereas the year 2005 did not show significant differences, despite a tendency to higher values during the breeding season (37% vs 32%).

Similarly to the cleavage rate, when data among years were compared for blastocyst rate, the 2007 reached the lowest values, with significant differences ($P<0.05$ and $P<0.001$, respectively).

In conclusion also the embryo *in vitro* production seems to be affected by season and year.





Notes

EFFECTS OF LINOLENIC ACID ON BOVINE OOCYTE MATURATION AND EARLY EMBRYO DEVELOPMENT IN VITRO

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Previous studies showed that changes in dietary polyunsaturated fatty acids affect the profile of fatty acids in tissues and fluids of the reproductive system and can influence reproductive functions. In dairy cows, dietary sources of omega-3 (n-3) fatty acids including linolenic acid (LNA; 18:3) which are naturally present in grass and linseed oil were proved to benefit fertility. However, little is known about their effect on oocyte or embryo development. In this study we investigated the effect of LNA on bovine oocyte maturation and early embryo development in vitro.

Experiment 1: Cow ovaries were collected from a local abattoir. Cumulus oocyte complexes (COCs; n≥20 in each group) were cultured in serum-free M199 medium supplemented with 0µM (control) or 50µM LNA for 24h. Cumulus expansion was then assessed and stage of nuclear maturation was determined by aceto-orcein staining. Prostaglandin E2 (PGE_2; an important mediator of oocyte maturation) and $PGF_2\alpha$ were analysed in spent media by radioimmunoassay. Treatment of COCs with LNA did not affect cumulus expansion but significantly increased the percentage of oocytes at MII stage (88.8±3.9 vs. 83.3±3.4; P=0.05). This was accompanied by a significant increases in PGE_2 (233±41.01; P=0.009) and $PGF_{2\alpha}$ (152±9.2; P=0.001) as a percentage of the control.

Experiment 2: Extracellular signal-regulated kinase (ERK1 and 2) were analysed to determine if LNA action on maturation is mediated by the mitogen activated protein kinase (MAPK) cascade. Proteins extracted from COCs matured in the presence or absence of LNA (n=25 in each treatment group) were immunoprobed with total and phosphorylated ERK1/2 antibodies. Density of bands was quantified. LNA treatment resulted in a tendency to increase the percentage (phosphorylated/total) of ERK1 (57±6.2 vs. 52±2.9) and ERK2 (64±2.3 vs. 60±2.7) with electrophoretic mobility shift of the bands.

Experiment 3: COCs (n=200, 4 repeats) treated with 0 µm (control) or 50 µM LNA during maturation were fertilized in vitro. Embryos were cultured in serum free synthetic oviductal fluid medium. Cleavage (day 2) and blastocyst rate (B; day 8) were recorded. To assess the blastocyst quality, total cell number (TC), inner cell mass (ICM), trophoectoderm (TE) and apoptotic cells (Ap) were counted by differential staining combined with TUNEL. LNA resulted in a significantly higher percentage of cleaved embryos and blastocyst rate and better quality embryos with higher number of ICM and TE cells and lower apoptosis (Table 1).

Table 1. Development and embryo quality of LNA treated COCs in vitro.

	Cleavage	B/Cleav.	Hatch/B	TC	ICM	TE	Ap	ICM/TE
	%			Cell no.				Ratio
Control	69.0±8.7	23.3±4.5	11.1±7.0	114±4.4	33±1.3	91±2.3	2.3±0.4	0.37±0.02
LNA	77.4±8.7	36.0±4.0	31.8±4.5	139±7.7	40±1.4	116±6.7	1.1±0.3	0.39±0.03
P value	0.027	0.008	0.026	0.004	0.018	0.042	0.001	0.706

In conclusion, treatment of bovine COCs with LNA during maturation improves maturation rate and subsequent early embryo development. This effect is associated with changes in ERK1/2 profile and increased prostaglandin production by cumulus cells.

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EFFECT OF OOCYTE COLLECTION METHOD AND BREED ON EFFICIENCY OF OOCYTE COLLECTION AND SUBSEQUENT IN VITRO BOVINE EMBRYO PRODUCTION

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In vitro production of embryos is either based on ultrasonic guided transvaginal oocyte collection (Ovum Pick Up program (OPU); in vivo aspiration) or on collection from ovaries upon slaughter (Genetic Recovery program (GR); ex vivo aspiration). Number of retrieved oocytes is in general lower with in vivo aspiration due to the limited resolution of the OPU equipment. In a previous study (Mullaart et al, Theriogenology, 2003) we already showed that the number of small (<2mm) follicles /oocytes left in the ovary upon OPU is substantial. In this study we compare the efficiency of oocyte recovery between OPU and GR. This comparison was made for Belgian White Blue (BWB) and Holstein-Friesian (HF) breeds.

Immature Cumulus-Oocyte-Complexes (COCs) were obtained either by OPU (twice weekly) or by GR. COCs were matured in CM199/FCS/LH/FSH/(cysteamine), fertilised with frozen-thawed gradient separated semen and subsequently cultured for 7 days in SOFaaBSA. Number of transferable embryos was recorded at Day 7 and 8. Data set covered a production period of 12 years (1995-2007). Data were analysed by Chi-square.

The number of retrieved COCs per session was significantly higher with GR compared to OPU in both breeds (see Table 1). However, with OPU the number of COCs was two times higher for BWB as compared to HF, whereas this difference was absent with GR. Overall (both breeds together) cleavage and embryo production rate were significantly lower with GR. Within OPU, cleavage and embryo production rate was significantly higher in BWB resulting in a higher number of embryos per session.

Table 1. Effect of collection method on oocytes recovery and embryo production in HF and BWB

Collection technique	Breed (n donors)	Sessions	COC n	COC/ Session	% Cleavage D4	% Embryos D7	embryo/ session
OPU	BWB (29)	136	1,991	14.7	54[a]	24[a]	3.47
	HF (709)	9,089	62,769	6.9	57[b]	17[b]	1.22
	Total	9,225	64,769	7.0	59[c]	18[c]	1.27
GR	BWB (26)	26	1,051	40.4	50[a]	14[a]	5.73
	HF (254)	254	9,977	39.3	47[a]	11[a]	4.33
	Total	280	11,028	39.4	47[d]	11[d]	4.46

[a,b]: For each collection technique, values in columns with different superscript are significantly different, P<0.05
[cd]: between collection techniques, values in columns with different superscript are significantly different, P<0.05.

These results show that a higher number of COCs can be retrieved by OPU from BWB compared to HF. However, this is not caused by a higher number of follicles present in the ovaries of BWB (i.e. number of COCs recovered by GR was the same in both breeds), suggesting that there are more large follicles present in the BWB ovary. The extra COCs collected by GR originate from smaller follicles. This will affect the overall quality of the retrieved batch of COCs, which is confirmed by the lower cleavage and embryo production rate found in this study with GR.

Notes

BOVINE OOCYTE QUALITY IS REFLECTED IN ZONA PELLUCIDA ARCHITECTURE WHEN ASSESSED BASED ON G6PDH ACTIVITY

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Oocyte quality profoundly affects monospermic fertilisation, early embryonic survival, the establishment and maintenance of pregnancy and even fetal development. Therefore, identification of reliable predictors of oocyte quality will be important for efficient embryo production and infertility treatment. Brilliant cresyl blue (BCB) staining has been proved to be one of the most efficient non-invasive tools for oocyte quality assessment in different animal species. We have previously demonstrated that molecular and subcellular characteristics of bovine oocytes selected according to this test were extremely different. Since BCB+ oocytes showed over-expression of ZP3 transcript compared to BCB− counterparts. Here, we would like to investigate if oocyte quality is also reflected in zona pellucia architecture when assessing based on G6PDH activity.

Cumulus oocyte complexes (COCs) were recovered from slaughterhouse ovaries by aspiration. In the first experiment, COCs were first incubated with 26 μM BCB for 90 min. Treated oocytes were then divided into BCB+ (coloured cytoplasm, low G6PDH activity) and BCB− (colourless cytoplasm, increased G6PDH activity) based on their ability to metabolize the stain. After denudation of cumulus cells, value for the birefringence peak (PV-Mean), the birefringence peak combined with the surface of the birefringence (CV-Mean) and the thickness of zona pellucia (WG-Mean) for oocytes of each group were analysed using polarized light microscopy and OCTAX polar AIDE-Software. In the second experiment, oocytes were allocated into three groups: control and BCB treated which was divided into BCB+ and BCB−. Following IVM of the previously mentioned groups, all oocytes were fertilized in vitro and cultured till day 8.

There was a clear difference ($P \leq 0.05$) for CV-Mean (21.156 vs. 24.125), PV-Mean (41.364 vs. 45.719) and WG-Mean (0.524 vs. 0.538) between immature oocytes which stained (BCB+) and those which did not (BCB−). The cleavage rate was significantly higher ($P \leq 0.05$) for control (73.4%) and BCB+ oocytes (74.1%) than for BCB− group (65.6%). BCB+ oocytes yielded a significantly higher blastocyst rate than the control or BCB− oocytes (29.9, 23.0%, and13.2, respectively; $P < 0.05$).

In conclusion, a significant relationship was found between oocyte quality classes assessed according to BCB test and zona pellucida architecture. This could implicate that, the zona pellucida architecture could also be considered as a simple and efficient non-invasive tool to screen oocytes and embryos for their developmental competence.

Notes

CRYOTOPS VERSUS OPEN-PULLED STRAWS (OPS) AS CARRIERS FOR THE VITRIFICATION OF BOVINE OOCYTES: EFFECTS ON EMBRYO DEVELOPMENT

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The experiment was designed to assess the effectiveness of cryopreserving bovine MII oocytes using cryotops as the carrier system for vitrification. We examined the developmental competence of oocytes after: (i) vitrification in open-pulled straws (OPS method); or (ii) vitrification in <0.1 µl medium droplet on the surface of a specially constructed fine polypropylene strip attached to a plastic handle (Cryotop method). Both cow and calf oocytes were used.

Three experimental groups were established according to whether the oocytes were vitrified in OPS (OPS group), cryotops (cryotop group) or left untreated (CTR group). Twenty two hours after the onset of maturation, sub-groups of 2-4 oocytes were pre-equilibrated in 1 mL of Hepes-TCM 199 with 20% FCS (HM), 10% DMSO, 10% ethylene glycol (EG) and 1 µM Taxol for 30 sec. The oocytes were then transferred to a 20-µl drop of HM with 20% DMSO, 20% EG, 0.5 M of sucrose and 1 µM Taxol, loaded into OPS or cryotops and immersed in liquid nitrogen. Oocytes were thawed by plunging the OPS or cryotops for 5 min into 0.25 M sucrose in HM, and then placed for 5 min each in 0.15 and 0 M sucrose in HM. After vitrification and warming, the oocytes were fertilized and cultured *in vitro*. Cleavage and blastocyst rates were determined on Days 2 and 7 after fertilization, respectively.

Oocytes in the Cryotop group exhibited a significantly higher cleavage rate compared to the OPS group for both cow (46.1% vs 31.5%) and calf (46.4% vs 20.2%) oocytes. These cleavage rates were, nevertheless, significantly lower than those recorded for the control oocytes (66.9% and 65.7% for the cow and calf oocytes, respectively). Blastocyst rate/cleavage rate ratios were also significantly higher in the Cryotop group (5.3% and 3.8%) than the OPS group (0%) for cow and calf oocytes, respectively. Both the cow and calf oocyte vitrification groups showed a lower blastocyst/cleavage rate ratio than their respective control groups (33.5% and 29.8% for cow and calf oocytes, respectively).

These findings suggest the cryotop system is a more efficient carrier for vitrification than OPS for the cryopreservation of bovine oocytes.

Notes

COMPARISON OF THE COMPOSITION AND MORPHOLOGY OF THE ZONA PELLUCIDA OF EQUINE AND PORCINE OOCYTES

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In equine species, *in vitro* fertilization rates are lower than 30%. The equine spermatozoa bind to the zona pellucida (ZP) but do not penetrate this envelope. The equine ZP is a major barrier to successful *in vitro* fertilization. On the contrary, in porcine species, *in vitro* fertilization rates are higher than 70% and *in vitro* polyspermy rates range from 50 to 70%. The porcine spermatozoa penetrate easily the ZP. We hypothesized that the composition and morphology of equine and porcine ZP are different.

We first compared the composition of the equine and porcine ZP. To identify the major glycoproteins composing the equine and porcine ZP, we performed phylogenetic analysis. Our results showed that the porcine ZP contains 3 glycoproteins ZPA, ZPB and ZPC, whereas the equine ZP contains 4 glycoproteins ZPA, ZPB, ZPC (60-72% similarity) and ZP1.

Then, we compared the morphology of equine and porcine ZP before and after *in vitro* maturation of oocytes. Equine and porcine immature oocytes were collected from slaughtered mares and sows. Half were fixed and half were matured *in vitro* and fixed. Some immature and in vitro mature oocytes were prepared and analysed by transmission electron microscopy (TEM) after serial ultra-thin sections. Some others were prepared and analysed as a whole by scanning electron microscopy (SEM). We could not evidence any modification of the equine and porcine ZP during *in vitro* maturation using SEM or TEM. When using TEM, no obvious difference was observed between the equine and porcine ZP whatever the maturation stage. However, when using SEM, we observed a difference between equine and porcine ZP: porcine ZP has a net-like structure with numerous large pores and hollows whereas equine ZP has a rough surface with only few pores and without a net-like structure.

In conclusion, the composition and morphology of equine and porcine ZP are different. This may be related to the different competence of the spermatozoa to penetrate the ZP.

Notes

ZONA PELLUCIDA BIREFRINGENCE IN BOVINE OOCYTES AND ZYGOTES IS ASSOCIATED WITH PREIMPLATATIVE DEVELOPMENTAL COMPETENCE

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To raise the efficiency of bovine IVP, noninvasive parameters to predict developmental competence of oocytes and zygotes would be useful. Therefore we tested the correlation of the zona birefringence at different timepoints with subsequent development. MII oocytes were denuded and activated parthenogenetically (5 mM Ionomycin for 4 min followed by 2 mM 6-DMAP for 3.5 hours) or fertilized before denudation. Subsequently, birefringency of MII oocytes (directly after parthenogenetic activation) and zygotes (24h after start of IVF) were measured. Values for the birefringence peak (PV-Mean) and the birefringence peak combined with the surface of the birefringence (CV-Mean) of the zona pellucida were measured using polarized light microscopy and OCTAX polarAIDE-Software. To allow tracking of subsequent development, embryos were cultured individually in a Well of Well (WoW) culture system until Day 7 in CR1aa medium (5% CO_2, 38,7°C, humidified air).

Measuring the birefringence of activated MII oocytes (n=365) did not indicate any differences between those which cleaved within 48h and those which did not. However, significant differences ($p<0.05$) in terms of PV-Mean (41.4 vs. 43.3) and CV-Mean (21.3 vs. 22.6) were observed at MII stage between oocytes which subsequently reached the blastocyst stage at day 7 and those which did not. Additionally, zygotes that cleaved within 48h had significant lower CV-Mean (24.7 vs. 27.1) and PV-Mean (47.1 vs. 51.5) values compared to zygotes that did not cleave ($p<0.05$) and zygotes which developed to the blastocyst stage at day 7 were significantly lower in terms of CV-Mean (24.3 vs. 25.4) and PV-Mean (45.5 and 47.9) compared to zygotes that did not reach that developmental stage, too.

In conclusion we successfully established a non invasive technique to predict the developmental competence of bovine MII oozytes and zygotes with lower values for PV-Mean and CV-Mean being correlated with higher developmental competence. Identification of zygotes with high developmental competence will offer great benefit for human IVP and identification of favourable MII oozytes will increase the efficiency in nuclear transfer experiments.

Notes

QUALITY OF BOVINE BLASTOCYSTS DEVELOPED FROM BCB STAINED AND NON-STAINED OOCYTES

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The aim of the experiment was to determine the affect of BCB staining on oocytes developmental competence by assessing the quality of bovine blastocysts on the basis of caspase-3 activity and DNA fragmentation. Bovine blastocysts were produced following IVM/IVF/IVC of immature oocytes selected for the experiment by combine evaluation of COCs morphology and G6PDH activity (BCB staining). Embryo culture to the blastocyst stage was carried out in the co-culture with Vero cells, in B2 medium, at humidified atmosphere of 5% CO_2 in air.

A total number of 182 blastocysts (88 BCB+ and 94 Control) was used for assessment of caspase-3 activity (PhiPhiLux G_2D_2, Calbiochem). Additionally 25 blastocysts (10 BCB+ and 15 Control) were used for TUNEL assay (DeadEnd^{TM} Fluorometric TUNEL System, Promega).

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 and 58.2% respectively for BCB+ and Control), the lowest activity of the estimated enzyme was seen in early and middle stage blastocyst (43.8 vs. 29.4% for BCB+ and Control). Comparing the blastocyst rates with low and high caspase-3 activity within each of the analyzed groups (Table 1), the significant difference was observed only in the BCB+ group ($P<0,05$, test χ^2).

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

Fluorescence intensity	Blastocysts BCB+ (%)	Blastocysts Control (%)
+	37 (42.0)^{a*}	45 (47.9)^{a}
++	51 (58.0)^{a*}	49 (52.1)^{a}

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

Both BCB+ and Control blastocysts contained TUNEL positive cells (30,4% vs. 17,8% respectively) as measured by DCI (Death Cell Index). The high statistical difference was found between both analyzed groups of blastocysts ($P<0,001$, test χ^2).

In conclusion, BCB staining of immature oocytes seems to have a detrimental affect on the quality of the blastocysts in terms of the apoptosis progression.

Notes

DNA FRAGMENTATION IN FROZEN SEMEN SAMPLES OF FIGHTING BULLS

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The objective of this study was to analyse the DNA Fragmentation Index (DFI) and spermatic viability in frozen-thawed seminal samples from fighting bulls.

The functional integrity of the spermatic membrane is essential to fertilization, but it does not contribute in further processes of embryo development, while DNA integrity is mainly involved in the initial stages of embryonic development. An adaptation of the sperm chromatin dispersion (SCD) test has been used to determine DNA fragmentation. This test is based on the differential response of sperm nucleus to a desproteinization treatment. Sperm cells containing fragmented DNA show a large peripheral halo of chromatin dispersion after staining with a fluorochrome. However, when DNA is not fragmented within the sperm nuclei, a restricted spreading of DNA close to the flagellum can be observed. The sample observations were performed by a fluorescence microscope. Moreover, sperm viability was assessed using a supravital stain based on the red/green emission of two fluorescent dyes, acridine orange (AO) and propidium iodide (PI), respectively.

We determined the basal DFI in 53 fighting bull semen samples, resulting in 7.88±6.27 and lower and upper limits 1-34 %, respectively. The vitality average was 23.09±15.80, the lower and upper limits being 1-60 %, respectively. A negative correlation between both parameters was observed when relating DNA fragmentation and vitality of bull sperm samples.

The results of the study are evidence of the high individual variability for both parameters, probably due to external individual factors that are being studied. We suggest that DFI should be introduced as a routine analytical parameter for semen analysis, as damaged DNA could contribute negatively to fertility.

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Notes

BIRTH AND CHARACTERISTICS OF BOVINE CLONE OFFSPRING: PRELIMINARY RESULTS

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In the perspective of agronomical applications of animal cloning and in the context of the evaluation of clone products by national and international agencies, clone offspring are of major interest. They are indeed more likely to enter the human food chain than the clones themselves, which will be used as genitors. Information on clone offspring, however, is still limited, especially in species with a long inter-generation interval such as cattle. In the present study we report data on calves born from cloned females at the INRA experimental unit. Characteristics of these offspring are compared to that of clones or control animals born and raised in the same farm.

Currently, 39 clone offspring have been obtained after AI of cloned heifers from 3 different genotypes, with semen from different 6 Holstein sires used in both groups. All calves were delivered naturally at term with a mean easy calving score of 1.58 and a normal pregnancy length of 282 days. There were 20 males and 19 females, resulting in a normal male /female sex ratio of 51/49. Male calves were euthanized during the first few weeks after birth according to the INRA moratorium and only a group of 18 Holstein single born female offspring were further considered and compared to female AI controls (n= 68) or clones (n=50).

The mean birth weight of the female offspring was 40.28 ± 3.32 kg (range 35-44), not significantly different from that of AI controls: 40.57 ± 5.55 kg and significantly lower ($P<0.05$) and less variable than that of the clones: 49.27 ± 10.93 kg. No case of large offspring syndrome was observed. The mean birth weight of the males was 45.55 ± 6.66 kg (range 38-61).

Post natal survival in the clone offspring group was normal as 17/18 (94.4%) remained alive and healthy by 6 months of age. This was comparable to survival in AI control group 64/68 (94.1%) and higher than for clones at the same age: 35/50 (70.0%). Animals were weighed every month. There were no statistical differences between the clone offspring group and the AI controls raised in the same conditions, with a total body weight of 188.25 ± 15.17 kg *vs* 185.30 ± 18.94 kg by 6 months of age and a daily weight gain of 0.820 and 0.803 kg/day, respectively.

None of the clone offspring presented any of the pathologies observed in clones. The oldest heifers are now mature and their reproductive function is being evaluated. Moreover, some animals are already used as recipients in an embryo transfer programme.

Notes

EFFECT OF FOLLICLE DIAMETER ON THE APOPTOSIS INCIDENCE IN IMMATURE PREPUBERTAL GOAT OOCYTES

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Follicle diameter has been extensively used to select the oocytes according to their developmental competence, although it does not indicate whether a specific follicle is healthy or atresic. Apoptosis in cumulus cells has been related with impaired enclosed oocytes (Zeuner et al., 2003). Annexin-V and TUNEL (Tdt-medaited dUTP nick-end labelling) stainings have been used to study apoptosis by changes membrane phosphatidylserine redistribution (early apoptosis) and DNA strand breaks (late apoptosis), respectively. Our objective is to determine the apoptosis incidence in immature prepubertal goat oocytes according to follicular diameter (< 3 mm and > 3 mm) and control group.

Ovaries were recovered from prepubertal goats (1 to 2 month old) slaughtered at a local abattoir. Oocytes from follicles > 3mm in diameter were obtained by aspiration while the oocytes from < 3 mm follicles and the control oocytes (not selected by their follicular diameter) were recovered by slicing technique. After oocyte selection, oocytes were washed twice in mPBS and denuded. Early and late apoptosis were detected by Annexin-V staining (Annexin-V-Fluos staining Kit, Roche) and TUNEL assay (In Situ Cell Death Detection Kit, Roche) following manufacturer's instruction, respectively. In early apoptosis study, the oocytes were classified in (1) viable oocytes, no Annexin staining, (2) early apoptotic oocytes, an homogeneous Annexin positive signal in the membrane; and (3) necrotic oocytes, which showed propidium iodide positive red nuclei, indicative of membrane damage. In late apoptosis study, oocytes were classified (1) non-apoptotic oocytes, TUNEL negative nuclei (red); (2) apoptotic oocytes with fragmented nuclei, TUNEL-positive (green).

The results of Annexin-V and TUNEL staining in immature prepubertal goat oocytes are presented in the next table. Oocytes from follicles > 3mm presented significantly higher late apoptosis than oocytes from follicles < 3mm. Oocytes from control group were less apoptotic (early and late) than oocytes from > 3mm and < 3 mm follicles.

Follicle diameter	Annexin-V staining			Tunel Assay	
	Oocytes	Apoptotic (%)	Necrotic (%)	Oocytes	Apoptotic (%)
> 3mm	88	36 (40.91) [a]	12 (13.64)	98	42 (42.86) [a]
< 3mm	95	32 (33.68) [a]	8 (8.42)	194	47 (24.23) [b]
Control	117	23 (19.66) [b]	14 (11.97)	191	25 (13.09) [c]

Values in the same column with different superscripts ([a,b,c]) differ significantly ($p < 0.05$)

In conclusion, a higher rate of early and late apoptosis has been observed in oocytes from follicles > 3 mm in diameter. It could be due to these oocytes become atretic in ovaries without ovulation cycle. The lower apoptosis found in oocytes from control group could be due to the short time between manipulation of ovaries and analysis of oocytes.

Notes

DEPENDENCE OF APOPTOSIS OCCURRENCE AT THE BLASTOCYST STAGE ON THE SIMULTANEOUS FUSION AND ACTIVATION OF PORCINE NUCLEAR TRANSFER OOCYTES RECONSTRUCTED WITH YO-PRO-1-ANALYZED FETAL FIBROBLAST CELLS

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The aim of our study was to examine whether the simultaneous physical fusion and activation of porcine somatic cell nuclear-transferred oocytes affects both the preimplantation developmental competences 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 fetal fibroblast cells, which had been classified as non-apoptotic on the basis of vital DNA YO-PRO-1 dye-mediated diagnostics. Then, nuclear transfer-derived oocytes were artificially stimulated with the use of simultaneous fusion and electrical activation (SF-EA). In the SF-EA protocol, electric pulses that 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^{-1} 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 NCSU-23/BSA/FBS medium for 6-7 days. At the end of the *in vitro* culture period, embryos at the blastocyst stage were analyzed *intra vitam* for 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 392/449 (87.3%) reconstituted zygotes were selected to *in vitro* culture. Out of 392 cultured nuclear-transferred embryos, 319 (81.4%) were cleaved. The percentages of cloned embryos that reached the morula and blastocyst stages yielded 237/392 (60.5%) and 120/392 (30.6%), respectively. As measured in relation to a total number of nuclear transfer-derived blastocysts analyzed for apoptosis, the frequency of the embryos, in which annexin V-eGFP-positive cells were detected, was 28.6% (24/84). In conclusion, using the SF-EA protocol the abilities of non-apoptotic fetal 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

OCT-4 EXPRESSION IN *IN VITRO* CULTURED OVINE BLASTOCYSTS

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The Oct-4 POU transcription factor has been shown to play a key role in early embryonic development and cell differentiation. It is also required for the maintenance of pluripotency in the inner cell mass (ICM) of blastocyst. Oct-4 expression, during embryo development, undergoes a down-regulation as the differentiation progresses in different species. In mice Oct-4 is expressed in the ICM, but not in the trophoblast (TB), for this reason it is used as pluripotency marker. In bovine, porcine, caprine, rhesus monkey, equine and human, Oct-4 is detected both in the ICM and in TB. However, the expression is higher in the ICM than in TB thus that can be used as a pluripotency marker. To our knowledge, in sheep the presence of this gene has not yet been studied. The aim of this study was to examine the expression of Oct-4 in ovine blastocysts produced *in vitro*.

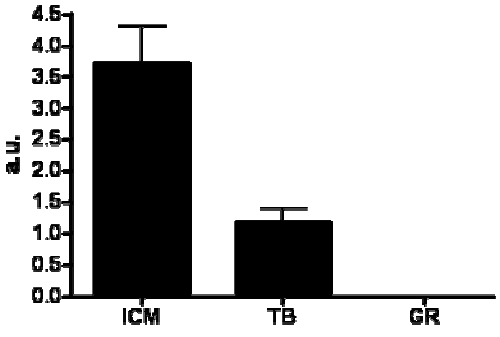
Oocytes were obtained from ovaries of slaughtered adult ewes, matured in vitro in TCM199 supplemented with 4 mg/ml BSA for 24 h and then fertilized with fresh ram semen. Zygotes were cultured for 6-7 days in 20 µl droplets of SOF supplemented with 4 mg/ml fatty acid free BSA at 39°C in an atmosphere of 5% CO_2,7% O_2 in air. On the third and fifth day of culture (day 0= fertilization day) 4 mg/ml fatty acid-free BSA was added to the culture. Forty blastocysts were divided in two groups: 10 blastocysts (EB) were used for sequencing and 30 blastocysts were biopsied (BB) in order to obtain ICM and TB. EB and BB were snap frozen in LN_2 for mRNA isolation. The cDNA produced by Reverse Transcriptase PCR (RT-PCR) from EB was synthesized and amplified by PCR using Oct-4 bovine primers to obtain a sequence of 290 bp (Table 1). The ovine sequence was aligned to the bovine sequence by dint of NCBI-blast and a high similarity was detected (98%). On the strength of new ovine sequence, quantitative expression for Oct-4 in ICM and TB was performed by Real Time PCR. The ovine Oct-4 was expressed in the ICM and in TB, but the level of expression in ICM was significant higher than in TB (Table 2).

In conclusion, Oct-4 is present in *in vitro* produced ovine embryos and this contributes to define their quality. Moreover the different level of expression in ICM and TB makes possible to use the presence of Oct-4 as a marker of cellular pluripotency.

Table 1 Ovine OCT-4 Sequence (290 bp).

CAATTTGCCAAGCTCCTAAAGCAGAAGAGGATCAC
CCTAGGATATACCCAGGCCGATGTGGGGCTCACCC
TGGGGGTTCTCTTTGGAAAGGTGTTCAGCCAAACGA
CTATCTGCCGTTTTGAGGCTTTGCAGCTCAGTTTCA
AGAACATGTGTAAGCTGCGGCCCCTGCTGCAGAAG
TGGGTGGAGGAAGCTGACAACAACGAGAATCTGCA
GGAGATATGCAAGGCAGAGACCCTTGTGCAGGCCC
GAAAGAGAAAGCGGACGAGTATCGAGAACAAGGT
GAGAGGCAA

Table 2 Gene expression: ICM, TB and Granulosa Cells (GR) negative control.



Notes

HIGH EFFICIENT PRODUCTION OF PORCINE TRANSGENIC NUCLEAR-TRANSFERRED EMBRYOS USING ANNEXIN V-eGFP-DIAGNOSED ADULT DERMAL CELLS UNDERGOING LIPOFECTION

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The purpose of our study was to determine the *in vitro* developmental potential of porcine nuclear transfer-derived embryos reconstructed with *pWAPhGH-GFPBsd* transgene-lipofected sow ear skin-retrieved fibroblast cells, which had been analyzed for apoptosis through the live-plasma membrane fluorescent tagging. Frozen/thawed fibroblast cells, which had been *in vitro* cultured up to a total confluency after 2-6 passages, were used for analysis. To detect the early-apoptotic changes in the fibroblast cells, single nuclear donor cell suspension was labeled with the conjugate of annexin V and eGFP protein. The source of recipient cells were *in vitro*-matured gilt or sow oocytes. Maternal chromosomes were eliminated by chemically assisted microsurgical technique. Fibroblast cell-ooplast couplets were simultaneously fused and activated. Reconstructed embryos were cultured in NCSU-23/BSA/FBS medium for 6-7 days. The rates of cleavage and development to morula/blastocyst stages were examined on Days 2 and 6/7, respectively. After fluorescent analysis of adult dermal fibroblast cells, it was shown that a relatively high proportion (ranging from 20 to 30%) of donor cells exhibited ultrastructural late-apoptotic or necrotic changes. In contrast, from among the morphologically normal cells the extremely low rate (ranging from 0 to 2%) of the cells emitted the annexin V-eGFP-derived green fluorescence, but the other ones did not emit this biochemiluminescence. It can suggest that the former subpopulation of the cells was early-apoptotic, and the latter was non-apoptotic. A total of 285 enucleated oocytes were successfully fused with non-apoptotic transgenic nuclear donor cells and intended to be *in vitro* cultured. Out of 285 reconstructed oocytes, 199 (69.8%) NT embryos were cleaved. The frequencies of cloned embryos, that reached the morula and blastocyst stages, were 144/285 (50.5%) and 51/285 (17.9%), respectively. In conclusion, the lipofection efficiency of *in vitro* cultured porcine adult dermal fibroblast cells as estimated by nuclear donor live-fluorescent evaluation based on expression index of eGFP reporter transgene was nearly 100%. Moreover, our results demonstrate that the morphological criteria commonly used for cell viability classification are a sufficient selection factor for qualitative evaluation of nuclear donor cells to somatic cell cloning. It was also found that porcine cloned morulae and blastocysts exhibited approximately 100% index of xenogeneic eGFP gene transcriptional activity, which revealed the live diagnostics of emission intensity for green fluorescent protein-derived biochemiluminescence.

Notes

THE RELATIONSHIP BETWEEN THE NUMBER OF CL AND EMBRYO QUALITY

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The objective of this study was to evaluate the efficiency of the superovulatory treatment on the ovary response and the quality of the flushed embryos. Superovulation was induced with twice application of PGF2α in 11-days intervals, followed by FSH administration (twice daily within 4 days). The non-chirurgical recovery of embryos was conducted every 7th day after the 1st insemination by the standard method. There was evaluated the number of corpus luteum (CL) on both ovary by rectal palpation. Donors were separated into groups based on the numbers of CL subsequently: 1st group n=16 donors with 1-5 CL; 2nd group n=32 donors with 5-10 CL; 3rd group n=27 donors with more than 10 CL. Further, the quality of all flushed embryos was analyzed. Average number of suitable/total embryos was 1.44/1.94, 4.25/7.25, 7.15/12.74 in the first, second and third group respectively. There was the significant difference between groups (ANOVA, $F=10.98$, $p<0.001$). Tukey multiple comparisons of means showed the difference between the first and the other two groups ($p<0.02$). The second and third groups were not significantly different ($p=0.078$). 74.2 % of suitable embryos was recovered in the first group (1-5 CL) and in the other groups there was the percentage of suitable embryos lower (58.6%; 56.1%). We assume that the larger number of CL (embryos) increases the number of degenerate or non-fertilized embryos because of possible competition. Due to the unbalanced counts of the animals in the groups, further studies are to be in progress to confirm our hypothesis based on these preliminary data.

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Notes

FREEZING OF SEMEN FROM ENDANGERED ASTURIANA DE LA MONTAÑA BULLS IN PLANT ORIGIN LIPIDS LIPOSOMES

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Two different preparation protocols of plant lipids liposome's for bull semen cryopreservation were tested: 1. Soya bean lipids were homogenized by high pressure of 800 bars (HPH) and then mixed with 8% glycerol (Y2); 2. Lipids were homogenized together with glycerol by 800 bars HPH (L1). Bioxcell extender was used as control. Semen was collected 3 times from 3, endangered Asturiana de la Montaña bulls with an artificial vagina and processed by standard freezing protocol. Sperm motility and movement characteristics were analyzed microscopically, immediately after collection, after dilution and after 4, 24, 48 and 72hr of storage at 4^0C by CASA. Membrane functionality was evaluated by the Hypoosmotic Swelling Test (HOST). Membrane integrity (percentage of viable spermatozoa) was evaluated by fluorescent microscopy with a dual staining system (propidium iodide and 6-carboxyfluorescein diacetate). Primary and secondary abnormalities and acrosome integrity were determined by direct count of 100 sperms from random fields of an immobilized smear, under 1000x magnification. Data were compared between extenders and bulls by 2-way analysis of variance. Percentages were transformed by arc sine transformation before ANOVA. Total and progressive sperm motility at 0hr after dilution ranged from 90 to 70% and was no different between extenders and bulls. There was no difference between bulls in total and progressive motility after 24hr of cold storage, however both were significantly higher ($P<0.05$) for Control (62.4±14.7 and 41.4±14.9) and Y2 (70.1±12 and 33.8±7.0) extenders than for L1 extender (22.5±17 and 1.2±1.3) accordingly. Average post-thaw sperm motility were no different between bulls in either extender, but motility for Bioxcell (48.1±14.6) and Y2 extenders 43.2±13.0) were significantly higher ($P<0.05$) than for L1 extender (18.7±8.8). There were no differences between bulls for all kinematics semen parameters curvilinear (VCL), straight line (VSL), average path (VAP) velocities and linearity (LIN) and straightness (STR) evaluated by CASA before and after freezing. However all were lower ($P<0.05$) for L1 extender and not different between Control and Y2 extenders. Sperm movements derived from heads (VCL) and linearity of sperm (LIN) both closely related to field fertility were in the range of 90.9±2.1 and 63.0±5.5 for Control, 99.1±3.4 and 49.4±3.5 for Y2 and 21.8±2.2 and 29.9±4.0 for L1 extenders accordingly. In terms of membrane stability, L1 showed a significantly lower ($P<0.05$) post-thawed viability (19.6±0.9) than Control (51.5±3.1) and Y2 (41.3±3.1). Percentage of sperm primary abnormalities were significantly lower ($P<0.05$) for Y2 (3.8±0.9) than for Control (7.5±0.9). There were no differences between extenders for secondary abnormalities and the remaining tested parameters.

In summary, zwitteronic Soya bean lipids are effective egg yolk substitute for the cryopreservation of Asturiana de la Montaña bull's semen, however lipids-glycerol liposomes were less efficacious.

This work was performed in collaboration with ASEAMO.

Notes

GENE EXPRESSION PROFILE OF BOVINE CUMULUS CELLS DERIVED FROM OOCYTES MATURED EITHER IN VIVO OR IN VITRO

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Bidirectional communication of the oocyte and its enclosed cumulus cells is a prerequisite for the oocyte to attain full developmental capacity during the course of its growth and maturation. Although it is also well established that the environment in which the oocyte matures has an influence on both the competency and transcript expression profile of the oocyte, it is not known if this could be due downstream effects of perturbation of the transcript profile of its adjacent cumulus cells. Therefore, the aim of this study was to compare the transcript profiles of cumulus cells derived from cumulus-oocyte complexes (COCs) matured in vitro (n=78; low developmental competence) or in vivo (n=76; high developmental competence). Using a previously validated combined synchronization and superstimulation protocol in vitro matured COCs were recovered from the ovaries of beef heifers just prior to the expected time of the LH surge and matured in vitro, while in vivo matured COCs were recovered 20 h later, just prior to ovulation.

A custom-made cDNA microarray containing granulosa/cumulus ESTs from four subtracted libraries with 2278 clones was used over six replicates of target and dye swap hybridizations. A total of 64 genes were differentially expressed ($P \leq 0.05$; FDR$\leq$ 5%) between the two groups. Transcript abundance of key genes associated with cumulus expansion (TNFAIP6), regulation of oocyte maturation (INHBA and FST) and anti-apoptosis/signal transduction (YWHAZ and PRMT1) were up-regulated in in vivo derived cumulus cells, while cumulus cells derived from in vitro matured COCs were enriched mainly with genes involved in response to stress (HSPA5 and HSP90AB1), hypoxia (TRA1) and cell cycle arrest (PPM1G). Quantitative real-time PCR has confirmed the transcript abundance of 8 out of 10 genes selected for validation.

The data presented here revealed that previously reported differences in developmental potential between oocytes matured in vitro or in vivo is also accompanied by transcriptome profile differences in the corresponding cumulus cells.

Notes

THE INFLUENCE OF EGF, BSA AND PROGESTERONE ON BITCH OOCYTE NUCLEAR MATURATION IN VITRO

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The modern methods of assisted reproductive technology find small reference in relation to dogs. There is no significant progress in production of dogs' embryos in vitro. The main cause of failures is low ability of dog oocytes to finish of the maturation process. Percentage of dogs oocytes maturated *in vitro* does not cross 20% while at other animal species it means: goats, cattle and pigs this percentage amounted: 94, 74.5 and 58% respectively. The aim of the present investigation was to verify the influence of hormonal and proteins (progesterone, BSA, EGF) IVM medium supplements on IVM rate for oocytes collected from anestrous bitches. Following ovariohysterectomy ovaries were maintained in physiological saline and transported to the laboratory. Ovaries were thinly sliced with a scalpel blade to release the COCs into a 0,9 % NaCl solution. Oocytes with more than 2 layers of intact cumulus cells and dark cytoplasm were cultured at DME/F-12 supplemented with, sodium pyruvate (25 mM), penicillin G (100 IU/ml), streptomycin (100 µg/ml), FSH (2µg/ml), LH (10 µg/ml) and estradiol (1 µg/ml). The basic IVM medium was further supplemented either with (at 1 group) 100ng/ml EGF and 3% BSA, and at the 2 group 100ng/ml EGF, 3% BSA and 2,0µg/ml progesterone (P4). Incubation endure for 48 h at 38.5°C in a humidified atmosphere of 5% CO2 in air. After this time cumulus cells were mechanically removed from the oocytes by vortexing. Oocytes were placed at propidium iodide (PI) solution. PI is a fluorescent nucleic acid label DNA. By this method we was able to observe the presence of polar body, with is a visible sign of completion of nuclear maturation. Percentage of oocytes finished the maturation process was higher in 2 (EGF+BSA+P4) group than in 1 (EGF+BSA) and control group and approximate respectively 14, 8 and 4%.

In conclusion, addition of progesterone, EGF and BSA to culture media increased the rate of resumption to completion of nuclear maturation

Notes

IMPROVEMENT OF EMBRYO RECOVERY RATES BY MODIFIED FLUSHING TECHNIQUES IN HOLSTEIN CATTLE AT A COMMERCIAL EMBRYO TRANSFER STATION

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During the last 30 years an embryo recovery rate (RR) of about 65% - 75 % was reported after flushing of superovulated cattle. This indicates that the collection techniques might be suboptimal and that some embryos might be left in the reproductive tract. The aim of our study was to improve the recovery rate in commercial embryo transfer (ET) with the support of uterotonic drugs, which should promote the release of embryos by myometrial contractions.

After superovulation with 630 IU FSH (Folltropin-V®) in a decreasing dose for 4 days twice daily and oestrus induction with 500 µg Cloprostenol (Estrumate®) and 25 mg Dinoprost (Dinolytic®), 174 Holstein cows (1 - 12 lactations) were divided at random into five experimental groups: Group A (n=36) got a luteolytic dose of Dinoprost (Dinolytic®: 25 mg in 5 ml i.m.) 12 to 16 h before flushing as well as 10 IU Oxytocin (10 IU in 1 ml i.v. Oxytocin Albrecht®) at the beginning of the flush, Group B (n=34) was treated with Dinoprost and placebo (1 ml i.v.: 0.9 % saline solution), Group C (n=37) received placebo (0.9 % saline solution: 5 ml i.m.) and Oxytocin, and Group D (n=31) got a placebo twice. Group E (standard control) included 36 animals. Embryo collection (EC) was done by a single technician. Each uterine horn was flushed separately with 400 ml DPBS on D7 of pregnancy. Thirty minutes after the first flush EC was repeated with the same amount of medium to control the effectiveness of the drugs in Groups A-D, and to collect possibly remaining embryos. In order to rule out the risk of a drug induced embryo expulsion, the vagina of every animal was flushed after finishing the normal EC procedures. Embryos/oocytes in the different flushings were counted and classified according to the standards of the IETS. Corpora lutea were counted ultrasonically (7.5 MHZ, Sonovet 2000) after EC, and RR was determined.

After the first uterus flushing the embryo recovery rate was 78.1 %, 81.2 %, 74.7 %, 72.4 % and 73.9 % in Group A-E, respectively. The RR enhancement via double flushing was more noticeable in the placebo group D with an increase of 6.7 % (increase comparing 1st flush to 2nd flush: Group A: 3.5 %, B: 2.3 %, C: 3.3 %). After double flushing a total RR (1st + 2nd flush) of 81.6 %, 83,5 %, 78.0 %, 79.1 % was reached in Group A-D, respectively, while in the standard control 73.9 % of the embryos/oocytes were collected. Since the number of additionally collected embryos/oocytes was significantly higher in Group D in comparison to Group A-C ($P < 0.05$) the hypothesis is supported, that an uterotonic treatment improves the embryo recovery rate in the 1st flush. No embryos were collected from the vagina on D7. Embryo quality and the pregnancy rates after transfer of the embryos into the final recipients showed no differences between the different groups.

It can be concluded, that an uterotonic support especially with Dinoprost enhances embryo recovery rates in commercial embryo transfer. Therefore it is recommendable to treat the donors with a luteolytic dose of Dinoprost 12 - 16 hours before the embryo collection.

Notes

AUTHOR INDEX

AUTHOR INDEX

S

T

V

W

Y

Z