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European Embryo Transfer Association

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Dr Yvan HEYMAN

Special Celebration

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KUOPIO, Finland, 10th and 11th September 2010

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Dr. Yvan HEYMAN

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Dr. Yvan HEYMAN
A.E.T.E. Medalist 2010

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Yvan Heyman was born the 8th of August 1948 in the city of Coulommiers located about 60km east from Paris in the middle of the Brie province, otherwise known for its rich agriculture and its famous round and creamy cheese which size can be almost as large as a cart 's wheel. His parents were farmers that had left Belgium one year before. Yvan Heyman grew up with his brother and four sisters in the middle of corn and sugar beet fields and became rapidly familiar with the management of a dairy herd. Although he was offered the possibility of working with his father to exploit new lands around the farm he preferred to study biochemistry and its application to biology in Paris and started his career as a laboratory technician in 1969. From this first experience he acquired a practical turn of mind, a precious quality within a research team. After two years, he decided to become a scientist and spent three hard working years to complete his Masters degree in Physiology, the sesame for starting a doctorate. At that time National Service was mandatory for young men in France and he spent the years 1974 and 1975 in the French West Indies at an INRA Station studying the zootechnical performances of local (Créole) breeds, a rich experience that brought him in contact with the innovative problematic of heat and environmental effects on reproductive physiology. Back to the continent, he spent one year at the SERSIA (http://www.sersia.fr/), which is the 1st French exporter of cattle and goat genetics, created by the French breeders association. This was at the time when the technique of embryo transfer in cattle, pioneered by Prof Rowson in Cambridge, UK, had just become an economical reality with the first commercial ET Company created in the US, concomitantly with the International Embryo Transfer Society (IETS). In France, Pr. Charles Thibault had convinced INRA to reinforce research activities in the field of reproductive technologies and embryonic development. The senior author of these lines had just joined the small group headed by Drs Dumesnil du Buisson and Jacques Testart. The period was already becoming scientifically and socially tumultuous with the first press articles announcing the era of test tube babies and the technological breakthrough of embryo freezing in the mouse. Yvan Heyman was the best suitable person to be recruited to bring a dose of wariness in the whirl of ideas prevailing at that period in the lab. The experience he had acquired in the management of an experimental cattle herd during his stay in the West Indies was also an asset for the lab facing increasing needs in terms of animals. In those days, the European Union was actively supporting reproductive biotechnologies in livestock through its first RTD research programs. Improving twinning in cattle was considered as a means to increase meat production in developed western countries. It was the style of the time and we were at the beginning of year 1977 when Yvan Heyman joined the lab.

Yvan first got involved in the development of a new non-surgical embryo transfer technique in cattle and the results published the same year in Theriogenology

(Theriogenology,7,189) contributed to the replacement of surgical techniques which had at that time still the favor of private companies, mainly in the US. He then played a key role in demonstrating that cattle twins could be produced after bisection and cervical transfer of freshly recovered blastocysts. Two additional papers published in the Veterinary Record Journal respectively in 1980 (Vet Rec. 107, 152) and 1982 (Vet Rec. 110,126) provided evidence that rates as high as 50% could be obtained in "on-farm" experiments, providing a strictly controlled management of both superovulation and synchronization of the cell cycles of donors and recipient females.

The first steps of Yvan as a scientist were thus marked by the objective of facilitating the diffusion and application of technical and physiological knowledge relative to embryo transfer in mammals. It was thus not surprising that he became involved at that period in the conception of the AETE in Lyon, with the support of Pr Pierre Mauléon and the Foundation Charles Mérieux.

During the 80's, Yvan got involved in studying embryo's life in vitro and its consequences on blastocyst development after transfer in vivo. Embryo freezing attracted him for a while but his two major scientific and technical contributions were directly related to basic embryology. A judicious use of cattle trophoblast cells as an auxiliary for in vitro culture media revealed that this epithelium could markedly improve both the cleavage rate of in vitro fertilized embryos and the number of embryos reaching the blastocyst stage (a result published in the Journal of Reproduction and Fertility in 1984), but also the full term in vivo development of blastocysts. For that he co-cultured or co-transferred cattle embryos together with trophoblastic vesicles obtained after crushing out the inner cell mass cells of blastocyts, thereby preventing their implantation in the recipient's uterus (a result published in Theriogenology 1987). This approach led to the concept of "in vivo coculture". It was a first evidence that extra embryonic tissues were contributing to the cross-talk between the embryo and the uterine environment at a time when cellular contact are established between two organisms of different genetic constitution. Today and more than twenty years later, we still know very little on the embryo-to-mother molecular balance that underlies this crosstalk. That immune mechanisms are only a part of the story has been evidenced only recently with the discovery that the peri-implantation period in cattle can exert long lasting effects, not only on the development of the conceptus but, importantly enough, also during the postnatal life. Yvan was very active in developing the production of frozen trophoblast vesicle coculture to be used as an "on farm" technique. Clearly, the transfer of technologies from the bench to the farm has been his first drive: his down-to-earth, no-nonsense attitude was essential for an early dialogue between scientists and practitioners, which is crucial to the harmonious development of science in agriculture!

Then came cloning. Yvan has been, since the very beginning, and still is, actively involved in the adventure: cloning cattle embryos from blastomeres between years 1991 (Rec.Med.Vet., 167, 315) and 1993 (C.R. Acad. Sci. 316, 487), cloning cattle embryos from short term culture of embryonic cells in 1994 (Theriogenology 42, 695), cloning cattle embryos from diploid germ cells in 1996 (Theriogenology, 46, 871), combining cloning with donor embryo sexing in 1997 (J. Reprod. Fertil. 113, 343), combining cloning with transgenesis in 1998 (Transgenic Res.,7, 331-341), and finally contributing the same year to the birth of "Marguerite", the first mammalian somatic clone born in Europe (C.R. Acad. Sci. Paris, 321, 735). With Marguerite, the controversy about the authenticity of Dolly that was still prevailing within the scientific community one year after the announcement of its birth, took a new turn. As outlined by the journal Nature (1998 392:113), it confirmed that differentiated somatic mammalian cells can be reprogrammed to make them totipotent. Ten years later, Yvan has been involved in the birth and breeding of more than one hundred cattle

clones. Meanwhile and because of the social perception and acceptability of cloning, his contribution to research during that period has been markedly changing.

Prudentes ut serpentes, simplices ut columbae (to be sly as a fox, to remain simple as a dove) is the motto of Yvan's birthtown. Strikingly enough, this motto perfectly applies to his present contribution to research.

Prudentes (wariness) is more than appropriate at a time when any new information coming from research with clones is immediately negatively considered and most often misinterpreted. This is for instance the case with the potential risks associated with the consumption of food product from cloned animals. Yvan organised the first international workshop on the quality of products from cloned cattle, held at INRA in 2004 and sponsored by OCDE (proceedings published in Cloning and Stem Cells 6(2), 2004). He spent three years (2002-2005) organizing and managing a multidisciplinary and integrated research program on the quality and security of products obtained from clones including their offspring and an analysis on the welfare of clones and of their foster dam. The results, published in 2007 (Theriogenology, 67: 134; Animal 1, 963), provided the scientific substrate to the IETS recommendations for the Health Assessment and Care for Animals Involved in the Cloning. FDA and the European Food safety Authority (EFSA) reports on the safety of cloned animal products have adopted most of these recommendations and Yvan is still involved in generating data and contributing to the EFSA risk assessments updates for products from clones and their offspring. As a consequence, Yvan is now a member of the French National Food council.

Simplices (simplicity) is mandatory when explaining to the public why research with animals is needed and how it is now possible to prevent animals from suffering by a careful management associated with a meticulous assessment of experimental protocols. Yvan has also been for many years an appreciated member of the Ethical and animal experimentation committee at INRA Jouy en Josas.

Wariness and simplicity but also generosity and frankness reflect the general attitude of Yvan in life. He is a wonderful lab and office partner for all those who work with him and he, together with his wife Martine, a scientist at the French Medical Research Institute (INSERM), are friends close to our hearts.

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FROM NON SURGICAL EMBRYO TRANSFER TO SOMATIC CLONING IN CATTLE: TECHNICAL CHALLENGES AND HURDLES TO THE USE OF REPRODUCTIVE BIOTECHNOLOGIES

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Introduction

The first successful embryo transfer reported in animal reproduction was achieved in Cambridge more than one century ago by Walter Heape. He showed that rabbits from one breed can be gestated in the reproductive tract of other females of another breed without being affected by the uterine environment (Heape 1897). Since this pioneering era, embryologists working mainly on laboratory species have developed embryo transfer for their basic research on reproduction and extended their experimental embryology to farm animals (sheep, pigs and cattle) during the second half of the 20th century, convinced that embryo transfer could be of practical value to agriculture. The first calf derived from surgical embryo transfer was born in Wisconsin (Willet 1951) and practical non surgical methods have been developed since the middle of the 1970s leading to commercial activity and the embryo transfer industry, mainly in cattle. Currently, almost a million bovine embryo transfers are performed worldwide each year according to the International Embryo Transfer Society (Thibier 2007) and more than 100 000 in Europe (S. Merton AETE 2009). However, more than one century after Heape's success, embryo transfer is not limited to a routine reproductive biotechnology but is central to investigations to determine how profoundly embryos, fetuses and offspring can be affected by their earliest in vivo or in vitro environment.
In this review we give a retrospective look on the development of embryo transfer in cattle and related technologies and focus on examples of difficulties that were encountered to achieve embryo transfer between the first laboratory attempt and its application in breeding.

1) The way to successful non surgical embryo transfer in cattle

For all the cattle ET practitioners, it is now obvious that the technique of choice to transfer blastocysts to recipients is non surgical. It must be remembered, however, that the first commercial application of embryo transfer in cattle was for the importation of "exotic breeds", i.e., European breeds, towards the USA and Canada in the 1970's. This was an economically favourable period and surgical methods of embryo recovery and transfer perfected for sheep were then adapted to cattle by the group of Tim Rowson and colleagues at Cambridge.

Nevertheless, attempts to transfer embryos through the cervix were performed during the early 1950s, before developing surgical methods for ET in cattle. These efforts were made difficult by problems of uterine infection and contractions resulting in egg expulsion (Lamming and Rowson, 1952, Harper et al. 1961). The manipulation of the cervix was then considered as a real hurdle for non surgical embryo transfer as it could induce uterine

constriction expelling ova into the vagina. In Japan, Sugie et al. (1965) used a transvaginal approach to bypass the cervix. They also insufflated the uterine lumen with CO2 to make sure that the needle used to deposit the embryo was indeed in the lumen. CO2 was also believed to prevent the expulsion of the embryo. Sugie designed and used a special apparatus to transfer the eggs to the uterus and it resulted in the birth of a calf. In France, research on cattle embryo transfer was conducted at INRA by the group of Pr Charles Thibault and a transvaginal method for bovine embryo transfer was proposed by Testart et al, (1975).

It was not until the middle 1970's that transcervical methods were "rediscovered" for both recovery and transfer and progressively replaced surgical methods completely. One main point was that the activity of the myometrium is dependant upon the stage of the estrous cycle. Electric and mechanical activity of the cow myometrium was shown to be high during the oestrogenic phase and low during the luteal phase when progesterone is secreted (Ruckebush and Bayard, 1975). The initial attempts to transfer cattle embryos non-surgically were performed with early stage embryos (8-32cell). These were transferred to D4 recipients, in which progesterone level is still low. Advances came from different groups in Europe (Irlande, Netherlands, Germany and France) who combined the use of the modified AI Cassou gun and the transfer of more advanced embryos (compacted morula and blastocysts) at a later stage. The first significant and detailed results were presented in a meeting organised by the Commission of European Communities in 1977 in Galway (Sreenan 1977, Heyman et al. 1977).

We achieved pregnancies after unilateral or bilateral cervical transfer at the blastocyst stage (Renard et al 1977) and investigated the optimal timing for non surgical embryo transfer. Pregnancy rates over 50% were obtained when blastocysts were transferred non-surgically between D7 and D13 (Heyman et al 1977). Brand and Drost (1977) also reported successful embryo transfer by non surgical methods at the same time.At that time, one of the objectives was to improve twinning in cattle by transferring an additional embryo to previously inseminated recipients (Renard et al 1979; Heyman et al 1980; Sreenan et al 1981).

Another major advance that had a real impact on the development of embryo transfer was the onset of the use of prostaglandins F2α and its analogues to synchronise estrus and superovulation treatments in cattle (Phillipo and Rowson, 1975). This helped reducing the costs of embryo transfer: before that time, reliance on naturally cycling animals required the maintenance of very large and expensive recipient herds.

The average pregnancy rate following single non surgical transfer (30%) remained significantly lower, however, than that obtained following surgical methods (60%), according to Brand and Akabwai (1978). This was explained by the unilateral relationship of the conceptus and the ovary bearing the corpus luteum. The importance of the location of the embryo deposition in determining the survival rate of the embryo has been nicely demonstrated by Newcomb et al (1980), using surgical transfer of two embryos at the tip or the base of the uterine horn ipsilateral or contralateral to the CL (Newcomb et al, 1980). The fetal survival rate was significantly greater when the embryo had been transferred to the tip rather to the base of the ispilateral horn. They concluded that "in any event, nonsurgical transfer is unlikely to produce as high a pregnancy rate as the surgical transfer of embryo to the uterine tip, unless embryos can be delivered to this site without trauma". Consequently several technical improvements were designed for non-surgical transfer. The diameter of the classical insemination gun was adapted for young heifers and its length was extended to reach as deep as possible inside the uterine horn. The tip was also modified to become less traumatic. Special devices were developed by the French company IMV, featuring a smooth tip and two subterminal orifices in order to prevent the obstruction of the aperture by mucus during the progression of the ET gun through the cervix. Furthermore, a thin disposable

sanitary sheet was designed to cover the device during progression through the vagina in order to prevent the introduction of bacteria from the vagina to the endometrium.

Attemps were made to sustain the corpus luteum in recipients after non surgical transfer in order to improve luteotrophic/antiluteolytic signal and embryo survival. There was some evidence that human chorionic gonadotropin treatment could improve embryo survival after single embryo transfer to an unsuitable site (contralateral uterine horn) (Christie et al. 1979). However, further comparative studies did not show any improvement of pregnancy rates when recipients were treated with progesterone or hCG after nonsurgical transfer to the ispilateral horn (Heyman 1985).

Finally, the technique developed due to the strong demand from farmers for non surgical transfer, together with improved skill of ET technicians, adapted equipment and selection of recipients. The use of heifers vs multiparous cows as recipients was a key factor in the achievement of efficient pregnancy rates after routine transfer of fresh embryos throughout Europe and the world. In commercial groups in France, a mean pregnancy rate of 55.8% was reported for on-site non surgical transfer of single fresh embryos (Ponsart et al. 2004).

Retrospectively, the embryo transfer methods have been easily accepted by farmers, breeder organisations and on a larger scale by the society. This may be due to the fact that the non surgical methods developed both for the recovery and the transfer were not invasive nor traumatic for the animals. Furthermore, innovative techniques such as ET were perceived as a real progress thirty years ago

2) Freezing embryos

Embryo freezing has been a very important step for the development of embryo transfer in cattle, especially in the context of a high variability of in vivo embryo production through superovulation. With cryopreservation, not only the number of recipients could be adjusted to the number of superovulated embryos available from the selected donors, but the embryos could be stored and transferred at another time of the year when recipients were available. Moreover, embryos could be shipped easily for international exchange. Nowadays, embryo cryobanks have become a reality for domestic as well as laboratory and endangered breeds and species.

The birth of offspring after mammalian embryo freezing in liquid nitrogen was first achieved in the mouse species by D Witthingham et al. (1972) but practical methods for freezing bovine blastocysts were set up about 10 years later. These methods were derived from the initially so-called “slow freezing procedure” through adaptation and simplification. The choice of the cryoprotectant, optimal cooling and thawing rates, the removal of cryoprotectant by a non permeating compound such as sucrose have led to the development of efficient methods for the freezing of bovine blastocysts in straws. Storage, transport, and on farm transfer of frozen embryos became nearly as easy as a routine AI (in straw freezing:sucrose dilution by Renard et al 1982). Once the technical problems were solved, the freezing method was widely used despite a slight decrease in pregnancy rate compared to the transfer of fresh embryos. The use of embryo cryopreservation has probably benefited from the previous experience in the large use of frozen semen for AI.

The efficiency of embryo cryopreservation has been, however, impaired by the wrong way of selection of embryos to be frozen after flushing. Two decades ago, freezing was mainly used to store the excess embryos when there was a shortage of synchronous recipients prepared for

an ET programme. Then, practitioners used to transfer directly to recipients the best quality fresh embryos (grade 1) and freeze the lower quality ones (grade 2 and 3). It is known that the freezing /thawing process can damage a small proportion of embryonic cells and a better overall calf production is obtained when then best quality embryos are frozen and the lower quality ones are transferred as fresh.

About duration of storage in liquid nitrogen, clearly, there have been no deleterious effects caused by long term storage of bovine blastocysts. So far, no abnormalities have been reported after the birth of calves resulting from the transfer of frozen thawed embryos.
Currently, in straw freezing/thawing method using ethylene glycol as cryoprotectant or the glycerol-sucrose dilution are the most widely used. The conditionning of frozen embryos in plastic straws is a safe and practical way for storage as the straws can be labelled with the complete identification code according to the standard proposed by the International Embryo Transfer Society (see Manual of the IETS). Commercial companies are routinely freezing thousands of bovine blastocysts in straws using this "classic" method for export and non surgical transfer to recipients with a high efficiency.

However, if the slow freezing thawing procedure is efficient for good quality embryos (in vivo produced grade 1 compact morulae or blastocysts), this method remains unsatisfactory for early cleavage stage embryos or for manipulated embryos (cultured, biopsied, bissected…) that have a limited number of cells and fragile cellular membranes which are more sensitive to dehydration/rehydration and ice crystals formation during the freezing process. Therefore, another approach, vitrification, is being developed. Vitrification is based on the use of very rapid cooling rates associated with high cryoprotectant concentrations in order to avoid ice crystal formation in the solid phase (Rall and Fahy 1985). This method was initially tested on cattle embryos by A Massip et al.(1986) and is currently improved for in vitro produced embryos (see workshop by C Joly et al. in this conference).

3) In vitro embryo production

In bovine embryo transfer activity, the low production of blastocysts through the superovulation of selected donors is the main limiting factor. Despite the progress during the past decades in the design and use of superovulating drugs and treatment schedules, the mean number of transferable embryos that can be expected from a superovulated cows remains low (about 6 embryos) and extremely variable (0-50). This mean number has only slightly improved during the last 10 years if we compare the data from the AETE statistics 1998 (X = 5.53 transferable embryos per flushed donor) and 2008 (x = 5.97 transferable embryo per flushed donor).

Fortunately, active research was developed since the first successful in vitro fertilization of a bovine oocyte (Brackett et al. 1982) but it took another ten years to achieve consistent in vitro embryo production by combining in vitro maturation of oocytes recovered from antral follicles of ovaries collected at slaughterhouse, in vitro fertilisation with frozen sperm and in vitro culture of the fertilized egg up to the blastocyst stage. This achievement results from basic studies on key criteria for the induction of full oocyte maturation (Moor and Warnes 1978) and studies on suitable in vitro microenvironments in which temperature, gas phase and nutrient conditions are compatible with the normal function of both the oocyte and its associated somatic compartment. The conditions for sperm capacitation and selection for IVF have also been optimized and various culture systems have been used for the in vitro development of the zygotes into blastocysts. Complex media have often been associated with co-culture systems to mimic the in vivo situation and overcome the in vitro block at the time

of the embryonic genome activation. In our laboratory, we still use for experimental purposes a coculture sytem in microdrops of B2 medium seeded with Vero cells (Menck et al 1997) but simple and efficient culture media such as SOF are routinely used for commercial in vitro production of bovine embryos.

A major step for the practical development of IVP bovine embryos has been the recent progress in the technology of ultrasound scanning that enables the visualisation of the small follicles at the surface of the ovaries in live animals. This visualisation combined with the transvaginal puncture and aspiration of follicles to recover the oocytes has led to the development of the Ovum Pick Up (OPU) method (Pieterse et al 1991). The technical parameters for OPU have been defined (Bols et al., 1996) and the procedure for ovum pick up is now considered as a relatively non-invasive and repeatable technique in cattle. OPU is now currently used to generate IVP embryos from valuable donors and has really become an alternative to superovulation, especially in some countries such as The Netherlands (Merton 2009). Each OPU session on a cow results in nearly 2 IVP blastocysts and can be repeated twice a week on the same animal. Therefore, in a short period of 2 weeks, a donor can produce more IVP embryos than in vivo after one superovulation treatment. However, this "productivity" is counterbalanced by some limitations. The main hurdle for IVP is probably the quality of in vitro produced embryos which is slightly different from that of their in vivo counterparts. Even if the pregnancy rates obtained after direct transfer of IVP embryos is similar to that of fresh in vivo ones, the in vitro produced embryos are more sensitive to freezing. In vitro culture conditions are not yet optimal and many differences exist between in vitro and in vivo bovine blastocyts at the cellular level in terms of metabolic profiles and morphology (Thompson 1997, Leese et al 1998) as well as for gene expression (Wrenzycki et al, 1999).

Furthermore, the use of IVP embryos may result in some possible long term effects such as the large offspring syndrome which has been reported after the birth of calves that were derived from IVP embryos cultured in media supplemented with special batches of serum (Walker et al, 1996, Kruip and den Daas 1998) Now, in commercial programmes where the culture conditions are more controlled using the SOF system without high levels of serum or BSA, pregnancies are normal and the incidence of LOS is very reduced (Galli and Lazzari, 2003)

Nevertheless, bovine embryo production through OPU and IVP opens the possibility of fertilisation "à la carte" as each oocyte recovered from one OPU session on a donor can be fertilized individually by sperm from different bulls, thus offering large possibilities for genetic programmes.

4) Embryo splitting and embryo sexing

The micromanipulation of embryos to separate blastomeres and produce semi-embryos able to develop into pairs of monozygotic twins was initially achieved in sheep by Willadsen (1979). However the original method was difficult to perform as it required the inclusion of the groups of blastomeres into agar chips and subsequent intermediate in vivo culture into the oviduct of sheep before transfer to a definitive recipient. A simplified approach has been developed at INRA to generate pairs of monozygotic twin calves after cervical transfer in the cow (Ozil et al 1982). This method consists in a simple bisection of the embryo at the blastocyst stage directly through the zona pellucida using a microblade. The bisected embryo is then transferred as a pair to the uterine horn ipsilateral to the CL of synchronous recipient heifers, resulting in relatively high twinning rates (30 to 50%). Surprisingly, it is important to

note that embryo splitting has not been developed in subsequent years to produce twins by the cattle embryo transfer industry. The reasons are linked to the low acceptance of twin pregnancies by the farmers and the associated risks at calving. Embryo transfer groups also also limited their investment in equipment for micromanipulation and training of technicians to acquire the necessary skills. In fact, in the bovine embryo transfer industry, embryo splitting by micromanipulation has only been used occasionally when an elite donor cow failed to superovulate and produced for example only one single transferable embryo. Through the bisection of this single blastocyst, the transfer of two semi-embryos to separate recipients became possible, thus improving the chance to get an offspring from the valuable donor.

The possibility to determine the sex of an embryo before its transfer to recipients induced a revival of interest for embryo micromanipulation techniques. Since the 1990's, accurate methods for embryo sexing were established. They are based on the identification of the presence (or not) of a Y chromosome in the DNA of an embryo biopsy, using specific Y chromosome specific DNA probes. The diagnostic has to be performed in an acceptable time frame in order to allow the survival of the biopsied embryo until transfer. Important technical advances such as the removal of a small biopsy (1 to 5 cells), the amplification of the DNA sequence by PCR after hybridisation with the probe and the localisation of the male specific bands after electrophoretical separation, have been achieved and can be performed rapidly. The result is obtained within a 2 hours period, which is compatible with a commercial embryo transfer activity.

Despite the very good accuracy of the embryo sexing diagnosis (nearly 100%), the use of embryo sexing in practice remains limited, mainly due to the extra costs for embryo biopsy and sexing but also to the fact that biopsied embryos are more sensitive to freezing /thawing procedure than intact ones. Furthermore, PCR is a very sensitive technology that can be influenced by foreign cell material, therefore the use of embryo sexing under farm conditions has to be performed very carefully. In fact embryo sexing has now opened the way to further prenatal diagnostic with the revolution of genomic tools for marker assisted selection.

5) Cloning by somatic cell nuclear transfer

Since the birth of Dolly the sheep (Wilmut et al 1997) a real breaking-out has occurred in the field of reproductive biotechnologies as the possibility for mammals to be reproduced without sexual contribution of gametes was first demonstrated. In cattle, somatic cell clones produced by nuclear transfer have been obtained in many countries over the world since 1998 (Cibelli et al, 1989, Vignon et al, 1998). Despite the remaining low efficiency of the technique due to increased embryonic and fetal loss, it is possible to obtain viable and apparently normal animals from valuable cows or bulls. A few thousands heads of cattle have already been obtained worldwide after SCNT, both by research groups and by private companies involved in the pharmaceutical industry. The potential applications of cattle cloning for the breeding industry to recover the genetics of outstanding progeny tested bulls, animals with a rare phenotype or survivers of endangered breeds have been reviewed (Faber et al, 2004, Heyman et al, 2005). Furthermore, transgenic animals can now be obtained by nuclear transfer from cultured somatic cells which have been targeted during in vitro culture , thus opening new dimensions in animal breeding and pharmaceutical industry (Brem 2008)

During the recent years, important progress has been achieved on the different steps of the technique of nuclear transfer and on the biology of the somatic nucleus remodelling after its

introduction into the recipient cytoplasm. Even if this emerging technology is still poorly efficient compared to other assisted reproductive technologies, it may already be useful for specific applications in agriculture. The degree to which clones can be used for agricultural purposes will depend on decisions that regulatory authorities will make regarding the health of these animals and the safety of food products derived from clones and their offspring. Other hurdles that are currently influencing the acceptance of this technology are the issue of public perception and the growing importance of ethical concerns.

The concerns related to risks associated with the consumption of food products derived from livestock have been addressed in different national and international instances (AFSSA in France, EFSA in Europe and FDA in the United States). Our laboratory at INRA has contributed to this multidisplinary research programme by assessing the quality and safety of bovine clones and their products (Heyman et al, 2007), as well as the health care and well being of the clones (Chavatte-Palmer and Lee 2010). In conclusion, no significant food consumption risks have been identified in cattle and the issues documents on safety of food from clones have been released by the FDA on January 15, 2008 mentioning that: “meat and milk from clones of adult cattle , pigs and goats and their offspring are as safe to eat as food as food from conventionally bred animals”.

The FDA could not identify any toxicological hazard of concern for human consumers, but in the same time, FDA asked the industry to maintain a voluntary moratorium on delivering milk and meat from cloned animal and their offspring to the food industry. A few months later, the European Food Safety Authority (EFSA) adopted its final scientific opinion on animal cloning with similar conclusions as the FDA. Nevertheless, the issue of the health and welfare of surrogate dams and of clones was raised. In the same time, the European Group of Ethics (EGE) published a negative opinion relating its serious doubts as to whether cloning animals for food supply is ethically justified.

In France, a working group of representatives of the society concluded that the recurring social controversy linked to the use of GM products (even if clones are not genetically modified animals!) should lead to great caution concerning the use of animal cloning. In fact the public perception of cloning appears completely "out of sync" when compared with other methods of reproduction. Cloning is considered as a prototype technique and there is a risk that, despite the confirmed safety of the products, introducing products from cloned animals in the food chain might lead to a strong and long lasting deterioration of the image of the meat and milk sectors.

Recently (3 sept 2008), the European parliament voted massively a resolution calling the European commission to ban cloning for food production.

The acceptance/rejection of such new biotechnology varies between countries and the perception of cloning in Japan may be quite different than in European countries.

Altogether, the example of cloning clearly shows that even if live offspring have been obtained by nuclear transfer of somatic cells from valuable donors, it is not sufficient for a technology to be utilised by the industry. Concerns on how farm animals are produced, potential risks for consumers, animal health and welfare and ethical concerns are of growing importance in the development of an emerging technology.

Concluding remarks

Among the reproductive biotechnologies that have been developed in the cattle industry, embryo transfer has been well accepted since its beginning as it was considered as a new tool for genetic improvement to increase production. The use of non surgical techniques for

embryo recovery and transfer at the farm level helped reducing the costs and enabled the diffusion of the technique. Embryo freezing was also rapidly applied as it really facilitated the application of ET. In vitro embryo production was more difficult to develop at the practical level due to a higher degree of complexity (combination of ovum pick up, oocyte maturation, fertilisation and culture), but it is now well regulated and accepted. It is not yet the case for cloning and transgenesis which are very promising but in the same time raise problems of possible long term effects. Further multidisciplinary research work is needed to warrant the safety of these biotechnologies regarding health, efficiency and costs. These technologies must also be accepted by the society for the generation of new products and new animals.

With the development of modern genomic tools, embryo transfer and its related technologies will probably enter a new era as they facilitate genomic selection in the breeding industry. They are important also in basic science as the combination of embryo transfer methods with the modern "omics tools" is used to better understand the dialogue between the embryo and the recipient uterus. Gene expression profiles show that the endometrium responds differently according to the type of embryo (in vivo or in vitro produced) (Bauersachs et al 2009). The fact that the endometrium can be considered as a biosensor (Mansouri et al., 2009) opens the way to a better selection of the manipulated embryos before their transfer and improved pregnancies and outcomes.

References

1) Bauersachs S., Ulbrich S., Zakhartchenko V., Minten M., Reichenbach M., Reichenbach HD., Blum H., Spencer T., Wolf E. (2009). The endometrium responds differently to cloned versus fertilized embryos. PNAS 106:5681-5686.

2) Bols PE., Van Soom A., Ysebaert MT., Vandenhede JM., de Kruif A. (1996). Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and development capacity of bovine oocytes. Theriogenology 45: 1001-1014.

3) Brackett BG., Bousquet D., Boice ML., Donawick WJ., Evans JF., Dressel MA. (1982). normal development following in vitro fertilization in the cow. Biol. Reprod. 27:147-158.

4) Brand A., Drost M. (1977). Embryo transfer by non surgical methods. In : Betteridge KJ. Ed., Embryo transfer in farm animals: A review of techniques and applications. Monograph N°16. Canada Department of agriculture, Ottawa, pp 31-34.

5) Brand A. and Akabwai D. (1978) Some aspects of non surgical embryo transfer in cattle. Vet. Sci.Com 2:23-37.

6) Brem G. (2008) Generation of recombinant antibody transgenic farm animals. In proceedings 24th AETE meeting12-13 sept 2008, Pau, France, pp 7-18.

7) Chavatte-Palmer P. and Lee R. (2010). Health care and well being of animal clones. In Manual of the International Embryo Transfer Society, 4th edition, chapter 10, pp106-113.

8) Christie W., Newcomb R., Rowson LEA., (1980). Non surgical transfer of bovine eggs: investigation of some factors affecting embryo survival. Vet.Rec. 106 (9): 190-3.

9) Cibelli JB, Stice S, Golueke PJ., Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JL. (1998). Cloned transgenic calves produced from non quiescent fetal fibroblasts. Science 280, 1256-1258.

10) European Group of Ethics in science and new technologies to the European commission, (2008). Ethical aspects of animal cloning for food supply. Opinion N° 23, January 16.

11) Faber DC., Ferre L., Metzger J., Robl JM., Kasinathan P. (2004). Agro-economic impact of cattle cloning. Cloning Stem Cells 6:198-207.

12) FDA release documents on the safety of food from animal clones January 15, (2008). www.gov/cvm/cloning.htm

13) Galli C. Lazzari G. (2003). In vitro production of embryos in farm animals. Proceedings 19th Meeting AETE Rostock 12-13 sept 2003, pp 93-102.

14) Harper, MJK., Bennett,JP, Rowson ,LEA., (1961). A possible explanation fort he failure of non surgical ovum transfer in the cow. Nature190, 789.

15) Heape W., (1897) further note on the transplantation and growth of mammalian ova within a uterine foster-mother. Proceedings of the Royal Society of London Biological Sciences 62, 178-183.

16) Heyman Y., Renard JP., Ozil JP., du Mesnil du Buisson F. (1977) Cervical embryo transfer at different stages in cattle . In EEC conference on "Control of reproduction in the cow. EEC conference Galway 27-30 sept 1977, pp 330-335.

17) Heyman Y, Renard JP., Chupin D., Du Mesnil du Buisson F. (1980) Utilisation of cervical embryo transfer for twin production in beef and dairy herds. In proceedings 9th International Congress on Animal Reproduction, Madrid 16-20 june 1980

18) Heyman Y. (1985). Factors affecting the survival of whole and half embryos transferred in cattle. Theriogenology 23: 63-75.

19) Heyman Y, Chavatte-Palmer P., Vignon X., Richard C. Renard JP. (2005) le clonage somatique : un état des lieux chez les bovins et les petits ruminants. INRA Prod .Anim. 18 :339-354.

20) Heyman Y., Chavatte-Palmer P., Berthelot V., Fromentin G, Hocquette JF, Martignat L, Renard JP (2007) Assessing the quality of products from cloned cattle: an integrative approach. Theriogenology 67:134-141.

21) Heyman Y, Chavatte-Palmer P, Fromentin G, Berthelot V, Jurie C, Bas P, Dubarry M, Mialot JP, Rémy D, Richard C, Martignat L, Vignon X and Renard. JP (2007) Quality and safety of bovine clones and their products. Animal 1: 963-972.

22) Kruip TAM., den Daas JHG. (1997). In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 47:43-52.

23) Lamming, GE., Rowson LEA., (1952). superovulation and ovum transplantation in cattle. In: Proceedings of the second International congress on Animal Reproduction, Vol 1.AI, Copenhagen, pp144-153.

24) Leese HJ., Donnay I., Thompson JG. (1998). Human assisted conception: a cautionary tale. Lessons from domestic animals. Hum.Reprod. 13 (suppl. 4) 184-202.

25) Mansouri-Attia N., Sandra O., Aubert J., Degrelle S., Everts R., Giraud-Deville C., Heyman Y., Galio L., Hue I., Yang XZ., Tian C., Lewin H., Renard JP. (2009) The endometrium as an early sensor of in vitro manipulation technologies. PNAS 106:5687-5692.

26) Massip A., Van der Zwalmen P., Scheffen B., Ectors F. (1986) Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo-Letters 7: 270-273.

27) Menck MC, Guyader-Joly C., Peynot N., LeBourhis D., Lobo RB., Renard JP. Heyman Y. (1997). Beneficial effect of vero cells for developing IVF bovine eggs in two different coculture systems. Reprod Nutr.Dev. 37:141-150.

28) Merton S. (2009). Overall bovine embryo transfer activity in Europe in 2008. Proceedings of the 25th AETE meeting Poznan 11-12th September 2009, p 85

29) Moor RM., Warnes GM. (1978). Regulation of oocyte maturation in mammals. In Control of ovulation (eds DB Crighton, GR Foxcroft, NBHaynes, GE Lamming) 1978 pp 156-176.London: Butterworth

30) Newcomb R., Christie W., Rowson LEA. (1980). Fetal survival rate after the surgical transfer of two bovine embryos. J.Reprod.Fert. 59, 31-36.

31) Ozil JP., Heyman Y., Renard JP. (1982). Production of monozygotic twins by micromanipulation and cervical transfer in the cow . Vet.Rec. 110: 126-127.

32) Phillipo P. and Rowson LEA. (1975). Prostaglandins and superovulation in bovine. Ann.Biol.Anim.Bioch.Biophys., 15,223.

33) Pieterse MC., Vos P., Kruip T., Wurth YA., van Beneden T., Willemse AH et al. (1991). Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 35:19-24.

34) Ponsart C., Marquant-Leguienne B., Humblot P. (2004) Les biotechnologies de l'embryon bovin, evolution et perspectives. In proceedings Rencontre Recherches Ruminant 2004 pp 361-368.

35) Rall WF., Fahy GM. (1985). Ice free cryopreservation of mouse embryos at – 196°C by vitrification. Nature 313: 573-575.

36) Renard JP., Ozil JP., Heyman Y. (1979). The use of embryo transfer in the field for increased calf crop in beef and dairy cattle. Anim. Reprod.Science.

37) Renard JP, Heyman Y , Ozil JP . (1982) Congélation de l'embryon bovin : une nouvelle methode de decongélation pour le transfert cervical d'embryons conditionnés une seule fois en paillettes. Ann.Med.Vet. 126, 23-32.

38) Ruckebush Y. and Bayard F. (1975). motility of the oviduct and uterus of the cow during the oestrus cycle. J.Reprod.fertil. 43:23-32.

39) Sreenan, JM., Diskin MG., Mc Donagh T. (1981) Induction of twin-calving by non-surgical embryo transfer: a field trial.Vet.Rec. 109, 77-80.

40) Sugie T., (1965). Successful transfer of a fertilized bovine egg by non surgical techniques. J. Reprod. Fertil. 10, 197-201.

41) Testart J., Godard Siour C., du Mesnil du Buisson F. (1975) Transvaginal transplantation of an extra egg to obtain twinning in cattle. Theriogenology, 4: 163-168.

42) Thibier M., (2007) New records in the number of both in vivo derived and in vitro produced bovine embryos around the world in 2006. IETS Newsletter 2007 (4), 15-20.

43) Thompson J. (1997). Comparison between in vivo derived and in vitro produced pre-elongation embryos from domestic ruminants. Reprod.Fertil.Dev. 9:254-341.

44) Vignon X, Chesné P, LeBourhis D, Fléchon JE, Heyman Y, Renard JP.(1998). Developmental potential of bovine embryos reconstructed with enucleated matured oocytes fused with cultured somatic cells. C.R.Acad.Sci.,Paris, 321:735-745.

45) Walker SK., Hartwich KM., Seamark RF. (1996). The production of unusually large offspring following embryo manipulation: concept and challenges. Theriogenology 45:111-120.

46) Willadsen S. (1979). A method for culture of micromanipulated sheep embryos and its use to produce identical twins. Nature 277: 298-300.

47) Willett EL., Black WG., Casida LE., Stone WH., Buckner PJ.(1951). Successful transplantation of a fertilized bovine ovum. Science 113:247.

48) Wilmut I., Schnieke AE., McWhir J., Kind AJ., Campbell KHS. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-813.

49) Witthingham DG., Leibo SP. , Mazur P. (1972). Survival of mouse embryos frozen to -196 and -269°C. Science 178:411-414.

50) Wrenzycki C., Herrmann D., Carnwath JN., Niemann H. (1999). Alteration in the relative abundance ofgene transcripts in preimplantation bovine embryos cultured in medium supplemented with serum or PVA. Mol.Reprod.Dev. 53:8-18.

National Statistical Data of Bovine Embryo Transfer Activity in Europe (2009).

TABLE: 1 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **AUSTRIA** ***A.E.T.E*** ***2010***

Data collected by
Dr. Lukas Kalcher

Total number of approved E.T. teams in the country	8
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	118	B/A=	10.9
	Embryos collected	**B**	1290	C/A=	7.5
	Embryos transferable	**C**	883	C/B=	68.4%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	883	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	196		
In vivo	Frozen	**I**	389		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	585	H+I+J+K=	
Number of frozen stored embryos		**M**	315		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	66.5%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	312
Number of calves born from *in vitro* embryos	
Total	312

TABLE: 2 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **BELGIUM** ***A.E.T.E 2010***

Data collected by Dr. Peter Vercauteren & Dr. Isabelle Donnay

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	624	B/A=	5.9
	Embryos collected	**B**	3686	C/A=	4.3
	Embryos transferable	**C**	2674	C/B=	72.5%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	2674	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	560		
In vivo	Frozen	**I**	2590		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3150	H+I+J+K=	
Number of frozen stored embryos		**M**	2114		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	82.2%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 3 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **CROATIA** ***A.E.T.E 2010***

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**	6	B/A=	14.5
	Embryos collected	**B**	87	C/A=	6.0
	Embryos transferable	**C**	36	C/B=	41.4%
In vitro *(OPU)*	Nb of oocyte donors		17		
	Nb of OPU sessions		68		
	Nb of transferable embryos	**D**	148		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	148	=(D+E)	
Total number of transferable embryos		**G**	184	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3		
In vivo	Frozen	**I**	46		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	49	H+I+J+K=	
Number of frozen stored embryos		**M**	33		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	93.9%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 4 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **CZECH REPUBLIC** ***A.E.T.E 2010***

Data collected by
Dr. Jirina Peteliková

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	255	B/A=	11.6
	Embryos collected	**B**	2966	C/A=	6.1
	Embryos transferable	**C**	1556	C/B=	52.5%
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**	6		
Total i*n vitro* embryos		**F**	6	=(D+E)	
Total number of transferable embryos		**G**	1562	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	779		
In vivo	Frozen	**I**	940	54.7% frozen	
In vitro	Fresh	**J**	6		
In vitro	Frozen	**K**		100% frozen	
Total embryos transferred		**L**	1725	H+I+J+K=	
Number of frozen stored embryos		**M**	827		
% of *in vitro* embryos transferred		**N**	0.3%	(J+K)/L=	
% of frozen embryos transferred		**O**	54.5%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 5 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **DENMARK** ***A.E.T.E 2010***

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**	325	B/A=	9.8
	Embryos collected	**B**	3178	C/A=	7.0
	Embryos transferable	**C**	2290	C/B=	72.1%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	2290	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1244		
In vivo	Frozen	**I**	889		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2133	H+I+J+K=	
Number of frozen stored embryos		**M**	1040		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	41.7%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	1796
Number of calves born from *in vitro* embryos	
Total	1796

TABLE: 6 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **Estonia** ***A.E.T.E 2010***

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**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**	0	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**	0	=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	21	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	21	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	5
Number of calves born from *in vitro* embryos	
Total	5

TABLE: 7 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **FINLAND** ***A.E.T.E 2010***

Data collected by Dr. Marja Mikkola

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	436	B/A=	11.7
	Embryos collected	**B**	5104	C/A=	7.9
	Embryos transferable	**C**	3433	C/B=	67.3%
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**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	3433	=(C+F)	
Number of sexed embryos			11		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1038		
In vivo	Frozen	**I**	3092	% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	4130	H+I+J+K=	
Number of frozen stored embryos		**M**	2284		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	74.9%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 8 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **FRANCE** ***A.E.T.E 2010***

Data collected by Dr. Claire Ponsart

Total number of approved E.T. teams in the country	20
Number of teams providing data	17

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	6030	B/A=	8.8
	Embryos collected	**B**	52827	C/A=	5.0
	Embryos transferable	**C**	30442	C/B=	57.7%
In vitro *(OPU)*	Nb of oocyte donors		61		
	Nb of OPU sessions		64		
	Nb of transferable embryos	**D**	142		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	67		
Total i*n vitro* embryos		**F**	209	=(D+E)	
Total number of transferable embryos		**G**	30651	=(C+F)	
Number of sexed embryos			495		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	14747		
In vivo	Frozen	**I**	14471	49.5% frozen	
In vitro	Fresh	**J**	124		
In vitro	Frozen	**K**	9	6.8% frozen	
Total embryos transferred		**L**	29351	H+I+J+K=	
Number of frozen stored embryos		**M**	11905		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	49.3%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	15000
Number of calves born from *in vitro* embryos	58
Total	15058

TABLE: 9 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **GERMANY** ***A.E.T.E 2010***

Data collected by Dr. Hubert Cramer

Total number of approved E.T. teams in the country	45
Number of teams providing data	23

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2445	B/A=	10.8
	Embryos collected	**B**	26504	C/A=	6.6
	Embryos transferable	**C**	16098	C/B=	60.7%
In vitro *(OPU)*	Nb of oocyte donors		150		
	Nb of OPU sessions		1506		
	Nb of transferable embryos	**D**	3271		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	3271	=(D+E)	
Total number of transferable embryos		**G**	19369	=(C+F)	
Number of sexed embryos			861		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5114		
In vivo	Frozen	**I**	8952	63.6% frozen	
In vitro	Fresh	**J**	1072		
In vitro	Frozen	**K**	586	35.3% frozen	
Total embryos transferred		**L**	15724	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	10.5%	(J+K)/L=	
% of frozen embryos transferred		**O**	60.7%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 10 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **GREECE** ***A.E.T.E 2010***

Data collected by
Dr. Samartzi Fonteini

Total number of approved E.T. teams in the country	2
Number of teams providing data	1

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**	0	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**	0	=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
Number of sexed embryos			0	
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**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L=

Number of E.T. calves born (2009)

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

TABLE: 11 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **HUNGARY** ***A.E.T.E 2010***

Data collected by Dr. Ference Flink

Total number of approved E.T. teams in the country	3
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	69	B/A=	7.5
	Embryos collected	**B**	519	C/A=	4.1
	Embryos transferable	**C**	280	C/B=	53.9%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	280	=(C+F)	
Number of sexed embryos			128		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	43		
In vivo	Frozen	**I**	283		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	326	H+I+J+K=	
Number of frozen stored embryos		**M**	183		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	86.8%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	504
Number of calves born from *in vitro* embryos	
Total	504

TABLE: 12 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **IRELAND** ***A.E.T.E 2010***

Data collected by Dr. Pat Lonergan

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	338	B/A=	10.8
	Embryos collected	**B**	3639	C/A=	5.5
	Embryos transferable	**C**	1855	C/B=	51.0%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	1855	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	675		
In vivo	Frozen	**I**	1059		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1734	H+I+J+K=	
Number of frozen stored embryos		**M**	1180		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	61.1%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 13 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **ISRAEL** ***A.E.T.E 2010***

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**	130	B/A=	3.1
	Embryos collected	**B**	406	C/A=	1.0
	Embryos transferable	**C**	116	C/B=	29.0%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	116	=(C+F)	
Number of sexed embryos			0		
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**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**		(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 14 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **ITALY** ***A.E.T.E 2010***

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**	1922	B/A=	9.6
	Embryos collected	**B**	18367	C/A=	5.9
	Embryos transferable	**C**	11341	C/B=	61.7%
In vitro *(OPU)*	Nb of oocyte donors		62		
	Nb of OPU sessions		105		
	Nb of transferable embryos	**D**	275		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	1367		
Total i*n vitro* embryos		**F**	1642	=(D+E)	
Total number of transferable embryos		**G**	12983	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	8500		
In vivo	Frozen	**I**	2000	19.0% frozen	
In vitro	Fresh	**J**	20		
In vitro	Frozen	**K**	1986	99.0% frozen	
Total embryos transferred		**L**	12506	H+I+J+K=	
Number of frozen stored embryos		**M**	5660		
% of *in vitro* embryos transferred		**N**	16.0%	(J+K)/L=	
% of frozen embryos transferred		**O**	31.9%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 15 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **LUXEMBOURG** ***A.E.T.E 2010***

Data collected by Dr. Aline Lehnen

Total number of approved E.T. teams in the country	2
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	42	B/A=	11.1
	Embryos collected	**B**	465	C/A=	5.1
	Embryos transferable	**C**	213	C/B=	49.8%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	213	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	54		
In vivo	Frozen	**I**	104	65.8% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	99		
Total embryos transferred		**L**	257	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	38.5%	(J+K)/L=	
% of frozen embryos transferred		**O**	79.0%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 16 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: THE NETHERLANDS ***A.E.T.E 2010***

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**	2788	B/A=	11.6
	Embryos collected	**B**	32456	C/A=	6.9
	Embryos transferable	**C**	19138	C/B=	59.0%
In vitro *(OPU)*	Nb of oocyte donors		164		
	Nb of OPU sessions		1679		
	Nb of transferable embryos	**D**	2260		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	117		
Total i*n vitro* embryos		**F**	2377	=(D+E)	
Total number of transferable embryos		**G**	21515	=(C+F)	
Number of sexed embryos			232		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	4604		
In vivo	Frozen	**I**	11877	72.1% frozen	
In vitro	Fresh	**J**	1167		
In vitro	Frozen	**K**	736	38.7% frozen	
Total embryos transferred		**L**	18384	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	10.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	68.6%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 17 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **NORWAY** ***A.E.T.E 2010***

Data collected by Dr. Eiliv Kummen

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	12	B/A=	7.5
	Embryos collected	**B**	90	C/A=	5.7
	Embryos transferable	**C**	68	C/B=	75.6%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	68	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	27		
In vivo	Frozen	**I**	120		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	147	H+I+J+K=	
Number of frozen stored embryos		**M**	41		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	81.6%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	70
Number of calves born from *in vitro* embryos	
Total	80

TABLE: 18 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **POLAND** ***A.E.T.E 2010***

Data collected by Dr. Jedrzej Jaskowski

Total number of approved E.T. teams in the country	7
Number of teams providing data	6

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	76	B/A=	8.7
	Embryos collected	**B**	659	C/A=	5.7
	Embryos transferable	**C**	432	C/B=	65.6%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	432	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	225		
In vivo	Frozen	**I**	269		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	494	H+I+J+K=	
Number of frozen stored embryos		**M**	182		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	54.5%	(I+K)/L=	

Number of E.T. calves born (2009)

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

TABLE: 19 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **PORTUGAL** ***A.E.T.E 2010***

Data collected by
Dr. Joao N Chagas e Silva

Total number of approved E.T. teams in the country	5
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	28	B/A=	13.2
	Embryos collected	**B**	370	C/A=	6.6
	Embryos transferable	**C**	185	C/B=	50.0%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	185	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	111		
In vivo	Frozen	**I**	103		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	214	H+I+J+K=	
Number of frozen stored embryos		**M**	74		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	48.1%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	83
Number of calves born from *in vitro* embryos	
Total	

TABLE: 20 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **ROMANIA** ***A.E.T.E 2010***

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**	23	B/A=	8.4
	Embryos collected	**B**	185	C/A=	4.8
	Embryos transferable	**C**	109	C/B=	59%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	109	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	47		
In vivo	Frozen	**I**	116		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	163	H+I+J+K=	
Number of frozen stored embryos		**M**	62		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	71.2%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	22
Number of calves born from *in vitro* embryos	
Total	22

TABLE: 21 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **SPAIN** ***A.E.T.E 2010***

Data collected by Dr. Julio De la Fuente

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	687	B/A=	9.8
	Embryos collected	**B**	6744	C/A=	4.9
	Embryos transferable	**C**	3293	C/B=	48.8%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	3293	=(C+F)	
Number of sexed embryos			26		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	641		
In vivo	Frozen	**I**	1557		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2198	H+I+J+K=	
Number of frozen stored embryos		**M**	2091		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	70.8%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	1016
Number of calves born from *in vitro* embryos	1
Total	1017

TABLE: 22 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **SWEDEN** ***A.E.T.E 2010***

Data collected by Dr. Johanna Geust

Total number of approved E.T. teams in the country	2
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	64	B/A=	5.3
	Embryos collected	**B**	339	C/A=	3.1
	Embryos transferable	**C**	196	C/B=	57.8%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	196	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2		
In vivo	Frozen	**I**	50		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	52	H+I+J+K=	
Number of frozen stored embryos		**M**	196		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	96.2%	(I+K)/L=	

Number of E.T. calves born (2008)

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

TABLE: 23 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **SWITZERLAND** ***A.E.T.E 2010***

Data collected by Dr. Rainer Saner

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	438	B/A=	11.4
	Embryos collected	**B**	5002	C/A=	8.0
	Embryos transferable	**C**	3510	C/B=	70.2%
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**	0	=(D+E)	
Total number of transferable embryos		**G**	3510	=(C+F)	
Number of sexed embryos			1		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	586		
In vivo	Frozen	**I**	2132	78.4% frozen	
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	3	100% frozen	
Total embryos transferred		**L**	2721	H+I+J+K=	
Number of frozen stored embryos		**M**	2924		
% of *in vitro* embryos transferred		**N**	0.1%	(J+K)/L=	
% of frozen embryos transferred		**O**	78.5%	(I+K)/L=	

Number of E.T. calves born (2009)

Number of calves born from superovulated embryos	1400
Number of calves born from *in vitro* embryos	2
Total	1402

TABLE: 24 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **TURKEY** ***A.E.T.E 2010***

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**	0	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**	0	=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	14	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**		H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2009)

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

TABLE: 25 EMBRYO TRANSFER ACTIVITY IN 2009

COUNTRY: **UK** ***A.E.T.E 2010***

Data collected by Dr. Ian Kippax

Total number of approved E.T. teams in the country	39
Number of teams providing data	

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**	8347	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**	0	=(D+E)
Total number of transferable embryos		**G**	8347	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**		(fresh + frozen)
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	4857	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 (2009)

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

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2009

I. EMBRYO PRODUCTION

(Data collected from 22 countries)

In vivo* produced embryos (superovulation)	
- number of flushed donors	16,856
- number of transferable embryos	98,148
- mean number per flushed donor	5.82
***In vitro* produced embryos:**	
From OPU	
- number of OPU sessions	3,422
- number of transferable embryos	6,096
- mean number per session	1.78
From slaughterhouse collected ovaries	
- number of transferable embryos	1,557
Total *in vitro*	7,653
Total number of transferable embryos	114,148
Embryos sexed:	1,754

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

(S. Merton, AETE, Kuopio, Finland 2010)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2009

II. EMBRYO TRANSFERS

(Data collected from 23 countries)

***In* vivo produced embryos ***	**Number of embryos transferred** 90,270 (39,142 fresh / 51,074 frozen)
***In vitro* produced embryos**	5,808 (2,389 fresh / 3,419 frozen)
Total number of embryos transferred	100,678
Proportion of IVF embryos transferred	5.8%
Proportion of frozen embryos transferred	56.7%

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

(S. Merton, AETE, Kuopio, Finland 2010)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2009

Species	Embryo Production	Embryo Transfers	Countries
Sheep	197	143	Turkey Czech Republic
Swine	716	20	Czech Republic
Goat			
Horse	1024 (incl. 60 IVP)	1037 (incl. 60 IVP)	Czech Republic France Hungary Italy Netherlands

(S. Merton, AETE, Kuopio, Finland 2010)

INVITED LECTURES

USE OF FLOW CYTOMETERICALLY SEX-SORTED SEMEN IN SINGLE AND SUPEROVULATING COWS AND HEIFERS.

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Introduction

The ability of flow cytometry to accurately detect the difference in DNA content of X- and Y-chromosome-bearing sperm was first reported by Garner and coworkers (1983). The validation of this technology through live births of rabbits of the predicted sex was reported by Johnson et al. (1989). Another ~15 years would pass before the technology became sufficiently developed to consider as a viable option for commercial application in bovine animal breeding. Even then, equipment expense, sorting speeds and efficiency dictated that commercial application would only be possible with extremely low (2.1×10^6) sperm numbers per insemination dose (Amann, 1999; Garner and Seidel, 2002). Furthermore, the highly invasive nature of the procedure imparts detrimental effects on sperm viability and quality (Schenk et al., 1999). Together these factors contributed to the reduced conception rates with sex-sorted semen, which at the time of initial commercialization were reported as ~75% of that obtained with conventional semen (Seidel et al., 1999). Thus, despite 20+ years of research and development, the economic return on investment from use of sex sorted semen by the dairy producer was still questionable at the time of market introduction in 2005 (Olynk and Wolf, 2006; Fetrow et al., 2007). However, a conservative and cautious marketing approach not to oversell expectations resulted in wide-spread and rapid customer acceptance. Within 3 years, all major AI organizations in the US were offering sex-sorted semen in their product lines.

Commercial application of sex-sorted semen has largely focused on use in virgin heifers to capitalize on the more favorable conception rates compared to those of lactating cows. Continuing research has focused on improving conception rates through increased sperm dosages (DeJarnette et al., 2008; Schenk et al., 2009) and limited trials have investigated utility in superovulated lactating cows (Schenk et al., 2006). Despite recommendations to the contrary, considerable anecdotal data exists from producer application in lactating cows (DeJarnette et al., 2009) and superovulation (Potter et al., 2009). This manuscript will provide an update to a previous publication (DeJarnette et al., 2009) wherein on-farm records of Holstein dairy herds that have experiences with commercial application of sex-sorted semen were summarized and reviewed. In addition, a brief review of research attempts to improve conception rates of sexed sorted semen in heifers and lactating cows will be provided.

Conception rates

At the time of U.S. market introduction, existing published research (Garner and Seidel, 2002) suggested that sexed-sorted semen should be expected to yield conception rates in virgin heifers that range from 70 to 90% of that obtained with conventional semen. From the time of market introduction, Select Sires, Inc. (Plain City, Ohio, USA, 43064) has compiled conception and calving data as voluntarily reported by participating producers. Conception rates in this updated report (Table 1) are similar to those previously published (DeJarnette et

al., 2009). First service conception rates in virgin Holstein heifers average 47% (n = 41,452) which is approximately 84% of that obtained with conventionally processed semen (56%, n = 33,572) in the same 78 herds. As previously reported, these field results appear somewhat greater than the anticipated 70-75% of conventional reported in controlled research trials. However, the authors again warn that these results are likely by biased by the preferential use of sex sorted semen at first service in heifers exhibiting robust signs of estrus. By default, conventional semen usage is directed to a disproportionate percentage of repeat services and (or) females exhibiting questionable signs of estrus. Therefore the 84% of conventional semen estimate may be somewhat inflated. Interestingly, first service conception rates with sexed semen in Holstein cows are only 70% of that achieved with conventional semen. Though conception rates with conventional semen illustrate typical declines with increasing service number, conception rates with sexed sorted semen actually exhibited a numeric increase with advancing service number, which is likely an artifact of selective management decisions regarding which repeat service cows are "eligible" for re-insemination with sexed semen. By default, all other cows receive conventional semen.

Table 1. Conception rate (LS Means) of sexed and conventional semen by age and service number. (n = total number of animals evaluated)

			Service no.		
Age	Herds	Semen type	1st	2nd	≥ 3rd
Cows	56	Conventional	34% (33,202)	33% (19,111)	29% (27,739)
		Sexed	24% (5,180)	26% (2,826)	30% (698)
Heifers	78	Conventional	56% (33,572)	54% (19,830)	47% (19,701)
		Sexed	47% (41,452)	44% (11,344)	39% (4,629)

The suggestion that fertility estimates are biased in herds using sexed semen is supported by a large-scale blind field trial (DeJarnette et al., submitted for publication), wherein 2.1 and 3.5 x 10^6 sexed sperm dosages were compared to a 15 x 10^6 conventional sperm dosage (blind control) across 7 Holstein sires in Holstein heifers (n = 6,268) and cows (n = 5,466). Among heifers (Figure 1), the sexed-sperm dosages achieved comparable conception rates in 6 of the 7 sires evaluated. Across sires, sex-sorted semen achieved conception rates that were 75% of that obtained with the blind conventional semen treatment. Among cows (Figure 2), results were strikingly similar to those in heifers with conception rates of the 2.1 x 10^6 sexed sperm dosage averaging 75% of that achieved with the conventional sperm dosage.

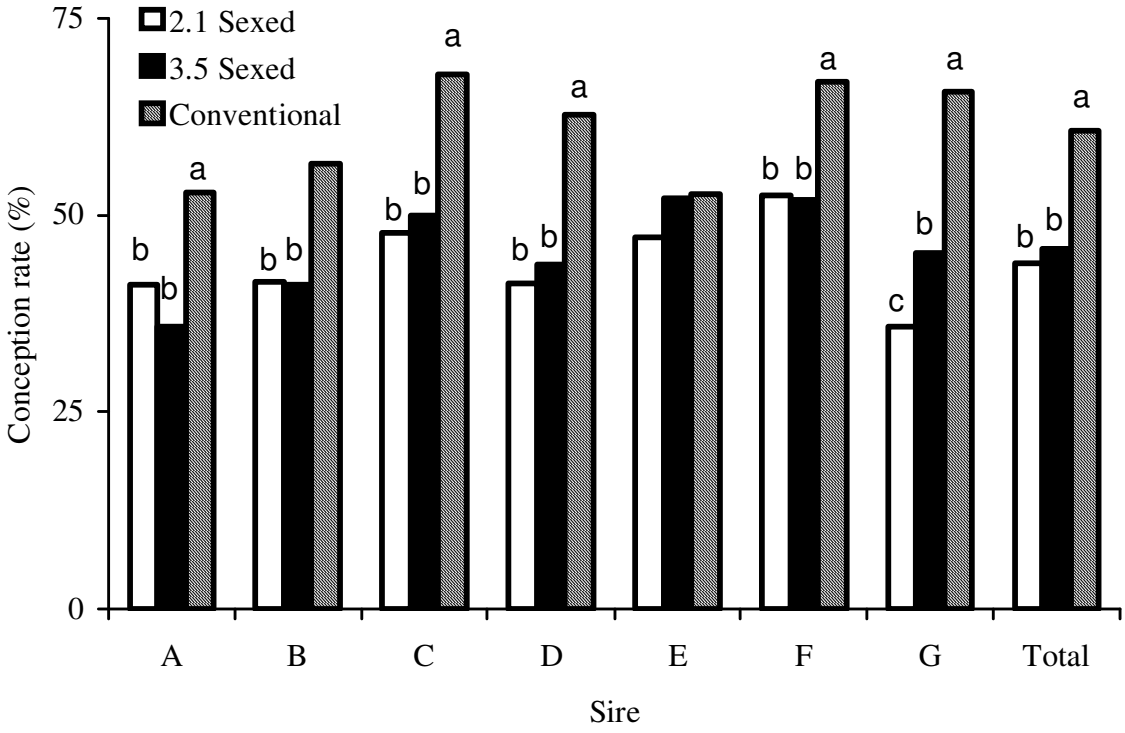

Figure 1. Effects of sire and sexed sperm dosage on conception rates in virgin Holstein heifers.
[abc] Values within sire or total lacking common superscripts differ, $P < 0.05$.
Sire effect, $P < 0.001$.
Sperm dosage effect, $P < 0.001$.
Sire by sperm dosage interaction, $P < 0.01$.

A similar survey of U.S. Dairy Herd Improvement herds (Norman et al., In press) also documented considerable evidence for the on-farm bias in sexed semen application. That study noted that among heifers, 82% of sexed semen usage was reported at first service and among cows, 61% of sexed semen usage was reported at first service in primiparous cows. These observations are relatively consistent with our present data (Table 1). In contrast however, the first service conception rate of sexed semen used in virgin heifers was only 41% (70% of conventional), while the conventional semen contemporary group achieved first service conception rates of 59%. Obviously, there are a wealth of possibilities to explain the discrepancies among these two data sets, not the least of which are the differences in herds and (or) geographic regions included. Although there was no specific prerequisite for selection of herds in the DeJarnette database, as a voluntary program, herds achieving below normal acceptable levels of success may have been somewhat less inclined to contribute data. In contrast, the data of Norman et al. (In press) were provided on a non-voluntary basis. It is important to acknowledge that all sexed semen data included by DeJarnette et al. (2009) were processed at a single location (Select Sires and Sexing Technologies, Plain City, Ohio). Previous research has documented that the fertility potential of conventional semen supplied by A.I. laboratories can vary significantly (Kuhn and Hutchison, 2007). Additionally, the highly invasive nature of the sorting procedure combined with the low sperm numbers per dose would likely exacerbate and magnify the relative importance of otherwise trivial differences in semen processing, cryopreservation procedures, and (or) on-farm training and technical expertise in semen handling and AI procedures (Salisbury and VanDemark, 1961; den Daas et al., 1998). Thereby, the lower conception rates of sexed semen reported by

Norman et al. (In press) may be a reflection of variance across sex sorting laboratories. These observations are supported by European studies indicating comparable relative differences in non-return rates of sexed vs. conventional semen and similar warnings of confounding biases in sexed semen application (Borchersen and Peacock, 2009; Frijters et al., 2009).

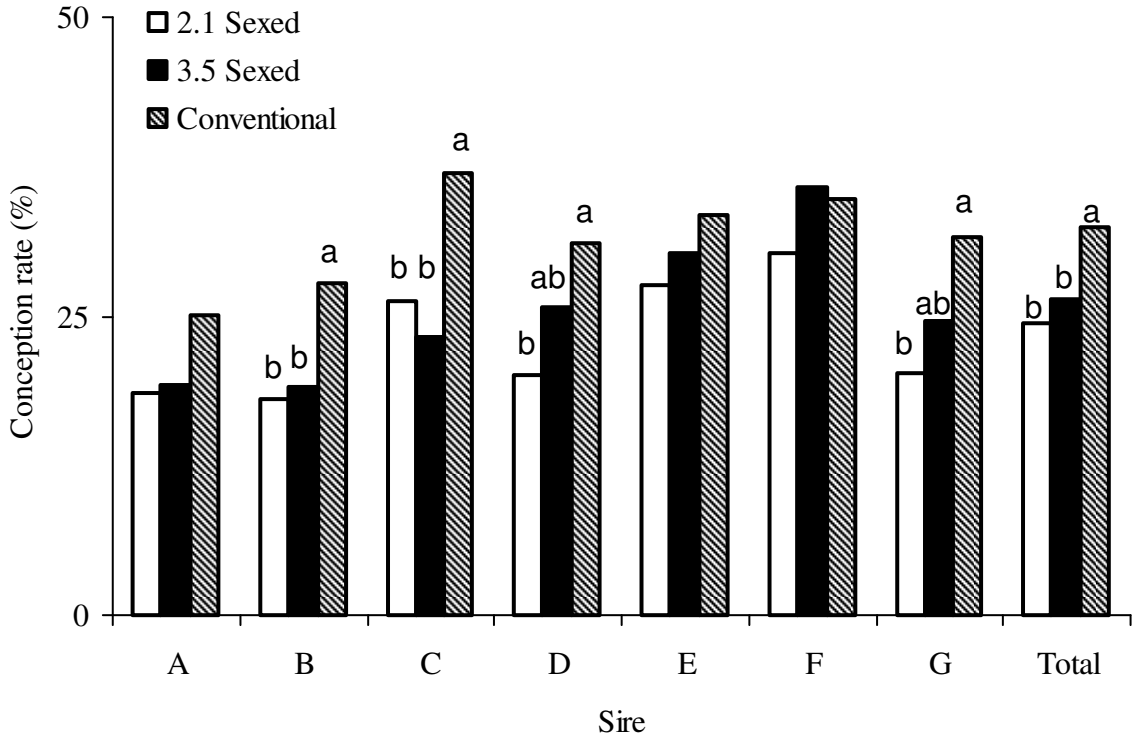

Figure 2. Effects of sire and sexed sperm dosage on conception rates in lactating Holstein cows.
[abc] Values within sire or total lacking common superscripts differ, $P < 0.05$.
Sire effect, $P < 0.001$.
Sperm dosage effect, $P < 0.001$.
Sire by sperm dosage interaction, $P = 0.64$.

Sire conception rates

The sire effect was identified in early research as a major contributor to overall success with sexed sorted semen (Seidel, 2007). However, DeJarnette et al. (2009) found that estimates of sire fertility based on use of conventional semen to be of little value for predicting success with sex sorted semen ($r = 0.35$), which is consistent with observations of others (Abdel-Azim, 2010). Similarly in the blind field trial of DeJarnette and coworkers (Personal communication), there was no association of conception rates with sexed and conventional semen when used in virgin heifers (Figure 3; $R^2 = 0.04$ vs. 0.19 for 2.1 vs. 3.5 x 10^6 sexed sperm dosages, respectively). Furthermore, the two sexed sperm dosages only demonstrated a moderate correlation with each other ($R^2 = 0.48$) when used in heifers. In contrast, the relationship between conception rates of sexed and conventional was much greater when used in cows (Figure 4; $R^2 = 0.66$ vs. 0.42 for 2.1 vs. 3.5 x 10^6 sexed sperm dosages, respectively) as was the relationship of conception rates for the two sexed dosages to

each other (R^2 = 0.73). Perhaps most interesting was the observation that the relationship of sire conception rates for each sperm dosage when used in heifers vs. use in cows (Figure 5). The relationship across cows and heifers was relatively high for sexed sperm dosages (R^2 = 0.78 vs. 0.66 for 2.1 vs. 3.5 x 10^6 sexed sperm dosages, respectively) but more moderate for the conventional sperm dosage (R^2 = 0.46).

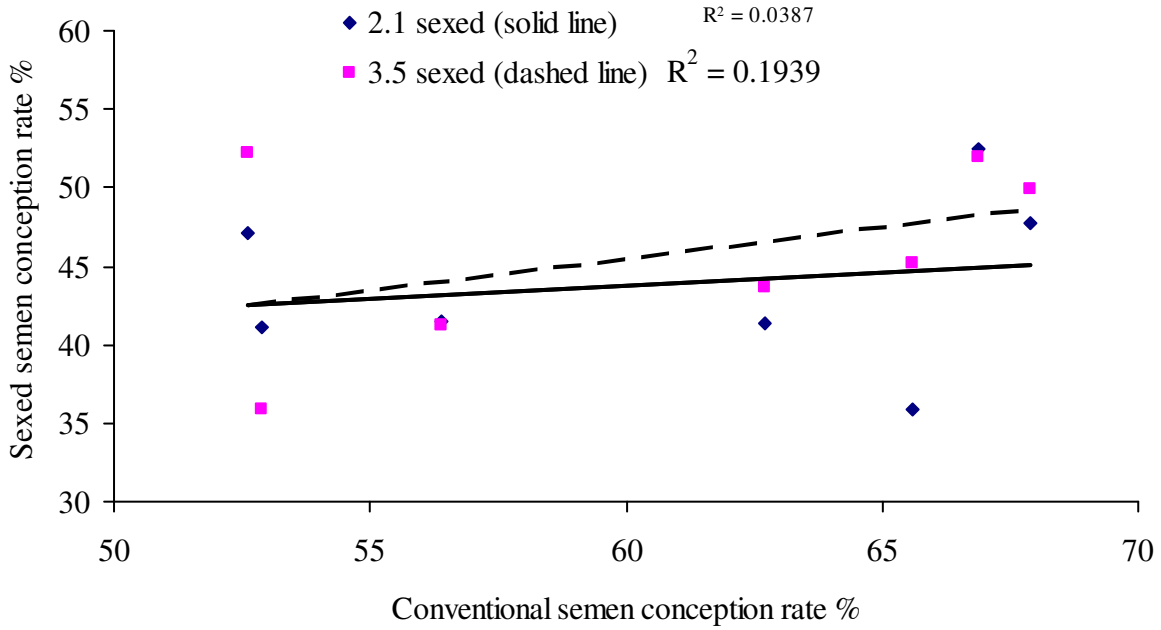

Figure 3. Relationship of conception rates in Holstein heifers to 2.1 and 3.5 x 10^6 sexed sperm and 15 x 10^6 conventional sperm dosages across 7 sires.

Collectively, the above observations appear to reflect and support the hypotheses of compensable semen quality traits as posed by others (Salisbury and VanDemark, 1961; den Daas et al., 1998; Saacke et al, 2000). These principles predict that some attributes of semen quality are compensable and will respond to increasing dosage with greater fertility. The magnitude of this response varies by sire (semen sample) as a function of the percentage of sperm that are deficient for compensable traits. For each sire (semen sample), a threshold exists beyond which further increases in sperm numbers do not increase conception potential and maximum fertility is thereby limited by these uncompensable traits. The importance of the female population to these principles has been often discussed but seldom studied. In theory, more fertile virgin heifers with smaller and uncompromised reproductive tracts would require a lower threshold sperm dosage to achieve maximum fertility and are thereby less sensitive to sperm dosage. In contrast, cows, having larger reproductive tracts and inherently lower fertility potential, are predicted to require greater threshold sperm numbers for maximum fertility potential. The legitimacy of these hypotheses are supported by the observation of lower correlations of sire fertility estimates among sperm dosages within heifers (Figure 3) wherein a greater proportion of the differences in sire fertility among dosages are likely a

function of uncompensable traits. Whereas in cows (Figure 4) compensable semen traits continue to play a more measureable role due to greater sperm number thresholds and greater agreement was observed among sire fertility deviations across dosages. Perhaps most impressive (Figure 5) was the observation that the conception rates of both heifers and cows do reflect the influence of compensable semen quality traits among sires within all sperm dosages, however, the magnitude of the association distinctly diminishes with increased sperm dosages (2.1 x 10^6 sexed vs. 3.5 x 10^6 sexed vs. 15 x 10^6 conventional).

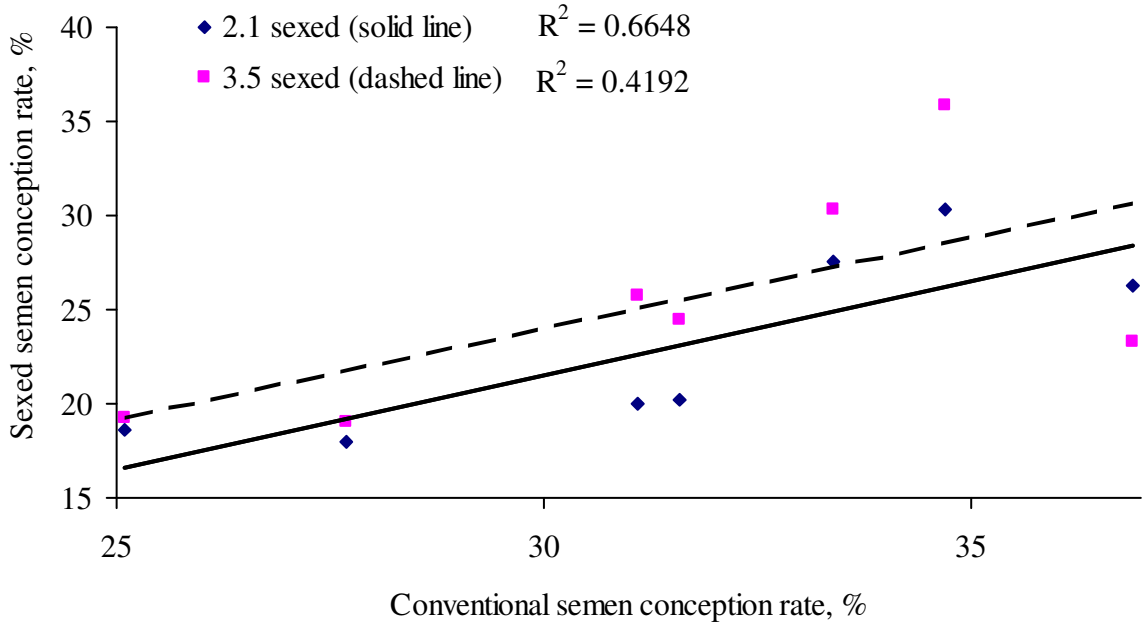

Figure 4. Relationship of conception rates in lactating Holstein cows to 2.1 and 3.5 x 10^6 sexed sperm and 15 x 10^6 conventional sperm dosages across 7 sires.

Other factors influencing conception rates

Aside from the obvious factors of herd, parity, sire, and service number, other factors implicated as influencing conception rates of sex sorted semen include timing of AI and fixed time insemination. Macmillan and Watson (1975) demonstrated that a major component of the variance in sire fertility estimates is likely function of post-thaw sperm longevity in the female reproductive tract. When insemination occurs too late relative to ovulation, conception potential is limited by the ability of the unfertilized ovum to maintain functional competence until sperm arrive and fertility differences among sires (semen samples) are likely minimized. In contrast, when insemination occurs too early relative to ovulation, conception potential is limited by the viable lifespan and other unknown traits of the sperm in the inseminate (Dalton et al., 2001). It would seem logical to assume that due to the highly invasive nature of the sorting procedure (Schenk et al., 1999), sex sorted sperm will likely have compromised post-thaw survival compared to non-sorted contemporaries thereby making conception potential more sensitive to deviations in appropriate insemination timing, especially if inseminated too early. Sá Filho and coworkers (submitted for publication) studied the effects of the interval from onset of estrus to AI on conception rates of Jersey heifers bred to sex-sorted semen. Conception rates were greatest when AI occurred 16 to 24

hours after the onset of estrus (54%, n = 398) and declined with earlier (38%, n= 106) and later (46%, n = 134) insemination timings. In similar studies using conventional semen in Holstein cows, conception rates were comparable for insemination times from 4 to 16 hours after the onset of estrus and declined more dramatically for late insemination than for earlier insemination (Dransfield et al., 1998). Therefore, the observations of Sá Filho and coworkers wherein slightly later AI was less compromising to conception than early AI, tends to supports the theory that sexed semen is more sensitive to timing of insemination than is conventional semen.

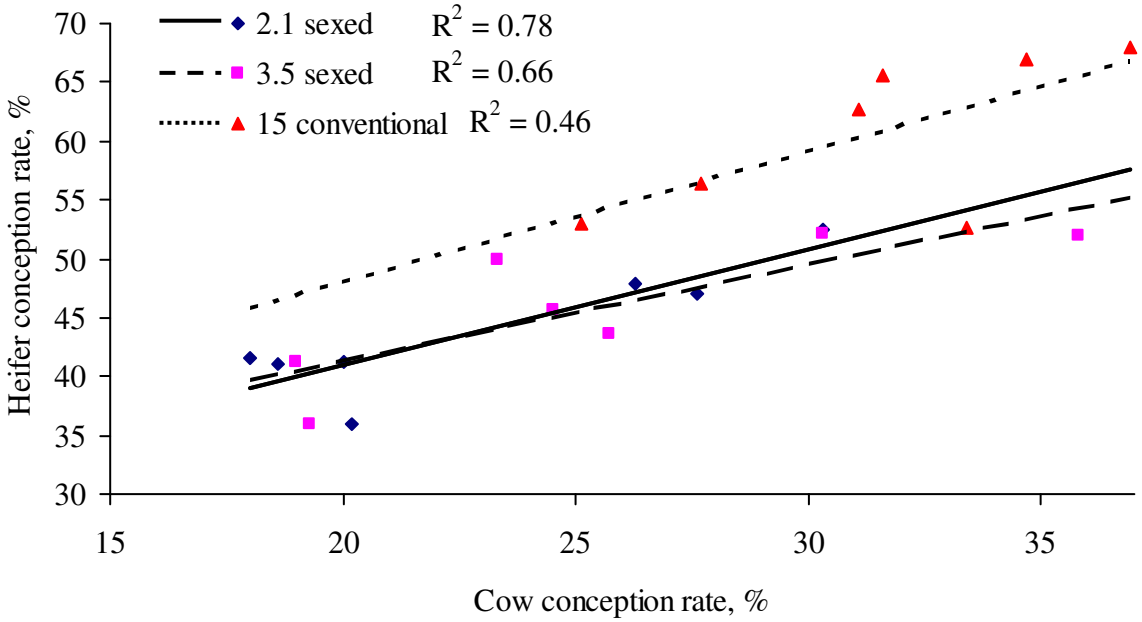

Figure 5. Relationship of sire conception rates (n = 7) among Holstein heifers and cows for 2.1 and 3.5 x 10^6 sexed sperm and 15 x 10^6 conventional sperm dosages.

With the commercial introduction of sex sorted semen it was widely recommended that producers should avoid using sexed semen in conjunction with fixed time AI. The primary basis for this recommendation was to minimize the opportunity for the inherent variation in success often associated with timed AI programs (Nebel and DeJarnette, 2006) to cast a negative perception among producers toward this novel but expensive product offering. However, it could easily be argued that in many herds, fixed-time AI is a more appropriate and successful application of sexed semen than estrus based AI. This supported by considerable evidence that suggests our ability to predict time of ovulations (i.e., optimum time to AI) is much more accurate and repeatable in an ovulation synchronization program (Pursley et al., 1997) than in estrus detection based AI programs (Senger, 1994). Based on studies of sire fertility in relation to insemination timing (Macmillan and Watson, 1975; Dalton et al., 2001), DeJarnette (2006) speculated that timed control of ovulation and AI may be a valuable tool to minimize variation in sire fertility and may indeed be the optimum scenario for use of a semen product known to have a limited viable life span. Therefore it is not necessarily surprising that relative conception potential of sexed semen used in fixed time AI of virgin heifers was in the normal range of 70% to 80% of conventional (Thatcher et al., 2009). Though the results of Thatcher et al. (2009) are easily argued as acceptable, they do not necessarily represent optimal. The denominator to success in a timed AI program always

includes animals that had nearly zero opportunity to conceive (i.e., anestrous, failure to respond, failure to receive all injections, etc.). Thus within timed AI programs, the sub-population of animals observed in estrus have significantly greater conception rates than do animals that did not display evidence of estrus activity (Perry, 2006). These observations tend to support the present recommendation that encourages the use of estrus synchronization in conjunction with use of sexed semen to facilitate estrus detection among responding and presumed fertile females, while non-responders are inseminated with less expensive and (or) more readily available semen products.

The above observations tend to imply that sorting technology adaptations which can be deemed less invasive, damaging, and detrimental to sperm longevity are promising areas of study to enhance fertility potential of sex sorted semen. However, this has largely been the focus of 20+ years of research to date with only nominal evidence of meaningful gains (Schenk et al., 2009).

Inter-estrus intervals

Shortly after the commercial introduction of sexed-sorted semen, considerable rumor and speculation circulated relative to the incidence of early embryonic mortality and delayed inter-estrus intervals as result of sex-sorted semen. This speculation and concern was not surprising as it was anticipated that producers would closely scrutinize all aspects of this premium priced but fertility compromised product. Furthermore, trials using sex-sorted semen in IVF and (or) superovulation provide evidence to suspect embryo quality could be compromised, thereby increasing the risk early embryonic mortality (Sartori et al., 2004; Schenk et al., 2006; Potter et al., 2009). Delayed inter-estrus intervals would have negative implications on the economic value of sexed sorted semen beyond that of reduced conception rates as a function of increased days (and associated expenses) to conception and calving when compared to normal length inter-estrus intervals.

The effect of sexed semen on inter-estrus intervals was assessed in the 54 herds reporting $\geq$ 50 services to both sexed and conventional semen (DeJarnette et al., 2009). Heifers that failed to conceive to sexed semen were actually determined to have a greater probability of returning to estrus in a normal 18 to 24 day interval than heifers that failed to conceive to conventional semen (Table 2). There are several possible explanations for this observation. Reduced sperm viability in sex-sorted samples may tend to increase rates of fertilization failure which would tend to facilitate a normal inter-estrus interval. Additionally, preferential use of sexed semen in heifers showing definitive signs of estrus would facilitate normal inter-estrus intervals compared to heifers displaying questionable signs of estrus, which by default, receive a disproportionate percentage of breeding to conventional semen. However, neither explanation should be interpreted to imply that early embryonic mortality is not an issue with sexed-semen. To the contrary, embryonic mortality was implicated as a contributing factor to the reduction in conception rates from use of sexed semen both in vivo (Sartori et al., 2004; Schenk et al., 2006; Potter et al., 2009) and in vitro (Palma et al., 2008) studies. Collectively however, these data imply that any increase in embryonic mortality due to use of sexed semen is likely occurring prior to maternal recognition and thereby does not appear to negatively influence inter-estrus intervals.

Table 2. Inter-estrus interval in heifers (% LS Means) after an unsuccessful AI to sexed or conventional semen. (n = total animal evaluated)

	≤17 d	18 to 24 d	25 to 35 d	36 to 48 d	49 to 90 d
Conv. semen	10.7 (2,065)	63.7 (12,340)	9.7 (1,882)	11.0 (2,137)	4.9 (946)
Sexed Semen	7.5 (1,381)	69.6 (12,857)	8.8 (1,631)	9.6 (1,778)	4.4 (818)

Data were obtained from 54 herds reporting ≥ 50 inseminations to both sexed and conventional semen.

Sex ratios

Across all breeds and parities, 88% of all singleton births reported to be conceived from use of sexed semen were female offspring. Where date of conception and date of calving could be used to validate a gestation length within a normal 265 to 295 d interval, 89% of offspring were female (Table 3). Where gestation length was < 265 d or > 295 d, the percentage of female offspring resulting from use of sexed semen was considerably less the 90% perhaps indicating errors in identification of sire of conception. Interesting was the observation that when sire identity was reported as unknown and gestation length was less than or greater than a normal 265 to 295 day interval, 93% to 95% of the offspring were reported as female. This highlights typical issues of on-farm data integrity and (or) accuracy that may occur through errors in service sire identity at AI, sire of conception at pregnancy diagnosis, and perhaps dam of calf in often crowded maternity pens with sometimes infrequent observations. Therefore, the reported incidence of female calves (88 to 89%) is likely biased downward slightly due to these inherent errors in on-farm data integrity.

Among twin births conceived from use of sexed semen (n = 423), 74% were female-female calving, 16% were female-male, and 10 were male-male. In comparison, only 25% of twins born from use of conventional semen (n = 2766) were female-female, 41% were female-male, and 34% were male-male. With consideration for the incidence of free-martins and bull calves, only 25% of twin born offspring from conventional semen have the possibility to become productive members of the herd, compared to 74% for sex sorted semen. The incidence of twinning in Holstein heifers in present data (≤ 1%) was similar for both sexed and conventional and consistent with that reported in a large-scale regional DHIA analysis of twinning (Silva del Rio et al., 2007).

Table 3. Percentage of heifer calves reported from singleton births by reported calf sire and by gestation length.

	Gestation length (d)				
Semen source	< 265	265 to 295	> 295	Unknown	Total
Sexed	78% (757)	89% (33,314)	57% (400)	87% (10,809)	88% (45,280)
Conventional	42% (2,079)	49% (80,905)	32% (1,451)	50% (31,116)	49% (115,551)
Herd bull	54% (1,738)	51% (13,469)	44% (996)	49% (5,209)	50% (21,412)
Unknown	93% (224)	60% (5,786)	95% (357)	49% (2,014)	60% (8,381)

Data were obtained from 158 herds reporting calves born from sexed semen. Because some herds did not report calving data for conventional semen or herd bulls, these data are provide as reference only but with no statistical comparisons.

Gestation length

The effects of calf sex and semen type on gestation length of singleton births among Holstein cows and heifers are presented in Table 4. Irrespective of calf sex, heifers tend to have a slightly shorter gestation length than cows. Within each age group (heifers vs. cows), male calves had a gestation length ~ 1 to 2 d longer than did female calves. Within each parity and calf sex classification, the gestation length for sexed vs. conventional semen was nearly identical (P = 0.71). In previous reports of these data (DeJarnette et al., 2009), mean gestation length among herds ranged from 275 to 281 d while gestation length for births occurring during the months of Oct through May (278 ± 0.15, n = 22,889) averaged 1 d longer than for those occurring during the months June through September (277 ± 0.15, n = 10,232). Among sires with ≥ 20 calves, gestation length by sire (adjusted for calf sex) ranged from 273 to 282 d. All observations are consistent with previous reports of sources of variance in gestation length (Fisher and Williams, 1978; Norman et al., 2007).

Table 4. Effects of calf sex and semen type on gestation length of Holstein cows and heifers.

		Semen Type	
Parity	Calf sex	Conventional	Sexed
		Mean ± SEM (n)	
Cows	Female	278 ± 0.05 (14,446)	278 ± 0.14 (1,694)
	Male	280 ± 0.06 (18,141)	278 ± 0.47 (232)
Heifers	Female	277 ± 0.04 (24,947)	277 ± 0.04 (26,736)
	Male	278 ± 0.05 (24,162)	278 ± 0.19 (3,485)

Age at calving

DeJarnette et al. (2009) reported that age at calving was influenced by herd, semen type, season within herd, and the herd by semen type interaction. It was clear in that study that preferential use of sexed semen at first and second services was resulting in a younger average age at calving for heifers bred to sexed vs. conventional semen. Updated trends across 158 herds are presented in Figure 6 and are consistent with prior reports.

Stillbirth analysis

Due to the preferential use of sexed semen that results in a younger age at calving and the documented impact of both calf sex and age at calving on the incidence of stillbirth (Cole et al., 2007), it is very important to simultaneously account for these confounding factors when assessing the impact of semen type on the incidence of stillbirth. DeJarnette et al. (2009) observed a 3-way interaction between semen source, calf sex, and heifer age at calving. After adjusting for age at calving, semen type had no affect on the incidence of stillbirth among female calves. However, male calves born from 90% purity, female-biased, sex sorted semen had an increased incidence of stillbirths that was only partially explained by age at calving. The updated analyses of these data are presented in Figures 7 and 8. The incidence of stillbirth remains unaffected by semen type among female calves. With the much larger sample size, the magnitude of the stillbirth anomaly in male calves has slightly diminished, but has not abated and remains abnormally high even after adjusting for the effects of age at calving. It is important to highlight that despite this increased incidence of stillbirths among male calves born to sexed semen (~20%), the total incidence of male calves born from use of sexed semen is so small (~10%) that there is on effect on the overall incidence of stillbirths among virgin heifers (~10%). These results are consistent with those of similar population surveys of Dairy Herd Improvement records (Norman et al., in press). However, the

magnitude of the increased incidence of stillbirth among males calves born from sexed semen was considerably less in that study (16% vs. 11% for sexed vs. conventional, respectively) than in the present study. Additionally, because the data of Norman et al. (In press) were not adjusted for the confounding effect of age at calving, the effects of semen type on the incidence of stillbirth in that study are likely over-estimated. Furthermore the incidence of stillbirth for both calf sexes among lactating cows is nearly identical for sexed and conventional semen (Figure 9). Thus, the source to this observational anomaly remains purely speculative and may be as much attributable to inconsistencies in data reporting as due to biology (DeJarnette et al., 2009).

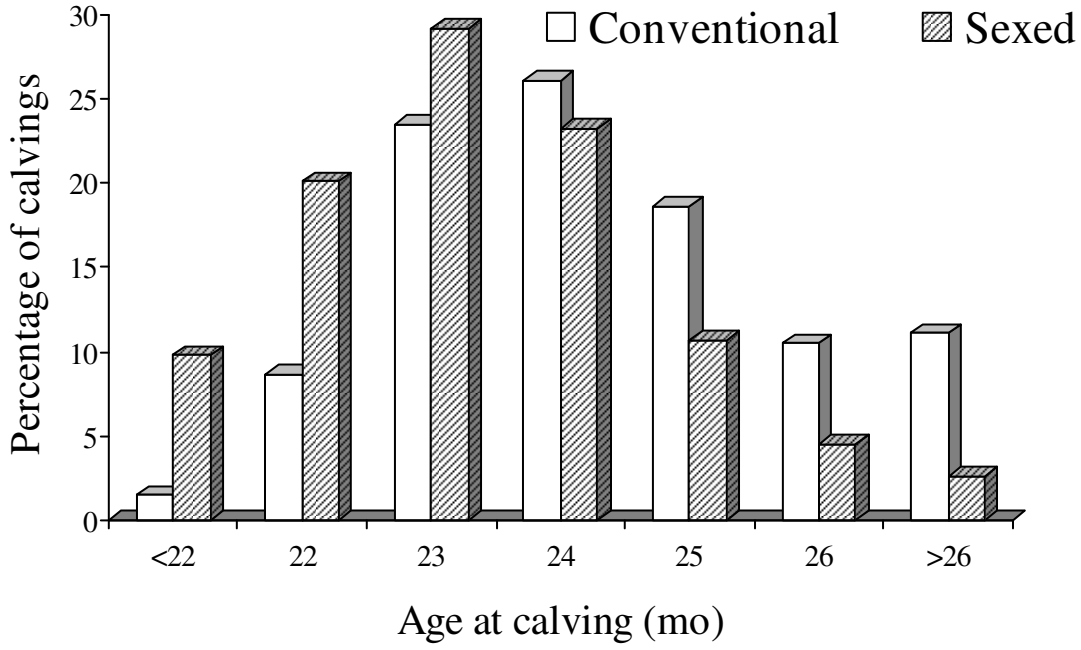

Figure 6. Distribution of age at first calving by semen source of conception (sexed vs. conventional) in 158 herds using both sexed and conventional semen.

Sexed semen in superovulated females

A summary of references comparing the effects of sexed semen and conventional on fertilization rates and embryo quality in superovulated cows and heifers are presented in Table 5. By in large, these studies indicate fertilization rates and the percentage of transferable embryos is favored by conventional over sex-sorted semen. However, the sex bias differential (90% vs. 48% of desired sex for sexed vs. conventional semen respectively) typically results in similar to slightly greater numbers of transferable embryos of the desired sex. In conditions where there are extreme differences in value of calf gender and recipient expenses are high, absence of conception to a fetus of the undesired sex could perhaps be of significant economic advantage.

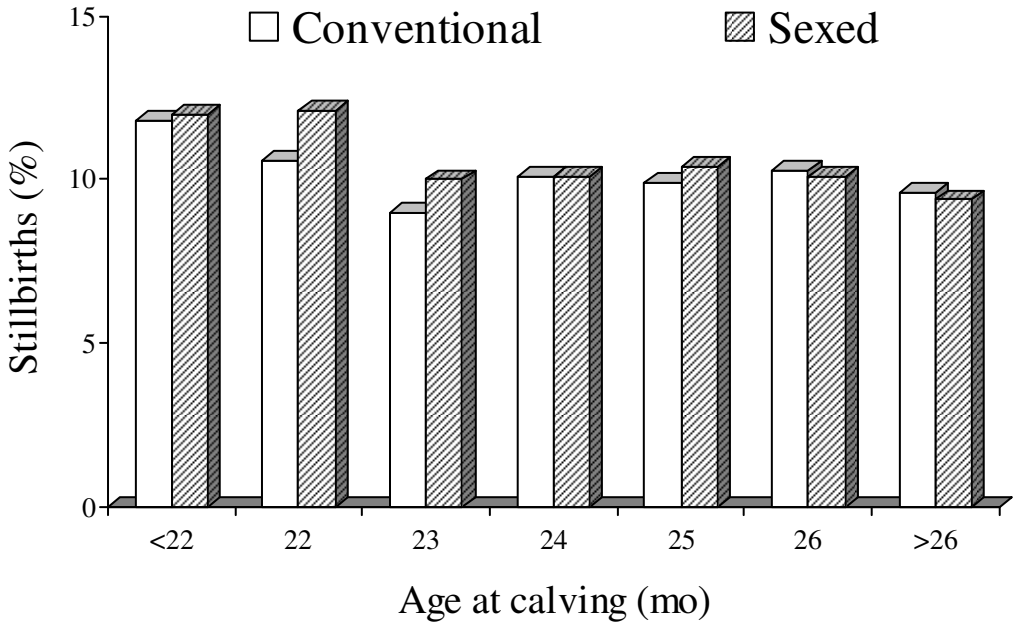

Figure 7. Effects of age at calving and semen type (sexed vs. conventional) on the incidence of stillbirths among female calves in 143 herds of Holstein heifers. Total calvings for sexed semen = 33,201 and for conventional semen = 30,043.

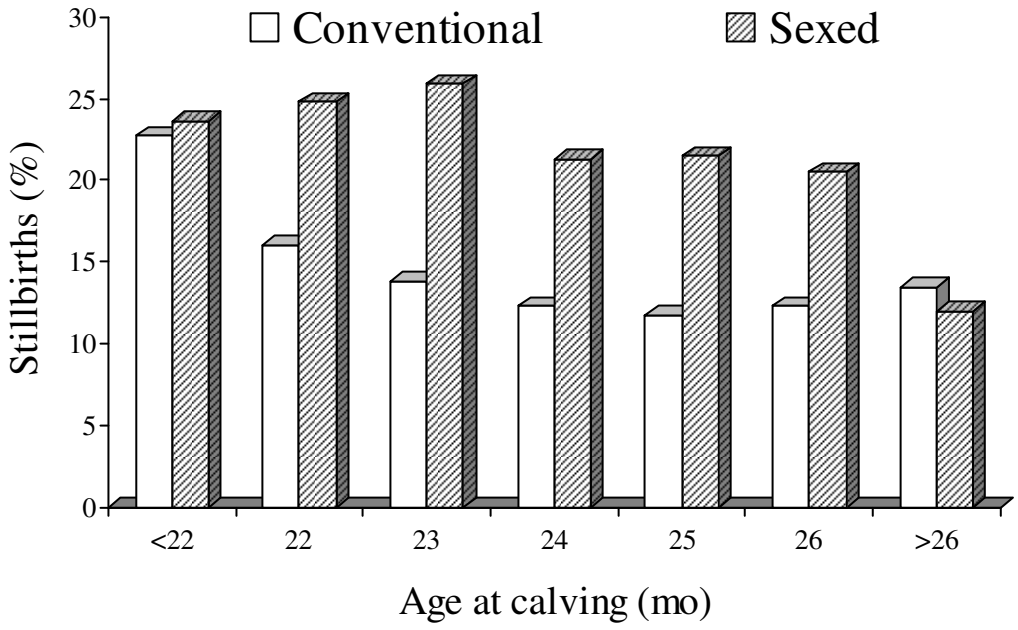

Figure 8. Effects of age at calving and semen type (sexed vs. conventional) on the incidence of stillbirths among male calves born in 143 herds of Holstein heifers. Total calvings for sexed semen = 4,375 and for conventional semen = 29,476. All age groups were different ($P < 0.05$) except <22 months and >26 months.

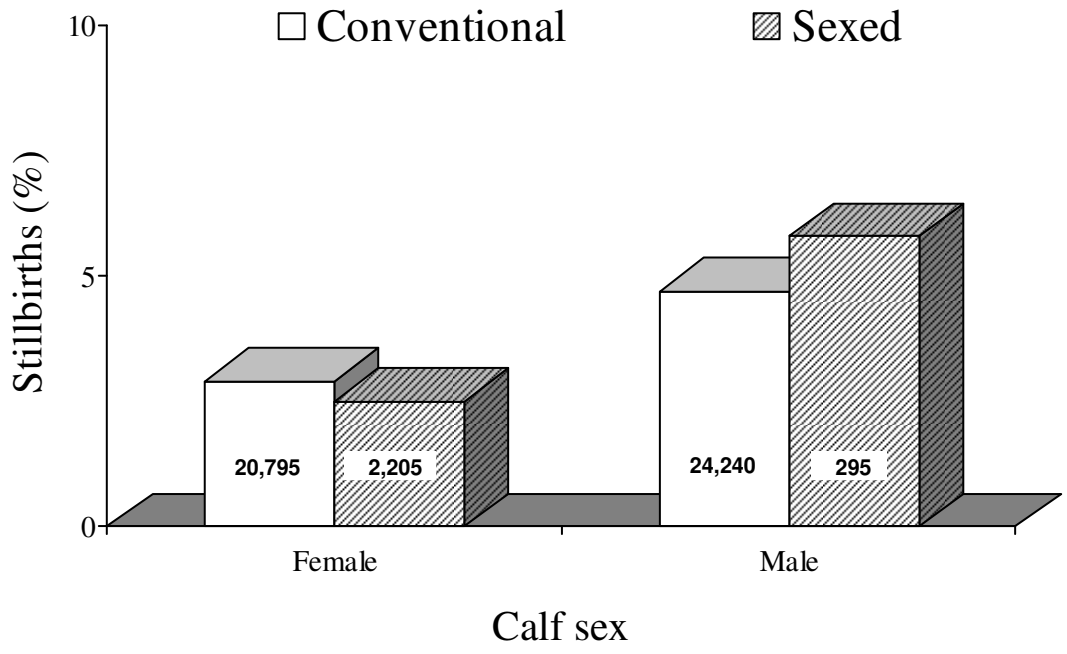

Figure 9. Effects of calf sex and semen type (sexed vs. conventional) on the incidence of stillbirths among in 69 herds of lactating Holstein cows.

Conclusion

Sex-sorted semen has been widely and rapidly implemented by dairy producers world-wide to increase the economic efficiencies of dairy production. However the highly invasive and inefficient sorting process continues to require premium prices for a product that is of compromised quality and conception potential. Although analysis of on-farm records indicates sexed-semen achieves conception rates that are ~80% of that obtained with conventional semen, these results are likely biased by preferential use of sexed semen at first service and among heifers displaying definitive signs of estrus. Despite numerous technological attempts to improve conception rates with sex sorted semen, sire selection remains one of the most effective, yet one of the most poorly understood methodologies. Though early embryonic mortality is likely a contributor to reduced conception rates with sexed semen, there is no evidence to suggest this imparts a negative effect on inter-estrus intervals. The percentage of female calves born from use of sexed semen remains steady at 89 to 90% and gestation lengths of both sexes are comparable to conventional semen. After adjusting for age at calving, there was no effect of semen type on the incidence of stillbirths within female calves or across all calvings. However, among the 10% of male calves born from sexed semen, the reported incidence of stillbirths in virgin heifers was greater than that reported for conventional semen and this effect is only partially explained by differences in age at calving. A sound explanation for this observation remains unclear. Though generally not recommended, in most studies, superovulation and sexed semen tends to results in similar numbers of transferable embryos of the desired sex, even though the total number of transferable embryos is reduced as a function of both fertilization failure and compromised embryo quality. Preferential and biased use of sexed semen in on-farm applications dictates that caution must be exercised when summarizing, analyzing, and comparing fertility and calving data of sexed and conventional semen in both on-farm and population analyses. The data contained herein are no exception.

Table 5. Effects of sexed and conventional semen on fertilization rates and embryo quality in superovulated cows and heifers.

Reference [a]	Parity	Semen type	Dosage (x10^6)	No. flushes	Fertilized %	Transferable as a percentage of fertilized ova%	Mean no. transferable embryos/flush[a]	Mean no. female transferable embryos/flush[a]
Sartori et al., 2004	Heifers	Sexed	20	25	55	48	2.2	2.0
		Conventional	20	14	88	72	5.4	2.6
Schenk et al., 2006	Heifers	Sexed	2	21	50	51	3.1	2.8
		Sexed	10	21	56	55	3.8	3.4
		Conventional	40	20	76	78	7.3	3.5
	Cows	Sexed	2	9	30	40	3.5	3.1
		Sexed	10	9	42	34	4.1	3.7
		Conventional	40	9	61	62	11.0	5.3
Potter et al., 2009	Heifers	Sexed	6.3	34	86	64	6.6	5.9
		Conventional	40+	104	90	73	7.9	3.8
	Cows	Sexed	6.3	10	67	51	4.1	3.7
		Conventional	40+	255	77	67	6.2	3.0
Hayakawa et al., 2009								
Exp. 1 & 2	Heifers	Sexed	10	24	91	69	9.6	8.6
		Conventional	20+	31	92	76	10.7	5.1
Exp. 3 & 4	Heifers	Sexed	5 to 10	52	79	71	3.9	3.5
	Cows	Sexed	5 to 10	85	59	56	3.8	3.4
Larson et al., 2010	Cows	Sexed	8.4	32	47	84	4.2	3.8
		Conventional	60	32	72	84	6.5	3.1

[a] For each reference, data were converted (weighted mean) to a uniform presentation format with fertilization rate as a percentage of all ova and transferable (Grade 1 & 2) as a percentage of fertilized ova. The number of transferable and female embryos per flush was then extrapolated from the common overall mean of total ova per flush (sexed and conventional combined) and assuming 48% and 90% female calves for conventional and sexed semen, respectively.

References

1) Abdel-Azim, G. 2010. Effect of synchronization and semen sorting on artificial insemination bull fertility. J. Dairy Sci. 93:420-425.

2) Amann, R. P. 1999. Issues affecting commercialization of sexed sperm. Theriogenology 52:1441-1457.

3) Borchersen, S. and M. Peacock. 2009. Danish A.I. field data with sexed semen. Theriogenology 71:59-63.

4) Cole, J. B., G. R. Wiggans, and P. M. VanRaden. 2007. Genetic evaluation of stillbirth in United States Holsteins using a sire-maternal grandsire threshold model. J Dairy Sci. 90:2480-2488.

5) Dalton, J. C., S. Nadir, J. H. Bame, M. Noftsinger, R. L. Nebel, and R. G. Saacke. 2001. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. J. Dairy Sci. 84:2413-2418.

6) DeJarnette, J. M. 2006. Commercial application of biotechnology in male reproduction. Proc. Applied Reprod. Strategies Beef Cattle. St. Joseph, MO pp 291-305.

7) DeJarnette, J. M., R. L. Nebel, and C. E. Marshall. 2009. Evaluating the success of sex-sorted semen in U.S. dairy herds from on farm records. Theriogenology 71:49-58.

8) DeJarnette, J. M., R. L. Nebel, C. E. Marshall, J. F. Moreno, C. R. McCleary and R. W. Lenz. 2008. Effect of Sex-Sorted Sperm Dosage on Conception Rates in Holstein Heifers and Lactating Cows. J. Dairy Sci. 91:1778-1785.

9) DeJarnette, J. M., C. R. McCleary, M. A. Leach, J. F. Moreno, R. L. Nebel, and C. E. Marshall. Effects of 2.1 and 3.5 x 10^6 sex-sorted sperm dosages on conception rates of Holstein Cows and Heifers. J. Dairy Sci. Submitted for publication.

10) den Daas, J. H. G., G. de Jong, L. M. T. E. Lansbergen, and A. M. van Wagtendonk-de Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J Dairy Sci 81:1714-1723.

11) Dransfield, M. B., R. L. Nebel, R. E. Pearson, and L. D. Warnick. 1998. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. J. Dairy Sci. 81:1874-1882.

12) Fetrow, J., M. Overton, and S. Eicker. 2007. Sexed semen: Economics of a new technology. The Bovine Practicioner 41:88-99.

13) Fisher, L.J., and C. J. Williams. 1978. Effect of environmental factors and fetal and maternal genotype on gestation length and birth weight of Holstein calves. J Dairy Sci. 61:1462-1467.

14) Frijters, A. C. J., E. Mullaart, R. M. G. Roelofs, R. P. van Hoorne, J. F. Moreno, O. Moreno, and J. S. Merton. 2009. What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology 71:64-67.

15) Garner D. L., L. A. Johnson, S. Lake, N. Chaney, D. Stephenson, D. Pinkel, and B. L. Gledhill. 1983. Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. Biol Reprod 28:312-21.

16) Garner D. L. and G. E. Seidel, Jr. 2002. Current status of sexing mammalian spermatozoa. Reprod 124:733-43.

17) Hayakawa, H., T. Hirai, A. Takimoto, A. Ideta, and Y. Aoyagi. 2009. Superovulation and embryo transfer in Holstein cattle using sexed sperm. Theriogenology 71:68-73.

18) Johnson, L. A., J. P. Flook, and H. W. Hawk. 1989. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol. Reprod. 41:199-203.

19) Kuhn, M. T. and J. L. Hutchison. 2007. Prediction of dairy bull fertility from field data: Use of multiple services and identification and utilization of factors affecting bull fertility. J. Dairy Sci. 91:2481-2492.

20) Larson, J. E., G. C. Lamb, B. J. Funnell, S. Bird, A. Martins, and J. C. Rodgers. 2010. Embryo production in superovulated Angus cows inseminated four times with sexed-sorted or conventional, frozen-thawed semen. Theriogenology 73:698-703.

21) Nebel, R. L. and J. M. DeJarnette. 2006. Artificial insemination programs for dairy heifers. Proc. Dairy Cattle Reprod. Council Denver, CO pp 59-67.

22) Norman, H. D., J. L. Hutchison, and R. H. Miller. Use of sexed semen and its effect on conception rate, calf sex, dystocia and stillbirth of Holsteins in the United States. J. Dairy Sci. In press.

23) Norman, H.D., J. R. Wright, M. T. Kuhn, S. M. Hubbard, J. B. Cole. 2007. Genetic and environmental factors that affect gestation length. J Dairy Sci. 90(Suppl. 1):264(Abstr. T73).

24) Olynk, N. J. and C. A. Wolf. 2006. Expected net present value of pure and mixed sexed semen artificial insemination strategies if dairy heifers. J. Dairy Sci. 90:2569-2576.

25) Palma, G. A., N. S. Olivier, C. H. Neumüller, F. Sinowatz. 2008. Effects of sex-sorted spermatozoa on the efficiency of in vitro fertilization and ultrastructure of in vitro produced bovine blastocysts. Anat. Histol. Embryol. 37:67-73.

26) Potter, S. R., B. J. Paus, J. M. DeJarnette, and R. L. Nebel. 2009. A case study of experiences with use of sex-sorted semen in superovulated Holstein cows and heifers in a single herd. J. Anim. Sci. Vol. 87, E-Suppl. 2/J. Dairy Sci. Vol. 92, E-Suppl. 1:79 (Abstr.).

27) Pursley, J. R., M. R. Kosorok, and M. C. Wiltbank. 1997. Reproductive management o lactating dairy cows using synchronization of ovulation. J. Dairy Sci. 80:301-306.

28) Saacke R. G., J. C. Dalton, S. Nadir, R. L. Nebel, and J. H. Bame. 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 60-61: 663-677.

29) Salisbury, G. W., and N. L. VanDemark. 1961. Significance of semen quality. In: Physiology of reproduction and artificial insemination in cattle. 1st ed. W. H. Freeman and Co. San Francisco, p. 359-379.

30) Sartori, R., A. H. Souza, J. N. Guenther, D. Z. Caraviello, L. N. Geiger, J. L. Schenk, and M. C. Wiltbank. 2004. Fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm. Anim. Reprod. 1:86-90.

31) Schenk, J. L., D. G. Cran, R. W. Everett, and G. E. Seidel, Jr. 2009. Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. Theriogenology 71:717-728.

32) Schenk, J. L., T. K. Suh, D. G. Cran, and G. E. Seidel, Jr. 1999. Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology 52:1375-1391.

33) Schenk, J. L., T. K. Suh, and G. E. Seidel, Jr. 2006. Embryo production from superovulated cattle following insemination with sexed sperm. Theriogenology 65:299-307.

34) Seidel, Jr, G. E. 2007. Overview of sexing sperm. Theriogenology 68:443-446.

35) Seidel, Jr, G. E., J. L. Schenk, L. A. Herickhoff, S. P. Doyle, Z. Brink, R. D. Green, and D. G. Cran. 1999. Insemination of heifers with sexed sperm. Theriogenology 52:1407-1420.

36) Senger, P. L. 1994. The estrus detection problem: new concepts, technologies, and possibilities. J. Dairy Sci. 77:2745-2753.

37) Silva del Rio, N., S. Stewart, P. Rapnicki, Y. M. Chang, and P. M. Fricke. 2007. An observational analysis of twin births, calf sex ratio, and calf mortality in Holstein dairy cattle. J. Dairy Sci. 90:1255-1264.

38) Thatcher, W. W., M. B. Rabaglino, C. A. Risco, M. J. Thatcher, I. H. Kim, and J. E. P. Santos. 2009. Timed AI programs in dairy heifers. Proc. Dairy Cattle Reprod. Council. Minneapolis, MN/Boise, ID pp 57-70.

LIFE IMAGING OF REPRODUCTIVE PROCESSES IN THE OVIDUCT

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1. Introduction

The oviduct plays a vital role in ensuring pick-up and transport of the ovum, transport of spermatozoa, fertilization, and development and transport of the early embryo [1, 2]. Although the male inseminates many thousands or even millions of spermatozoa, successful fertilization only occurs when the oocyte and the spermatozoa meet in the ampulla at the appropriate time [3]. After fertilization, a precise timing of the transport is essential for the early embryo to obtain the capacity for implantation [4]. The transport of the oocyte and embryo in the oviduct is achieved by a) ciliary beating of the oviductal epithelial cells and b) contraction of the oviductal smooth muscle [5, 6, 7]. Up to now, especially the role of ciliary tubal transport is still unclear, as excision of a part of the ampulla and re-implantation in reverse direction sporadically results in pregnancies [8] and women with immotile cilia (Kartagener`s syndrome) may or may not be fertile [9].

The basic events of fertilization and early embryonic development have been extensively investigated and are similar in all mammals. However, up to now the interactions of the gametes and the early embryo with the oviductal epithelium have not been elucidated. This is mainly due to two facts: first, the oviduct is localized in the mesosalpinx and integrated within the bursa ovarica so that it is difficult to investigate in vivo. Second, there are several shortcomings of in vitro oviductal cell cultures. Thus, the integrity of synthetic activity and ciliary function are lost during in vitro culture [10] leading to the loss of essential signal transduction pathways. Digital videomicroscopy and live cell imaging for the first time enable to analyze sperm transport, oocyte transport, gamete interaction, and early embryonic development under in vivo or in situ conditions thus providing new insights into events essential for the success of reproduction.

2. Sperm transport and sperm storage in the oviduct

In most mammals, millions of spermatozoa are inseminated, but only thousands reach the isthmus of the oviduct. In mammals with vaginal insemination, the cervix is the first great barrier and serves for sperm selection because only vigorously motile spermatozoa can pass the highly hydrated cervical mucus [11,12, 13]. The second anatomical barrier for sperm passage is the uterotubal junction (UTJ). Thus, only thousands of spermatozoa reach the isthmus.

As soon as the spermatozoa enter the tubal isthmus, they are trapped and held in a sperm reservoir (reviewed by [14,15]. In the sperm reservoir the sperm heads bind to the ciliated cells of the epithelium thus maintaining fertility until ovulation . This binding is mediated by species-specific carbohydrate moieties on the epithelium. This involves fucose in the cow [16], mannose in the pig [17], galactose in the horse [18] and sialic acid in the hamster [19]. The factors maintaining fertility of spermatozoa within the oviduct for several days are still unknown. However, live cell imaging has shown that viability of spermatozoa is maintained by direct contact with the oviductal epithelium rather than by interaction with oviductal secretions [20,21,22]. Using oviductal explants, it has been shown that the hormonal state of the oviductal epithelium does not affect sperm binding [23, 24, 25]. Additonally, in in vitro

studies both oviductal cells from the ampulla and the isthmus bind spermatozoa [26]. The reservoir in vivo, however, is confined to the isthmus, because it is the first region encountered by spermatozoa entering the oviduct. Using a digital videomicroscopic analysis system in cows we recently showed that only vital spermatozoa bind to the oviductal epithelium [27]. Generally the density of sperm binding is much higher in fresh semen as compared to frozen semen. Also, after applying the swim-up procedure in frozen thawed semen, binding density can clearly be improved. However, distinct inter-individual differences in binding density during videomicroscopy occur which are correlated with fertility [27].

3. Transport of the oocyte and gameto-maternal interaction

When ovulation occurs, the COC is picked up by adhesion at the cilia of the infundibulum and slides over the surface of the infundibulum in the direction of the ostium [28, 29]. When the COC reaches the ostium, the pick-up process slows while the COC, which is too large to pass directly through the opening, churns for several minutes in the ostium. During this process the cumulus matrix becomes compacted so that it can enter the lumen of the infundibulum [28, 29]. Adhesion between the cumulus cell matrix and the tips of the cilia is essential to move the complex over the surface of the infundibulum [28, 30]. Thus, unexpanded COCs with little cumulus matrix cannot adhere to the infundibulum and are not picked up even though normally beating cilia are present [28]. The COC is guided to the ampulla by ciliary beating and the current of the oviductal fluid. Using digital videomicroscopy in cows we documented that - as soon as the mature COC enters the ampulla – it immediately and firmly attaches to the oviductal epithelium [27] . This attachment is so strong that it can only be reversed by destroying the cumulus cells. Immature COCs also bind to the oviductal epithelium pointing to the fact that the adhesion of the COC to the oviductal epithelium is not dependent on maturation. However, when a degenerated COC enters the ampulla it floats within the oviductal lumen implying that the oviduct is able to select vital oocytes. When denuded oocytes are put into the living oviduct, they turn and move around slightly because of the ciliary beating indicating that the cumulus cells and their intercellular matrix are essential for the attachment of the COC to the oviductal epithelium [27]. Videos demonstrating the behaviour of the COC in the ampulla can be viewed at http.//www.biolreprod.org/cgi/content/abstract/biolreprod.108.073874 [29]. (supplemental files, Movies 4, 5 and 6).

4. The event of fertilization

As soon as a vital COC is in the oviduct, the spermatozoa are released from the reservoir and migrate to the oocyte. The detachment from the endosalpingeal epithelium is achieved by sperm hyperactivation [31, 32]. Additionally capacitation-induced changes in the sperm head reduce binding affinity to the oviductal epithelium [33, 34, 26]. The spermatozoa leaving the reservoir first move into the middle of the oviductal lumen. Although the current of the oviductal fluid is stronger in the middle of the tube, it seems to be easier to move forward there because near the oviductal epithelium the presence of mucus or – if there is locally less mucus - strong whirls inhibit straight sperm movement [27]. In each case, hyperactivation strongly supports movement of the spermatozoa through the viscoelastic mucus. However, contractions of the oviductal smooth muscles are a vital support for the spermatozoa, helping them to move against the strong current caused by ciliary beating [27]. Recently first evidence has been provided that spermatozoa in the oviduct are able to up-regulate the local prostaglandin system in the oviduct thereby enhancing oviductal contractions. Thus, spermatozoa may be able to accelerate their own transport to the fertlization site [35].

Spermatozoa are guided to the oocyte by two mechanisms: thermotaxis (a long-range mechanism) and chemotaxis (a short-range mechanism). In rabbits, it has been shown that capacitated spermatozoa tend to swim towards warmer temperatures [36]. In the ampulla, i.e. in closer proximity to the oocyte, chemotactic mechanisms may guide the spermatozoa to the oocyte [37, 38]. It has been shown that mammalian spermatozoa accumulate in a gradient of follicular fluid accompanying the oocyte into the oviduct [39, 40]. However, the chemotactic agent in follicular fluid has not been identified nor has its presence in the oviduct been proved. Odorant receptors unique to spermatozoa are localized at the base of the flagellum and have been demonstrated in humans [41], dogs [42] and rats [43]. Placing human spermatozoa in a gradient of the odorant bourgeonal causes them to orient into the gradient [44]. However, this odorant cannot physiologically be detected in the female genital tract. Thus, the precise signal transduction factors guiding the spermatozoa to the oocyte remain to be identified. As during in vitro fertilization most spermatozoa move aimlessly, the oocyte and the cumulus cells alone are unable to guide them. Our videomicroscopic studies provided strong hints that the interaction between cumulus cells and oviduct may result in a signalling cascade which direct the spermatozoa to the COC. Thus, the interaction of the COC with the oviductal epithelium seems to be essential for initiating a signal transduction pathway resulting in sperm guidance. This is supported by the fact that the ovulatory ampulla in humans contains a significantly larger percentage of spermatozoa than the non-ovulatory ampulla [45].

Spermatozoa reaching the COC penetrate the cumulus oophorus. Binding to the zona pellucida glycoproteins induces spermatozoa to undergo the acrosome reaction. After penetration of the zona pellucida the spermatozoon enters the perivitelline space, the extracellular region between the zona and the egg plasma membrane. The initial gamete contact is between the tip of the spermatozoon and the egg plasma membrane, followed by a lateral attachment between the side of the spermatozoon and the egg. Membrane fusion occurs with the spermatozoon positioned in a perpendicular or parallel direction to the egg surface [46]. As soon as fertilization has occurred the presumptive zygote detaches and continues its migration down the oviduct [27].

5. Embryonic transport and first embryo-maternal communication in the oviduct

The transport of the early embryo in the oviduct is achieved by a) ciliary beating, b) smooth muscle contraction and c) flow of tubal secretions [47, 48]. Using videomicroscopy in cows and mice a system was established to visualize mechanisms of tubal transport and to quantify average and maximum speed of transport using small polystyrene beads. First it was shown that there are distinctly different mechanisms of transport in the ampulla and in the isthmus. In the ampulla ciliary driven transport takes place in the depth between the folds. Similar to the dynabead particles, the cumulus-oocyte-complex tends to move into the deep folds and settles down [27]. For the COC this is the first contact with the oviductal epithelium, thus creating appropriate conditions for the first communication between COC and maternal genital tract. In the isthmus particles are rapidly transported to the apical ridges of the folds – this clearly differs from what is observed in the ampulla. If some particles settle down in the isthmus in the depth between the folds, they are quickly removed by ciliary beating and guided back to the stream of the other dynabeads [27]. Thus, the isthmus exclusively serves for rapid transport of the presumptive zygote to the uterus. Before fertilization the particle transport speed is not significantly different in the ipsilateral and contralateral ampulla and isthmus, respectively. However, after fertilization the speed of transport is significantly lowered at the site of the embryo [27]. Thus, in the course of its migration down the oviduct, i.e. days 1 to 3.5 of bovine pregnancy, the embryo is able to locally down-regulate speed of transport thus creating appropriate conditions for the first

embryo-maternal communication. As soon as the embryo leaves the isthmus and enters the uterus, the speed of transport in the oviduct caused by ciliary beating is up-regulated again [27].

The comparison of the ipsi- and contralateral oviduct under in vivo conditions is a valuable tool for evaluating local effects of the embryo and for investigating embryo-maternal interaction. As shown in the bovine, the embryo is able to induce local changes in vascularisation. Whereas in the contralateral ampulla and isthmus the A. tubae uterinae is running straight and parallel to the oviduct, the A. tubae uterinae of the ipsilateral oviduct at day 1 to 3 p.c. is strongly wound especially at the site of the embryo [27]. Generally the wall of the ipsilateral oviduct is thicker, more edematous and more transparent as compared to the contralateral oviduct [27]. The early embryo is not only able to locally change the periphery of the oviduct, but also induces changes in the inner surface. Thus the embryo induces the formation of secretory cells during its migration down the oviduct [17] thereby ensuring optimal microenvironment and nutrition during the first days of the embryo`s life [49, 50]. These effects occur 24 to 48 hours after fertilization implying that signal transduction cascades are rapidly initiated by local signals of the embryo.

6. Conclusions

The numerous new insights gained by videomicroscpy and live cell imaging help to essentially improve understanding of the basic mechanisms of gamete transport, fertilization and embryo-maternal interaction [27]. Videomicroscopy additionally enables direct investigations of the influence of drugs or analyses of the effects of smoking on gamete interaction [51] thereby obtaining valuable hints how to proceed with proteomic and genomic approaches and how to improve the technologies of assisted reproduction.

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8. References

1) Menezo Y, Guerin P. The mammalian oviduct: biochemistry and physiology. Eur J Obstet Gyecol Reprod Biol 1997;73:99-104.

2) Greve T, Callesen H. Rendez-vous in the oviduct: implications for superovulation and embryo transfer. Reprod Nutr Dev 2001;41:451-459.

3) Talbot P, Shur BD, Myles, DG. Cell adhesion and fertilization: Steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. Biol Reprod 2003;68:1-9.

4) Pulkinnen MO. Oviductal function is critical for very early human life (1995). Ann Med 1995;27:307-310.

5) Halbert SA, Tam PY, Blandau RJ. Egg transport in the rabbit oviduct: the roles of cilia and muscle. Science 1976;191:1053-1053.

6) Halbert SA, Becker DR, Szal SE. Ovum transport in the rat oviductal ampulla in the absence of muscle contractility. Biol Reprod 1989;40:1131-1136.

7) Croxatto HB. Physiology of gamete und embryo transport through the fallopian tube. Reprod Biomed Online 2002;4:1160-1169.

8) McComb PF, Halbert SA, Gomel V. Pregnancy, ciliary transport and the reversed ampullary segment of the rabbit fallopian tube. Fertil Steril 1980;34:386-390.

9) McComb P, Langley L, Villalon L, Verdugo P. The oviductal cilia and Kartagener´s syndrome. Fertil Steril 1986;46: 412-416.

10) Bongso A, Chye NS, Sathananthan H, Lian NP, Rauff M, Ratnam SS. Establishment of human ampullary cell cultures. Hum Reprod 1989;5:486-494.

11) Chatdorung K, Kampa N, Axner E, Linde-Forsberg C. Investigation on cervical patency and uterine appearance in domestic cats by fluorescopy and scintigraphy. Reprod Dom Anim 2002;7:275-281.

12) Barros C, Vigil P, Herrera E, Arguello B, Walker R. Selection of morphologically abnormal sperm by human cervical mucus. Arch Androl 1984; 12(Suppl):95-107.

13) Katz DF, Slade DA, Nakajima ST. Analysis of preovulatory changes in cervical mucus hydration and sperm penetrability. Adv Contracep 1997;13:143-151.

14) Suarez SS. Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol 2008;52:455-462.

15) Rath D, Schuberth HJ, Coy P, Taylor U. Sperm interactions from insemination to fertilization. Reprod Dom Anim 2008;43 (Suppl 5):2-11.

16) Lefebvre R, Lo MC, Suarez SS. Bovine sperm binding to oviductal epithelium involves fucose recognition. Biol Reprod 1997; 56:1198-1204.

17) Green CE, Bredl J, Holt Wv, Watson PF, Fazeli A. Carbohydrate mediation of boar sperm binding to oviductal cells in vitro. Reproduction 2001;122:305-315.

18) Lefebvre R, De Mott RP, Suarez SS, Samper JC. Specific inhibition of equine sperm binding to oviductal epithelium. Equine Reprod VI, Biol Reprod Mono 1995;1:689-696.

19) De Mott RP, Lefebvre R, Suarez SS. Carbohydrates mediate the adherence of hamster sperm to oviductal epithelim. Biol Reprod 1995;52:1395-1403.

20) Murray SC, Smith TT. Sperm interaction with fallopian tube apical membrane enhances sperm motility and delays capacitation. Fertil Steril 1997:68:351-357.

21) Dobrinski I, Suarez SS, Ball BA. Intracellular calcium concentration in equine spermatozoa attached to oviductal cells in vitro. Biol Reprod 1996; 54:783-788.

22) Smith TT, Nothnick WB. Role of direct contact between spermatozoa and oviductal epithelial cells in maintaining rabbit sperm viability. Biol Reprod 1997;56:83-89.

23) Suarez SS, Redfern K, Raynor P, Martin F, Philips DM. Attachment of boar sperm to mucosal explants of oviduct in vitro: possible role in formation of a sperm reservoir. Biol Reprod 1991;44:998-1004.

24) Thomas PGA, Ball BA, Brinsko SP. Interaction of equine spermatozoa with oviduct epithelial cell explants in affected by estrous cycle and anatomic origin of explant. Biol Reprod 1994;51:222-228.

25) Baillie HS, Pacey AA, Warren MA, Scuadamore IW, Barratt CL. Greater numbers of human spermatozoa associate with endosalpingeal cells derived from the isthmus compared with those from the ampulla. Hum Reprod 1997;12:198-1992.

26) Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Hum Reprod Update 2006;12:23-37.

27) Kölle S, Dubielzig S, Reese S, Wehrend A, König P, Kummer W. Ciliary transport, gamete interaction and effects of the early embryo in the oviduct: Ex vivo analyses using a new digital videomicroscopic system in the cow. Biol Reprod 2009;81:267-274.

28) Talbot P, Geiske C, Knoll M. Oocyte pick-up by the mammalian oviduct. Mol Biol Cell 1999;19:5-8.

29) Talbot P, Shur BD, Myles DG. Cell adhesion and fertilization: Steps in oocyte transport, sperm-zona pelucida interactions, and sperm-egg fusion. Biol Reprod 2003; 68:1-9.

30) Lam X, Gieseke C, Knoll M, Talbot P. Assay and importance of adhesive interaction between hamster (Mesocricetus auratus) oocyte-cumulus complexes and the oviductal epithelium. Biol Reprod 2000;62:579-588.

31) DeMott RP, Suarez SS. Hyperactivated sperm progress in the mouse oviduct. Biol Reprod 1992;46:779-785.

32) Ho HC, Suarez SS. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction 2001;122:519-526.

33) Fazeli A, Duncan AE, Watson PF, Holt WV. Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. Biol Reprod 1999;60:879-886.

34) Lefebvre R, Suarez SS. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. Biol Reprod 1996;54:575-582.

35) Kodithuwakku SP, Miyamoto A, Wijayagunawardane PB. Spermatozoa stimulate prostaglandin synthesis and secretion in bovine oviductal epithelial cells. Reproduction 2007;133:1087-1094.

36) Bahat A, Tur-Kaspa I, Gakamsky A, Giojalas LC, Breitbart H, Eisenbach M. Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. Nat Med 2003;9:149-150.

37) Eisenbach M. Mammalian sperm chemotaxis and its association with capacitation. Dev Gen 1999;25:87-94.

38) Babcock DF. Development: Smelling the roses? Science 2003; 299:1993-1994.

39) Cohen-Dayag A, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proc Natl Acad Sci USA 1995;92:11039-11043.

40) Hong CY, Chao HT, Lee SL, Wei YH. Modification of human sperm function by human follicular fluid: a review. Int J Androl 1993;16:93-96.

41) Spehr M, Gisselmann G, Poplawski A, Riffell JA, Wetzel CH, Zimmer RK, Hatt H. Identification of a testicular odorant receptor mediating human sperm chemotaxis. Science 2003;299:2054-2058.

42) Vanderhaeghen P, Schurmans S, Vassart G, Parmentier M. Olfactory receptors are displayed on dog mature sperm cells. J Cell Biol 1993;123:14441-1452.

43) Walensky LD, Roskams Aj, Lefkowitz RJ, Snyder SH, Ronnett GV. Odorant receptors and desensitization proteins colocalize in mammalian sperm. Mol Med 1995; 1:130-141.

44) Spehr M, Schwane K, Riffell JA, Barbour J, Zimmer RK, Neuhaus EM, Hatt H. Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. J Biol Chem 2004;279:40194-40203.

45) Williams M, Hill CJ, Scudamore I, Dunphy B, Cooke I, Barratt CL. Sperm numbers and distribution within the human fallopian tube around ovulation. Hum Reprod 1993;8:2019-2026.

46) Gaddum-Rosse P. Mammalian gamete interactions: what can be gained from observations on living eggs? Am J Anat 1985;174:347-356.

47) Croxatto HC. Physiology of gamete and embryo transport through the Fallopian tube. RBM online 2002;4:160-169.

48) Brüssow KP, Ratky J, Rodriguez-Martinez H. Fertilization and early embryonic development in the porcine fallopian tube. Reprod Domest Anim 2008; 43 Suppl 2:245-251.

49) Buhi WC, Alvarez IM, Kouba AJ. Oviductal regulation of fertilization and early embryonic development. J Reprod Fertil Suppl. 1997;52:285-300.

50) Lee KF, Yeung WS. Gamete/embryo-oviduct interactions: implications on in vitro culture. Hum Fertil (Camb) 2006;9:137-143.

51) Talbot P, Riveles K. Smoking and reproduction: the oviduct as a target of cigarette smoke. Reprod Biol Endocrinol 2005;3:52.

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REPRODUCTIVE TECHNOLOGIES AND GENOMIC SELECTION IN THE BOVINE

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Introduction

During the last decades, progresses in reproductive physiology and improvements in embryo based reproductive biotechnologies allowed to develop a rather complete "tool box" including reproductive techniques used either for commercial purposes and/or in the frame work of breeding schemes. They present today various degrees of efficiency (Ponsart et al., 2004) and for most of them continuous improvements may be expected in the future. Used alone or in combination, their development is influenced in many different ways including ethics and general acceptance, consumer demand for specific products, regulation changes and also changes related to the evolution of breeding strategies.

The recent development of genomic selection induces dramatic changes in the way genetic selection schemes are to be conducted (Humblot et al., 2009a, Boichard et al, 2010). Due to these present and expected evolution in the organisation of selection and associated needs, the value of the various reproductive techniques used today for commercial purposes and in genetic schemes should be revisited.

The new context and corresponding needs for genomic based selection schemes

Marker Assisted Selection (MAS) has been developed recently and was based in the past few years on a limited number of micro satellite analyses for a few Quantitative Trait Loci. Selection was performed by combining this first generation of genomic information with conventional indexes issued from quantitative genetics. The progress of the knowledge of the bovine genome and of DNA analyses together with the refinement of the position of markers in relation with genes of interest allows animal breeding companies to use today (or in the very near future) sets of thousands of genetic markers to select animals (Ducrocq 2010,Fritz 2010). The evolution of genomic techniques will probably make available the use of the complete genome information for selection purposes in a few years. Today, a lot of progresses have been achieved for the Holstein breed in which all the characters previously evaluated in classical selection by quantitative genetics can now be evaluated from genomic information. For instance in France, recent development allows to make this evaluation by using several hundreds of markers/character instead of 30 QTL/character in the previous MAS evaluation (Fritz 2010). Due to those technical evolutions, application to small breeds can now be expected (Ducrocq 2010) and efforts should be made to get also appropriate phenotypic information for those breeds which have not been studied as intensively as the Holstein breed.

In parallel with those technological evolutions attempts are made in different countries to reinforce the value of the genomic information by including more and more animals in the evaluation and selection process (Chesnais 2009, Ducrocq 2010). Consequently, more reliable estimates can be obtained for the desired traits while genetic variability is better preserved. Candidates will have to be produced from parents of different pedigree's (maximum of families within a breed) and at the same time breeding should be organised in a way to maximize the variability of the next generation. The potential advantages of genomic selection programmes run on these principles have been shown recently by Monte-Carlo simulations on full-size breeding schemes (Colleau, 2009). This work demonstrated that by multiplying the number of candidates by 3 (2400 young male calves evaluated by MAS, leading to 80 in use, instead of 800 calves leading to 130 progeny tested and 15 in use under classical selection) it was possible at the same time to increase genetic progress dramatically (+80% when compared to the classical breeding scheme) while decreasing inbreeding rate (-23%).

Due to its costs and to the fact that the genetic value of a given future sire is known with enough precision from genomic analyses, progeny testing is on the way to be considerably reduced or even suppressed (as reported and recommended by Boichard et al, 2010) For some traits, such as fertility the precision associated to genomic indexes is or will be much better than with classical selection (Fritz et al. 2009,Unpublished, Barbat et al., 2010, Figure 1).

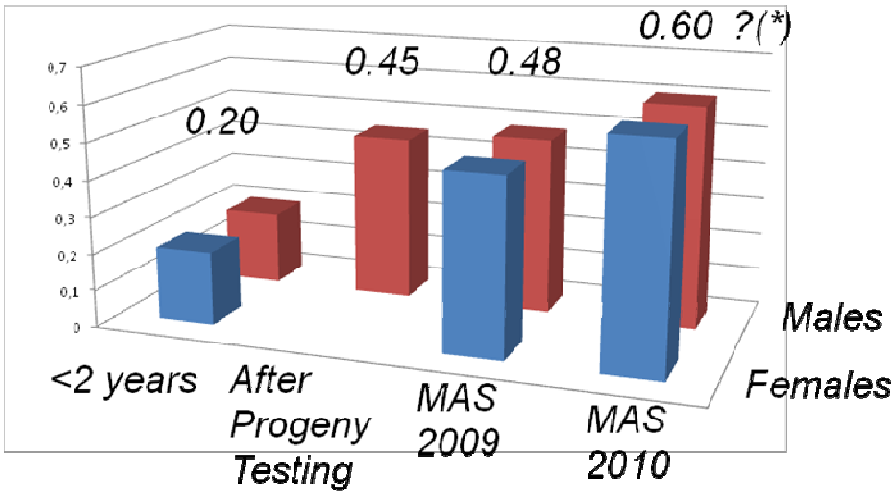

Figure 1: Accuracy of fertility Estimated Breeding Value of young animals (<2years) and after progeny test without molecular information, and of animals with MAS information (MAS 2009, MAS 2010) obtained before 2 years of age (Barbat et al., 2010).

Questions are raised about the need to keep a common reference basis in different populations to optimize the evaluation process and evaluate the changes induced by genomic selection. This is illustrated by the agreement that has been found recently in the consorsium "Eurogenomics" (a group of breeding companies from France, The Netherlands, Germany and Denmark, Finland and Sweden to gather and share the genomic information for evaluation of breeding values from a common reference basis including 16 000 Holstein sires. Research

is made also on computational methodologies to define the best way to analyse and use the huge quantity of information issued from genomic analyses of a very large number of animals (Ducrocq 2010).

For all traits of interest, these changes highlight the **importance of the phenotypic information** that must be unified from large number of animals in the reference basis and which becomes one of the main bottleneck in the process.

How can reproductive physiology and reproductive techniques help to meet the needs of genomic selection and commercial production within this context ?.

Reproductive physiology.

In an attempt to improve numerous traits by genomic selection the knowledge of the relationships between genome information and the phenotypic criteria is of crucial importance. Following first studies (Adjaye 2005, Adjaye et al., 2007, Evans et al., 2008, Ko 2004) the development of micro arrays (dedicated or generic) helped to characterise the relationships between genotype and phenotype. More recently high throughput technologies for DNA sequencing and RNA analysis becomes more and more affordable and are now currently used in research programs aiming to study relationships between genotype and phenotype and gene expression. With such objectives, phenotyping (animal models, precise criteria and methods) becomes the main bottleneck to achieve this goal. As a consequence, there will be a need for research aiming to phenotype for new critical traits and/or to improve the precision of the phenotypes for existing traits. For instance for reproductive traits, it is possible by using progesterone and pregnancy associated proteins measurements to characterise relatively well pregnancy failures (Humblot 2001). This has been used in a genomic programme aiming to screen (from a large data basis) for the existence of candidate mutations that may explain difference in fertility between progeny groups (Humblot et al., 2009b). However, for such an approach, methods allowing to distinguish no fertilization from early embryonic mortality were/are still lacking and such developments would be most valuable to find new markers for fertility. Similarly a lot of information and new physiological markers for fertility may be issued from investigations made to better characterize reproductive function. For instance, due to the strong relationships existing between oocyte growth and maturation and subsequent embryonic development, (Humblot 2009c) the programs aiming to study links between follicular growth, oocyte quality and the presence of genomic markers by using proteomics, lipidomics and metabolomics looks particularly appropriate to find new markers for fertility (Dalbies Trann et al., 2009).

Use of embryo based biotechnologies

One of the most important features for the new selection procedures will be to considerably increase the number of candidates submitted to genomic selection to maximize the chances of getting interesting individuals that will be positively evaluated for a large number of traits. As mentioned before this will allow to increase the selection pressure for those traits. Also, it will be possible to use bulls for AI at a younger age to lower generation interval. Finally, the use of groups of bulls with a favourable genomic index will improve the precision of indexes when compared to the use of a very limited number of older sires as it was the case in the past. This may be also favourable to genetic variability if adequate and wise breeding schemes are implemented, otherwise shortening the generation interval may lead to increase also inbreeding rate faster than in the past.

The way to produce these large numbers of animals becomes critical and in this context, AI alone, looks insufficient to generate enough animals in a given period of time and the

efficiency of MOETs and OPU-IVP looks more and more critical to produce these large number of animals to be genotyped. With these "intensive" embryo based reproductive techniques it is relatively easy to increase the number of candidates by increasing the number of flushes in MOETs. When compared to MOET's, the number of produced embryos in a given period of time can even be multiplied by 2 or 3 (Merton et al., 2003, Ponsart et al, 2004) by the use of repeated OPU-IVF sessions leading to produce around 70 calves /donor and per year. Females of various origins can be collected to preserve genetic variability and this technique presents additional advantage if different bulls are used for different OPU sessions or even within a session (Merton et al., 2003, Ponsart et al, 2004).

A lot of research has been done to improve in vitro culture systems in a way they mimic oviduct fluid environment (Synthetic Oviduct Fluid, SOF). SOF based cultured systems are the most currently used today and overall development rates into blastocysts of 30-40% are achieved by most teams. The effect of a previous superovulation on fertilisation and subsequent embryonic development is still controversial as some authors reported detrimental effects (Wurth et al., 1994, Rizos et al., 2002) whereas other studies shown similar embryonic development rates under those 2 conditions (Ponsart et al., 2004). Whatever the type of treatment of the donor female and type of culture system, it has been shown very clearly from most studies that there is a significant decrease in embryo production when oocytes are matured in vitro in standard medium compared to in vivo conditions (Wurth et al., 1994, Hendriksen et al., 2000, Rizos et al., 2002, Dieleman et al., 2002, Humblot et al., 2005). This emphasizes the roles of the final steps of oocyte growth and maturation in subsequent embryo development which have been also illustrated by epidemiological studies made under in vivo conditions showing relationships between some factors influencing these steps and embryonic mortality (Humblot et al., 2009c). There is probably a lot of progresses than can be achieved in vivo and in vitro embryo production by optimizing the conditions under which the oocytes are growing within follicles in donor females. Handling at time of collection and therafter as well as in vitro maturation looks also as critical steps to be optimized since dramatic metabolic changes occurring very quickly after oocyte punction were recently reported (Gilchrist et al., 2010).

Despite the above mentioned limitations and potential margins for progress, the work that has been done in the past 15 years to improve oocyte collection and in vitro producing systems led to make those systems viable and used with profit by the most advanced breeding companies to produce more embryos in their genetic schemes (Merton et al., 2003, 2009, Van Wagtendonk de Leeuw, 2006). However i) there is a risk with these techniques to significantly increase inbreeding if bull dams are overexploited (Colleau 2010, personal communication) and ii) due to the new needs in relation with the implementation of genomic selection, additional strong limitations exist for giving birth to a very large number of candidates that would be genotyped after birth.

Effectively, one of the main bottleneck met by breeding organisations in Europe is the limited availability of female recipients. This is reinforced by the fact that the efficiency of embryo transfer is much lower when using cows instead of heifers as recipients (Ponsart et al., 2004). In addition to this, high costs will be induced by the transfer of a very large number of embryos into recipients that must be maintained pregnant until birth of progeny and the valorisation potential of the non selected calves will be low. When producing these candidate animals on farm, the amount of field work in relation with embryo transfer and in vitro production will be even heavier than today and will generate high logistic costs. Finally, this

process may increase the contractual cost with individual farmers especially due to the potential existence of very interesting candidate identified by genomics.

For these reasons, genotyping the embryos and selecting them before transfer appears as a attractive scenario to maximize the chances to find interesting individuals for multiple traits while transferring a "reasonable" number of embryos.

Embryo Typing

The interest of embryo typing for breeding companies has been discussed even before the emergence of the new techniques for genomic selection that includes today x thousands of markers (Merton et al., 2003). As soon as MAS based on a limited number of micro satellites could be used, advantages were found due to its potential value for screening the embryo for several traits. At the same time, embryo sexing could be used at a very low cost during the process of genomic analyses. Doing typing and selection early in life was also expected to be a solution to shorten the generation interval and to limit the costs for producing the high number of calves and associated costs of the still existing progeny testing to achieve multi character selection. Today the potential advantages of combining intensive embryo production and genotyping are even higher.

Results reported initially in the literature (Peippo et al., 2007, Guignot et al., 2008) were based on the typing for a limited number of markers. Peippo et al., 2007 have shown that it was possible to genotype embryonic biopsies for a limited number of micro satellites and to get subsequent pregnancies after transfer of the corresponding biopsed embryos. Similarly, Guignot et al., 2008, reported the possible use of embryo genotyping for a very limited set of markers to screen for sensitivity to scrapie combined with sex determination. The most recent published results are issued from the programme "TYPAGENAE" in which the efficiency of embryo typing was tested from a set of 45 micro satellites corresponding to the first generation of MAS (Lebourhis et al., 2008, 2009, Humblot et al., 2008).

In a first step, in vitro produced embryos were used to assess the accuracy and repeatability of embryo based genotyping. Day 6 embryos were biopsied and each blastomere from the biopsy was submitted to embryonic cloning to reconstitute full blastocysts (Lebourhis et al., 1998). A mean of 2 full blastocysts were obtained from cloning blastomeres and more than 95% of the embryos survived in culture following biopsy. The results of typing obtained from the reconstituted blastocyst and the donor embryo were subsequently compared from a total of 41 samples. The proportion of successfully typed samples was >90%. The typing of the cloned embryos corresponded well to the typing of the original embryos and genotypes were fully compatible with the genotypes of the parents. The error rate, when considering differences between the different types of samples was 3% and all errors were due to the lack of identification of one of the alleles (drop-out).

From a second series of experiments, the typing results between biopsies of 10-20 cells performed at the blastocyst stage and the rest of the embryo were compared. Whole Genome Amplification (WGA) was applied on cell extracts from the biopsy before typing. From 60 samples, 95% were genotyped and a similar rate of drop out was observed when compared to analyses made from full embryos (2-3%).

Another set of 40 samples was used to evaluate the minimum number of cells to be biopsied before pre amplification. WGA was performed on all samples and allowed genotyping in 98% of the cases with very low drop out rates from biopsies of 8-10 cells. This rate was much higher in biopsies containing less than 5 cells (Humblot et al., 2008, 2009a, Figure 2).

From further series, the correspondence between results of embryo typing and of typing realised in foetuses and young calves was found excellent (13 couples embryo/calf or foetus with same typing results). Those first results obtained with biopsies issued from *in vivo* or *in vitro* embryos produced in station and biopsies performed in a central laboratory were completed by a set of results were biopsies were made on farm following the collection of *in vivo* produced embryos (Lebourhis et al., 2008). Typing was made by using the usual set of 45 micro satellites markers which was completed by the analysis of a complementary set of 13 Single Nucleotide Polymorphism (SNP) markers. From 57 biopsied embryos, the total detection rate was higher for SNP's than for micro satellites (70.2% vs 31.6%; $p<0.01$). The detection rates of the markers were not significantly affected by embryo stage, biopsy size or sex of the embryo. However, from these series of biopsies made under farm conditions the proportions of markers detected where much lower than when the biopsies were prepared in laboratory and immediately followed by whole genome amplification suggesting that the conditions of preparation and/or transportation may affect the efficiency of the system.

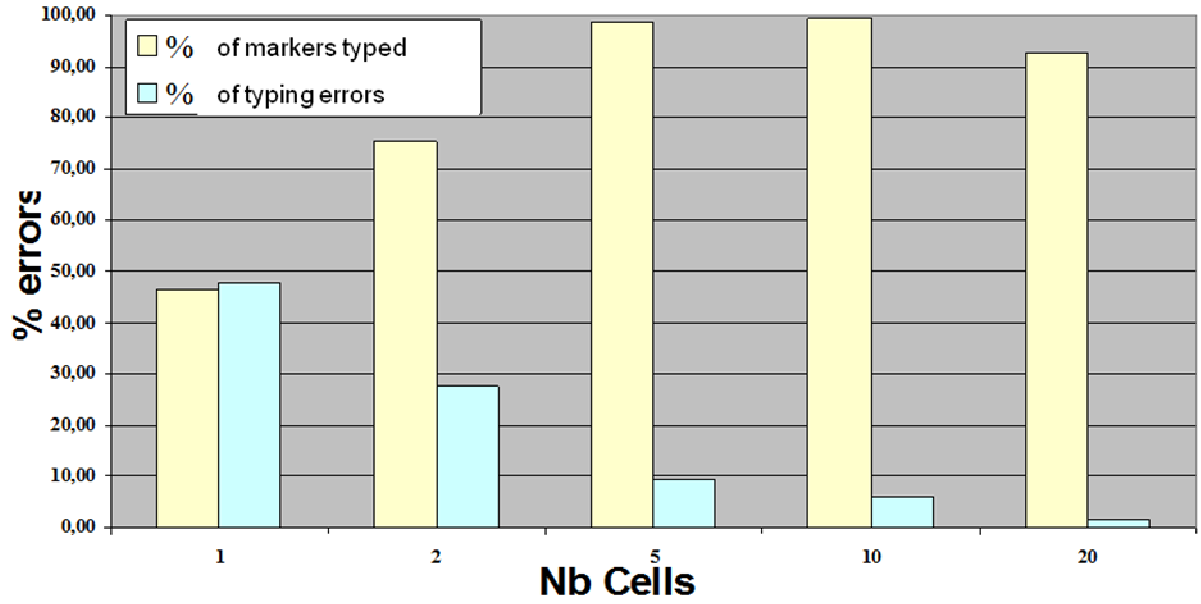

Figure 2: Effect of the number of cells of the biopsy on the percentage of detection of microsatellites (markers typed) and on the percentage of typing errors (Humblot et al., 2008, 2009a).

Additional experiments were carried out to evaluate and compare the developmental ability of biopsed embryos after *in vitro* culture and the pregnancy rates following transfer of *in vivo* produced embryos previously biopsed and frozen. Embryo survival following the biopsy of *in vitro* produced embryos was not different from the rate observed for non biopsed embryos from the same series of production that were used as controls (58/64 vs 18/20; 90%). From subsequent series, the embryonic development *in vitro* following biopsy of *in vivo* and *in vitro* produced embryos were not different (62/70; 89% vs 41/44; 93.2%). These results indicate that the effects of the biopsy by itself on subsequent embryonic development are very limited whatever the system used to produce embryos.

Pregnancy rates following the transfer on farm of fresh biopsied *in vivo* produced grade 1 and 2 (IETS classification) embryos were over 60% (Lacaze et al., 2008, Ponsart et al.,

2008,Table 1). Ponsart et al., (2008) reported pregnancy rates of 50% or more following of frozen biopsied embryos on farm and this percentage was close to 60 when transfers were made in station (54/90; frozen). In addition to this, from more recent series in station, it has been shown that grade 3 embryos may be used as well as pregnancy rates following transfer of those where not different when compared to results obtained with grade 1 and 2 (Gonzalez et al., 2008).

Table 1: Pregnancy rates following transfer of biopsied embryos on farms in different French programs since 2005

Author	Year of transfer	Type of embryos	N	Pregnancy rate
Ponsart et al., 2008	2002-2007	Fresh	1333	63.3 %
		Frozen	669	52.0 %
Lacaze et al., 2008	2005-2008 (Aubrac)	Frozen	132	55.3 %

When considering these results, the typing from biopsied *in vivo* produced embryos looks realistic as the development rates and pregnancy rates following transfer of biopsied and frozen embryos does not seems too much affected by the biopsy itself. In addition those results show that most of the embryos and even grade 3, could probably be kept in the process and this would allow to genotype most of them. Despite good pregnancy rates have been reported with frozen *in vitro* produced embryos in many countries (Ponsart et al., 2004), improvements are probably still necessary for those, because of the selection usually applied before and after freezing by most teams and lack of references on pregnancy rates following direct transfer of large numbers of biopsied and frozen *in vitro* produced embryos.

Calculations have been performed to estimate the genetic and economical advantages of using embryo typing in association with MOETs when compared to use of conventional embryo transfer alone. In a first study, simulations based on the use of the first generation of MAS markers were made from real series of observations obtained from females included as donors in genetic schemes and performances of their sons evaluated at various ages (Colleau et al., 2008). Those simulations have shown that the use of embryo typing is associated with very significant advantages at time of early evaluations (until 1 year of age) that disappears at the time of final evaluation. This indicates that when using this first generation of typing method (limited number of micro satellites), the embryo typing scenario suffered both from the lack of precision of the genetic information from young donor females and from the lack of precision of the genotyping evaluation which was used to select those embryos early in life. These defaults are much less important now and almost disappear today i) due to the better knowledge associated to young parents allowed by the accumulation of genetic information through generations and ii) will become completely negligible/inexistent with the gain of precision obtained from the use of the 54k SNP chip or from future Whole Genome Evaluation. Other types of simulations based on the costs induced by different scenarios to produce the same number of bulls of a same genetic value revealed that substantial gains can be raised with the help of embryo typing if the whole set of reproductive techniques is well controlled.

Additional economical and genetic simulations should be performed in this new context of using high density markers chips to precisely evaluate the costs and advantages for the

genetic schemes of such procedures based on embryo typing. Limitations may be encountered in relation with the technical feasibility of using amplified DNA together with the last generation of high density markers chips. However, preliminary studies from limited numbers of biopsies and typing have shown that the use of pre amplified DNA is compatible with the typing from those chips (Lebourhis et al., 2010, unpublished). This must be verified with the next generation of chips that will include 600 x thousands of markers. Cost efficiency of the whole system must be verified also by simulations made from different scenarii. If needed, alternatives may be found by using other types of chips fully compatible with the analysis of pre amplified DNA allowing a pre screening of the embryos at a very low cost before performing full genotyping in calves.

Other reproductive Techniques

To a certain extent, sperm sexing can help to limit the number of embryos to be produced for this purpose and may be used in combination with in vitro fertilisation – in vitro production (IVF-IVP) procedures. Use of semen sexing in association with IVF-IVP may allow also to avoid some of the present limitations of the use of semen sexing in selection schemes in relation with the high number of sperm that must be discarded and the large individual variation associated with the sexing process by flow cytometry (Rath 2008).

Finally, when considering the need to maximize genetic variability and due to strong limitations in reproductive efficiency, cloning do not appear today as an interesting tool in the frame work of selection schemes. However, beside selection schemes driven by breeding associations / companies, individual farmers that may get access to genomic selection, may be interested in the duplication of their best animals with the help of cloning for commercial purposes in the countries allowing the use of this process. Some applications may result from the use of transgenesis associated to cloning, however such technical options especially in the EU context, will face strong limitations in relation to ethics that will probably limit their use to types of production different from agronomics.

Impact of genomic selection on the use of reproductive techniques and more specifically ART

Genetic Schemes

Artificial Insemination (AI), Multiple Ovulation and Embryo Transfers (MOETs) and/or - depending on countries - Ovum Pick Up associated with *in vitro* Embryo Production (OPU–IVP) were already used in the past to generate the future sires to be widely used following selection through highly effective but very costly progeny testing programmes. The changes in breeding strategies and use of reproductive techniques associated with the needs of genomic selection are on the way. They result from the organisation of selection schemes which are already today completely different. As shown before, efficiency of embryo transfer, OPU and IVF will be critical and these techniques will be probably more used than in the past to increase the number of candidates. There is a large phenotypic variability between individual females for in vivo and in vitro production (Guyader Joly et al., 2008, Merton et al., 2009).Taking into account the genetic index of donor females on OPU-IVP production may also be used to optimize the results (Merton et al., 2009). On top of this, the potential value of the genotyped animals will probably lead breeding associations / companies to adopt strategies allowing them to control the production of genomic selected animals. This will lead them to reinforce the use of embryo based reproductive techniques MOETs and IVP in nucleus herds to give birth of previously (pre) selected animals within a given structure / company and not on farm. In this context, the success of embryo typing before transfer may be

more and more critical for the breeding organisations and some of the companies involved in Eurogenomics have already started to include embryo typing in their selection process.

Commercial activity

As soon as genotyping will be extended, farmers will have access to the corresponding information in females. This will probably induce a strong rise of the demand for ET and even OPU and IVF from farmers wishing to optimize the valorisation of their best females within their herds and/or for commercial purposes.

Conclusion

In the new context of genomic selection, there is still a lot of work for the reproductive physiologist to study gene expression and identify markers and networks of genes in relation with fertility. As far as selection for fertility is concerned, more precise phenotyping is needed for particular reproductive events and more especially for precocity of reproductive traits that has not been well characterized so far. More generally for all production traits and functional traits, in the present context showing very impressive improvements induced by the intensive use of MAS, it is likely that the use of a set of intensive reproductive techniques together with embryo typing will bring very significant advantages to breeding organisations able to monitor all those techniques with efficiency. However, strategies must be developed to use all these techniques in a way they contribute to maintain genetic variability. It is clear that the emergence of the new methods for genomic selection makes all improvements related to embryo production *in vivo* or *in vitro* and associated techniques very attractive for breeding organisations and companies willing to valorise as much as possible the advantages of genomic selection. There will be probably also some changes in relation with commercial activity due to valuable genomic information becoming available in females that may lead individual farmers/companies to make a larger use of semen sexing and embryo related technologies.

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References

1) Adjaye, J. (2005) Whole-genome approaches for large-scale gene identification and expression analysis in mammalian preimplantation embryos. Reprod Fertil Dev, 17, 37-45.

2) Adjaye, J., Herwig, R., *et al.* (2007) Conserved molecular portraits of bovine and human blastocysts as a consequence of the transition from maternal to embryonic control of gene expression. Physiol Genomics, 31, 315-27.

3) Barbat A., Le Mézec P., Ducrocq V., Mattalia S., Fritz S., Boichard D., Ponsart C., Humblot P. (2010). Female fertility in French dairy breeds: current situation and strategies for improvement. J Reprod Dev., 56:S15-S21.

4) Boichard D. Ducrocq V., Fritz S., Colleau J.J. (2010). Where is dairy cattle breeding going? A vision of the future. 41th Interbull International Workshop. March 4-5 2010, Paris, France. Interbull Bulletin 41. http//www/interbull.org

5) Chesnais J. (2009). Genomic selection : how i twill affect the dairy producer. In proceedings CETA/ACTE & AETA Joint Scientific Convention, Montréal, Québec, Canada, p90.

6) Colleau J.J., Fritz S., Ponsart C., Le Bourhis D., Lacaze S., Tissier M., Amigues Y., Druet T., Malafosse A., Boichard D., Humblot P. (2008). The value of embryo typing in a marker-assisted dairy cattle breeding scheme (In French) Renc. Rech. Ruminants. 15 427-430

7) Colleau J.J., Fritz S., Guillaume F., Baur A., Dupassieux D., Boscher M.Y., Journaux L., Eggen A. Boichard D. (2009). Simulating the potential of genomic selection in dairy cattle breeding (In French). Renc.Rech.Ruminants 16, 419

8) Dalbies Tran R., Humblot P., Eggen E., Duranthon V. (2009). OVOAGENAE2 – La régulation de l'expression génique dans l'ovocyte, enjeu pour l'élevage bovin. In Proceedings 7th Seminar AGENAE, Oct 2009, Tours, pp95-96.

9) Dieleman S.J., Hendricksen P.J.M., Viuff D., Thomsen P.D., Hyttel P., Knijn H.M., Wrenzycki C., Kruip T.A.M., Niemann H., Gadella B.M., Bevers M.M., Vos P.L.A.M. (2002). Effects of in vivo prematuration and in vitro final maturation on developemental capacity and quality of pre-implantation embryos. Theriogenology, 57:5-20.

10) Ducrocq V. (2010). Le projet AMASGEN vers la sélection génomique du futur. BulletinTechnique de l'Insémination Artificielle, 135 :18-19.

11) Evans, A. C., Forde, N., O'Gorman, G. M., Zielak, A. E., Lonergan, P., and Fair, T. (2008) Use of microarray technology to profile gene expression patterns important for reproduction in cattle. Reprod Dom Anim, 43 Suppl 2, 359-67.

12) Fritz S. (2010). Perspectives d'utilisation de la selection génomique chez les bovins laitiers. BulletinTechnique de l'Insémination Artificielle , 135 :15-17.

13) Gilchrist R.B., Albuz F.K., Thompson J.G. A new approach to in vitro maturation (IVM) and embryo in vitro production. In : 36th annual conference of the I.E.T.S., Cordoba (Argentine), 9-12 January 2010. p. 293 (Abstr. 272).

14) Gonzalez C., Le Bourhis D., Guyader Joly C., Moulin B., Heyman Y., Humblot P. (2008). Pregnancy rates after single direct transfer of biopsed frozen-thawed bovine embryos according to quality. Proceedings 24th AETE, Abstract p158.

15) Guignot F., Baril G., Dupont F., Cognie Y., Folch J., Alabart J.S., Poulin N., Beckers J.F., Bed'hom B., Babillot J.M., Mermillod P. (2008). Determination of Sex and Scrapie Resistance. Genotype in Preimplantation Ovine Embryos. Molecular Reproduction Development, Published online in Wiley InterScience, DOI 10.1002/ mrd.20940

16) Guyader Joly C., Ponchon S., Gonzales C., Marquant leguienne B., Clement L., Dalbies Tran R., Mermillod P., Humblot P. (2008). Identification of contrasted phenotypes in the bovine from repeated in vivo and in vitro embryo production following superovulation. Reprod Fertil Dev. 2008, 20:131-132 (Abstract).

17) Hendriksen P.J.M., Vos P.L.A.M., Steenweg W.N.M., Bevers M.M., Dieleman S.J. (2000). Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology, 53:11-20.

18) Humblot P. (2001). Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology, 56:1417-1433.

19) Humblot P., Holm P., Lonergan P., Wrenzycki C., Lequarré A.S., Guyader-Joly C., Herrmann D., Lopes A., Rizos D., Niemann H., Callesen H. (2005). Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes. Theriogenology, 63(4) : 1149-1166.

20) Humblot P., Le Bourhis D., Amigues Y., Colleau J.J., Heyman Y., Fritz S., Gonzalez C., Guyader-Joly C., Malafosse A., Tissier M., Ponsart C. (2008). Combined use of reproductive technologies for genomic selection in the bovine. In : 20th European AI Vets Meeting, November 19-20th . Utrecht pp. 4-10.

21) Humblot P., Le Bourhis D., Amigues Y., Colleau J.J., Heyman Y., Fritz S., Gonzalez C., Guyader-Joly C., Malafosse A., Tissier M., Ponsart C. (2009a). Combined use of reproductive technologies for genomic selection in the bovine. In proceedings CETA/ACTE & AETA Joint Scientific Convention, Montréal, Québec, Canada, p103.

22) Humblot P., Noé G., Ponsart C., Gatien J., Ledoux L., Fritz S., Boichard D (2009b). Mesures phénotypiques et étude du polymorphisme de gènes candidats de QTL de fertilité femelle en race Prim Holstein. In Proceedings 7th Seminar AGENAE, Oct 2009, Tours, pp79-80.

23) Humblot P., Freret S., Ponsart C. (2009c). Epidemiology of Embryonic Mortality in Cattle; practical implications for AI and Embryo production. In proceedings CETA/ACTE & AETA Joint Scientific Convention, Montréal, Québec, Canada, 17-19 sept. 2009.

24) Ko, M. S. (2004) Embryogenomics of pre-implantation mammalian development: current status. Reprod Fertil Dev, 16, 79-85.

25) Lacaze S., Ponsart C., Discala D., Richet L, Valadier A., Pailhous C., Humblot P. (2008) Use of embryo sexing and MOET in Aubrac cows to save the dairy genetic type. Proceedings of the 24th AETE ; Abstract pp 182.

26) Le Bourhis D., Chesne P., Nibart M., Marchal J., Humblot P., Renard J.P., Heyman Y. (1998). Nuclear transfer from sexed parent embryos in cattle, efficiency and birth of offsprings. J. Reprod. Fertil., 113:343-348.

27) Le Bourhis D., Amigues Y., Charreaux F., Lacaze S., Tissier M., Morel A., Mervant G., Moulin B., Gonzalez C., Humblot P. (2008). Embryo genotyping from in vivo biopsed bovine embryos. Proceedings of 24th AETE ; Abstract pp188.

28) Le Bourhis D., Amigues Y., Charreaux F., Lacaze S., Tissier M., Guyader-Joly C., Mervant G., Moulin B., Vignon X., Gonzalez C., Humblot P. (2009). Embryo genotyping from In vivo biopsied bovine embryos after whole genome amplification. Reproduction, Fertility and Development, 2009, 21. p.192 (abstr. 187).

29) Merton J.S., de Roos A.P.W., Mullaart E., de Ruigh L., Kaal L., Vos P.L.A.M., Dieleman S. (2003). Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology, 59:651-674.

30) Merton J.S., Ask B., Onkundi D.C., Mullaart E., Colenbrander B., Nielen M. (2009). Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up in-vitro production embryo-production program. Theriogenology, 72:885-893.

31) Peippo J., Viitala S., Virta J., Ra¨ Ty M., Tammiranta N., Lamminen T., Aro J., Myllyma¨ Ki J., Vilkki J. (2007). Birth of correctly genotyped calves after multiplex marker detection from bovine embryo microblade biopsies. Mol. Reprod. Dev.,74:1373-1378.

32) Ponsart C., Marquant Leguienne B., Humblot P. (2004). Evolution and future trends for use of embryo based biotechnologies in the bovine. In proceedings 11th Rencontre Recherche Ruminants (3R). Paris (France). p. 361-368.

33) Ponsart C., Salas Cortes L., Jeanguyot N., Humblot P. (2008) Le sexage des embryons a le vent en poupe ! BulletinTechnique de l'Insémination Artificielle, 129, p 30-31.

34) Rath D. (2008). Status of sperm sexing technologies. In proceedings 24th AETE meeting. Pau (France). P.89-94.

35) Rizos D., Ward F., Duffy P., Boland M., Lonergan P. (2002). Consequences of bovine oocyte maturation fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev., 61:234-248.

36) Van Wagtendonk de Leeuw A.M. (2006). Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. Theriogenology, 65:914-925.

37) Wurth Y.A., Merton S., Kruip Th.AM. (1994). In vitro maturation and fertilization limit the embryo production rate and in vitro embryo development diminishes embryo viability. Thesis, Utrecht, The Netherlands.

SHORT COMMUNICATIONS

METABOLIC STRESS IMPAIRS FOLLICULAR GROWTH IN SUPEROVULATED HEIFERS

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Fertility in high producing dairy cows has declined over the last decades. Metabolic stress around parturition and during the early post partum period has been postulated to be one of the major causes resulting in delayed first oestrus postpartum hence prolonged calving intervals.

To study the impact of metabolic stress on ovarian follicular function via hormonal analysis of follicular fluid and follicular growth, 12 cycling heifers were at random allocated either to a group (n=6) that received a control diet (ad libitum grass silage) or to an experimental group (n=6) that was fasted during the superovulation (SO) treatment for four days until ovariectomy, to induce metabolic stress during final follicular growth and development (Jorritsma et al., 2003). Heifers were synchronized with a CIDR® intravaginal device (Pfizer AH) during 7 days and prostaglandin injection (5 ml Enzaprost® CEVA AH) one day before CIDR® removal. On day 9 of the synchronized cycle the dominant follicle of the growth wave was removed, 2 days before the standard SO protocol was started (twice daily decreasing doses of 40, 30, 20 and 10 mg Folltropin-V® Bioniche AH). To suppress a spontaneous LH surge, cows received a CIDR® device during SO. To collect ovarian tissue at 22 hours after the LH peak by ovariectomy, a controlled LH surge was induced by a GnRH injection (5 ml Fertagyl® Intervet SP AH) at the time of CIDR® removal (Vos et al., 1994). Directly after ovariectomy ovaries were transported to the laboratory at 37°C. For each animal follicular growth was determined, by counting the number of follicles of >8 mm (defined as presumptive follicles). The size category of follicles was based on the volume collected after puncturing (8-<10mm, 10-<12mm, >12mm). To determine the quality of the follicle, follicular fluid of each individual follicle was analyzed for estradiol (E2) and progesterone (P4) concentrations. Healthy follicles at 22 hours after the LH peak were defined as follicles showing low E2 and high P4 > 0.5 µmol/L, (E2/P4 <1) (Dieleman et al., 1983). Statistical analysis was performed by generalized linear model for grouped data ($P<0.05$, mean ± SEM).

The total number of follicles per animal (follicle size>8mm) did not differ between the control (18 ± 2.8) and fasted group (19.8 ± 2.9). The number of large follicles (>12mm) developed during the situation of metabolic stress was however significantly lower (2.7 ± 0.9) compared to the control situation (6.0 ± 1.7). No difference was observed for the percentage of healthy follicles per animal between fasted (32 ± 9.2%) and control (56 ± 13%).

These data show that follicular growth during metabolic stress of heifers was impaired after a SO treatment. Although not significantly different, which might be due to the wide variation in SO response, a reduced number of healthy follicles was observed in the experimental group possibly indicating a negative influence of metabolic stress on follicular function and quality. Whether metabolic stress affects the metabolic composition of the follicular fluid and as a consequence influences oocyte quality hence embryo quality is under current investigation.

Project is sponsored by Pfizer Animal Health.

Notes

EFFECT OF DURATION OF STORAGE ON THE ABILITY OF FRESH BOVINE SPERM TO PENETRATE ARTIFICIAL MUCUS

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The main obstacle limiting the more widespread use of fresh semen in AI is its short shelf life. We have previously reported that sperm retain the ability to fertilise oocytes following fresh storage for up to 4 days, although differences exist between sires (Ward et al., AETE Proceedings 2005, p196). However, addition of sperm directly to oocytes in IVF clearly does not mimic the events that occur in vivo after AI, where sperm have to traverse the female reproductive tract in order to reach the oocyte. The aim of this study was to examine the ability of fresh stored sperm to penetrate artificial mucus in vitro as a proxy for its ability to traverse the reproductive tract, in an effort to understand the conflicting in IVF results with results after AI where fertility declines significantly after 2 days storage. Fresh and frozen semen from the same ejaculate was obtained from 4 sires and diluted in a Caprogen-based (fresh) or Biociphos (frozen) extender. Day 0 was defined as the day of semen collection. The ability of sperm from each bull to penetrate artificial mucus composed of 60 % MAP-5 and 40% PBS (modified from Gillan et al., Anim Reprod Sci 2008;103:201–214) was assessed from Day 0 to Day 4 post collection (n=4 replicates). Glass capillary tubes (n=2/bull/replicate) were filled with artificial mucus and incubated with sperm stained in 1% Hoechst 33342 for 10 min at 37^{o}C. The number of sperm was subsequently counted at 10 mm intervals along the tube between 30 and 90 mm. Data were analysed by ANOVA. The ability of fresh sperm to penetrate artificial mucus declined significantly compared to that of frozen sperm from the same ejaculate after Day 2 post collection. Thus, one of the principle reasons for the decline in fertility observed in vivo with fresh stored semen is likely due to the ability to travel through the reproductive tract rather than the ability to fertilise oocytes.

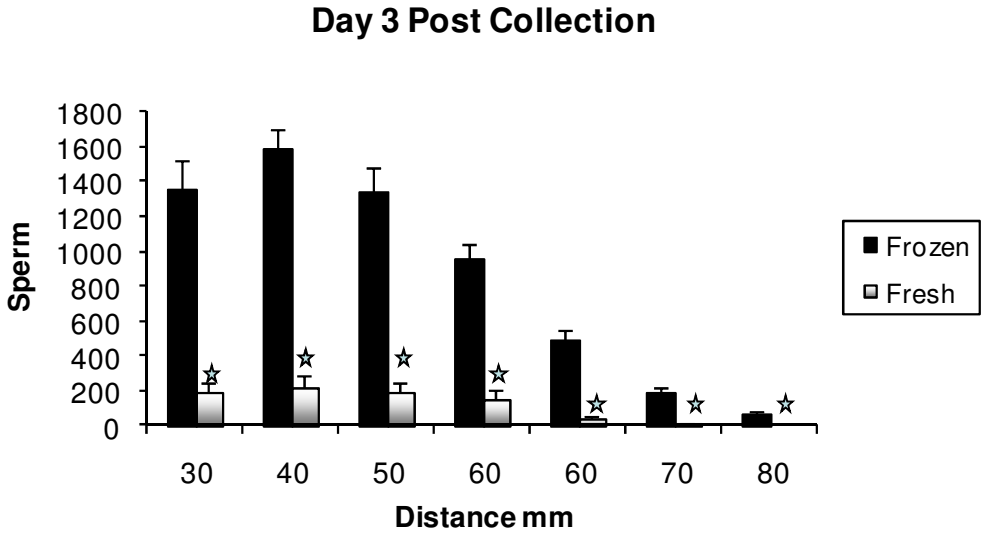

Figure 1. Number of fresh and frozen bovine sperm migrating through artificial mucus after storage for 3 days at room temperature.

Notes

EFFECT OF A DEFINED OR AN UNDEFINED IN VITRO MATURATION MEDIUM ON CLEAVAGE RATE AND EMBRYO DEVELOPMENT IN SHEEP

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Ovine oocytes matured *in vitro* are compromised in their developmental capacity compared with *in vivo* matured counterparts. While the causes of these differences are not fully elucidated, it is known that *in vitro* maturation (IVM) can be influenced by culture media composition and culture conditions used for IVM as well as by variations in oocyte quality. Follicular fluid (FF) is a natural medium of nuclear and cytoplasmic maturation of mammalian oocytes *in vivo*, suggesting that it may be suitable for oocyte culture. Epidermal growth factor (EGF) and follicle stimulating hormone (FSH) are also known to play a key role in nuclear and cytoplasmic maturation, and cysteamine (Cys) enhances glutathione synthesis, which is known to protect cells from oxidative damage. The aim of the present study was to compare the efficiency of a defined IVM medium (TCM199 supplemented with 100 μM Cys and 10 ng/ml EGF; Cys+EGF) with an undefined medium (TCM199 supplemented with 132 μg/ml pFSH and 10% FF; FSH+FF) for *in vitro* production (IVP) of ovine embryos, using abattoir-derived oocytes (170 COCs) from Rasa Aragonesa ewes.

Cleavage rates were 74.3% (52/70) and 80.0% (80/100) for Cys+EGF and FSH+FF groups, respectively (fresh semen; $2 \cdot 10^6$ spermatozoa/ml). Blastocyst rates at day 8 were 40.4% in Cys+EGF and 33.7% in FSH-FF groups, with an increase of 5.8 and 3.7%, respectively, when compared with blastocyst rates at day 7. Final yield of embryo IVP was 30.0% (21/70) when Cys+EGF medium was used for IVM, and 27.0% (27/100) when using FSH+FF medium. Day 7 and 8 good morphological quality blastocysts were vitrified, and some of them were transferred to synchronized recipients, reaching 9.1% (1/11) and 10.0% (1/10) lambs born in Cys+EGF and FSH+FF groups, respectively. Differences were not significant between groups for any of the studied variables.

Under the experimental conditions of the present study, TCM199 supplemented with cysteamine and EGF is a convenient, defined, maturation medium for IVP of embryos from abattoir-derived oocytes that provides an efficiency equivalent to that achieved using supplementation with FF and FSH. Further studies are needed to elucidate the role of different factors in IVM in sheep and to check the efficacy of this defined medium with oocytes from different sources.

Notes

LOSS OF MIMT1 EXPRESSION, A NOVEL MUTATION IN THE MATERNALLY IMPRINTED PEG3 DOMAIN, CAUSES ABORTIONS AND STILLBIRTHS IN CATTLE (BOS TAURUS)

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The development of fetus and placenta are affected by imprinted genes. The imprinted genes are not functionally equivalent and one of the alleles, the paternal or maternal allele becomes epigenetically inactivated based on parental origin. In the fetus the imprinted genes have a mono-allelic expression. A maternally imprinted gene has a paternal expression; this means that only the allele coming from the father is expressed (functional) in the embryo and fetus. Approximately half of the fetuses can be adversely affected when the maternally imprinted gene is defective in one of the two alleles in the male. Approximately 200 mammalian genes are imprinted and the transcription is epigenetically repressed based on its parent of origin. Known imprinted genes are clustered in discrete chromosomal regions called imprinted domains.

A bull, which semen can be used to produce for instance 500 – 100.000 pregnancies is a delicate target for a natural mutation in a maternally imprinted gene.

In this study we describe a new mutation in the PEG3 domain that caused stillbirths and abortions in 44% of the fetuses in cattle (Bos taurus) that inherited the mutation from a bull which semen was used in artificial insemination of more than 1500 cows. The corresponding average percentage of late abortions and stillbirths for artificial insemination bulls of Ayrshire breed is 5%. The stillborn calves were of approximately 50% size (20 kg) compared with the average for the breed (40 kg). The lungs were not inflated in the dead calves.

The most probable genetic model was a mutation in a maternally imprinted area. Initially the proband and 19 half siblings sired by the proband were genotyped (Illumina BovineSNP50). The analysis revealed a candidate region in BTAU 18, in the area between 51446347 and 64727406 bp. In this area is the imprinted PEG3 domain. The expression of three maternally imprinted genes (PEG3, MIMT1 and USP29) was studied from fetal brain tissue and from cotyledons using eight half-siblings. Four of these half-siblings had the sick haplotype and four had the normal haplotype. MIMT1(MER1 repeat-containing Imprinted transcript 1, a RNA gene) was expressed only from the four fetuses with the healthy haplotype.

The reason for the killer effect was a 100kb deletion that cut out a piece of the MIMT1 gene which finally led to the late abortions and stillbirths.

Notes

USING THE LAB-ON-CHIP SYSTEM TO EVALUATE THE QUALITY OF PORCINE EMBRYOS COLLECTED FROM PREPUBERAL AND CYCLING GILTS

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Lab-on-Chip is a new system based on microfluidic technology, which may be a useful tool to assess mammalian oocyte and embryo quality and their developmental competence. The aim of this study was to analyze porcine embryo quality collected from prepuberal and cycling gilts after standard SO and AI programs.

Altogether, 10 cycling and 12 prepuberal German Landrace donor gilts were used. In cycling donor gilts, the estrus cycle was synchronized by 15 day-long feeding of Regumate® followed 24h later by stimulation of SO with 1500 IU eCG and 80h later with 500 IU hCG. Gilts were inseminated 24 and 38h after hCG. In prebuberal gilts SO was induced with 1500 IU eCG and 500 IU hCG 72h after eCG, and insemination was performed at 24 and 38h after hCG. Embryos were recovered surgically on day 5 with flushing the uterine horns. Embryos were analyzed after previous morphological evaluation in the Lab-on-Chip measurement setup which consists of a VIS/NIR light source (halogen lamp), miniature spectrometer (Ocean Optics, USA) and PC with original Ocean Optics software. Each embryo was introduced into the lab-on-a-chip by pipetting and capillary forces. After short-time measurement, the embryo was flushed back to a sterile transporting container for further treatment. The spectral characteristics were recorded, normalized, and processed under Origin (USA) software.

In this experiment, altogether 209 morulae were recovered and analyzed, and the light absorbance and transmission of each embryo was measured. Regarding their light transmission embryos were divided into two groups: class-I morulae displayed light transmission less than 60 %, while class-II morulae had transmission more than 60 %. Cycling gilts revealed 66 class-I and 53 class II embryos (55 *vs*. 45%). In prepuberal donors, 38 class-I and 52 class-II embryos were observed (42 *vs*. 58%).

These are the first results on biophysical characteristics of embryos using Lab-on-Chip system detection. Differences in absorbency may be related both to embryo development and donor age. These differences have to be confirmed in ongoing embryo transfer.

Notes

INHIBITION OF SERINE PROTEASES BY THE SPECIFIC INHIBITOR AEBSF HAS A NEGATIVE INFLUENCE ON SPERM MOTILITY AND *IN VITRO* FERTILIZATION (IVF) IN PIGS

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Proteases and protease inhibitors play a crucial role in many physiological and pathological processes, including reproduction. Spermatozoa have a specialized organel, the acrosome, that contains different enzymes like hyaluronidase and proteolytic enzymes. It is commonly accepted that these enzymes are involved in the penetration of the cumulus oophorus and the zona pellucida (ZP). The most studied acrosomal protease is acrosin, a trypsin-like serine protease. Acrosin is linked with secondary binding to the ZP as well as with dispersion of acrosomal content and zona lysis. Proteases can fulfill multiple functions in different pathways and protease inhibitors may therefore inhibit not only proteases present on the sperm membranes or in the acrosome, but also proteases involved in intracellular signaling. The aim of this study was to evaluate the effect of a serine protease inhibitor on fertilization parameters during *in vitro* fertilization in pigs. We used 4-(2-aminoethyl)benzene-sulfonyl fluoride hydrochloride (AEBSF), an irreversible serine protease inhibitor which is presumed to be active intracellular and has been used in cell culture.

Ovaries of prepubertal gilts were collected at a local slaughterhouse. After *in vitro* maturation during 44h, oocytes were transferred to droplets of fertilization medium with 0, 100 or 250 µM AEBSF and inseminated with frozen-thawed epididymal semen giving a final ratio of 600 sperm per oocyte. After a co-incubation period of 2 or 6 hours, oocytes were vortexed in HEPES-buffered medium to remove the protease inhibitor, washed four times in culture medium and cultured for 22 hours. Subsequently, presumed zygotes (total n = 850) were fixed overnight with 4% paraformaldehyde and stained with 10 µg/ml bis-benzimide (Hoechst 33342) to assess fertilization and polyspermy and to calculate the sperm penetration index (mean number of penetrated spermatozoa per fertilized oocyte). Sperm motility was evaluated via Computer Assisted Sperm Analysis (CASA) before and after 2, 4 and 6 hour incubation in fertilization medium with 0 or 250 µM AEBSF. Toxicity on spermatozoa and oocytes was assessed by using SYBR-PI and Hoechst-PI staining, respectively.

Presence of AEBSF during pig IVF (100 and 250 µM) resulted in a significant reduction of all fertilization parameters compared to the control group ($P<0.05$). In case of 6 hour incubation, 100 µM and 250 µM resulted in 50% resp. 91% reduction of fertilization rate compared to the control group. Gamete membrane integrity was not influenced by AEBSF but sperm motility was remarkably inferior in the AEBSF treated groups (-41%, -67% and -84% after 2, 4 and 6 hour incubation, respectively). We suggest that the decline in fertilizing capacity of the spermatozoa is mediated through an AEBSF-induced decrease in sperm motility, possibly in combination with inhibition of acrosin. Sperm motility is in part regulated by phosphorylation of flagellar proteins. This pathway starts with the activation of cAMP, of which the main downstream target is protein kinase A. Protein kinase A is most likely involved in the phosphorylation process. Our hypothesis is that AEBSF inhibits protein kinase A, thus impeding phosphorylation of flagellar proteins and sperm motility, resulting in hampered sperm penetration of cumulus cells, ZP and oocyte.

This study was supported by Research Foundation-Flanders, grant number 1.1.450.09.N.00

Notes

X-CHROMOSOME INACTIVATION IS PARTIALLY ACCOMPLISHED BETWEEN DAYS 7 AND 14 DURING BOVINE EMBRYO DEVELOPMENT

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In eutherian mammals, X-chromosome inactivation (XCI) is required to ensure an equal transcriptional level for X-linked genes in both sexes. In mice, XCI occurs before the blastocyst stage, but very little is known in other species. In a previous study, we found that in cattle 88 % of the expressed X-linked genes were upregulated in female blastocysts, suggesting that at this stage XCI is far from being accomplished. The aim of the present study was to determine whether XCI occurs in the posthatching and early embryo elongation periods in cattle. For this purpose, we analyzed the expression level of 6 X-linked genes known to display sexual dimorphism at the blastocyst stage in male and female Day 14 embryos.

Forty in vitro produced bovine blastocysts were transferred on Day 7 to the ipsilateral uterine horn of two synchronized recipients. Seven days after embryo transfer, the recipients were slaughtered and 19 Day 14 embryos of a similar size were selected for the analysis. PolyA mRNA and cDNA synthesis and DNA extraction was performed individually. Embryo sexing was performed based on the expression of the Y-linked gene YZRSR2 and the result was confirmed on DNA by a PCR for BRY/Sat. The mRNA abundance relative to the housekeeping H2AZ of 6 X-linked genes: brain-expressed X-linked 1 (BEX1), calpain 6 (CAPN6), spermidine/spermine N-acethyltransferase 1 (SAT1), brain-expressed X-linked 2 (BEX2), the nonannotated transcript X24112, and ubiquitin-conjugating enzyme E2A (UBE2A) was determined on 11 male and 8 female Day 14 embryos. Statistical differences were assessed by ANOVA.

Two of the genes (SAT1 and UBE2A) analyzed showed an upregulation in female embryos compared with male, whereas no sex-related differences were observed in the other four, suggesting that from Day 7 to Day 14, a partial XCI occurs and that XCI timing differs greatly among species.

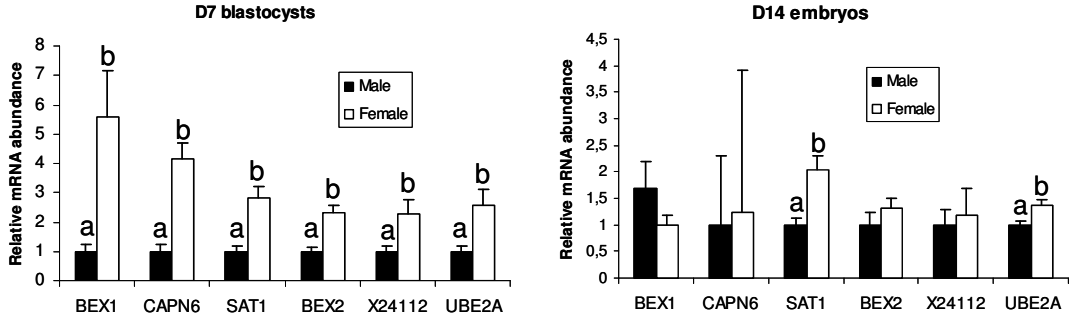

Figure 1. Relative mRNA abundance of six X-linked genes in male (black bars) and female (white bars) Day 7 (left) or Day 14 embryos (right).

Notes

THE FOETAL AND POSTNATAL EFFECTS OF PERICONCEPTIONAL HYPERGLYCAEAMIA USING A RABBIT MODEL

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The aim of this study was to analyze the effects of maternal hyperglycaemia during the periconceptional period on fetal and post natal development using a rabbit model.

Adult New-Zealand female does were used. Diabetes was induced (group D, N=22) by IV alloxan injection and glycaemia was subsequently maintained between 3 and 5 g/l with 2 daily subcutaneous insulin injections. Controls (N=19) did not receive any treatment (group C). Does were mated one week after induction of diabetes and embryos were collected after sacrifice on Day 4 post-coitum. In a first experiment, embryos from D (n=11) and C (n=13) group were transferred to the right and the left horn of 3 female recipients, respectively. Recipients were euthanised on Day 28. In a second experiment, 68 embryos were collected from 17 D females and 98 from 16 Controls. They were transferred to 26 non-diabetic recipients (6-7 embryos per doe). Foetal and placental development was monitored by ultrasound. At birth, litters were equilibrated in number and sex. 15 D and 7 C pups were euthanized at weaning (one month). The remaining pups were allocated to one of two feeding groups: control diet (groups Dc and Cc) or cafeteria diet (Dcaf and Ccaf) and sacrificed at 5 months of age. Body weight, adiposity (by impedancemetry and absorptiometry), and glucose metabolism were monitored until sacrifice.

Foetal development as assessed by ultrasound was not significantly different between D and C groups. Fœtal, placental and fœtal organs weights did not differ at 28 days of pregnancy, but for brain weight which was significantly lower in D foetuses (0.86 ± 0.1 g vs 1.05 ± 0.08 g, $p<0.05$). There was no significant difference in litter size at birth (gestation length 31-32 days), but birth weight was significantly increased in D offspring (211 ± 6 g vs 194 ± 6 g, $p<0.05$). Pups caught up rapidly and there was no significant difference in weight after 14 days any more. At one month of age, adiposity, plasma insulin and leptin concentrations were not different between groups. In contrast, in male D offspring, mean fasting plasma glucose was significantly lower (1.7 ± 0.2 g/l vs 2.1 ± 0.02 g/l, $p<0.01$), plasma IGF1 was significantly increased (0.41 ± 0.03 vs 0.45 ± 0.04, $p<0.05$) and kidney/body weight ratio was significantly reduced. Experiments are still being conducted for animals kept after weaning and results are therefore yet incomplete. So far, body weight becomes significantly different between D and C groups and according to diet from 12 weeks of age (puberty, $p<0.005$, ANOVA). Fasting glycaemia is significantly higher in animals fed the cafeteria diet at 3 months of age (1.24 ± 0.03 g/l vs 1.11 ± 0.06 g/l, $p<0.04$) but there is no difference between C and D.

These data suggest that maternal hyperglycemia during the periconceptional period affects glucose metabolism and organ development in offspring, with sexual dimorphism. More investigations are on-going to evaluate glucose homeostasis and kidney function in adult offspring and to possibly identify associated epigenetic marks.

Notes

STUDY ON STIMULATORY ACTIVITY OF SYNTHETIC LAMPREY GNRH-III ON GONADOTROPIN RELEASE AND FOLLICLE DEVELOPMENT IN GILTS

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Based on the supposition that lamprey GnRH-III (lGnRH-III) could have FSH releasing activity in swine, as recently demonstrated in barrows (Kauffold et al., RDA 2005), and which possibly influence estrus induction, synthetic lGnRH-III (peforelin, Maprelin® XP10, Veyx-Pharma, Schwarzenborn, Germany) was used in cyclic gilts. The secretion of reproductive hormones FSH, LH, estradiol and progesterone was analyzed, and follicle growth and ovulation recorded.

Altogether 24 German Landrace gilts were treated after 18-day long synchronization of the estrus cycle with Regumate® as follows: 48 h after the last Regumate® feeding they were stimulated either with 150 µg Maprelin® XP10 (group Maprelin, n = 6), 50 µg Gonavet Veyx® (Veyx-Pharma; group GnRH, n = 6), 850 IE Pregmagon® (IDT Biologica Dessau-Roßlau; group eCG, n = 6) or saline (group Control, n = 6). Additionally, in eight gilts the concentrations of FSH and LH were analyzed after application of 150 µg Maprelin® XP10 (n = 3), 50 µg Gonavet Veyx® (n = 3) or saline (n = 2) at mid-cycle (d10 of the estrus cycle). Blood samples were collected via implanted jugular vein catheters after drug application. Ovarian features were judged endoscopically at the end of the Regumate® feeding and on days d5 and d6 post injection.

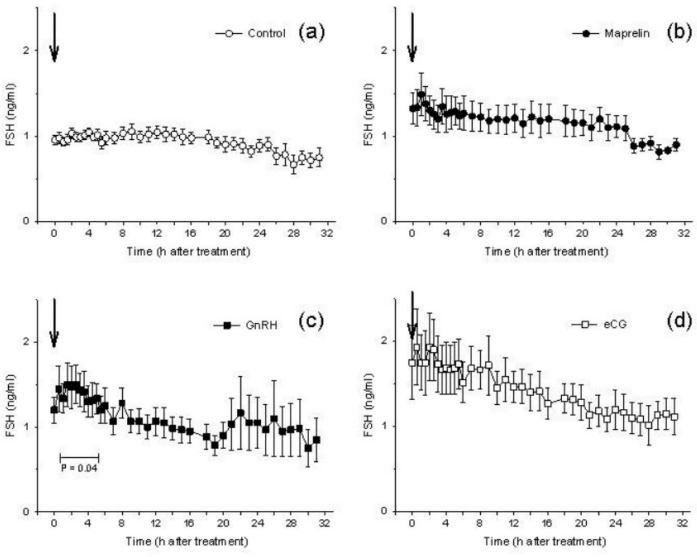

Fig. 1 Mean (± SE) FSH concentrations in Control (a) and in Maprelin (b), GnRH (c) and eCG (d) treated gilts at the preovulatory period

Marperlin® XP10 did not selectively stimulate FSH release in gilts; neither at the preovulatory period (Fig. 1b) or at mid-cycle. Differently, GnRH induced FSH release (Fig. 1c), however less compared to LH secretion. Due to the direct action on the ovary, eCG did not stimulate the release of pituitarian hormones FSH and LH. LH secretion was induced by GnRH both during the follicular phase and mid-cycle. Increased estradiol concentrations during d2 and d5 after Regumate® in all groups of gilts indicate preovulatory follicle growth. Equine CG provoked a significant higher number of ovulatory follicles (22.3 ± 1.0, $p<0.01$) compared to Maprelin (17.5 ± 1.3), GnRH (15.2 ± 1.1) and Control (17.7 ± 1.2) groups. Between 83 to 100 % of gilts ovulated to d 6 post injection.

Notes

PARTIAL DELAY IN MUSCLE MATURATION IN BOVINE CLONE OFFSPRING

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As cloned cattle exhibit a delay in muscle maturation until puberty (Jurie et al 2009), the present study aimed to examine the muscle properties of their offspring (F1) at 8, 12 and 18 months of age. Repeated biopsies of the *semitendinosus* muscle were collected on 10 F1 heifers born after AI of cloned cows at the experimental farm of INRA. Muscle properties of these F1 were compared to those of 9 female clones and 8 AI control heifers previously biopsied at the same ages. All animals were female Holstein, raised under the same conditions in the same farm. Biopsy samples were stored at -80°C until analysis. The contractile type of fibres was determined from the proportion of the different myosin heavy chain (MyHC) isoforms separated by SDS-PAGE. The metabolic type of muscle was characterized from the activities of two oxidative enzymes, isocitrate dehydrogenase (ICDH) and cytochrome-c oxidase (COX) (µmole/min per g muscle). Results are presented in table 1.

Table 1. Contractile and metabolic characteristics of semitendinosus muscle from control, cloned and cloned offspring (F1) heifers

	Age (months)	Control			Cloned			F1		Effect
		Mean	s.e.		Mean	s.e.		Mean	s.e.	
Contractile characteristics										
MyHC I (%)	8	6.9 ^{b}	1.0		13.9 ^{a}	2.6		9.5 ^{b}	0.8	0.03
	12	8.0 ^{b}	0.5		10.4 ^{a}	0.7		7.4 ^{b}	1.0	0.04
	18	13.0 ^{ab}	1.1		15.2 ^{a}	1.5		9.3 ^{b}	1.7	0.04
MyHC IIa (%)	8	16.3 ^{b}	2.0		27.9 ^{a}	2.3		23.1 ^{a}	1.7	0.002
	12	16.7 ^{b}	1.4		24.7 ^{a}	1.6		17.2 ^{b}	2.5	0.01
	18	23.4	2.5		26.5	1.9		23.1	2.0	NS
MyHC IIx (%)	8	76.8 ^{a}	2.8		58.2 ^{b}	4.7		67.4 ^{a}	2.3	0.004
	12	75.3 ^{a}	1.8		64.9 ^{b}	2.0		75.5 ^{a}	2.9	0.007
	18	63.7	2.7		58.3	3.0		67.6	3.3	NS
Oxidative metabolism										
ICDH activity	8	0.54 ^{b}	0.07		1.16 ^{a}	0.08		1.39 ^{a}	0.22	0.002
	12	0.71	0.08		0.96	0.09		0.82	0.10	NS
	18	0.98	0.11		0.98	0.04		0.99	0.18	NS
COX activity	8	4.2 ^{b}	0.9		9.9 ^{a}	0.9		11.4 ^{a}	1.6	0.003
	12	6.1	1.0		8.3	0.9		9.4	2.2	NS
	18	10.4	1.5		8.4	0.7		12.3	2.5	NS

Data were analysed separately for each time of biopsy using the GLM procedure of SAS. a, b LSmeans, within each age, with different superscript are significantly different at $P<0.05$.

The proportion of MyHC I and MyHC IIx in the muscles of F1 was not significantly different from those of controls at 8- and 12- months of age. However, F1 had different muscle contractile properties compared to clones. At 8 months of age, F1 had greater ICDH and COX activities than controls, but not later on. The muscles of cloned offspring F1 were more oxidative than those of controls, thus a delay in muscle maturation was only partially found in clone offspring.

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Notes

ANALYSIS OF SPECIFIC MATURATION GENE CANDIDATES IN IMMATURE AND MATURE SHEEP OOCYTES SELECTED BY BRILLIANT CRESYL BLUE (BCB) TEST

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Brilliant Cresyl Blue (BCB) stain determines the activity of glucose-6-phospahate dehydrogenase (G6PDH), an enzyme which activity decreases as the oocytes reaches their growth phase. In goats and cows, BCB is considered a useful test in order to select oocytes with higher developmental competence. The aim of this study was to analyze the expression of 10 maturation gene candidates in sheep oocytes selected by BCB test before and after *in vitro* maturation (IVM).

Cumulus oocyte complexes (COC) were exposed to 13 µM BCB for 1 h before IVM and classified according to oocyte coloration: oocytes with blue cytoplasm or fully grown oocytes (BCB+) and oocytes not colored or growing oocytes (BCB-). COC were then matured in conventional TCM199 medium for 24 h. Groups of 15 denuded oocytes were taken before (T0) and after maturation (TIVM) and placed in 100 µL Trizol until use. Total RNA from 4 samples per condition was extracted and reverse transcription and RT-PCR analysis using SYBR Green was performed. Relative expression (RE) was calculated using GAPDH as the internal control gene.

No significant differences in RE of genes were found between BCB+ and BCB-. However, significant increases in RE was observed in matured oocytes, when compared to immature oocytes for *CPEB1* (cytoplasmic polyadenylation element binding protein 1), *ATP1A1* (alpha 1 polypeptide), *COX1* (Cytochrome c oxidase subunit), *EEF1A1 (*elongation factorA1), *NASP* (nuclear autoantigenic sperm protein), *ODC* (ornithine decarboxylase), *H2AFZ* (histone H2A.Z). Peroxiredoxin (*PRDX1*) RE decreased in matured oocytes and expression of cyclin B1 (*CCNB1*) and calcium-binding protein (*S100A10*) remained unchanged during IVM.

In conclusion, expression of studied genes is sheep oocytes was affected by IVM, but did not reflect differences in BCB status.

Notes

IDENTIFICATION OF HOUSEKEEPING REFERENCE GENES FOR STUDIES OF GENE EXPRESSION IN ELONGATING BOVINE EMBRYOS

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The conceptus (embryo/fetus and associated extraembryonic membranes) and endometrium reciprocally interact throughout pregnancy. This area of reproductive biology is particularly important in ruminants due to relatively high levels of pregnancy loss during the peri-implantation period. Ruminant embryos differ from those of primates and rodents in that they undergo a prolonged period of elongation in the uterus prior to implantation. Because elongation cannot be achieved in vitro this stage of development is only infrequently studied. Quantitative real-time PCR (qPCR) is a highly specific and sensitive tool to compare mRNA expression levels of specific genes in biological tissues. Not only are the RNA quality, the reverse transcription, the reagents and the protocol of critical importance, but the analytical method and most importantly, the embryo developmental stage used can influence the results dramatically. All data must be normalized for technical differences between samples. There are limited data available in the literature relating to reference genes when early conceptuses are studied for specific gene expression analysis. Therefore, the aim of the present study was to assess a set of 7 stable reference genes for use in the study of gene expression in Day 13 bovine conceptuses originating from blastocysts derived in vivo or in vitro. Day 7 bovine blastocysts were produced either in vitro by maturation, fertilization and culture or in vivo by superovulation, AI and non-surgical embryo recovery. Blastocysts were transferred in groups of 10 to synchronized recipients ipsilateral to the corpus luteum. Day 13 embryos were recovered by flushing the uterus at slaughter, measured and individually snap frozen in liquid nitrogen. In order to identify stably expressed genes for normalization of quantitative data in early elongating bovine conceptuses, transcription levels were examined of 7 frequently used reference genes (*H2AFZ, ACTB, 18S rRNA, GAPDH, SDHA, HPRT1* and *YWHAZ*). Analysis of transcription profiling using geNorm software revealed that the expression of *H2AFZ* and *ACTB* is highly regulated by the system of embryo production used, while the relative abundance of *18S rRNA* and *GAPDH* transcripts were highly stable (Figure 1). Moreover, a pair-wise variation between samples indicated that the use of more than three control genes is unnecessary for the analysis. Using the geometrical mean, we conclude that *GAPDH* and *18S rRNA* are the most stable genes that can be used for normalization of qPCR data in experiments with bovine early conceptuses produced either in vivo or in vitro.

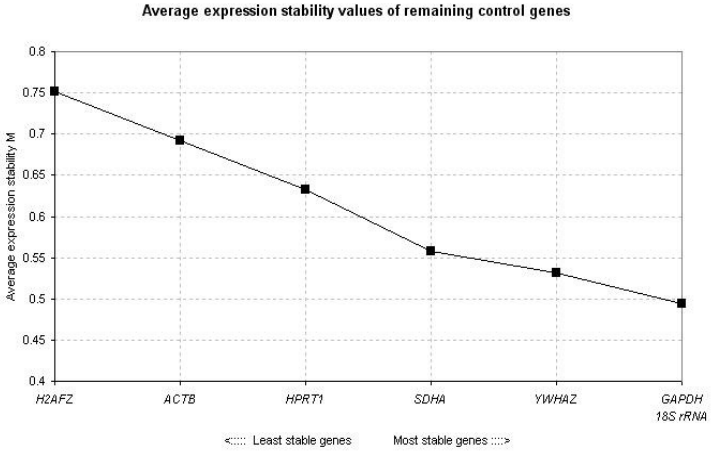

Figure 1. Gene expression stability of candidate reference genes in early elongating conceptuses analyzed by geNorm program which proceeds to the stepwise exclusion of the genes whose relative expression levels are more variable among samples. Lower values of M corresponded to the most suitable genes for normalization.

Notes

EFFECTS OF LEPTIN ON IN VITRO EMBRYO DEVELOPMENT OF PREPUBERTAL BOVINE OOCYTES

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Leptin has been shown to exert positive effects during the maturation of adult bovine oocytes, influencing blastocyst development, apoptosis, and the transcript levels of developmentally important genes. The present study was performed to analyze the differential effects of leptin on prepubertal bovine oocytes and cumulus cells. In particular, we evaluated whether leptin treatment (0, 10, 100 or 1000 ng/ml) of oocytes during maturation improves their developmental capacity after fertilization (Experiment 1) and whether it affects the incidence of apoptosis in cumulus oocyte complexes (COCs) (Experiment 2). COCs were matured in serum-free medium containing 1 mg/ml polyvinyl alcohol (PVA) and 0, 10, 100 or 1000 ng/ml leptin or in medium supplemented with 10% foetal calf serum as a positive control. In Experiment 1, COCs were matured as described above, fertilized and cultured *in vitro.* The proportions of oocytes that cleaved and developed to the blastocyst stage were assessed at Days 2 and 8 post-insemination, respectively. In Experiment 2, COCs were matured as described above and fixed in 4% paraformaldehyde for TUNEL analysis to detect DNA fragmentation characteristic of apoptosis. Addition of leptin during oocyte maturation had no effect on cleavage rate after fertilization: control: 68.6%; LEP-0: 62.9%; LEP-10: 66.9%; LEP-100: 63.4%; LEP-1000: 60.9%. Likewise, no significant differences in blastocyst rates were observed when oocytes were matured in presence of 0 (8.4%), 10 (9.3%), 100 (6.7%), 1000 (8.2%) ng/ml leptin compared to control (9.4%). In Experiment 2, different concentrations of leptin had no effect on the proportion of TUNEL-positive cumulus cells, with percentages of apoptotic cells (~ 6.06%) similar to the control (4.96%). In conclusion, the addition of the leptin to the *in vitro* maturation medium of prepubertal bovine oocytes did not enhance oocyte development potential or reduced the percentages of cumulus cell apoptosis.

Notes

EFFECT OF ENUCLEATION PROCEDURES ON *IN VITRO* DEVELOPMENT AND BLASTOCYST QUALITY OF MOUSE CLONED EMBRYOS TREATED WITH VALPROIC ACID

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Somatic cell nuclear transfer (SCNT) is a very inefficient process due to the incompetence of the enucleated oocytes to remove epigenetic marks of the transferred nucleus and reprogram it into a totipotent embryonic state. To help the cytoplasts on playing this role, histone deacetylase inhibitors (HDACi), like valproic acid (VPA) have been included in SCNT protocols, enhancing cloning efficiency. However, up to date, only cytoplasts prepared by mechanical enucleation (ME) have been used in the studies with the HDACis. In our study, we wanted to compare *in vitro* embryonic development and blastocyst quality between mouse cloned embryos treated with VPA that were prepared from ME, chemically-assisted (AE) and chemically-induced (IE) enucleated oocytes.

Oocytes and donor somatic cells were collected from B6CBAF1 (C57BL/6JxCBA/J) female mice. All micromanipulations were performed in H-CZB medium with 5 µg/ml cytochalasin B (CB), using piezo-actuated micropipettes. A total of 268 oocytes were prepared by ME. In AE, oocytes were treated with 0.3 µg/ml nocodazole (NOC) in KSOM for 30 min and cortical protrusions, present in 97.8% of the treated oocytes (n= 221), were mechanically removed. A total of 242 and 188 cytoplasts prepared by ME and AE, respectively, were successfully reconstructed. In experiments of IE, SCNT was performed by reverse-order. Oocytes were first individually injected with a cumulus cell nucleus. Afterwards, they were pre-activated with 7% ethanol for 5 min and treated with NOC for 15 min in KSOM. At 90 min post-activation (p.a.), completely or partially extruded second polar bodies, observed in 90.2% of the injected oocytes (n= 319), were mechanically-aspirated. Regardless of the enucleation procedure, oocytes were cultured for 2-3 h post-SCNT in KSOM and then for 6 h in Ca2+free CZB medium supplemented with 10 mM SrCl2 and CB, in the continuous presence of 4 mM VPA. At the end of the treatment, all embryos were washed and cultured *in vitro*. Embryos that reached blastocyst stage at 96 h p.a. were differentially stained for counting of inner cell mass (ICM) and trophectoderm cells. Parthenogenetically-activated oocytes were used as control. Results of cloned embryos are shown in table 1.

Embryonic development rates of cloned embryos were very similar between ME and AE groups, and significantly lower than control. The highest rates of blastocyst formation were achieved with AE-cytoplasts, although no differences were found compared to ME. The mean number of ICM cells in cloned blastocysts was equivalent between all groups and control (17), however, blastocyst obtained from parthenogenetic oocytes had a significantly higher total cell number (83). Studies are currently being performed to determine *in vivo* development potential of the cloned embryos.

Enucleation method	No. of oocytes with pronuclei	SCNT-embryos developed to (%)			Blastocysts		
		Two-cell	Morula	Blastocysts	No.	Mean cell number (± SD)	
						Total	ICM
ME	224	210 (93.6)[a]	151 (66.8)[a]	73 (32.5)[a,b]	35	63 (12)[a]	16 (4)[a]
AE	175	161 (91.8)[a]	110 (63.6)[a]	64 (36.5)[a]	33	59 (14)[a]	16 (4)[a]
IE	264	217 (82.3)[b]	117 (44.5)[b]	68 (25.3)[b]	31	67 (15)[a]	15 (5)[a]

[a–b] Values with different superscripts within the same column differ significantly ($p < 0.05$).

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Notes

EFFECT OF MEDIA SUPPLEMENTATION ON GENE EXPRESSION OF IN VITRO PRODUCED BOVINE EMBRYOS

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Media composition during maturation and culture can influence embryo quality by affecting morphology, metabolism, cryotolerance, lipid content and expression profiles of specific transcripts. The aim of this study was to compare the effect of media supplementation with either fetal calf serum (FCS) or bovine serum albumin (BSA) on the relative expression of genes related to metabolism and development in in vitro produced bovine blastocysts. A total of 431 immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of heifers and cows collected at slaughter. COCs were matured for 24 h in TCM-199 supplemented with 10 ng/ml epidermal growth factor and either 10% FCS or 4 mg/ml BSA. For IVF, matured COCs were inseminated with frozen–thawed percoll-separated bull semen. At 20 h post insemination, presumptive zygotes from each group were denuded and cultured in synthetic oviduct fluid (SOF) supplemented with either with 5% FCS or 4 mg/ml BSA. Day 7 blastocysts were snap frozen in groups of ten and stored at -80 ºC until extraction of poly(A)mRNA. Relative mRNA abundance was measured by quantitative RT-PCR for nine genes related to glucose metabolism (*SLC2A1*), lipid metabolism (*ACSL1*, *ACCA*, *FADS1* and *CYP51*) and embryo development (*IGF2R*, *UBE2A*, *DNMT3A* and *TDGF1*). Statistical analysis was performed by ANOVA.

Supplementation with BSA during both IVM and IVC resulted in a lower blastocyst yield than supplementation with FCS group, although the difference was not significant (37.3±3.8% vs 29.3±4.9% for FCS and BSA, respectively). Significant differences in relative mRNA abundance ($p<0.05$) were observed for *IGF2R*, *FADS1*, *CYP51* and *TDGF1* (Fig. 1). In conclusion, media composition in IVM and IVC, in particular, the presence of serum, can alter gene expression patterns, especially for imprinted genes (*IGF2R*) and genes related with lipid metabolism.

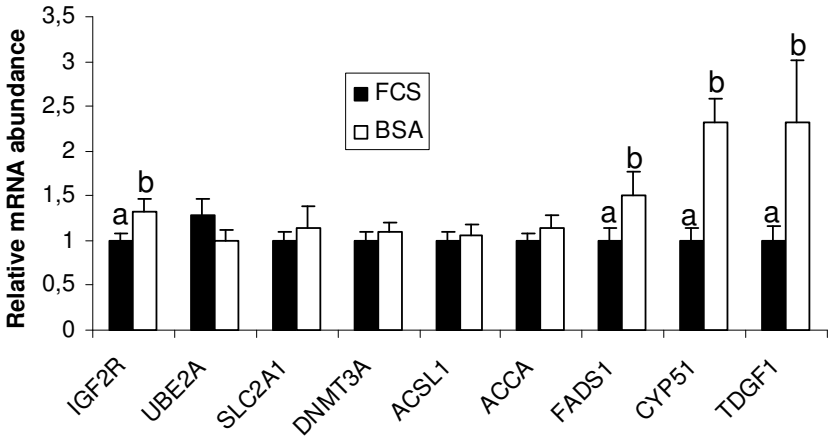

Figure 1: Relative abundance of various developmentally important gene transcripts in bovine blastocysts produced in synthetic oviduct fluid in the presence of FCS or BSA in IVM and IVC.

Notes

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SUPEROVULATION BY SPLIT FSH DOSE IN BEEF COWS

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The aim of the present study was to determine the efficiency of a superovulatory treatment which aims to minimize the number of FSH-applications to two days (split dose), to reduce the number of interventions in the beef cattle, where the management turns out to be more complicated than in dairy cows.

Sixty pure breed beef cows (Blonde D´Aquitaine) were randomly assigned to two groups and treated with two different superovulatory treatments.

Animals in the Control Group (n=30) received a CIDR device (Pfizer, Spain) for 12 days. At the same time of CIDR insertion and 5 days later, all animals received 2 mL (0.15 mg) of PGF (Veteglan, Calier, Spain). A 5 mL of GnRH (Fertagyl, Schering-Plough, Spain) was administered twice, 36 hours after second PGF and 24 hours after CIDR removal. Superovulation was started 36 hours after the first GnRH application using 13 mL (650 iu) FSH (Pluset, Calier, Spain) given as injections twice daily in 8 decreasing dosages. Luteolysis was induced with 0.15 mg PGF given twice 12 hours before and at the time of CIDR removal.

The experimental group (n=30) received the same treatment except for the FSH administrations that were substituted on Day 8 by a 162.5 iu (25% of the total FSH dose) intramuscular injection, a 325 iu (50%) subcutaneous retro scapular injection and, 48h later, a 162.5 iu (25%) in a subcutaneous retro scapular injection. Likewise, only one 0.15 mg PGF was given at the time of CIDR removal.

Animals from both groups were inseminated with frozen-thawed semen 12 and 24 hours after the onset of standing oestrus. Embryos were recovered by nonsurgical flushing 7 days later.

In total, superovulated animals from the control group delivered 348 embryos (11.23 ± 8.00) and 156 transferable embryos (5.20 ± 4.34). The superovulation treatment in donor animals from the experimental group resulted in totally 337 embryos (11.60 ± 5.42) and 223 transferable embryos (7.43 ± 5.70). Data were analyzed using One-way ANOVA (Student-Newman-Kleus). No significant differences were found between groups neither in the total numbers of embryos nor in the numbers of transferable embryos.

In conclusion, in beef cows superovulation treatments using either a 4 days (8 doses of FSH – control group) or 3 days application scheme (split dose – experimental group) result in similar collection rates and numbers of transferable embryos.

Notes

PIGLETS BORN AFTER TRANSFER OF PIG MATURED OOCYTES VITRIFIED USING OPS METHOD

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Recently considerable progress has been achieved in the vitrification of porcine embryos, although there has been no offspring born from the vitrified oocytes in this species. Moreover the problem of using media with protein (FCS or BSA) for oocytes and embryo cryopreservation has not been definitively resolved. The objective of the experiment was to examine the effect of vitrification solution with or without fetal calf serum (FCS) on in vivo survival of matured pig oocytes vitrified using OPS method.

Oocytes matured in vivo were recovered from the oviducts of superovulated gilts 32-34 h after hCG injection by flushing with Dulbecco's PBS solution. Morphologically normal oocytes at metaphase II were vitrified in following vitrification solution: group 1. – 2.5 M DMSO + 3.2 M ethylene glycol (EG) + 0.6 M sucrose (S) in HEPES/TCM-199 and 20% FCS or group 2. – 2.5 M DMSO + 3.2 M EG + 0.6 M S in TCM without FCS. The vitrification was performed in OPS straw using the method described by Vajta et al. () with some modifications. The straws were stored in the LN_2 for about 2 months. The warming solution was TCM-199 containing various concentrations of sucrose. Oocytes with normal morphology after warming were transferred to synchronized and inseminated recipient-gilts.

After transfer of 48 oocytes from group 1 and 67 from group 2 into 4 recipients, two gilts that received vitrified oocytes from group 1 were pregnant. As a results, 12 piglets were born, 4 of them were identified as originated from vitrified oocytes transferred to recipients. The identification was done through analysis of polymorphic dinucleotide repeat locus.

Notes

START UP OF MIDATEST OPU-IVP STATION: FIRST RESULTS OF EMBRYO PRODUCTION

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New genetic tools associated with Marker Assisted Selection (MAS) progressed very rapidly those last years. To optimize the use of genomic tools in breeding programs, the application of biotechnologies may be adapted to obtain high number of candidates (Humblot et al., 2010). Therefore, MIDATEST opened in October 2009 an OPU-IVF station in Denguin (South West of France) in collaboration with UNCEIA to produce in vitro embryos from high genetic merit females. In order to assess the efficiency and regularity of this new lab, the IVP results were recorded during two successive periods: period I (October to December 2009) and period II (January to March 2010). Oocytes from slaughterhouse ovaries as well as from two non pregnant Brown cows submitted to Ovum Pick Up (OPU) were used during both periods. Non pregnant Holstein heifers were only collected during the second period. Animals were stimulated with 5 injections of pFSH (Stimufol®, Reprobiol; total dose in heifers: 250 µg ; cows: 350 µg pFSH) 12 hours apart in decreasing doses. OPU was performed 12 hours after the last FSH injection. Oocytes were in vitro matured in M199 supplemented with FCS, FSH/LH, estradiol and EGF for 22 hours at 38.5°C. They were then fertilised with frozen – thawed semen in TALP medium using different bulls. Presumptive zygotes were cultured in SOF medium (Minitub) plus 1 % cow serum up to day 8 at 38.5 °C in 5% CO2, 5% O2 , 90% N2 atmosphere with maximum humidity. Number of blastocysts was recorded on day 6, 7 and 8. Embryo development was analyzed by Chi square analysis.

Cleavage and blastocysts development rates from slaughterhouse oocytes (table 1) were significantly higher ($p<0.001$) in period II compared to period I. Regarding oocytes issued from OPU sessions, the blastocysts rate was also significantly increased during period II compared to period I in Brown donor cows, with high numbers of blastocysts per session (Table 2). A breed effect was also observed. In period II, cleavage and blastocysts rates were significantly higher in Holstein heifers than in Brown cows, without any difference in numbers of blastocysts produced per OPU session.

Table 1: Cleavage, blastocyst of bovine oocytes from slaughterhouse ovaries

	Inseminated oocytes	Cleavage (%)	Blastocysts (%)
Period I	425	290 (68)[a]	67 (16) [a]
Period II	792	607 (77)[b]	281 (35) [b]

Table 2: Number of collected oocytes and blastocysts produced from OPU sessions

	Breed	OPU sessions	Collected oocytes	Cleavage (%)	Blastocysts (%)	Blastocysts /session
Period I	Brown cows	6	143	65 (45) [a]	11 (8) [a]	1.8 [a]
Period II	Brown cows	10	227	124 (55) [a]	49 (22) [b]	4.9 [b]
Period II	Holstein heifers	4	53	40 (75) [b]	24 (45) [c]	6 [b]

These results show a significant increase in embryo production from oocytes collected at slaughterhouse or by OPU during the second period. This confirms that even with skilled technicians and predefined validated procedures, a minimum period of 3 months with slaughterhouse ovaries is needed to stabilise embryo production and get used to equipment and materials. The efficiency has to be verified with systematic records of blastocysts rate together with media and procedures characteristics. To complete the start up, a minimum number of OPU training sessions have to be performed to settle different in vitro techniques.

Notes

CORRELATIONS BETWEEN ENZYME ACTIVITIES AND PARAMETERS OF BOAR SEMEN

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The aim of the study was to establish possible correlations between specific activities (U/mg protein) of the enzymes, involved in main metabolic sperm processes – alkaline phosphatase (AP), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT) and creatine kinase (CK), isolated from boar spermatozoa and the parameters of boar semen.

Sperm ejaculates from 18 boars were investigated, which were divided into 2 groups, depending on sperm parameters. The following parameters were accepted as a normal: concentration > 150 mln/ml; mobility > 75%; morphologic pathology < 12%; survival on 24 h > 50%; ejaculate volume - min. 200 ml; as a pathological – values below these parameters. The extenders "Sredets", BTS and BTS Gold were used for the semen dilution.

After separation of the sperm plasma, sediments were treated with distilled water and Triton-X 100 to obtain the extracts from spermatozoa.

Ejaculates with normal sperm parameters showed a positive correlation between mobility and concentration of sperm and AP (r = 0.63) and LDH (r = 0.66) activity in water-soluble extract as well as in Triton-X 100 extract. The positive correlation AP-GGT (r = 0.64) was only found in water-soluble extracts, whereas a correlation between AP- LDH (r = 0.83) was observed for both extracts.

Ejaculates with abnormal sperm parameters did not exhibit any correlation between semen parameters and the enzymes studied.

In semen diluted with BTS and BTS Gold extenders, the activity of LDH (r = 0.62) in Triton-X 100 extract corresponds with morphologic pathology spermatozoa. Water-soluble extracts from semen diluted with extender BTS concentration of spermatozoa positively correlated with GGT (r = 0.53). Using BTS Gold extender a correlation between CK and LDH and concentration of boar sperm was found.

In conclusion differences in the correlation between ejaculates with normal and abnormal sperm parameters and the presence of different isoforms of boar sperm enzymes were found. Despite using different extenders, AP and LDH could be used as biological marker of semen quality.

Notes

SEASONAL EFFECT ON MOET PROGRAM SUCCESS IN PROLIFIC ROMANOV SHEEP

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Multiple ovulation and embryo transfer (MOET), an indispensable tool for the implementation of genetic improvement and conservation programs of endangered livestock, is the method of choice for germplasm health control and exchange (Thibier and Guérin, 2000) and allows for accelerated genetic progress (Colleau et al., 1998). The success of ovarian response to superovulatory treatment is dependent on factors inherent to each individual animal, the breed being used, season of the year and nutritional status (Mitchell et al., 2002). Sheep are seasonal breeders and several reports indicate a seasonal effect on MOET performance. Romanov is known as one of the most prolific breeds such as Finnsheep, Booroola Merino, Barbados Blackbelly and British Milk Sheep and its reported that Romanov, are less influenced by season. No previous data exists on the response of this breed to ovarian stimulation and this experiment was therefore carried out to assess the effect of season (Fall, Winter, Spring) on the superovulatory response and embryo quality in the prolific Romanov breed. A total 17 Romanov donors were superovulated using FSH-p with 200 mg NIH-FSH-P1 (total of 20 ml) (Folltropin-V; Vetrepharm, Canada) applied in 8 decreasing doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, 1 ml i.m. at 12 h intervals, starting 60 h before sponge withdrawal. Donors recieved 1ml esturumate and 100 I.U. PSMG 36h prior sponge removal and an additional 200 I.U. PMSG were injected at sponge removal. Donors underwent intrauterine insemination with fresh diluted semen (10 x 107) 40 h after sponge removal. Ewes were tested for estrus and 6 or 7 d later were laparotomized and surgically flushed to recover embryos. The number of corpora lutea (CL), the total number of embryos and of viable embryos were recorded. Embryo recovery was performed according to a procedure previously described (Naqvi et al., 2000). The overall success rate of MOET programs depends on not only the ovulation rate achieved, but also on the fertilization and embryo recovery rate. In these trials, as it is shown in Table 1. non of the parameters related with program successs differed among seasons.

Table 1. Effect of season on superovulatory response and embryo production in Romanov ewes

Attributes	Fall	Winter	Spring
No. of ewes	6	4	7
Ovulation rate (no. of CL/ewe) mean ± S.E.	16.43± 2.37	18.67±3.63	20.14±2.38
Superovulatory response (no. of ewes>3CL)	6	4	7
Transferable embryo no.	9.29±2.59	14.33±3.95	8.00±2.59
Unfertilized egg no.	0.57±0.73	1.33±1.11	2.57±0.73
Embryo recovery rate % (no. of embryos/CL)	52.14	40.29	77.33

Notes

DESIGN OF A MINIMALLY INVASIVE SURGICAL APPROACH TO THE PIG OVIDUCT FOR IN VIVO OBTAINING OF OVIDUCTAL FLUID

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The obtaining of oviductal fluid from living animals has been approached for years by different methods with the goal of getting a precise knowledge of this milieu, useful for the development of in vitro reproductive technologies (Besenfelder et al., 1998). Chronical cannulation has demonstrated to produce adverse effects (Leese et al., 2008) and acute approaches by laparatomy cannot be repeated many times in the same animal. The present work was aimed at defining a minimally invasive surgical protocol to approach to the pig oviduct. This goal might enable in vivo sampling of oviductal fluid from the same single sow throughout the different phases of the estrous cycle.

Laparoscopy was used to approach the right and left oviducts in one Large White sow weighing 75 Kg. The animal was placed in lateral recumbence after having produced a pneumoperitoneum (10 mmHg CO_2). A 10 mm diameter trocar was aimed at a lateral localization to obtain a passage (port) for the laparoscopic optics. Right and left oviductal approaches required lateral recumbence combined with Trendelenburg, and three additional portals for the laparoscopic instruments, all in the abdominal wall: one at subcostal position, another near the paralumbar fossa and a third one between the previous two, like a triangle.

When approaching the oviduct, the infundibulum was manipulated to locate the abdominal opening. The mesosalpinx was sustained with an Endograsp® whereas fenestrated grasping forceps were used to introduce a 4 French catheter (Angiodynamics®) into the oviduct. A second catheter was also tried, a more rigid 4 French catheter (Cook®), so as to pass a guide wire through a microlaparoscopy sheath. Folding the oviduct wall over the catheter was more effective than directly pushing the catheter through the oviduct lumen.

This study demonstrates that a laparoscopic approach to the pig oviduct is feasible; however a trained laparoscopy surgeon is required. The technique requires placing the patient in lateral recumbence and using catheter of appropriate rigidity so as to progress within the oviduct lumen without the risk of boring the wall. A training period with laparoscopy simulators on postmortem reproductive apparatus is strongly recommended so as to reduce the surgery timing, gain the necessary skills and try with different surgical instruments.

Notes

FFECT OF DIFFERENT BATCHES AND CONCENTRATIONS OF PLANT PROTEIN ON DEVELOPMENTAL COMPETENCE OF PORCINE ZYGOTES CULTURED *IN VITRO*

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In a previous study we demonstrated the possibility of replacing bovine serum albumin with plant protein (PP, Animal Pharma B.V., Hengelo, The Netherlands) in porcine embryo culture medium (Gajda et al. 2009; *Reprod. Fertil. Dev*, 21(1): 157). The aim of the present study was to investigate the effect of four different batches of plant protein on development of cultured porcine embryos. Moreover we determined an optimal concentration of plant protein in porcine embryo culture medium. The experiment was done on pig zygotes obtained surgically from superovulated gilts at 24-26h after insemination. Morphologically normal zygotes were cultured *in vitro* in 5% CO_2 in air at 39^{o}C in NCSU-23 medium supplemented with 0.002 g mL^{-1} (group Exp 1), 0.004 g mL^{-1} (group Exp 2), 0.008 g mL^{-1} (group Exp 3) of PP for every PP (I, II, III, IV) batch or 0.004 g mL^{-1} BSA (control). Embryo quality criteria were cleavage (on Day 2 after *in vitro* culture), morula (on Day 4) and blastocyst (on Day 6 to 8) rates. The results are presented in table 1.

Table 1. Effect of different batches and concentration of plant protein (PP) on developmental competence of porcine zygotes.

PP batches	Treatment group	No. of zygotes cultured	No. of cleaved embryos (%)	No. of morulae (%)*	No. of blastocysts (%)*
I	Exp 1	22	18 (81.8)	14 (77.7)	10 (55.5)
II		10	10 (100)	10 (100)	3 (30)
III		10	9 (90.0)	8 (88.8)	4 (44.4)
IV		10	10 (100)	8 (80)	5 (50)
I	Exp 2	22	16 (72.7)	14 (87.5)	10 (62.5)
II		10	9 (90.0)	8 (88.8)	4 (44.4)
III		10	10 (100)	10 (100)	2 (20)
IV		10	10 (100)	10 (100)	4 (40)
I	Exp 3	22	18 (81.8)	16 **(88.8)**	13 **(72.2)**
II		10	10 (100)	10 (100)	5 (50)
III		10	10 (100)	10 (100)	0
IV		10	10 (100)	10 (100)	6 (60)
	Control	22	17 (77.3)	13 **(76.5)**	14 **(64.7)**

*as a percentage of cleaved embryos

In conclusion, this study indicates that there are differences between particular plant protein batches. This product isn't stabilized yet. The results demonstrated that the development of porcine embryo batch of plant protein with 0.008 g mL^{-1} concentration was higher than in control group with 0.004 g mL^{-1} of BSA.

Notes

ACCURACY OF TRANSRECTAL ULTRASONOGRAPHY: AN EVALUATION OF THE SUPEROVULATORY RESPONSE IN ANGLO-NUBIAN DONOR GOATS

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The use of embryo transfer (ET) has positively impacted the genetic gain in animals leading to a better performance. However, the success of embryo collections following different hormonal protocols still shows a high variability, especially in relation to ovarian response. Therefore, this study aimed to evaluate the accuracy of the transrectal ultrasound scan for the estimation of superovulation (SOV) in donor goats undergoing hormonal protocols of short duration. Pluriparous Anglo-Nubian goats (n = 08) were used as embryo donors in different periods: the rainy season and dry season. In experiment I (rainy season) all donors were consecutively introduced to the three different embryo collection procedures according to the treatment protocols of the three groups (GI, GII and GIII; cross-over). In GI (n = 08) estrus was synchronized by the use of CIDR for eight days. On the sixth day 0.10 mg cloprostenol was administered i.m. and SOV was started with 250 IU FSH-p followed by additional 5 i.m. applications in 12 hour intervals. GII (n = 08) animals were treated according to protocol GI including the i.m. application of 5 mg of estradiol benzoate (EB) at the time of CIDR insertion. GIII (n = 08) donors received a CIDR for 11 days. On the ninth day, SOV was initiated using the same scheme described for the other groups and 0.10 mg Cloprostenol injected i.m.. Estrus observations and inseminations were performed 12 and 24 hours after the CIDR withdrawal. 24 hours after the last insemination donors received again a CIDR. The embryos were flushed via the transcervical route on day 7 after estrus, and the viable embryos were directly transferred to recipient animals. Ultrasound examination was performed twice daily during estrus to evaluate the ovarian response to SOV. In experiment II we used the same methodology described for the first trial, but the programme was performed during the dry season. In the experiment I the use of ultrasound scanning of the ovaries for evaluation of SOV responses did not show a significant difference ($p \geq 0.05$) between groups (Means: GI 6.4±0.479, GII 7.0±0.327 and GIII 7.1±0.498). In the experiment II the number of follicle decreased significantly in GI (5.3±0.313, $p < 0.05$), whereas no difference ($p \geq 0.05$) between GII (6.6±0.263) and GIII (6.5±0.500) was found. Regarding the recovery of the total number of embryonic structures no difference ($p \geq 0.05$) between GI (7.1±0.718), GII (8.3±0.620) and GIII (8.6±0.844) was noted in experiment I. However, in experiment II a lower embryo recovery ($p < 0.05$) in GI (6.0 ± 0.500) was observed, but these results remained constant in the GII (7.3 ± 0.412) and GIII (7.4 ± 0.625). The accuracy of ultrasonographic evaluation of SOV ranged from 62.5 to 37.5% in females from GI and GII for both seasons: wet and dry. However, this rate only reached 25% for donors from GIII in experiments I and II, which was significant lower ($p > 0.05$) compared to animals from GI and GII. Data were analyzed with 5% probability by Tukey test. Therefore, we can conclude that the protocols of short duration are satisfactory to synchronize estrus in donor goats, as well as the BE favors the response to SOV during the dry season. It is still permissible to conclude that the ultrasonographic evaluation of the response to SOV in donor goats demonstrated a satisfactory accuracy, especially when follicular response is poor.

Notes

DEVELOPMENTAL ABILITY OF BOVINE OOCYTES AFTER IVM IN A COMMERCIAL DEFINED SERUM-FREE MATURATION MEDIUM

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Serum is a commonly used supplement for both oocyte maturation and embryo culture in bovine. Serum contains unknown factors and varying concentrations of beneficial substances supporting cumulus cell expansion and embryo development: energy substrates, amino acids, vitamins, growth factors, chelators of heavy metals. However, use of serum during in vitro maturation (IVM) results in either markedly stimulatory or inhibitory effects on embryonic development due to its biological source and the presence of undefined components affecting cellular processes. Moreover, it may contain some pathogens representing a source of medium contamination. StemAlpha.1 (ref 7001, www.stemalpha.com) is a commercially defined medium with non-animal and human-derived components specifically designed for the transport of human tissues.

The aim of this study was to examine the effect of StemAlpha.1-based serum free medium during IVM on embryo development in a serum-free embryo co-culture system.

Three replicates of slaughterhouse-derived oocytes were randomly divided into four IVM groups: **1/**M199 + 10% Fetal Bovine Serum (FBS); **2/**StemAlpha.1; **3/**M199 + 10% FBS on granulosa cell (GC) monolayers and **4/**StemAlpha.1 on GC. For each group, maturation medium was supplemented with 10 µg/ml FSH and 5 ng/ml EGF. After a 22 h maturation period, matured oocytes were incubated in fert-TALP with frozen-thawed semen for 18 h. Presumptive zygotes were cultured in drops of 50 µl B2-medium (CCD Laboratories, Paris, France) seeded with Vero cells. Culture droplets were renewed at day 4 post-insemination. Cleavage rates were assessed on day 2 post-insemination. Developmental data were recorded on days 6, 7 and 8 for morulae, blastocyst and expanded/hatched blastocyst stage embryos, respectively.

		Number of oocytes/embryos (%)				
IVM group	**n**	**Fertilized**	**Cleaved**	**Morulae D-6**	**Blastocysts D-7**	**Expanded & Hatched D-8**
1/M199-FBS	251	237 (94.4)	224 (89.2)	76 (30.3)	65 (25.9)	70 (27.9)[ab]
2/StemAlpha.1	251	234 (93.2)	218 (86.9)	74 (29.5)	62 (24.7)	72 (28.7)[ab]
3/M199-FBS + GC	251	237 (94.4)	221 (88.0)	87 (34.7)	71 (28.3)	90 (35.9)[a]
4/StemAlpha.1 + GC	255	242 (94.9)	228 (89.4)	80 (31.4)	58 (22.7)	70 (27.5)[b]

Different letters within columns differ significantly ($P<0.05$).

Fertilization, cleavage and embryo development on days 6 and 7 rates did not differ between oocytes matured in M199+10% FBS or in StemAlpha.1, with or without GC monolayers. The rate of expanded and hatched blastocysts on D-8 was significantly higher when COCs were matured in M199 +10% FBS on granulosa cell monolayers in comparison to the StemAlpha.1 + GC group.

In commercial embryo production, there is a demand for using solely non-animal-derived components. The commercially defined StemAlpha.1-based medium could provide a convenient system for in vitro maturation and experimental purposes, such as investigation of single molecules effects on specific pathways or embryo development.

Notes

EFFICIENCY OF DIFFERENT PROGESTERONE DEVICES (CIDR, PRID) FOR OESTRUS SYNCHRONISATION IN BOVINE EMBRYO TRANSFER RECIPIENTS

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As recipients play an important role in the success of embryo transfer programs this study was designed to compare the efficiency of two different progesterone applications (PRID alpha and CIDR) for oestrus synchronisation of heifer recipients. Sixty-eight Simmental heifers randomly received an intravaginal device (PRID alpha: 43 animals, CIDR: 25) for 7 days. One day prior to removal, a prostaglandin analogue (2 ml Cloprostenol, Estrumate®) was applied to all animals. Oestrus detection was carried out 48 to 72 hours following device removal. Suitability for embryo transfer based on rectal palpation of a corpus luteum (CL). Embryos were transferred 9 days following removal of device. Pregnancy diagnosis was performed 21, 30 and 42 days following oestrus. Blood samples were taken to analyze plasma progesterone concentrations 3 days before application of the intravaginal device, day of insertion of device and days 2, 6, 7, 8, 9, 10, 16, 23, 30, 39 and 51 following application of device.

Three animals out of 43 lost the PRID alpha and one out of 25 the CIDR. Average concentrations of progesterone in plasma were generally similar between treated heifers. However, during the first two hours after insertion of device, animals carrying a PRID alpha device had a higher concentration of progesterone in plasma than those having received a CIDR. Oestrus was observed in 95 % (38 out of 40) and 100 % (24 out of 24) of animals that were treated with PRID alpha and CIDR, respectively. Of these animals 89.5 % (34 out of 38) and 92.0 % (22 out of 24) in the group which received PRID alpha and CIDR, respectively were classified as suitable for embryo transfer. Although there were no differences in pregnancy rates at day 21, rates were significantly lower at day 30 and 42 in those animals treated with PRID alpha (16 out of 34, 47 %) than in animals treated with CIDR (14 out of 22, 64 %).

This study reveals a significant influence of intravaginal device on pregnancy rates following timed embryo transfer.

Notes

MASSIVE DEREGULATION OF miRNAS FROM NUCLEAR REPROGRAMMING ERRORS AFFECTING REDIFFERENTIATION FOR PLACENTOGENESIS IN BOVINE SCNT PREGNANCY

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Somatic Cell Nuclear Transfer (SCNT) is a break-through technology with potential applications in agricultural and biomedical research. However, the technology is hindered by very low rates of live birth due to high incidence of placental abnormalities. The major source of these abnormalities is thought to be due to genetic and epigenetic modifications arise from improper reprogramming of the donor cell after nuclear transfer, which leads to post-implantation lethality to embryo with ultimate placental defects. MicroRNAs have been evidenced as an important modifier that regulates gene expression epigenetically at the post-transcriptional or transcriptional level. However, the role of miRNAs for genetic and epigenetic abnormalities in placenta derived from SCNT is unknown.

Here we aim to elucidate the difference in expression profile of miRNAs in the placenta (at day 50) derived from artificial insemination (AI), in vitro production (IVP), SCNT pregnancy and in the donor cells (fibroblast from the same bull used to generate AI & IVP derived pregnancies) by quantifying 377 miRNAs along with their processing machinery genes. The study identified a massive deregulation of miRNAs and their processing genes in the placenta from cloned Pregnancy. Most of the miRNAs were found as not to be well reprogrammed and affected as large cluster residing in the specific chromosomal location. Of which, most interesting chromosomal location is Btau: 4.0:21:66000000-66044000 (44kb) harboring 4 miR clusters (bta-miR-379, bta-miR-495, bta-miR-539 and bta-miR-453) comprising 38 miRNAs and 3 important families of miRNAs (miR-329, miR-379 and miR-154 family) consisting of 25 miRNAs. Furthermore, cell specific expression of some interesting miRNAs in the expanded blastocysts of different sources by whole mount in situ hybridization and temporal expression of selected miRNAs in blastocyst, expanded blastocyst and elongated embryo (at day 15) derived from AI, IVP and SCNT by real time RT-PCR identified that the major difference in miRNAs expression arises at redifferentiation stage for placentogenesis.

The preliminary results of this study lead us to pre-conclude that aberrant re-programming of miRNA molecules during redifferentiation for placentogenesis leading to abnormalities of the placenta from cloned conceptus. Ongoing study on the relationship between miRNAs and epigenetic regulation will further elucidate the detailed mechanism of abnormal placentogenesis in cloned pregnancy which could be useful for somatic cell nuclear transfer technology.

Notes

SEMEN QUALITY, TOTAL PROTEIN CONTENT AND PROTEIN PROFILES OF SEMINAL PLASMA OF ALPACA (*Vicugna pacos*) FED DIFFERENT DIETS

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Advances in reproductive technologies reveal seminal plasma (SP) as a nutritive-protective medium for spermatozoa suspended in it. This complex physiological fluid contains various organic and inorganic components among which, a variety of proteins adsorb into plasma membrane during epididymal maturation and at ejaculation, affecting spermatozoal membrane stability and functions including fertilization. The potential influence of SP proteins on male reproduction has drawn attention because of their association with semen freezability and breeding scores in ruminants. Despite the relevance of SP proteins with fertility, studies on their precise natures and factors affecting their quantities and qualities are limited in alpacas. Therefore, this study was conducted to evaluate changes in total protein (TP) content and protein profiles (PP) of SP along with semen quality of alpacas maintained with different diets.

Five alpaca males were used to collect semen with an artificial vagina. Semen was collected once in a week, over a period of 6 months from March to September, 2009. The study period had four sessions with four types of diets: **I**.hay; **II**.hay+ pasture grazing; **III**. Sheep concentrate (gross protein 16%, lipids 2.7%, fiber 10%, +pasture grazing; **IV**. horse concentrate (gross protein 12%, lipids 3%, fiber 11%,) + pasture grazing. After proper evaluation of semen quality, samples were centrifuged (3500 r.p.m., 40 min) to separate SP to perform biochemical study including gel electrophoresis. The SDS electrophoresis was performed using two concentrations of polyacrylamide (14% and 16%) in the gel of separation. The gels were colored with cromassie brilliant blue and the intensity was analyzed with Sante Dicom Viewer and Image J program.

In this study there were great variations in TP and PP of SP along with semen quality of individual alpacas. TP (g/dl) were 4.5±4.04, 3.21±3.08, 3.06±2.93 and 6.38±2.83 in the sessions I, II, III and IV, respectively. Motility and concentration of spermatozoa were lower (18.5±8.8%, 10.44±23.35X10^6/ml respectively) during session IV. Assessment of the electrophoretic profiles of SP proteins showed a total of 7 bands having molecular weights from 200 to 14.00 kDa. Surprisingly, all bands were not present in all alpacas SP. Semen from males fed a high quality diet markedly differed and showed protein bands with molecular weights from 10 to 14 kDa and 31 to 45 kDa compared to semen of the other males. Moreover, there were significant changes in the percentage and quantity of similar protein profiles during the four sessions. Protein fractions of low molecular weights (14.40 to 31.00 kDa) were highest during session **II** and this fraction showed a positive correlation with sperm motility and concentration throughout the study.

These results demonstrate that there are marked changes in semen quality, TP and PP of SP of alpaca depending on diets. In ruminants, SP proteins vary with individual animal, season of collection, temperature, nutrition and stress, and are originated from seminal vesicles, which are normally absent in camelides. The origin, composition and function of SP proteins remain a mystery in alpacas. For successful preservation of alpaca semen we should define good quality semen before processing by determining the biological and biochemical makeup of SP. This study could help us to define different protein profiles in alpaca SP and could lead us to find out functions and the specific sources of these proteins in future.

Notes

INFLUENCE OF SEX SORTED SEMEN ON EMBRYO PRODUCTION IN DAIRY CATTLE

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Sex sorted semen became more readily available for commercial embryo flushings at the beginning of 2008 in Finland after which use of sex sorted semen has increased both in artificial insemination (AI) and in embryo flushings. The data was collected between January 2008 and May 2010 containing flushings (n=83) obtained using sex sorted semen exclusively in AI and using at least two inseminations into the uterine horns. All donors had either two or three AIs 9 to 15 hours apart. Instructions to the farmers were to use 2+2+1 straws in AIs. Majority of the straws contained 2 million spermatozoa; individual straws contained 5 million spermatozoa. Otherwise the embryos were produced by standard MOET protocol. The numbers of recovered transferable embryos, unfertilized oocytes (UFO) and degenerated embryos were compared to the results of flushings obtained using unsorted semen during the same time period. Poisson and logistic regression analysis with generalized estimating equations was used to assess differences in mean amounts and proportions of different embryo types between treatment groups (flushings obtained using sex sorted semen/flushings obtained using unsorted semen) and between heifers and cows. Using generalized estimating equations in the modeling, the correlation between observations due to the fact that same bull was used for several cows and heifers were taken into account.

The average numbers of transferable embryos, degenerated embryos and UFOs in flushings obtained by sex sorted and unsorted semen and also percentages of all recovered embryos are presented in Table 1.

Table 1. The average numbers of transferable embryos, degenerated embryos and UFOs in flushings obtained by sex sorted and unsorted semen.

	Heifers (n=45) Sorted semen Embryos (% of all embryos)	Heifers (n=465) Unsorted semen Embryos (% of all embryos)	Cows (n=38) Sorted semen Embryos (% of all embryos)	Cows (n=211) Unsorted semen Embryos (% of all embryos)
Transferable	7.2 (55)[a]	7.8 (66)[b]	5.2[c] (47)[c]	8.5[d] (68)[d]
Degenerated	2.4 (18)	2.0 (17)	2.2 (20)	1.6 (12)
UFOs	3.6[e] (27)[e]	2.0[f] (17)[f]	3.7[g] (33)[i]	2.5[h] (20)[j]
All	13.2 (100)	11.8 (100)	11.1 (100)	12.6 (100)

[a,b]$P < 0.01$ [g,h]$P < 0.05$
[c,d]$P < 0.001$ [i,j]$P < 0.01$
[e,f]$P < 0.01$

In summary, in heifers, numbers of transferable embryos equate in flushings obtained by sex sorted semen and unsorted semen whereas number of UFOs was significantly increased using sex sorted semen. In cows, flushings with sex sorted semen produce significantly less transferable embryos and significantly more UFOs than flushings with unsorted semen.

Notes

THE EFFICIENCY OF OVUM PICK UP AND IN VITRO EMBRYO DEVELOPMENT IN CHAROLAIS AND HOLSTEIN-FRIESIAN COWS

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The aim of the present study was to investigate the efficiency of ovum pick-up (OPU) and subsequent embryo development of oocytes collected from two cattle breeds. OPU was performed in 17 nonlactating donor cows (9 Charolais and 8 Holstein-Friesian). Cows were stimulated with pFSH, twice a day during two days (Folltropin®, Bioniche). Four OPU sessions was performed per cow. 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 subsequently cultured in vitro for 9 days. In order to study the kinetics of early cleavage, the number of cleaved embryos was recorded at 24, 27, 30, 33, 36 and 48 hpi. Embryo yield was recorded on day 5, 6, 7, 8 and 9 of IVC. Data were analyzed by Chi-square test. 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. The percentage of cleaved embryos at 24 hpi was significantly higher in Charolais donor cows (41.1% vs. 29.4%, $p<0.05$), but overall cleavage rate assessed 48 hpi showed no differences between breeds. In terms of blastocyst rate at day 5, 6, 7, 8 and 9 of IVC, no statistical differences were observed between breeds. These results show that a higher number of oocytes can be retrieved by OPU from Charolais donor cows compared to Holstein-Friesian. However, there were no differences in developmental competence of retrieved oocytes between breeds.

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

Animal breed	Number of follicles punctured per animal	Number of recovered ooc ytes per animal	Oocyte recovery rate per animal	Number of grade 1-2 oocytes per animal
Charolais	12.07±0.8[a]	7.2±0.7[a]	58.6±0.08	5.9±0,6[a]
Holstein-Friesian	8.5±0.7[b]	4.6±0.4[b]	54.6±0.06	3.5±0.5[b]

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

Figure 1. Kinetics of first cleavage following IVF of oocytes aspirated from Charolais and Holstein-Friesian cows

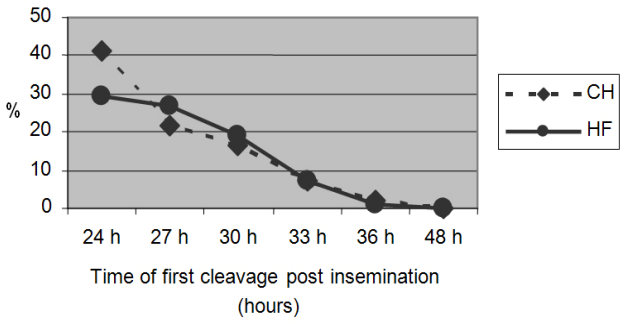
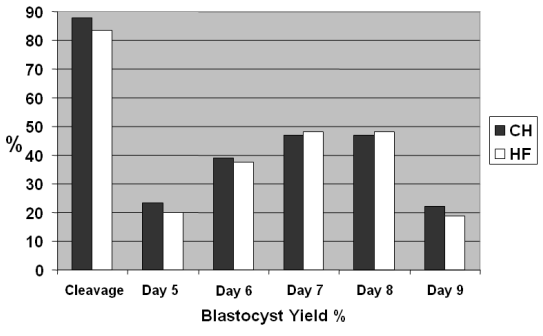


Figure 2. In vitro embryo development after IVF of oocytes aspirated from Charolais and Holstein-Friesian cows



Notes

ASSESSMENT OF PORCINE *ZONA PELLUCIDA* GLYCOPROTEIN 3 (pZP3) AND INTEGRIN BETA2 (ITGB2) mRNA AND PROTEIN LEVELS IN OOCYTES AFTER SINGLE AND DOUBLE EXPOSURE TO BRILLIANT CRESYL BLUE TEST

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The aim of the present study was to investigate the expression pattern of sperm-egg interaction molecules in oocytes after single and double exposure to BCB test.

In developmentally competent oocytes, assessed by determining the activity of glucose-6-phosphate dehydrogenase (G6PDH) using brilliant cresyl blue (BCB) test, real-time quantitative PCR reaction methods, western-blot and confocal microscopy analysis were applied to determine the transcript levels of porcine zona pellucida glycoprotein 3 (pZP3), and integrin beta 2 (ITGB2), as well as the levels of pZP3 and ITGB2 proteins.

We observed significantly higher levels of pZP3 mRNA in oocytes after single exposure to BCB test as compared to controls before and after IVM ($P<0.001$) and as compared to double staining ($P<0.05$). The level of ITGB2 mRNA was also statistically increased in gametes after single exposure to BCB test as compared to controls before IVM ($P<0.01$) and double staining ($P<0.05$). We did not detect any difference between oocytes after single exposure to BCB test and control oocytes after IVM. Western blot analysis demonstrated higher levels of pZP3 and ITGB2 protein in oocytes after single staining for BCB test as compared to controls both before and after IVM ($P<0.001$, $P<0.01$, respectively) and double staining ($P<0.05$). Confocal microscopic observations have revealed the same pattern of increased levels of pZP3 and ITGB2 expression after single exposure to BCB test. In both cases we detected specific cytoplasmic localization of both proteins. The ITGB2 protein has *zona pellucida* and membrane localization in control oocytes before IVM. After IVM and after single exposure to BCB test, ITGB2 was also strongly detected in the cytoplasm. In both cases, after double exposure to BCB test both proteins were detected only partially in the cytoplasm.

Our results suggest that (i) single exposure to BCB test may increase fertilization ability by up-regulation of sperm-oocyte interaction genes' expression, (ii) double exposure to BCB test may lead to degeneration of oocytes with inclusion of cytoplasm, and that (iii) the BCB staining test may be a reason of specific pZP3 translocation from the *zona pellucida* to the cytoplasm.

Notes

EFFECT OF SPIRULINA PLATENSIS AND SLENOPYRAN ON THE MOUSE OVARY ACTIVITY WITH RESPECT TO EMBRYO PRODUCTION

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The successful fertilization of the ovulated oocytes is a result of action of complex factors including the quality of germ cells as well as the whole physiological state of the female' organism. Our special scientific interest focuses on studies dealing with opportunities and mechanisms which may have a positive influence on the state of the female organism. The aim of the present work was to study the effect of two biological substances, microalgae Spirulina platentis and selenopyran (selenium organic compound), given to female animals before ovulation on the ovary state and the number and quality of in vivo produced embryos in mice.

Animal experiments were carried out in the accordance with the 86/609/CEE European Community regulation and Bulgarian National Law of veterinary medicine. 30 laboratory white mice of reproductive age were divided in three groups: Control group and two Experimental groups. The animals from the first experimental group received daily per os 0.01 ml 20% solution of Spirulina platentis per animal. The animals of the second experimental group were injected intraperitoneally (i.p.) in a 10 days interval over 30 days with an oil solution of the selenopyran (100 µg/kg body weight). Physiological parameters of blood such as a total protein, glucose content, selenium level and activity of GOT and GPT as well as the metabolic activity of ovaries estimating cytochrome C oxidase including histological traits were investigated. Embryo collection was performed using the standard protocol for superovulation in mice including natural mating. Embryos were counted and assessed under a light microscope.

The animals supplemented with biological active substances had a higher content of protein ($P<0.05$) and a lower level of glucose in the blood ($P<0.05$). The significant decline of GOT activity in both experimental groups confirms the good health status of the animals. The treatment of female animals with biological active supplements before ovulation also increases the embryo production in vivo. The highest number of embryos was recovered in the group treated with selenopyran compared to the control group and the Spirulina group (24 vs 17 vs 22). This effect is probably caused by the activation of the metabolic processes in the ovaries resulting in a higher quantity and better quality of ovulated oocytes. This assumption was confirmed by the histological analysis which revealed a higher number of ovulated follicles and yellow bodies as well as by higher activity of the cytochrom C oxidase in the ovaries of treated animals.

Notes

EFFECT OF LEPTIN DURING MATURATION ON IN VITRO DEVELOPMENT AND QUALITY OF BOVINE EMBRYOS

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Leptin plays a pivotal role in the regulation of body weight and energy expenditure, but next to this, evidence suggests that leptin plays also an important role in female reproduction. Several studies have been done to investigate if Leptin, added during in vitro maturation, can influence the outcome of in vitro production of bovine embryos. The results are variable, and limited information is available on the effect of Leptin on the quality of the embryo. The only study that we are aware of reported that maturation of bovine oocytes in the presence of Leptin reduced apoptosis (Boelhauve et al. 2005, BOR 73: 737-744).

The goal of this study is to investigate if we can improve the blastocyst rate of our in vitro production system by adding human recombinant Leptin during maturation. Furthermore we analyze the effect of Leptin on the quality of the embryos graded by IETS standards and on freezability by in vitro survival after thawing.

In our lab, in vitro production of embryos was performed using oocytes collected from slaughterhouse ovaries. Maturation was done in M199 with 10% FCS, FSH/LH and 1000ng/ml Leptin (n=1668) or without Leptin (n=1644) (Sigma, Missouri, USA). Fertilization and culture was performed in SOF_{AA} with BSA as described earlier (van Wagtendonk et al. 2000, Theriogenology 53: 575-597). At D8 the blastocysts rate and quality according to IETS standards were recorded (Table 1). From both groups approximately 50 embryos were frozen and upon thawing the survival rate was recorded after 24h (re-expansion) and 72h (hatching/hatched) (Table 1).

A significant increase of the blastocyst rate was found. The quality of the embryos was comparable in both groups.

Table 1. Number of oocytes, blastocyst rate at D8 with quality grades (IETS standards) and in vitro survival of blastocysts after freeze-thawing (re-expansion 24h after thawing and hatching/hatched 72h after thawing) in the control and the group matured with 1000ng/ml Leptin.

	Number Oocytes	% blastocyst D8	IETS grade (%)			Number blastocyst	In vitro survival upon thawing (%)	
			1	2	3		after 24h	after 72h
Control	1644	24 [a]	17	46	37	52	100	79
Leptin1000	1668	27 [b]	17	51	32	54	100	85

[a,b] Values with different superscript are significantly different (chi^2 test).

The addition of 1000ng/ml human Leptin had a positive effect on the blastocyst rate. The quality of the collected blastocysts was similar compared to the control group according to morphology and freezability.

Notes

ASCORBIC ACID EXPOSURE DURING BIOPSING AND FREEZING OF BOVINE *IN VIVO* EMBRYOS INCREASES CALVING RATE

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On dairy farms female calves are desired and sex diagnosis of embryo biopsies are often performed before transfer. Due to recipient management and embryo trade cryopreservation of embryos is an advantage. However, embryo viability is compromised after transfer of biopsied, frozen-thawed embryos. Lane et al., (2002) showed that a potent cellular anti-oxidant, ascorbic acid, improved survival of cryopreserved mice embryos. We aimed at investigating whether ascorbic acid supplementation during biopsing and freezing would enhance survival of biopsied frozen-thawed bovine *in vivo* embryos.

Day 7 embryos were produced by standard superovulation and embryo flushing of donor animals with high breeding value on Finnish dairy farms. The primary objective of the project was to produce female calves, whereas male embryos were utilized for *in vitro* studies (data not shown). Embryos were biopsied manually with a microblade: control embryos in Dulbecco´s PBS (DPBD), and ascorbic acid (AC) embryos in DPBS + 0.1 mM L-ascorbate (A4403; Sigma, St Louis, USA). Biopsies were analyzed for sex with Ampli-Y-kit (Finnzymes Oy, Espoo, Finland). Embryos were allowed to recover after biopsing for 1-1.5 h before freezing them either with 1.5M ethyleneglycol (EG) (control), or EG + 0.1 mM L-ascorbate (AC) using standard slow-freezing protocol. Frozen-thawed female embryos were transferred to recipient heifers or cows on dairy farms. Pregnancies were monitored rectally or with ultrasound depending on the routine used in each farm.

Pregnancy rates of 37 % have been reported after transfer of biopsied and frozen-thawed embryos (Hasler 2002). In this study, a pregnancy rate of 45 % was achieved for both experimental groups (Table 1). Calving rate was, however, lower due to high rate of abortions. AC-embryos exposed to L-ascorbate during biopsing and freezing resulted in higher calving rate compared to control embryos (31% vs 22%, respectively). This was due to the fact that control embryos were more often aborted (first heat after transfer later than three weeks) than AC-embryos (22% vs 14%, respectively). In conclusion, L-ascorbate may protect embryos from the detrimental effects of biopsing and freezing. Addition of L-ascorbate to biopsing and cryopreservation media resulted ten percentage units more calves compared to control embryos.

Table 1. Effect of ascorbic acid exposure on pregnancy and calving rates of biopsied cryopreserved *in vivo* bovine embryos after transfer

	Recipient animals n	Pregnant % (n)	Non-pregnant % (n)	Aborted % (n)	Calf delivery % (n)
Control	67	44.8 (30/67)	55.2 (37/67)	22.4 (15/67)	22.4 (15/67)
AC	65	44.6 (29/65)	55.4 (36/65)	13.8 (9/65)	30.8 (20/65)

The study was supported by Regional Council of Pohjois-Savo, Embryocenter Ltd and FABA breeding.

Notes

POSTIMPLANTATION DEVELOPMENT OF CLONED RABBIT EMBRYOS RECONSTRUCTED WITH FIBROBLAST CELL NUCLEI

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The aim of our study was to determine the postimplantation developmental competences of nuclear-transferred (NT) rabbit embryos. The source of nuclear donor cells were *in vitro* cultured adult dermal or foetal fibroblasts cells. Enucleated *in vivo*-matured rabbit oocytes were the source of recipient cells. A single somatic cell was introduced into a perivitelline space of the enucleated oocyte and membrane fusion of the donor cell and recipient oocyte was induced by 3 successive DC pulses of 3.2 kV/cm for 20 μs each. The reconstructed oocytes were incubated in B2 medium for 1 h and subsequently treated with 5 μM calcium ionomycin for 5 min to be artificially activated. Then, nuclear-transferred oocytes were incubated in 2 mM 6-dimethylaminopurine (6-DMAP) and 5 μg/ml cycloheximide (CHXM) for 1 h. The cloned embryos were cultured *in vitro* in 50-μl droplets of B2 medium for 22 h. Afterwards, dividing NT embryos were transferred surgically through the infundibulum into oviduct lumen of recipient females. Pregnancy was determined by ultrasonography on Day 14 after embryo transfer. A total of 97 embryos reconstructed with foetal fibroblast cell nuclei were transferred in to the oviducts of 6 pseudopregnant recipients; 4 of these (66.7%) females became pregnant. A total of 101 embryos reconstructed with adult skin fibroblast cell nuclei were transferred to 6 foster mothers and one of these (16.7%) became pregnant. All pregnancies were lost and no progeny was obtained. In conclusion, cloned rabbit embryos were characterized by the high intrauterine implantation rates, but by the failure in development to term.

Notes

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EFFECT OF PROLACTIN ON DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES SELECTED BY BRILLIANT CRESYL BLUE STAINING

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In previous studies we demonstrated that bovine prolactin (PRL) supplementation remarkably enhanced the proportion of late morulae and blastocysts obtained from oocytes aspirated from follicles of 3 - 5 mm diameter and matured in vitro (Kuzmina et al., 2001, Ontogenesis; 32:140-47). It was also shown that oocytes selected by brilliant cresyl blue (BCB) had different developmental competence (Alm et al., 2005, Theriogenology 63:2194-2205). BCB^+ oocytes had significantly higher blastocyst development than did BCB^- oocytes. The aim of the present study was to evaluate the influence of PRL supplemented maturation medium on the development of BCB selected bovine oocytes.

Before IVM compact cumulus oocyte complexes (COC) were incubated in BCB solution for 90 minutes. Treated oocytes were then divided into BCB^- (colorless cytoplasm, increased G6PDH) and BCB^+ (colored cytoplasm, low G6PDH) on their ability to metabolize the stain. The selected COC were matured in TCM 199 + 10% (v/v) heat-treated FCS and 10^6/ml granulosa cells (GC) either without (control) or with the addition of 50 ng/ml PRL (experimental group). After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols. The cleavage rate including status of chromatin (cytogenetic analysis by Tarkowsky, 1966) and the blastocyst rate was evaluated. Data were analyzed by Chi^2 test.

Both in the control and the experimental group the cleavage rates were significantly increased in the BCB^+ oocytes (75.9 and 63.1%) in comparison to the BCB^- oocytes (Tab. 1). The addition of PRL increased the number of embryos, in BCB^+ and BCB^- oocytes. The BCB^+ oocytes yielded a significantly higher proportion of blastocysts (37.6%) by the addition of PRL in comparison to the control (21.3%), and both BCB+ oocytes had significantly higher blastocyst development than did BCB- oocytes (19.5 and 4.5%). The number of nuclei in the blastocysts was significantly increased in BCB^+ oocytes of the PRL group (112.8 ± 2.3 vs. 100 ± 21.0). The addition of PRL improved the development and nuclei number in BCB^+ and BCB^- oocytes.

Table 1. Effect of prolactin on development of bovine oocytes selected by brilliant cresyl blue staining

Treatment during IVM	BCB	n oocytes	8-16 cells embryo n (%)	Blastrocyst n (%)
TCM 199+10% FCS + 10^6 GC	+	141	89 (63.1) [a]	19 (21.3) [a]
TCM 199+10% FCS + 10^6 GC	-	137	66 (48.2) [b]	3 (4.5) [b]
TCM 199+10% FCS + 10^6 GC +50ng/ml prolactin	+	133	101 (75.9) [c]	38 (37.6) [c]
TCM 199+10% FCS + 10^6 GC +50ng/ml prolactin	-	139	87 (62.6) [d]	17 (19.5) [d]

[a :b, a :c, b :d, c :d] $P<0.0$

Notes

CULTURE OF BOVINE EMBRYOS IN PRESENCE OF ERK AND GSK3 INIHIBITORS INCREASES NANOG EXPRESSION BUT DOES NOT ALLOW THE ESTABLISHMENT OF ES CELL LINES

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The derivation of true embryonic stem cells (ES cells) in large animals, with all the properties of mouse ES cells including chimera formation following blastocyst injection, is still to be accomplished. A recently published method for mouse and rat ES cells derivation, based on the use of specific inhibitors of signalling pathways, has offered a novel approach to renew the efforts in large animal species. Such molecules include SU5402 or PD173074 (FGF tyrosine kinase receptor inhibitors), PD184352 or PD032591 (ERK 1-2 kinases inhibitor) and CH99021 (GSK3 kinase inhibitor). Moreover, these compounds have been proven to affect early cell fate decision in the early mouse embryo switching the inner cell mass compartment from a nanog/gata6 salt and pepper expression pattern to a uniform nanog expression that facilitate ES cells derivation. In this study we applied the same culture procedure to in vitro produced bovine embryos to test the effect on the inner cell mass of the bovine blastocyst and on the subsequent derivation of bovine ES. We found that culture of early bovine embryos in presence of the inhibitor cocktail (0.8µM PD184352 or PD03259 and 3µM CH99021) in medium SOF did not affect the rate of development to the blastocyst stage. However gene expression analysis by real time PCR on pluripotency genes sox2, oct4, nanog and hypoblast marker gata6 indicated a decrease in the expression of gata6 and an increase in the expression of nanog in embryos exposed to inhibitors. We then isolated the inner cell mass of treated embryos and we plated them on inactivated feeder cells for ES cell derivation. We found that the highest concentration of inhibitors recommended in the mouse model (0.8µM PD032591 and 3µM CH99021) induced cell death following a few days of culture. Reducing the concentration of PD032591 allowed cell proliferation and the establishment of cell lines that maintain an ES-like growth pattern, were alkaline phosphatase and Sox2 positive - with a few Tuj1 positive cells - but Oct4 negative indicating a neural identity.

In conclusion, the inhibitors used in this study did not affect the rate of bovine blastocyst development and increased the expression of nanog. However, culture of isolated inner cell mass cells in presence of the ERK-GSK3 kinase inhibitors did not allow ES cell derivation.

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Notes

THE EFFECT OF STORAGE TIME, TEMPERATURE AND EXTENDERS ON NGUNI BULL SEMEN QUALITY

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The main purpose for extending semen is to increase the number of females serviced from one ejaculate. Moreover, a good extender does not only increase the volume of ejaculate but also provides nutrients for maintaining sperm survival as is limited to few hours in seminal plasma alone (Hafez, 1987; Webb, 1992). Therefore it is important to know appropriate extender to use and how long the spermatozoa can survive in that extender.

This trial evaluated the effect of temperature, storage time period and extenders on the quality of Nguni bull semen. Semen was collected from three bulls, twice a week using the electro-ejaculator. Semen samples were evaluated for the spermatozoa motility rate and survivability, ejaculate concentration and semen pH. Semen samples with sperm motility and survival rate greater than 60% were divided into three parts. The first part was used to evaluate the effect of temperature on semen quality, while the other two were either extended with egg yolk citrate (EYC) or egg yolk tris (EYT) at the ratio of 1:1 (37 °C). The first sample was stored at controlled temperature (either 5 °C or 25 °C) and then evaluated after 0, 3, 6, 9 and 12 hrs. The extended semen samples were also evaluated at similar storage times. The semen pH stored at 5°C was significantly lower at 0 and 3 h than semen stored at 25°C. At 6 and 9 h, the semen pH was similar for both storage temperatures however, at 12 h the pH was significantly lower in semen stored at 25°C than at 5°C. The sperm survival rate was similar at 0, 3, 6 and 9 h for both temperatures. At 12 h of storage, the sperm survival rate of semen stored at 5°C rate was significantly higher than at 25°C. At 0 and 9 h, the sperm motility was similar for the two storage temperatures. The sperm motility of semen stored at 5°C was significantly higher than of semen stored at 25°C at 3, 6 and 12 h. Immediately after extenders were added (0 h), the pH of semen extended with EYT (6.4±8.0) was significantly lower than that of undiluted semen (6.9±0.6) and semen extended with EYC. At 3 h of storage, semen diluted with EYC recorded a significantly higher sperm survival and motility rate compared to fresh semen. Both parameters were similar for the extended semen. The semen pH on the other hand was significantly different for the semen samples. At 6 h storage, the sperm survival rate of extended semen was significantly higher. The motility rate of semen extended with EYC was significantly higher than of semen extended with EYT and undiluted semen. At 9 and 12 h of storage, the sperm survival and motility rates were less and significantly different for the 3 groups - the highest being for semen extended with EYC and lowest for undiluted semen. At 9 h, the pH was not affected, but significantly lower in fresh semen at 12 h than in extended semen.

From the results it can be concluded that Nguni semen can be stored up to 6 h at 5°C with sperm survival and motility rates greater than 60% being recorded. Both extenders can be used to extend Nguni semen; however, if semen is to be used after 3 h, the egg yolk citrate is preferable.

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Notes

PREPUBERTAL OVINE OOCYTES DEVELOPMENTAL COMPETENCE IS AFFECTED BY OVARIAN MORPHOLOGY

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Ovarian morphology in prepubertal ovine oocyte is largely variable caused by the fluctuating antral follicle number exhibited in the ovarian surface. This characteristic is probably genetically determined and could affect the reproductive performances during reproductive lifespan.

Our aim was to investigate whether differences in follicle numbers, showed as soon as a few weeks after birth in lambs, can be related to oocyte developmental competence in vitro.

Prepubertal Sarda sheep (4–6 weeks old) ovaries were collected from the local slaughterhouse and classified into three groups according to the number of follicles larger than 2mm on their surface: A) <15 follicles; B) 16–30 follicles; C) ≥30 follicles. Cumulus oocyte-complexes (COCs) recovered from the three groups were in vitro matured, fertilized and cultured up to the blastocyst stage following a standardized protocol (Leoni et al., 2006). Blastocysts were vitrified/thawed and cultured in vitro for 24 hours to evaluate re-expansion and cell number after Hoechst staining.

Cleavage rate of fertilized oocytes was lower (chi square test: $p>0.01$) in the groups A (197/288, 68.4%) and B (1082/1355, 79.8%) compared to group C (1088/1411, 77.1%). Cleavage timing did not show differences between the groups. The blastocyst rate was not significantly different (chi square test: $p>0.05$) between the groups. Expanded blastocysts obtained in the groups A, B and C were 29 (19.02%), 189 (17.46%) and 207 (19.02%), respectively. Timing of blastocyst development was different between the groups ($p<0.01$), the developmental kinetic of the groups A and B was slower compared to group C. In fact, the rate of blastocysts produced at the 7^{th} day of in vitro culture was lower in the groups A (55.5%) and B (55.8%) compared to group C (71.0%). Blastocyst re-expansion after vitrification and warming did not show differences (chi square test: $p>0.05$) among the analysed groups. The rates of blastocysts that re-expanded the blastocoelic cavity after vitrification and thawing were 62% (17/27) in group A, 43.2% (58/134) in group B and 50.7% (66/130) in group C. Blastocysts of group C evidenced a higher cell number (ANOVA: $p<0.05$) than group A and B (Blastocysts in group C: 92.00±17.41 vs blastocysts in group A 76.4±26.5 and B 74.3±21.1, respectively; mean cell number±SE).

In conclusion, ovarian phenotype seems to reflect oocyte developmental competence in vitro, as suggested by differences obtained in cleavage rates, embryo developmental kinetic and cell number among the three experimental groups

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Notes

EFFECT OF CYTOPLASM VOLUME ON THE DEVELOPMENT OF CLONED PIG EMBRYOS

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The volume of oocyte cytoplasm is important in nuclear transfer in cattle (e.g. Peura et al. 1998 Mol Reprod Dev 50:185-91) and mouse (Sayaka et al. 2008 Zygote 16:211-222). However, no similar studies have been made in pig. Therefore two experiments were performed in pig using handmade cloning (HMC; Li et al. 2006 Clon Stem Cells 8:241-50) to investigate the effect of different cytoplasm volumes used in the first fusion between donor cell and first cytoplasm (Exp. 1) or in the second fusion between the reconstructed embryo and the second cytoplasm (Exp. 2). After enzymatic removal of the zona pellucida, matured oocytes were cut into two parts, and the parts without the first polar body were separated into two groups according to their size in relation to the initial oocyte: ~75% or ~50%. Following the two fusions and activation (Day 0) reconstructed embryos were cultured in-vitro for 6 days. To monitor development, both cleavage rates on Day 2 as well as blastocyst rates and cell numbers on Day 6 were recorded.

In Exp.1, the results after use of ~50% vs ~75% cytoplasm at first fusion (n=123 and n=118, respectively, 4 replicates) followed by ~75% and ~50% cytoplasm at second fusion (total volume ~125%) were not different in either cleavage and blastocyst rates or blastocyst cell numbers (data not shown). This result was used in Exp. 2 where the effect of varying the cytoplasm volume in second fusion was tested to obtain a final cytoplasm volume from ~75% to ~200% (Table). The results showed an effect of the final cytoplasm volume with the lowest quality at ~75% and the highest quality at ~200% in relation to control (~125%).

In conclusion, the final cytoplasm volume in the reconstructed embryos was important for the development of the HMC embryos, while the volume of cytoplast used for the first fusion of the two-step reconstruction was not critical.

Cytoplasm volume			Repli-	Reconstructed	Cleavage	Blastocyst	Cell
1st fusion	2nd fusion	Final	cates	embryos	rate (N)	rate (N)	number (n)
~75%	0	~75%	7	160	69.3± 2.4^{b} (7)	25.7± 4.2^{bc} (7)	29 ± 3^{c} (11)
~50%	~50%	~100%	6	138	88.6± 1.6^{a} (6)	33.2± 4.0^{b} (6)	53 ± 4^{b} (9)
~75%.	~50%	~125%	10	210	93.0±1.9^{a} (10)	50.3±3.4^{a} (10)	50 ± 4^{b} (13)
~75%	~75%	~150%	6	130	88.9± 1.0^{a} (6)	39.0± 4.2^{ab} (6)	57 ± 5^{b} (11)
~75%	~75% + ~50%	~200%	6	189	91.1± 1.7^{a} (6)	59.2± 4.4^{a} (6)	67 ± 4^{a} (18)

Different superscripts in the same column indicate significant differences: a vs b (P<0.05).
N = number of replicates; n = number of blastocysts.

Notes

COLONY FORMATION DURING CELL CULTURE AS A BIOLOGICAL MARKER FOR REPROGRAMMING ABILITY OF *XENOPUS* EGG EXTRACT

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Treatment with cytoplasmic extracts from *Xenopus laevis* eggs represents a suitable tool for cellular reprogramming (Hansis et al. 2004, Miyamoto et al. 2007). However, the biochemical activity and quality vary from batch to batch (Allen et al. 2007). This study was performed to evaluate if colony formation during cell culture may be used as an assessment marker for the efficiency of cloning when extract-treated cells are used as cell donors in nuclear transfer.

Extracts were prepared from 3 frogs by the same method (Higa et al. 2006). Fibroblasts grown on poly-L-lysine coated cover slips were permeabilized by 7 µg/ml digitonin on ice for 2 min, and then incubated on Day 0 with one of the extract batches in an ATP-regenerating system (ATP, GTP, creatine kinase, phosphocreatine and NTP) at 37ºC for 0.5 h. After resealing the membrane in DMEM supplemented with 2 mM $CaCl_2$ at 37ºC for 2 h (Håkelien et al. 2002), the remaining cells were cultured in ES medium (DMEM/F12 containing 5% FCS, 10% KnockOut Serum Replacement and 10 ng/ml LIF) for 8 days. The number of forming colonies was counted on day 7 after extract treatment. Colonies on day 7-8 were trypsinized and separated into single cells used for handmade cloning (Du et al. 2005). Non-treated cells cultured in DMEM were used as control group. Cleavage and blastocyst rates were analyzed with Chi-square test, and blastocyst cell numbers were analyzed with Duncan's multiple comparison (SAS version 9.2).

Batch 2 induced higher colony formation than the other two batches (56±6 vs 37±4 and 35±6, $P<0.05$). No colony formation was observed in control cultures. When comparing developmental rates of cloned embryos (Table), use of Batch 2-treated cells as somatic cell donors significantly increased blastocyst development.

In conclusion, assessment of colony formation may be a method to select between *Xenopus* egg extracts for pre-treatment of fibroblasts to increase the cloning efficiency.

Group	No. of embryos cultured (replicates)	Cleavage %±SEM (No.)	Blastocyst %±SEM (No.)	Total cell number of blastocysts ±SEM (No.)
Batch 1	55 (n=3)	96±2 (n=53)	44±6 (n=24)[a, b]	64±5 (n=15)
Batch 2	187 (n=8)	90±1 (n=166)	56±4 (n=109)[a]	70±2 (n=105)
Batch 3	80 (n=4)	92±2 (n=74)	43±6 (n=35)[b]	64±6 (n=14)
Control	242 (n=11)	89±2 (n=217)	45±4 (n=117)[b]	67±2 (n=109)

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

Notes

MEIOTIC COMPETENCE DETERMINES THE MORPHOLOGY AND LOCALIZATION OF MITOCHONDRIA IN BOVINE OOCYTES

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It is generally accepted that the meiotic competence of oocytes increases during follicular growth and is also influenced by follicular atresia. It has been documented that oocytes beginning to show signs of atresia have greater developmental competence than non-atretic oocytes. The present study was designed to compare the morphology and localization of mitochondria in oocytes at various stages of atresia that were derived from follicles of different size. Cyclic cows, examined for ovarian status, were used as oocyte donors. Oocytes recovered separately from medium (MF, average 6-10 mm) or small follicles (SF, average 3-5 mm) were assessed at GV or MII stages. Only cumulus-oocyte complexes useful for IVF were selected and, on the basis of their morphology, were classified as healthy, light-atretic and mid-atretic. Heavy-atretic oocytes or oocytes from larger follicles (avarage >10 mm) were excluded from the study. Half of the oocytes of each category were matured for 24 hours according to a standard protocol. The oocytes were denuded from cumulus cells, stained with MitoTracker Orange and fixed by paraformaldehyde. The morphology of active mitochondria (diffused, granulated or clustered) and their localization (peripheral or central) were evaluated by confocal microscopy. The results were expressed as mean percentages and analyzed by the Chi-square test.

Before maturation, a significantly higher ($P<0.05$) percentage of oocytes with granulated mitochondria was found for healthy than for light-atretic and mid-atretic MF oocytes (73.3 vs 44.4 or 38.7%). In contrast to MF oocytes, a significantly higher ($P<0.05$) percentage of oocytes with granulated mitochondria was found in light-atretic than in healthy and mid-atretic SF oocytes (53.8 vs 32.9 and 20.4%). After maturation, a significantly higher ($P<0.05$) percentage of oocytes with clustered mitochondria was detected for healthy than for light-atretic and mid-atretic MF oocytes (60.5 vs 35.5 and 8.0%). A significantly higher ($P<0.05$) percentage of oocytes with clustered mitochondria was found in light-atretic compared with healthy and mid-atretic SF oocytes (36.6 vs 17.2 and 2.9%). The percentage of oocytes with clustered mitochondria for light-atretic SF oocytes did not differ from that for light-atretic MF oocytes.

Before maturation, a significantly higher ($P<0.05$) percentage of oocytes with peripherally located mitochondria was observed in healthy and light-atretic, as compared with mid-atretic MF oocytes (36.6 and 20.0 vs 4.5%), but on the other side, in light-atretic and mid-atretic compared with healthy SF oocytes (62.5 and 63.2 vs 37.8%). Very high percentage of oocytes with centrally located mitochondria, without differences amongst healthy, light-atretic and mid-atretic oocytes, were detected in both MF and SF oocytes after maturation.

It can be concluded that bovine oocytes with different meiotic competence differ in the rate at which diffused, granulated or clustered mitochondria are present. Healthy oocytes from the medium follicles and light-atretic oocytes from the small follicles seem to be more competent in terms of mitochondrial granula or cluster formation before and after maturation, respectively. The specific changes in morphology of mitochondria during maturation can characterize the cytoplasmic maturity of oocytes better than changes in mitochondrial localization.

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Notes

ULTRASTRUCTURAL CHANGES IN RABBIT PREIMPLANTATION EMBRYOS CULTURED UNDER CONDITIONS OF HYPERTHERMIA AND THE HSP70 BLOCKAGE

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The aim of the study was examine effect of hyperthermia and the blockage of Hsp 70 on ultrastructural alterations in cytoplasmic organelles and the nucleolus of rabbit preimplantation embryos.

The morula stage embryos were cultured at 37.5°C (control) or 41.5°C (hyperthermia, HT) for 6h. In half of the embryos from both groups, Hsp70 was blocked by the incubation for 6 h in presence of an anti-Hsp70 in culture medium (4μg/ml). Thereafter, the embryos were fixed, embedded in Durcupan ACM and cut on the ultramicrotome. Ultrathin sections were contrasted with uranyl acetate and lead citrate and analyzed under JEMCX II (Jeol, Japan) electron microscope. The volume density of cellular components was measured from the electronograms of embryo sections, as a percentage value, by the point-count method (Weibel and Bolender, 1973). Statistical analysis of cellular components was done by Student's t-test.

In embryos exposed to hyperthermia the relative volume (%) of lipid droplets (4.86±0.35) and proportion of mitochondria with ringlet-like structure (0.46±0.09) were significantly increased compared to control (3.78±0.31; 0.19±0.04, resp.). The occurrence of cellular debris and slightly changed microvilli on the surface of trophoblastic cells were observed. In embryos exposed to anti-Hsp70 at 37.5°C the morphology of mitochondria was altered: they were increased and swollen. The volume of dense bodies in the cytoplasm was reduced (0.85±0.14 vs 2.0±0.18 in control) and the volume of mitochondria with annulated structures was increased from 14.5±0.55 to 22.06±1.15. In the embryos exposed to the antibody against Hsp70 at 41.5°C mitochondria were enlarged and swollen, the volume of flocculent vesicles (10.34±0.93) and lipid droplets (6.19±0.42) was increased compared to control (7.45±0.84). In the cytoplasm the volume of dense bodies (1.42±0.19) and vacuoles (0.71±0.13) was reduced compared to control (1.92±0.28 and 1.34±0.22 resp.). General organization of the cytoplasm was characterized as a segregation of cell organelles. Measurements of the nucleolar area (μm^2) on ultrathin sections confirmed significant enlarging in the embryos exposed to hyperthermia (7.02±0.35) compared to control (5.52±0.36). In the group exposed to an antibody against Hsp70 at 37.5°C nucleolar area was significantly diminished (4.29±0.28) in contrast to the control (5.52±0.36).

In conclusion, hyperthermia itself (at 41.5ºC) only slightly affects embryo organelles, but the blockage of Hsp70 by an antibody caused more extensive alterations in embryo ultrastructure. These results confirm an essential role of Hsp70 in embryo vitality.

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Notes

VIABILITY OF IN VITRO EMBRYO PRODUCTION IN SARDA SHEEP IS NOT AFFECTED BY SEASON

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It has been reported that the number and quality of *in vitro* produced embryos is season-related. Even where donor animals are identified and carefully managed, seasonal effects on fertility cause variation in embryo yields. This study was conducted to assess the effect of season on cleavage, blastocyst and lambing rates of *in vitro* produced ovine embryos during 2005 to 2007.

Ovaries of Sarda sheep were collected from a slaughterhouse. A total of 4404 oocytes were recovered and 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, and 1 □g/ml estradiol-17β. Matured oocytes were fertilized with fresh semen in synthetic oviductal fluid (SOF) with 20% heat inactivated estrous sheep serum. The presumptive zygotes were cultured for 6-7 days (blastocyst stage) in SOF medium supplemented with 1% BME, 1% MEM, 1mM glutamine and 8 mg/ml fatty acid-free BSA.

The embryos produced were vitrified and a total of 165 blastocysts (80 from the breeding season and 85 from the anoestrous season) were transferred in pairs into recipient ewes during the reproductive period.

There were no significant differences in cleavage rates between breeding and anoestrous season in any of the three years examined (84% vs 83%, 81% vs 80 % and 72% vs 73%, respectively).

On the contrary the blastocyst rate varied significantly between breeding and anoestrous seasons in 2006 and 2007 ($P<0.001$), while during 2005 there was only a tendency (37% vs 32%).

There were no differences in pregnancy and lambing rates for embryos produced during breeding vs anoestrous season respectively.

In conclusion, only the blastocyst rate and not the viability appeared to be affected by season, possibly due to variation in the number of developmentally competent oocytes.

Table 1. Lambing rate of vitrified embryos produced during anoestrous and breeding season.

Vitrified embryos	Transferred embryos	Recipient ewes	Pregnant ewes/recipient ewes (%) 40Days	Lambs born/transferred embryos (%)
in Anaestrous	85	42	35/42 (83.3%)	45/85 (52.3%)
in Breeding	80	40	34/40 (85.0%)	40/80 (50.0%)

This work was supported by the RAS, Biodiversità APQ5/2007 project.

Notes

RECOMBINANT BOVINE SOMATOTROPINE (rbST) IN SUPEROVULATED CHAROLAIS COWS

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The administration of rbST increases the concentrations of insuline and insuline like growth hormone factors (IGF-1) in plasma, increasing the number and growth of antral follicles, and this can increase the superovulatory response in cows treated with rbST. The objective of this study was to determine the effect of rbST administration on fertilization rate and embryo development in superovulated Charolais cows. Non-lactating cows with an average body condition score of 8 (scale 1 to 10) were superovulated with 300 mg of FSH (Folltropin-V-Bioniche, Animal Health, Canada, Inc) using a descendent protocol given in eight injections (50, 50, 40, 40, 30, 30, 10 and 10 mg) at 12 h intervals. The cows were inseminated with frozen semen from bulls of known fertility and were randomly assigned to the treatments: control group (n=11) and treated group (n=10) with 500 mg rbST (Boostin®-S-Shering Plough) subcutaneously in the ischiorectal fossa 48 h before artificial insemination (AI). The cows were flushed for embryo recovery through a non-surgical technique 7.5 d after AI and the structures obtained were classified according to its morphology and stage of development. The results were analyzed using a complete random design with the GLM procedure of SAS. The administration of rbST increased the number of transferable embryos (P=.09), degenerated embryos (P<.05) and the total number of structures recovered (P<.01), however, the number of unfertilized eggs (P=0.6) was similar between treatments (Table 1).

Table 1. Effect of rbST administration in superovulated Charolais cows

rbST (500 mg)	n	Transferable Embryos	Degenerated Embryos	Unfertilized Eggs	Total Structures
With	10	6.90 ± 4.25^{a}	6.30 ± 5.39^{a}	3.00 ± 3.74^{a}	16.2 ± 7.00^{a}
Without	11	4.27 ± 2.41^{b}	2.72 ± 1.67^{b}	2.27 ± 2.45^{a}	9.27 ± 3.46^{b}

[a,b] Values with different letters present significant differences between treatments.

The administration of rbST increased the number of transferable embryos, degenerated embryos and total structures as previously reported by Moreira *et al.* (Theriogenology 2002, 57, 1371-1387).

Notes

RECOMBINANT BOVINE SOMATOTROPINE (rbST) IN THE ESTRUS SYNCHRONIZATION OF CHAROLAIS COWS

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The administration of rbST increases the concentration of growth hormone in plasma during the period of treatment Gong *et al*. (Biology of Reproduction 1991, 45, 941-949) which amplifies the action of gonadotropins during the follicular development and ovulation (Izadyar *et al*.; Theriogenology 1996,45:279), due to an increase in the production of insulin like growth factor (IGF-1) at ovarian level (Yoshimura *et al*.; Endocrinology 1994,135:887-894). The objective of this study was to evaluate the effect of rbST administration on the incidence of estrus (IE), the time to estrus (TE), the size of preovulatory follicle (SPF) and the size of the corpus luteum (SCL) seven days after estrus in Charolais cows. Ninety three non-lactating cows were synchronized using the following scheme: day 0: insertion of an intravaginal device containing 1.9 g of progesterone (CIDR® Pfizer), day 7: administration of 500 µg of PGF2 (Celosil®, Shering Plough), day 8: removal of CIDR and day 9: administration of the treatments: control group (n=55) and treated group (n=38) with 500 mg of rbST (Boostin®-S-Shering Plough) subcutaneously in the ischiorectal fossa 24 h after CIDR removal. The results were analyzed using a complete random design with the GLM and GENMOD procedures of SAS. There were no significant differences between treatments for IE, TE and SPF (Table 1), but the SCL was higher (P<.08) in rbST treated cows.

Table 1. Effect of rbST administration in synchronized Charolais cows

rbST (500 mg)	n	IE (%)	TE (h)	SPF (mm)	SCL (mm)
With	38	95.0	50.60 ± 15.5[a]	12.8 ± 2.82[a]	17.4 ± 6.2[a]
Without	55	88.1	45.05 ± 15.4[a]	12.6 ± 2.79[a]	14.9 ± 6.7[b]

[a,b] Values with different letters present significant differences between treatments

The administration of rbST increased the size of the corpus luteum as previously reported by Lucy *et al.* (Theriogenology, 1994, 41:561-572).

Notes

FERTILITY RESCUE OF A HIGH MERIT RAM WITH LOW SPERM MOTILITY USING A SWIM UP TECHNIQUE IN A MOET PROGRAM.

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Differences in the fertility of selected males could have serious limitations on MOET programs. However, the use of some reproductive assisted technologies would improve the efficiency of genetically desirable rams. The aim of this study was to use in a MOET program a semen swim up technique together with intrauterine insemination in order to improve the efficiency of a ram with a high genetic merit (40 of pedigree's score) affected by an idiopathic low (score 1) sperm motility (scale from 0 to 5).

Four Sarda ewes were synchronized with an intravaginal sponge containing 40mg FGA for 12 days. Superovulation treatment was performed using 350 I.U. porcine FSH per sheep in eight decreasing doses twice daily starting 48 h before sponge removal. In order to get the sperm dose ready for a laparoscopic intrauterine insemination the semen was processed as follow: after collection by artificial vagina the ejaculate was assessed for motility, volume and concentration and then diluted with TRIS+egg yolk (30°C) to a volume of 3.3 ml with a concentration of 1.6 x 10^9 spz/ml. Following dilution, 0.5 ml of diluted semen were layered under 1 ml aliquots of the medium SOF+ 20% HSS (heat sheep serum) in six plastic tubes of 12x75 mm (IWAKI brand®, Japan). After 1 h at 37°C in incubator 5%CO_2, the supernatants containing the alive spermatozoa were removed and pooled together obtaining a final volume of 2 ml with a concentration of 360x 10^6spz/ml. Ewes were inseminated with a semen dose of 80x10^6spz/0.25ml per each horn (total dose 160x10^6spz) 48 h after sponge removal. Just before insemination the semen was scored according the standard parameters of motility (score 3.5) and viability (80%).

Embryos were recovered surgically by flushing at day 6 after intrauterine insemination. The quality of embryos was evaluated on scale from 1 to 3. Twenty fertile blastocysts were recovered after flushing and then vitrified. Because shortage of recipients only 10 embryos were transferred in pairs into 5 progestagen synchronized ewes during breeding season (November 2009).

The fertility rate was 80% (20/25) and all of the embryos (20/20) resulted to be of high quality (score 1-2). The lambing rate was 100% (10/10). Among all lambs borned 60% (6/10) were males and 40% (4/10) females. Thus, the new born animals will be part of the next genetic selection program.

In conclusion, the use of a swim up technique combined with intrauterine insemination makes possible to enhance the efficiency of rams with high genetic merit affected by low semen motility obtaining good results as well as in the more conventional MOET programs.

Notes

STAGE-DEPENDENT VIABILITY OF VITRIFIED GOAT AND BOVINE BLASTOCYSTS

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This work was designed to determine the ability of *in vitro* cultured blastocysts at different stages of development to survive the vitrification procedure using cryotop device.

Embryos from adult and prepubertal goat and bovine were classified as non-expanded, expanded, or hatching/hatched blastocysts. *In vitro* produced (IVP) blastocysts were obtained from prepubertal goat oocytes by slicing of slaughtered ovaries while adult goat oocytes were collected by laparoscopic ovum pick up (LOPU). In bovine, blastocysts were obtained from calf and cow oocytes recovered from slaughtered ovaries. Blastocysts were vitrified/warmed using the cryotop technique. Survival rates were assessed at 3 h post-warming.

Results showed a significant increase in survival rates at 3h when hatched blastocysts from adult goats were vitrified/warmed (57.1%) compared to non-expanded (0%) or expanded (36.7%) blastocysts. In prepubertal goats, significantly higher survival rates were obtained after warming expanded (80%) blastocysts compared to non-expanded (60%) or hatched (58%) blastocysts. When bovine embryos were vitrified, survival rates of 78.4% and 66.7% were observed after warming expanded and hatched cow blastocysts, while respective rates of 80% and 76.9% were observed for the calf blastocysts. Lowest survival rates were recorded for non-expanded blastocysts (51.2% and 54.5%, cow and calf blastocysts, respectively) compared to the other developmental stages.

Data analysis showed that the developmental stage of bovine blastocysts produced *in vitro* affect their survival rates after being vitrified/warmed. Among blastocysts produced *in vitro*, those cultured to the expanded and hatched stage before cryopreservation triggered the highest rates of survival after warming. Nevertheless, survival rates of prepubertal goat blastocysts were significantly higher than those obtained from adult goats, independently of the embryo developmental stage.

Notes

INFLUENCE OF COOLING DURING OVIDUCT TRANSPORTATION ON THE ESTABLISHMENT OF EQUINE OVIDUCT EPITHELIAL CELL VESICLE CULTURE

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Coculture of bovine embryos with oviduct epithelial cells (OEC) was widely used in the nineties to overcome the 8- to 16-cell block in cattle and is now still applied to investigate embryo-maternal interactions *in vitro*. Since horse embryos reside for a relatively long time in the oviduct (~6 days), we would like to establish an *in-vivo* like oviductal cell culture system to study embryo-maternal interaction in the horse. In the present experiment, the oviducts of 9 cyclic warmblood mares between the age of 4 and 20, were collected at a slaughterhouse. Per mare, one oviduct was transported on ice, and the other one at ambient temperature. The epithelial cells were obtained with a scalpel blade by scraping the ampullary-isthmic region of the longitudinally opened oviduct. After three steps of 10 minutes sedimentation in HEPES buffered wash medium, the harvested cellular material was washed in culture medium. Next, 20 µl of cell aggregates were seeded in 800 µl of DMEM/F12 culture medium, supplemented with 10 % of fetal calf serum, gentamycin and fungizone. The cell culture was incubated at 38.5 °C in 5 % CO_2 in air. Cellular integrity was confirmed by Trypan blue and SYBR14/PI staining. Epithelial vesicles were formed after 3 to 6 hours of incubation. The culture medium was refreshed the first and the fourth day after cell isolation. Every day, from day 0 to day 6, a sample of cell suspension was processed for histology. The cultured vesicles were assessed during 6 days by determination of the percentage of vesicles showing ciliary activity. In addition, the amount of debris and the homogeneity of colour of the vesicles were assessed. Furthermore, the effect of the age of the mare on the assessed parameters was investigated.

Trypan blue and SYBR14/PI demonstrated that approximately 99 % of the cells were intact at seeding. During the whole culture period, no vesicles attached to the bottom of the Petri dish, which is unlike the situation in cattle. Oviduct epithelial vesicles from oviducts transported on ice, showed a better ciliary activity (64.4 % of the vesicles were moving during the whole culture period), less central darkening (68 %) and less debris (18 %) in the cell culture, than OEC from oviducts transported at ambient temperature (respectively 41.6 %, 70 %, 34.4 %). Histology revealed no obvious differences between vesicles derived after cooled or non cooled transport of the oviducts. Polarized tall columnar epithelial cells, including apical cilia, microvilli (brush borders), secretory granules and basal nuclei were observed in both groups from day 0 to day 6 in the 2 groups. From day 2 of culture, tubular structures with a non-polarized simple cuboidal to a simple squamous epithelium without cilia, microvilli or secretory granules, were noticed at the same time. Moreover, statistical analysis revealed: the older the mare, the less ciliary activity, and the less vesicles showing central darkening. The age of the mare has no influence on the amount of debris.

In conclusion, it is recommended to transport oviducts for equine OEC culture on ice. From day 2, highly differentiated ciliated and secretory cells as well as less differentiated non polarized cells were observed. Further research to optimize the oviduct cell culture environment is ongoing.

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Notes

MATERNAL PERICONCEPTIONAL UNDERNUTRITION IN SHEEP: EFFECTS ON MALE LAMBS' PHYSIOLOGY AND REPRODUCTION

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Epidemiological studies in humans, as well as animal models, have shown that maternal environment at critical periods of fetal development can condition offspring adult health. Periconceptional undernutrition has been reported to induce pre-term delivery and to affect fetal adrenal and gonadal development as well as lamb survival and growth in sheep. The aim of this project is to evaluate the long term effects of maternal periconceptional undernutrition on offspring physiology and reproductive function. Post-natal development of male offspring is presented here.

One hundred and sixteen ewes of Merinos d'Arles breed were used and allocated to one of two group: control females (R100, N=52) were fed a normal diet adapted to their needs and restricted females (R50, N=64) received 50% of their dietary needs from 15 days prior to 30 days after natural breeding following oestrus synchronisation. Thereafter, both groups were fed to meet their nutritional requirements. Male offspring were weaned at 22 kilograms body weight and then raised until commercial slaughter at 35 kilograms body weight. They were weighed at birth and every week. Blood samples were collected monthly in the morning to monitor plasma leptin and cortisol concentrations. Organ weights were recorded at slaughter, and histological analysis was performed on testicles and adrenal. Data were analysed by Anova or Student's test.

Twenty-two live R100 and 34 R50 male lambs were obtained. There was no statistical difference between groups for pregnancy rates, prolificity, sex ratio and birth weight, but pregnancy was significantly longer in the restricted group ($p< 0.01$). Growth rate was not significantly different between groups. Males were slaughtered at a mean live weight of 38.9±2.9 kg, mean age of 147± 27 days. Plasma leptin concentrations were significantly lower in R50 at birth (6.15±0.8 vs 7.42±1.7 ng/ml, $p<0.001$) and tended to be higher in R50 at 4 months of age (10.61±2.1 vs 9.79±2.4 ng/ml, $p=0.09$). Basal cortisol concentrations were lower in R50 at 3 months of age (20.81±10.33 vs 28.21±5.49, $p=0.03$) but tended to be higher at 4 months of age (19.82±8.42 vs 14.58±5.01, $p=0.058$). There was no statistical difference for carcass, testicle and kidney weight. In contrast, carcass to live weight ratio and perirenal fat to live weight ratio were increased in R50 (54.4±1.7 vs 44.4±1.6%, $p<0.05$ and 9.46±2.7 vs 8.48±2.0 g/kg, $p<0.05$, respectively). Adrenals tended to be heavier in R50 lambs (2.03±0.6 vs 1.73±0.72g, $p=0.13$). Histological analyses revealed no significant differences for the area occupied by seminiferous tubules within the testicle and for the stage of meiosis. Adrenal corticomedullary ratio was not different between groups.

These results confirm the effects of periconceptional undernutrition on metabolic function. In contrast to previous studies, no effects were observed on testicular histology. The expression of genes involved in testicular differentiation is currently being studied by quantitative PCR.

Notes

BOVINE IVF EMPLOYING OPU-OOCYTES AND SEX-SORTED FROZEN/THAWED SPERM: INFLUENCE OF CO-INCUBATION TIME

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Epigenetic disturbances may occur in embryos if fertilization is delayed by asynchronous pronucleus development. Sex-sorted sperm have been successfully incorporated into IVF in cattle, but these sperm have altered patterns of motility and a reduced lifespan (*Morton et al., 2005*). The objective of this study was to investigate the influence of gamete co-incubation time on the performance of bovine IVF with OPU oocytes and sex-sorted/unsorted frozen-thawed semen.

Eighty four OPU sessions were carried out in 18 cyclic, dry and non stimulated *Holstein Friesian* and *German black pied* cows. OPU oocytes were matured in vitro 24h and fertilized with sex sorted sperm, by the Beltsville Sperm Sexing Technology (*Klinc & Rath, 2007*). Controls were fertilized with unsorted sperm from the same ejaculates. Fertilization was achieved by two different experimental protocols: **1**) short co-incubation time: 4, 8 and 12 h post insemination (hpi) and **2**) long co-incubation time: 18 and 24 hpi. After IVF, ova were fixed and stained to determine penetration (PEN), monospermy (MON), male pronucleus formation (MPF), performance (PERF, monospermic oocytes with 2 pronuclei from total matured oocytes), syngamy (SYNG) and synchrony of male and female pronuclei development (SYNC) (*Xu & Greve, 1988*). The number of viable oocytes obtained by OPU session (4.24) and maturation rate (82.98%) were similar to that reported under similar conditions (*Ruiz et al., 2009*).

In experimental protocol 1 (4, 8 and 12 hpi), co-incubation time affected fertilization. PEN increased progressively at 4 (9.09 and 25%), 8 (44.44 and 55.56%) and 12 hpi (65.38 and 69.57%) for sex-sorted and unsorted, respectively ($p<0.01$). Best rates ($p<0.01$) for MPF and PERF were obtained at 12 hpi with sex-sorted sperm (78.57 and 64.71%, respectively).

For long co-incubation time (18-24 hpi), PEN, MON, MPF, SYNG and SYNC did not differ between sperm treatment and hpi. PEN ranged from 80-85.2% (sex-sorted) to 87.5-100% (unsorted) and SYNG ranged from 33.4-16.1% (sex-sorted) to 14.3-12.4% (unsorted) for 18-24 hpi, respectively.

For short co-incubation time, PEN, MPF and PERF affected adversely, regardless of sperm treatment. In previous studies in cattle a reduction in sex-sorted sperm *in vitro* fertility was reported compared to unsorted (*Lu et al., 1999*), but we could not find differences in performance of bovine IVF with OPU oocytes between sperm treatments. PEN and SYNG are the most significant parameters with predictive value for the efficiency of bovine embryo production *in vitro* (*Machatkova et al., 2008*).

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Notes

EFFECT OF ROSCOVITINE AND TRICHOSTATIN A ON THE *IN VITRO* MATURATION OF PORCINE NUCLEAR RECIPIENT OOCYTES AND DEVELOPMENT OF NUCLEAR-TRANSFERRED EMBRYOS DESCENDED FROM FOETAL FIBROBLAST CELLS

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The aim of our study was to examine whether the inducible epigenetic modification, which was triggerred *via* trichostatin A (TSA) during *in vitro* maturation (IVM) of pig oocytes pre-treated with *R*-roscovitine (RSCV), affects the preimplantation developmental capability of cloned embryos. Cumulus-oocyte complexes (COCs) were pre-matured with 50 μM RSCV for 22 h. Afterwards, the COCs were incubated for 20 h in TC 199 medium supplemented with 0.1 IU/mL human menopausal gonadotropin (hMG), 10% porcine follicular fluid (pFF), 0.6 mM *L*-cysteine and 10 ng/mL recombinant human epidermal growth factor (rhEGF), followed by their continuous IVM for 22 to 24 h in the same medium enriched with 80 nM TSA. In the somatic cell cloning procedure, enucleated *in vitro*-matured oocytes were reconstructed with the genomic DNA of foetal fibroblast cells. 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 up to morula/blastocyst stages. The pre-treatment with RSCV prior to two-grade IVM in the TSA-depleted, and subsequently TSA-enriched medium led to the reaching meiotic maturity by almost all the pig oocytes selected for IVM as compared to a control (i.e., RSCV- and TSA-untreated) group (106/107; 99.1% *vs.* 84/102; 82.4%). It has been also found that the rates of cleaved embryos (94/99; 94.9%), morulae (71/99; 71.7%) and blastocysts (44/99; 44.4%) developing from NT oocytes that had been previously exposed to RSCV and TSA were significantly higher than in the RSCV- and TSA-untreated group (52/75; 69.3%, 38/75; 50.7% and 21/75; 28.0%, respectively). Reassumming, the abundance in a formation of morulae and blastocysts suggests the improved reprogrammability of epigenetic memory and thereby transcriptional activity for foetal fibroblast cell-inherited nuclear DNA in an epigenomically-matured cytoplasm of recipient oocytes undergoing sequential exposure to RSCV and TSA.

Notes

QUALITY OF IN VITRO PRODUCED OVINE EMBRYOS: GENE EXPRESSION, CRYOTOLERANCE, LAMBING RATE AND BODY WEIGHT

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Innumerable studies have demonstrated that modification of the post-fertilization culture environment may influence embryo quality. The aim of the present study was to evaluate the quality of ovine embryos produced with different supplements as Bovine Serum Albumin (BSA), Hyaluronic Acid (HA) and charcoal stripped Fetal Bovine Serum (chFBS) in in vitro culture in terms of gene expression, cryotolerance, lambing rate and lamb body weight.

Ovine cumulus oocyte complexes were matured in vitro in TCM199 supplemented with 4 mg/ml BSA for 24 h and 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 for group A(BSA4) and 8 mg/ml fatty acid free BSA for the groups B(BSA8), C(BSA8–HA), D(BSA8-chFBS). On day 3, 5 and 7 of culture (day 0=day of fertilization) 4 mg/ml BSA, 8 mg/ml BSA, 8 mg/ml BSA+6 mg/ml HA and 10% charcoal stripped FBS were added to the culture A, B, C, D respectively. Day 6-7 blastocysts from each group were snap frozen in LN_2 in groups of 10. Similarly as a control group in vivo derived embryos were used. Quantitative Real Time PCR was used for gene expression analysis. Eight genes related to imprinting, embryo development, apoptosis and DNA methylation were analyzed: *IGF2R* (Insulin-like growth factor receptor), *GRB10* (growth factor receptor-bound protein 10), *UBE2A* (Ubiquitin-conjugating enzyme 2A), *LAMA1* (Laminin alpha 1), *IL6* (Interleukin-6), *FGF4* (Fibroblast growth factor 4), *BAX* (BCL2 associated X protein) and *DNMT3A* (DNA methyltransferase 3a). Furthermore Day 6-7 blastocysts produced in vitro were transferred to recipient ewes either fresh or after vitrification/warming using the open pulled straw method. Pregnancy was confirmed by ultrasonography on day 40 and allowed to develop to term.

IGF2R and *UBE2A* were significantly upregulated ($P<0.05$) in all in vitro groups compared with in vivo. *UBE2A* was significantly upregulated ($P<0.05$) in group C compared with all other in vitro groups. *LAMA1* was significantly upregulated ($P<0.05$) in group A compared to all groups. Pregnancy rate did not differ either among groups or among vitrified or fresh. Lambing rate was similar for fresh groups, but among vitrified groups, it was significantly lower ($P<0.05$) for group D than for A and for vitrified group D than for fresh group D ($P<0.001$). The birth weight was higher for B and C within groups both fresh and vitrified, although there were no significant differences.

Our data confirm that the use of different supplements in culture media affects blastocyst quality in terms of cryotolerance, gene expression and body weight. Moreover the use of charcoal stripped FBS reduces the lambing rate when vitrified/warmed embryos are transferred, but not when they were transferred fresh. The transcription of the genes analyzed did not reflect this change in embryo quality.

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Notes

SUPEROVULATORY RESPONSE IN OLD DAIRY COWS

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Embryo transfer is an biotechnology widespread throughout in the world, however one of the problems that affects this biotechnology is the high variability in superovulatory response in cows subjected to ovarian stimulation to promote multiple ovulations.

This work was conducted at the Experimental Farm of Arcoverde belonging to the Agronomic Institute of Pernambuco, located in the northeast of Brazil. The aim of this study was to evaluate the superovulatory response of eight dairy cows with advanced age. Four Holstein cows and four 5/8 Girolando cows at the age of 10 to 14 years (average of 11.5 years) received progesterone implants (progesterone releasing device, day = 0) and were intramuscularly injected using 2 ml of estradiol benzoate. On Day five of the cycle the application of FSH was started to promote multiple ovulations. The Holstein cows received totally 500 IU of FSH while 5/8 Girolando cows were treated with 400 IU of FSH. The FSH amount was divided into eight dosages and injected over four days. The 5th application of FSH and the removal of the progesterone implants were done simultaneously. The 6th application of FSH was accombined by 2 µg of prostaglandin in all animals. Fifty-six hours after the removal of the implants all donors were artificially inseminated. The embryo collection was performed transcervically on Day seven. Viable embryos were directly transferred into recipients. Twenty-three days after embryo transfer was performed pregnancy was diagnosed using ultrasound.

In total, one hostein cow and two girolando cows did not respond to superovulatory treatment. The remaing Holstein cows provided 5 viable embryos, 3 degenerate complexes and 22 unfertilized oocytes. The 5/8 Girolando cows produced 2 viable embryos, 3 degenerate complexes and 4 unfertilized oocytes.

The embryos were transferred to seven recipients of which four were diagnosed pregnant 23 days later. The results of the present study let us conluded that old dairy cows produce a small number of viable embryos. Future studies should be conducted using a higher number of animals.

Notes

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GENERATION OF CLONED GOATS FOLLOWING CHEMICAL ACTIVATION OF OOCYTES RECEIVING ADULT SKIN-DERIVED FIBROBLAST CELL NUCLEI

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The purpose of our study was to determine the pre- and postimplantation developmental competences of caprine nuclear-transferred embryos reconstituted with adult ear cutaneous fibroblast cells. Fibroblast cells were cultured *in vitro* up to a total confluency state and then used for the somatic cell nuclear transfer (SCNT) as a source of nuclear donor cells. Enucleated i*n vitro*-matured oocytes were the source of recipient cells. Single nuclear donor cells were injected into a perivitelline space of previously enucleated oocytes. Fibroblast cell-ooplast couplets were simultaneously fused and activated with a single DC pulse of 2.4 kV/cm for 15 μsec. After a 1-h delay, the reconstructed oocytes were additionally activated by exposure to 5 μM/L calcium ionomycin for 5 to 6 min followed by treatment with 2 mM/L 6-dimethylaminopurine for 2 h. The SCNT-derived embryos were cultured *in vitro* in 50-μL droplets of B2 medium for 72 to 96 h. Afterwards, cloned embryos at morula stage were transferred into reproductive tracts of recipient does. Ultrasonographic diagnostics of gestation was performed between Days 35 and 42 after transfer of SCNT embryos into recipient surrogates. A total of 132/154 (85.7%) enucleated oocytes were successfully fused with nuclear donor cells and intended to be *in vitro* cultured. Out of 132 cultured SCNT embryos, 67 (50.8%) were cleaved and 56 (42.4%) reached the morula stage. After transfer of 56 cloned morulae to 7 recipient females (an average of 8 embryos per recipient), two does became pregnant (conception rate, 28.6%) and one of them delivered a total of 2 kids (3.6%). In conclusion, it has been shown that the adult dermal fibroblast cell nuclei were able to support the preimplantation development of cloned goat embryos to morula stage at a relatively high rate. Nonetheless, the capabilities of SCNT-descended morulae for both implantation *in utero* and development up to term decreased considerably.

Notes

CHANGES IN BODY CONDITION AND OVARIAN DOMINANT FOLLICLE STATUS OF EMBRYO RECIPIENT MARES THROUGHOUT THE BREEDING SEASON AND THEIR RELATIONSHIP WITH REPRODUCTION TRAITS

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The primary objective was to investigate the relationship between changes in body condition on reproductive traits of recipient mares in an embryo transfer program. Forty-five non-pregnant and clinically sound fertile mares were studied from August 2008 through April 2009. Body condition score (BC; 1-9 scale, thin to obese) was estimated monthly. Body weight (BW) and tail and rib s.c. fat depths (SF) were measured monthly. SF was determined by ultrasound. Dominant follicle diameter (DF) was measured daily during the follicular phase. Embryo transfers were conducted 6 d after ovulation and pregnancy diagnosis by 12 and 60 days post embryo-transfer (dpt). Analyses were performed with SAS® (SAS Institute Inc., Cary, NC, USA). BC classes were as follows: B1, <6.5; B2, 6.5 to 6.9; B3, 7.0 to 7.5; and B4, >7.5. BC and BW data were converted to percentage of the initial weight of each mare and grouped according to gain (GM) versus maintenance or loss (L) for analyses. DF diameter and growth rate were classified into <40 (S) and ≥40mm (LL) and < 2 (LR) and ≥2 (HR) mm/day, respectively. Proportions of pregnant mares at 12 and 60 dpt were compared by chi square and correlations by simple regression (Proc Reg, SAS®).

There was a positive correlation (P<0.0001) between BW and BC. A greater (P=0.08) proportion of mares that gained or maintained BC was pregnant at 12 dpt compared to the ones that lost BC (70.8%- 17 of 26 *vs* 52.9% - 9 of 17, respectively). Percent BC losses ranged from -3.40 to -20.1, averaging -9.5 ± 4.8 % and gains from 2.8 to 50%, averaging 14.0 ± 15.2%. Percent BW losses ranged from -1.1 to -11.9%, averaging -6.2 ± 2.7% and gains from 1.6 to 18.8 %, averaging 9.6 ± 5.8%. Dominant follicle growth rate class did not influence the proportion of pregnant mares at 12 (P=0.24) or 60 dpt (P=0.48). For LR and HR percentages pregnant were 52.9 (9 of 15) and 70.8% (17 of 26) at 12 days, and 77.8 (7 of 9) and 88.2% (15 of 17) at 60 dpt, respectively. BW and BC were positively correlated to the ultrasound measurements of subcutaneous fat depth (P<0.0001). Mares were grouped according to their ultrasound measurement changes (gain and maintenance versus loss), but no effect was observed on the proportion of pregnant mares on either day after embryo transplantation. In relation to DF growth rates, a greater (P<0.07) proportion of HR mares were pregnant at 60 dpt (88.2% - 15 of 17 *vs* 77.9% - 7 of 9). Thus, our results support the use of BC scoring to predict performance in equine embryo transfer programs. It has also been demonstrated that a faster growing dominant follicle was associated with better pregnancy maintenance ability of embryo recipient mares, under the conditions of this trial.

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Notes

CRYOPRESERVATION METHOD AFFECTS THE QUALITY OF IN VITRO PRODUCED BOVINE EMBRYOS AT THE MOLECULAR LEVEL

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The quality of in vitro produced bovine embryos has greatly been improved over the last couple of years. Nevertheless it still lags behind that of their in vivo generated counterparts.
The objective of the present study was to examine the effect of two cryopreservation methods on the quality of in vitro produced bovine embryos using a sensitive RT-qPCR assay.
Bovine blastocysts were produced in a standard IVP system using SOF (aa) medium. They were randomly divided into three groups, being either vitrified (vit), cryopreserved conventionally (con) or cultured until hatching without previous cryopreservation (ctl).
Post thawing, reexpansion and hatching rates were documented at 24h and 48h. Hatched blastocysts of all three groups were either stained using ethidium homodimer and Hoechst 33342 or used for RT-qPCR. The following gene transcripts known to play important roles during preimplantation development were analyzed, HSP70-1, SLC2A1, SLC2A3, TJP1, CDH1, DNMT3A, IFNτ, DSCII.

Reexpansion and hatching rates were similar in both groups of cryopreserved embryos (vit: 81.1% / 63.2%; con: 79.4% / 61.1%, respectively). Additionally, total cell numbers and live-dead-ratio of cells did not differ significantly (total cell numbers vit: 121.3 ± 21.2; con: 117.6 ± 20.4; ctl: 130.2 ± 25.6, respectively).
Significant differences were found in 4 of 8 gene transcripts (HSP70-1, SLC2A1, TJP1, DSC2) when comparing vitrified embryos to embryos of the control group and in 6 of 8 gene transcripts (HSP70-1, SLC2A1, SLC2A3, TJP1, DNMT3A, IFNτ) when comparing conventionally cryopreserved embryos to the embryos of the control group. These results indicate that vitrification might be the more favourable method to cryopreserve in vitro produced bovine embryos.

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Notes

CALCIUM CONCENTRATION IN VITRIFICATION SOLUTION AFFECTS SPONTANEOUS PARTHENOGENETIC ACTIVATION IN VITRIFIED OVINE OOCYTES.

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A large increase in intracellular calcium caused by cryoprotectants may decrease viability and explain the low efficiency of oocyte cryopreservation. Accordingly, dimethyl-sulfoxide and ethylene glycol have been shown to cause degeneration and parthenogenetic activation in vitrified ovine oocytes (Succu et al., Mol Reprod Dev 2007). The intracellular Ca^{++} increase, after exposure to CPAs, could mimic the activation process and reduce the male pronucleus formation in ovine and porcine oocytes (Tian et al., An Reprod Science 2007; Somfai et al., Cryobiology 2007).

The present study was designed to determine whether different calcium concentrations in the vitrification solutions could affect the spontaneous parthenogenetic activation of vitrified ovine oocytes.

In vitro matured ovine oocytes were vitrified (Succu et al., Mol Reprod Dev 2007) using vitrification media with different calcium concentrations in order to achieve five experimental groups: A (TCM 199+20%FCS,[Ca^{2+}] 9.9 mg/dl), B (PBS+20% FCS,[Ca^{2+}] 4.4 mg/dl), C (PBS without Ca^{++} and Mg^{++}+20% FCS,[Ca^{2+}] 2.2 mg/dl), D (PBS+0.4% BSA,[Ca^{2+}] 3.2 mg/dl) and E (PBS without Ca^{++} and Mg^{++}+0.4% BSA,[Ca^{2+}] 0.4 mg/dl).

After warming oocytes of the five experimental groups were cultured in synthetic oviductal fluid (SOF) containing 2% (v/v) oestrus sheep serum at 39°C and 5% CO_2, 5% O_2 and 90% N_2 atmosphere. After 28h oocytes were fixed with methanol, stained with propidium iodide and examined using an inverted microscope. Oocytes showing one or more pronuclei were considered as activated by parthenogenesis.

When the rates of spontaneous parthenogenetic activation were compared (Chi-square Test) no significant differences were observed between the groups A (41.2%) and B (35.3%). However, these rates were significantly higher than those of the groups C and E (12.7% and 11.5% respectively; $P<0.01$). Group D (25.3%) showed a significantly lower rate compared to group A ($P<0.01$) while no differences were shown neither between the groups B and D nor between the groups C and E. The oocytes vitrified in group C, finally, showed a significantly lower rate compared to group D ($P<0.05$). Moreover, Pearson test showed a positive correlation between calcium concentration in vitrification solutions and spontaneous parthenogenetic activation (correlation index 0.82; $P<0.001$).

In conclusion we have observed that the reduction of Ca^{++} concentration in the vitrification media determined a proportional reduction of parthenogenetic activation of ovine oocytes. This could suggest that also in sheep the initial activation and cell cycle progression in parthenogenitically activated oocytes is dependent on the level of intracellular calcium as already observed in mouse (Ducibella et al., Dev Biol 2002).

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Notes

FATE OF DONOR COWS AND HEIFERS AFTER EMBRYO FLUSHING

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Despite the intensive research in the field of embryo technology very little has been published about the effects of superovulation and embryo flushing procedures on the donor's fertility. The objective of the study was to discover the fate of the donor cows and heifers after embryo recovery attempt, especially from the fertility point of view.

The material was obtained from the Agricultural Data-Processing Centre Ltd., Finland, where milk recording and artificial insemination (AI) data for all the cattle in Finland are collected. All embryo recoveries during the years 1998–2003 were included. The data consisted of 2194 flushings, of which 1395 were performed in commercial farms and 799 in the ASMO Nucleus Herd (Ayrshire). Of the flushings, 905 were performed on cows and 1289 on heifers (1565 Ayrshire, 586 Holstein-Friesian and 43 other breeds).

On average, 5.3±5.1 transferable embryos were collected per recovery attempt. Subsequently, the animals were either flushed again (548 cases), inseminated again (1307), culled without AI (166) or used as embryo recipients (122). The time interval from flushing to the 1st AI was 35.6±27.4 d in commercial farms and 27.7±18.6 d in the ASMO herd. The time interval from flushing to conception was on average 66.5±57.0 days. In commercial farms, this was 69.2±60.7 days and in the ASMO herd 60.4±44.9 days. In heifers and cows the intervals were 66.0±55.9 and 67.8±58.0 days, respectively. On average, 2.0 AIs were needed per pregnancy.

Of the 1307 animals (405 cows/857 heifers) that were inseminated again 97 (69/28) never calved anew. Thirty-six (21/15) were culled due to infertility; in 24 (18/6) cases the reason was not reported. There were cases in which the fertility was compromised before the flushing (interval from calving to flushing >200 d, AIs before flushing), or after the flushing the interval from the 1st to the last insemination was less than 60 d and only 1–3 AIs were performed. After removing these cases 15 animals (6/9) were culled clearly due to infertility. Analysis of data (AIs, medical treatments, culling) regarding animals culled due to unknown reason revealed that 8 (6/2) were culled due to infertility. Thus, 1.8% (23/1307) of the flushed animals were culled due to infertility, 2.7% of cows and 1.3% of heifers.

In conclusion, superovulation and embryo flushing procedures do not seem to compromise the fertility of donor animals in practice, because nearly all animals that were intended to get pregnant conceived in fairly acceptable time period. In addition, the risk for culling due to infertility of donor animals did not seem to be increased.

Notes

EMBRYO TRANSFER FROM TWO CLONES OF THE SAME MARE: A WORLD FIRST

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Equine embryo transfer is today a common technique in equine industry. Somatic cloning in horses is available, but has a very low output, so this technique stays expensive, and then exceptional. Cloning has mainly given birth to males cloned, most often from geldings, to exploit the genetical potential of these animals. Since the birth of Prometea, the first cloned equid, very few mares have been cloned until now. Fertility of Prometea has been shown: she has foaled in 2008. Exploit cloned mares as embryo donors let contemplate a maximal diffusion of their precious genotypes, but is this possible? Two mares cloned from the same mare (same sample of skin fibroblasts), of the same age (transfer on the same day, and born respectively the 30 and the 31 of March 2007), breed in the same conditions, were followed in an insemination and embryo transfer center during the 2009 breeding season, to use them as embryo donors, with the same stallions.

The aim of this work was to test the embryo donor potential of mares cloned,and to compare the results obtained by two clones of the same mare.

The two cloned mares were gynaecologically monitored from May to August 2009. Three stallions were chosen as potential fathers, and were used successively during the season and for the two clones. For each exploitable cycle, mares were inseminated classically with fresh, cooled or frozen-thawed semen of one of the three selected stallions. Embryo recovery was done by uterine flush 7 or 8 days after ovulation, in accordance with the classical technique (Squires E.L. and coll., 1993). Non-surgical embryo transfers were performed in accordance with the classical technique (Squires E.L. and coll., 1993), in the hour after embryo recovery, on recipient mare ovulated two days after the donor mare. Pregnancy diagnoses were realised by ultrasound on day 7 after embryo transfer (day 14 of pregnancy) and regularly after.

The two mares cloned were exploited on 5 cycles. For the clone 1: an embryo has been recovered for 3 of the 5 attempts, and from 3 different stallions. For the clone 2: only one collection has been successful. The transfers of the 3 embryos of clone 1 were successful and the three foalings are expected for May, June and July 2010. The embryo transfer of clone 2 hasn't done a pregnancy.

These embryo transfers from 2 mares cloned of the same mare are a world first. It can be conclude that, firstly, a mare cloned can present a fertility to be used as an embryo donor, and then optimize the diffusion of its genotype. Secondly, two mares cloned from the same mare, of the same age, breed in the same conditions, don't present the same breeding performances. This difference could be linked to the influence of recipient oocytes and / or to other epigenetical phenomenons.

Reference: Squires E.L. (1993) Embryo Transfer. In : Equine Reproduction. Eds : McKinnon AO and Voss JL. Lea and Febiger, Pennsylvania USA pp 362-363.

Notes

SUPPRESSION OF NUCLEOPHOSMIN 1 IN BOVINE PREIMPLANTATION EMBRYO BY RNA INTERFERENCE

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Nucleophosmin 1 (B23), a member of nucleoplasmin family, is a multifunctional nucleolar phosphoprotein which is involved in ribosomal biogenesis, late rRNA processing, centrosome duplication or protein chaperoning. It is activated at 8-cell stage and participates in the establishment of the functionally activated nucleolus. The pre-EGA embryos lack functional nucleoli and hence it is supposed that protein synthesis is localized to maternally derived ribosomes. In vivo produced bovine embryos establish functional fibrillo-granular nucleoli at late 8-cell stage. Localization of nucleophosmin 1 is cell cycle dependent and reflects the nucleolar development. The expression of nucleophosmin 1 and other nucleolar proteins such as fibrillarin is used as marker of embryonic rRNA activation.

Bovine zygotes were microinjected with nucleophosmin 1 dsRNA (800 ng/μl) 20 h post fertilization. Two control groups were established – the uninjected group and a group injected with GFP dsRNA. After microinjection, embryos were cultivated in vitro and collected at specific developmental stages (late 8-cell stage, 16-cell stage, morula, blastocyst). The number of embryos that reached each developmental stage was counted and the quality of each embryo was determined. The expression of nucleophosmin 1 was characterized by a real-time RT-PCR.

After injection of nucleophosmin 1 dsRNA the level of mRNA was degraded by 97.9% ($p<0.001$) in comparison with uninjected control and by 94.6% ($p<0.001$) in comparison with GFP dsRNA injected control. No significant difference was found in the abundance of nucleophosmin 1 mRNA between the uninjected group and the GFP dsRNA injected group ($P>0.05$).

However, there was no decrease in protein staining intensity in nucleophosmin 1 dsRNA injected embryos and there was no difference in the localization in comparison to the control groups. No significant difference in embryo quality was found as well. This suggests the storage of maternal protein during preimplantation development and the substitutability of the embryonic nucleophosmin 1 by maternal protein.

The immunofluorescence analysis revealed that nucleophosmin 1 is dispersed through nucleoplasm during interphase in embryos before embryonic genome activation (EGA). However, we have found cytoplasmic localisation with only weak localization to the chromosomes during mitosis. As the nucleoli are being formed after EGA the presence of shell-like structures can be observed. Starting with the morula stage, protein is localized in numerous foci in nucleus, which reflects the establishment of matured functionally active nucleoli. These foci are comparable with the localization of another nucleolar protein - fibrillarin that is used as rRNA processing protein marker.

In conclusion, our results show that the de-novo synthesis of embryonic nucleophosmin 1 is not needed during bovine preimplantation development. The maternal protein is stored until the blastocyst stage and is capable of compensating the embryonic protein.

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Notes

CHARACTERIZATION OF CAPRINE SPERM MOTILITY ON SWIM-UP SELECTED FRACTION AND ITS RELATIONSHIP WITH IN VITRO FERTILITY

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In an attempt to develop a model for predicting in vitro fertility on the basis of the characterization of motile fraction selected by swim-up (SW), a retrospective study of a total of 14 experiments was made. The methodology consisted on the recovery and selection of oocytes from prepubertal goat ovaries and the subsequent *in vitro* maturation (IVM) and fertilization (IVF) as described by Romaguera et al. (2010, Theriogenology, D-10-00122R1). Briefly, fresh ejaculates from four bucks of proven fertility were collected using an artificial vagina and were immediately mixed in equal quantities. Spermatozoa were selected by the SW technique, heparin pre-treated and co-cultured with the IVM oocytes. At 24 hours post-insemination (hpi), presumptive zygotes were washed and cultured in synthetic oviductal fluid with 10% of fetal calf serum in groups of 10-15 embryos/drop for 8 days. Cleavage and blastocyst rates were recorded at 48 hpi and 8 days pi, respectively. To determine the motion characteristics of SW selected fraction, sperm samples (50 μL) were taken and analyzed by a computer-assisted sperm analysis system (CASA). Considering that the present results are still very preliminary, the stepwise multiple regression analysis seems to indicate that ALH is one of the most determinant motion characteristic on the IVF outcome. Moreover, in order to test the presence of separate sperm subpopulations, motility data were analyzed with a hierarchical clustering of variables based on a correlation or covariance matrix to select the sperm motility descriptors that better explain the spermatozoon kinetics, clustering the whole motile sperm population in four separate subpopulations, with significant differences in its distribution ($P<0.001$) among the SW selected fractions. Our results show that separate subpopulations of spermatozoa coexist in SW fraction, having to analyze still its potential relationship with IVF fertility.

Table 1. Characterization of caprine sperm motility on the swim-up selected fraction

Sperm motility descriptors	Whole population	Subpopulations			
		1	2	3	4
VCL (μm/sec)	109.5±15.0	103.3±13.0 ^{b}	141.7±13.9 ^{c}	57.5 ±23.6 ^{d}	202.5 ±13.0 ^{a}
VSL (μm/sec)	99.5±15.3	94.8±12.3 ^{c}	128.6±14.6 ^{b}	42.6 ±20.9 ^{d}	117.9 ±17.5 ^{a}
VAP (μm/sec)	104.4±15.3	98.9±11.8 ^{c}	135.6±12.1 ^{b}	48.4 ±20.9 ^{d}	189.9 ±0.4 ^{a}
LIN (%)	88.0±3.0	92.2±8.0 ^{a}	91.1± 9.2 ^{b}	74.0 ±24.5 ^{d}	88.5±0.4 ^{c}
STR (%)	93.0±2.0	96.0±5.9 ^{a}	94.9±7.5 ^{b}	85.3 ±18.5 ^{d}	94.0 ±9.1 ^{c}
ALH (μm/sec)	1.8±0.2	1.7±0.7^{c}	2.1±0.9 ^{b}	1.6 ±1.0 ^{d}	3.3 ±1.7 ^{a}
BCF (Hz)	8.6±0.8	8.9±2.7 ^{c}	9.61±3.2 ^{b}	6.7±3.2^{d}	9.9 ±3.2 ^{a}

Different letters in the same line show significant differences ($p \leq 0.005$) among subpopulation mean values (± SD).

Notes

EFFECT OF SUPEROVULATION ON THE QUALITY AND DEVELOPMENTAL COMPETENCE OF PORCINE OOCYTES MEASURED BY MICROFLUIDIC CHIP SYSTEM

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Stimulation of follicular growth by exogenous hormones as used for induction of superovulatory response may influence intrafollicular oocyte development. However, applicable methods to assess oocyte quality after hormonal stimulation are deficient. In a first study in pigs, we propose a new system based on Lab-on-Chip technology, which can be used to evaluate oocyte quality.

Altogether 12 cyclic German Landrace gilts, after 15-day long estrus synchronization with Regumate®, were stimulated with 850 IU eCG (Pregmagon®) to induce follicle growth. Oocytes were recovered by endoscopic OPU 64, 72, 80 or 88h after eCG. After morphological evaluation oocytes were stained using the developmental competence BCB test. BCB^-, BCB^+, as well as control cells without staining were evaluated using Lab-on-Chip system.

The Lab-on-Chip measurement setup consists of a VIS/NIR light source (a halogen lamp), miniature spectrometer (Ocean Optics, USA) and PC with original Ocean Optics software. The spectral characteristics were recorded, normalized, and processed.

We found several differences in light absorbency between BCB^+ and BCB^- oocytes within the evaluated time window. BCB+ oocytes displayed high absorbance intensity at the beginning (64h), decreased level at 72h and stabilized high absorbance from 80 to 88h. BCB- oocytes had low absorbance intensity at 64h, marginal decreased level at 72h, and increased level of absorbance intensity not until 88h, i.e. later than BCB+ oocytes. Furthermore, homogeneity of absorbance, intensity of absorbance and the dynamic of absorbance was always higher in BCB+ oocyte groups compared to BCB-.

From this first study we conclude (i) that light intensity measurement by Lab-on-Chip technology can be a promising tool to evaluate oocyte quality and (ii) BCB+ oocytes differs in absorbance intensity and dynamic from their BCB- counterparts, which is also reflected by specific ooplasmic parameters.

Notes

TRANSFER OF EMBRYOS TO OESTROUS RECIPIENT MARES TREATED WITH LONG-ACTING PROGESTERONE OR ALTRENOGEST

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The use of exogenous hormones to synchronise ovariectomised mares as embryo transfer recipients has been described previously. Likewise, anoestrous mares can be used as recipients by administering progesterone, with or without oestrogen, to achieve approximate synchrony with the donor mare. Furthermore, altrenogest treated asynchronous cycling mares have been used as recipients with varying degrees of success. This paper describes the administration of long-acting injectable altrenogest or progesterone-in-oil to oestrous mares in an attempt to use them as recipients.

Follicular development was monitored in donor and recipient mares by transrectal ultrasound. When the dominant follicle reached ≥ 35mm diameter in the donors they were inseminated with fresh, extended semen and concurrently administered 0.5ml Deslorelin[1] i.m to induce ovulation. Control recipient mares underwent the same treatment but were not inseminated. Treated recipients received either 5ml of 300mg/ml long-acting (LA) progesterone (LA P4)[1] or 1.5ml of 150mg/ml LA altrenogest (LA Alt)[1] i.m when serum progesterone concentrations were still < 1.0ng/ml and no follicle of ≥ 35mm diameter was present in the ovaries. These injections were repeated once weekly in mares receiving LA P4 and every 9 days for those given LA Alt. On day 8 after ovulation the donor mares were flushed and any Grade 1 embryos recovered were transferred non-surgically to either LA P4 or LA Alt-treated recipients that had ovulated 3 or 5 days previously (n = 6 in each group). Pregnancy diagnosis was performed 4 days after transfer by transrectal ultrasound examination of the uterus and repeated on alternate days thereafter to monitor embryonic development.

The percentages of recipient mares diagnosed pregnant 4 days after embryo transfer were the same (75%) in the untreated control and the treated recipient groups (n = 12 and 24 respectively) with no difference between LA P4 and LA Alt treatment regimes. However, whereas all 9 of the control mares remained pregnant and produced an embryo with a discernable heartbeat around days 14 – 16 after transfer, 10 of the 18 (55%) of the progestagen/progesterone treated recipients had lost their pregnancy by this stage. The pregnancies that failed were characterised ultrasonographically by small embryonic vesicles for gestational age that showed retarded growth prior to their disappearance.

In this experiment administration of LA P4 and LA Alt to non-ovulated recipient mares did not prevent pregnancy loss in a high percentage (55%) of them. This was not a consequence of any inability of the compounds to raise peripheral blood progesterone levels in the treated recipients and the retarded development and ultimate death of the embryos in a high proportion of the treated recipients suggests that the uterine environment in these animals could not support embryonic growth. It is proposed that the endogenous hormones associated with oestrus and ovulation alter the ability of the administered progesterone or altrenogest to bring about changes in the uterine environment.

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Notes

OVARIAN VOLUME AT THE START OF FSH-TREATMENT AND PREDICTION OF SUPEROVULATORY RESPONSE IN HOLSTEIN COWS

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Prediction of superovulatory (SO) response is mainly based on follicle counts. The present study was performed in order to investigate whether the pooled ovarian volume of both ovaries (pOV) at the start of FSH-stimulation is a suitable indicator to prognosticate the SO response in cattle. Holstein donor cows (n=196; >1st lactation) were grouped according to their ultasonographically determined pOV: Group 1 (n=59): <40 mm^3; Group 2 (n=52): 40-49 mm^3; Group 3 (n=48): 50-59 mm^3; Group 4 (n=37): >60 mm^3. Additionally ovarian follicles were counted (Sonovet 2000, 7.5 MHz). The animals were superovulated (630 IU FSH in decreasing doses), and luteolysis was induced with 500 µg Cloprostenol and 25 mg Dinoprost on the 4th day of FSH-stimulation (principally D13-16 of the cycle). Embryo collection took place on D7 of pregnancy. The results were recorded, and corpora lutea (CL) were counted ultrasonographically. Cows with larger pOV showed a better SO response than those with smaller ovaries (Group 1: 11.9±7.6 CL; Group 2: 13.3±7.6; Group3: 13.4±7.7; Group 4: 15.8±7.6 CL; r=0.14, P=0.05), and Group 1 vs. Group 4 differed significantly (P=0.02). Pooled ovarian volume at the start of superovulation itself was affected by the age of donors (P<0.001), and was significantly (P<0.001) correlated with the ovarian volume at the time of embryo collection, which reflects the number of CL. No significant correlation between pOV and the number of follicles ≥4 mm at the start of superovulation was found (P>0.5), although follicle counts were a hallmark for SO reaction in this study, too.

In conclusion, pOV-determination at the start of FSH-treatment can act as a quick direct as well as indirect indicator for the prediction of the superovulatory response.

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