19ème COLLOQUE SCIENTIFIQUE

19th SCIENTIFIC MEETING

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Professor Ian Wilmut

Special Celebration

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Rostock 12th and 13th September 2003

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Professor Ian Wilmut

A.E.T.E. Pioneer Award 2003

Ian Wilmut
A.E.T.E. Medalist 2003

Out of curiosity, if you type the name Ian Wilmut into an internet search engine, you will receive over 14,400 citations; all of them are post 1997 when Ian Wilmut stunned the world with the birth of Dolly, the first animal cloned from adult cells. In fact, Professor Ian Wilmut is known worldwide and not only amongst scientists but amongst people in general. Let us look back over several decades at this extraordinary career.

Ian Wilmut was born on July 7th of 1944. He is not a native from Scotland, like Dolly, but comes from Hampton Lucy in Warwickshire, England.

Since the beginning of his studies, he was interested in animal physiology. As an undergraduate at the University of Nottingham, where his mentor was the famous expert in reproduction, G E Lamming, he was seduced into embryology. He obtained a B.Sc. in Agricultural Science in 1967 and from then he probably knew that animal genetic engineering would become his life quest. He started working with pigs and for 4 years he prepared his PhD under the supervision of Professor "Chris" Polge FRS at the Darwin College of Cambridge. His Ph.D. degree was awarded in 1971 for research on the Deep Freeze Preservation of Boar Semen involving investigations into the influence of diluent composition, glycerol concentration and methods of thawing on survival of spermatozoa.

Cambridge was a mythical place for research on cryopreservation and Ian Wilmut achieved a MMB post-doctoral fellowship at the ARC unit of Reproductive Physiology and Biochemistry with Chris Polge and LEA Rowson to develop methods for deep frozen storage of mammalian embryos. After preliminary cryobiology work on mouse embryos (1972), he attempted to freeze cow embryos. He was already very successful at the beginning of his scientific career as he obtained the world's first calf from a frozen thawed embryo; this calf was named "Frosty". This result, published in 1973 (Veterinary Record 92:686-690), was obtained using late blastocysts recovered after hatching on Day 10 to 13 and slowly frozen in a combination of three cryoprotective agents (DMSO, sucrose and PVP) in glass test tubes. After 6 days storage in liquid nitrogen, blastocysts were thawed and cultured before surgical transfer to 11 recipients resulting in one calving. At the time, Ian Wilmut wrote: "It will be necessary to follow the calf and its progeny in order to be certain that no genetic damage was caused by the stress of freezing and thawing". Thirty years later, generations of cattle have proved to develop normally after freezing and all the embryo transfer practitioners, who are now routinely freezing thousands of bovine embryos each year, are thankful for this pioneering work on freezing. It has allowed great progress in the development of the embryo transfer industry and breeding procedures.

In 1973, Ian Wilmut got the position of Senior Scientific Officer in the AFRC Animal Breeding Research Organisation and moved to Edinburgh where he has lived ever since with his family in the Border countryside.

Initially, his research work on sheep and pigs was focussed on the identification of developmental and physiological causes of prenatal death. The novel experiments he conducted to investigate the influence of uterine environment on sheep embryo development using asynchronous transfers are a solid basis for successful embryo transfer in ruminants. These experiments also provided new understanding of the cause of embryonic death, implantation failures and the critical times when the uterine environment first modifies the embryo.(J.Reprod.Fert. 1981, 61:179-184).

He then became the leader of a research group that has been focussing on the factors regulating embryo development after nuclear transfer. This work led to the first birth of live lambs from embryo-derived cells and then to the birth of lambs derived from foetal and adult cells,

including Dolly. Subsequently, genetic changes were introduced into sheep by nuclear transfer from cultured modified cells, and he was co-leader of the successful project aiming to produce human therapeutic proteins in the milk of transgenic sheep. This opened the way to the development of new methods for the introduction of genetic changes into livestock by nuclear transfer.

In 2000, he became Head of the Department "Gene Expression and Development" at the Roslin Institute, but also serves as Scientific Advisor of Geron Bio-Med. The present objectives of the group are to determine the molecular mechanisms that are important for normal development of cloned embryos and to use that knowledge in biology, medicine and agriculture. Potential applications include the derivation of human cells for therapy and the provision of organs for xeno-transplantation.

To date, Ian Wilmut has published more than 100 refereed original publications and 45 chapters in books and conference proceedings from invited lectures he gave travelling all over the world. He is deeply involved in diffusion of scientific knowledge through his role in the editorial board of several journals and in scientific societies (President of IETS 1994, Society for Study of Fertility, Low Temperature Biology Society, and many others…). More recently he created a new scientific journal called "Cloning and stem cells" of which he has been Editor-in-chief since 1998. He co-wrote a book, entitled "The Second Creation", with Keith Campbell and Colin Tudge which was published in 2000.

Ian Wilmut, qualified as the father of Dolly, the first animal to develop after nuclear transfer from an adult cell, is recognized as a pioneer in the science of cloning but also as a very active participant in the public discussion on the social and ethical consequences of this new biotechnology. Since Dolly, his work has been recognized by many awards and honorary degrees. In 1999, he was made a member of the Order of the British Empire by Queen Elisabeth II and then was elected as Fellow of the Royal Society of Edinburgh (March 2000) and Royal Society of London (May 2002). In this context, the medal from our "Association Européenne de Transfert Embryonnaire" may appear quite modest but nevertheless the AETE is proud to dedicate this pioneer award to Ian Wilmut's entire career in embryology that contributed so much to the improvement of the reproductive biotechnologies in farm animals.

With such a busy life, we hope that Ian Wilmut can still spare some of his precious time for his hobbies as he enjoys walking in the mountains of Scotland, photography, classical music, and gardening in his small quiet village.

Yvan HEYMAN

LIVESTOCK CLONING: PAST, PRESENT AND FUTURE

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In principle, methods for mammalian nuclear transfer offer new opportunities in research as well as in livestock breeding and production. At present these benefits remain largely unfulfilled. The purpose of this paper is to review the development of the techniques and to discuss the limitations of the present procedures before considering possible avenues for improvement of the techniques so as to allow attainment of at least some of the practical benefits. It is also important to consider what needs to be known about the health and performance of cloned animals before they are produced for any of the potential applications.

So far there have been two periods in the history of livestock cloning. The first period began when Steen Willadsen first achieved mammalian cloning while he was working in Cambridge, England. At that time he described the birth of cloned lambs before he moved to the United States of America where he successfully applied the methods to cattle. At this time the donor nuclei were obtained from early embryos. The second era began when the group at Roslin Institute described the birth of offspring first from differentiated, cultured, embryo derived cells (Campbell et al., 1996b) and then later from fetal and adult sheep cells (Wilmut et al., 1997). Subsequently, essentially the same procedure was used to obtain offspring after transfer of nuclei from somatic cells of seven other adult mammals. The approaches used in these two methods of nuclear transfer will be contrasted before the effectiveness of the procedures is compared. Finally, in looking to the future, an analysis will be made of the molecular factors that limit development of cloned embryos.

Nuclear transfer from blastomeres

Undoubtedly attempts at nuclear transfer in mammals were stimulated by earlier research in amphibians. However, several technical challenges faced those considering the first experiments in mammals, including the enabling techniques for the recovery and culture of oocytes and embryos. Specifically, in relation to nuclear transfer, it was necessary to enucleate the recipient oocyte, to introduce the donor nucleus and to activate the reconstructed oocyte. Willadsen obtained enucleated oocytes by cutting the oocyte in half, knowing that one segment was very likely to be enucleated, although at that time he had no way of identifying that half. Either electric current or use of Sendai virus induced cell fusion in the first experiment. As the oocytes were comparatively aged at the time of micromanipulation it is likely that oocyte activation was induced by handling, although this was not appreciated at the time. While apparently simple with hindsight, these neat effective procedures were revolutionary at the time.

The outcome of these procedures was the birth of the first mammalian clones produced by nuclear transfer. Offspring were obtained first from 8-16 cell embryos, but later from morulae and early blastocyst. Indeed essentially this procedure also yielded offspring from the embryonic disc of day 9 sheep embryos. (Campbell et al., 1996b)

There were improvements to the method of nuclear transfer. The DNA specific fluorochrome (Hoechst 33342) was used to reveal the location of the metaphase spindle.(Tsunoda et al., 1988) Different groups have used this approach either to confirm enucleation guided by the presence of the first polar body or to reveal the spindle using extremely sensitive cameras. In this way the volume of the remaining cytoplast was greater and it was found that this lead to increased development of the reconstructed embryos.

It has long been recognised that if two cells are fused together the outcome depends upon the stage of the cell cycle of each cell (Campbell et al., 1996a). Many groups began to investigate the role of cell cycle in mammalian nuclear transfer and in particular to consider the influence of the activity variously known as Mitosis/Meiosis Promoting Factor (MPF). This complex of proteins acts to induce nuclear membrane breakdown after completion of DNA replication. At the time of ovulation the oocyte has a high level of MPF activity, but this declines after activation induced either by fertilisation, parthenogenetic activation or aging. It was established that transfer

of nuclei to oocytes at metaphase II is followed by nuclear membrane breakdown. This allows access to the chromosomes of licensing factors that induce DNA replication. In time, as the MPF activity decays the membrane reforms. Direct observation confirmed that DNA replication was induced regardless of the stage of the cell cycle of the donor nucleus. In these circumstances it was recognised that normal ploidy was only to be expected if the donor nucleus was awaiting DNA replication at the time of transfer. However, a second strategy was also available in which the recipient was activated before nuclear transfer and time allowed for the decay of MPF activity. In this case the nucleus determines whether or not DNA replication should occur. As a result it was expected that normal ploidy would be retained in all cases. This approach was simply a more systematic version of the procedure first used by Willadsen and formed the basis of methods used to produce cloned cattle, sheep, mice and rabbits. The methods of cattle cloning were developed for commercial use, but a variety of political and biological factors limited the extent of the application (see below).

Other research at this time revealed important differences depending upon the stage of the donor nucleus at the time of transfer. Specifically, there were benefits in selecting nuclei in G1 phase of the cycle for transfer to oocytes at metaphase II of meiosis, rather than using unselected cells for transfer to pre-activated oocytes. Unfortunately selection of cells in this phase is technically challenging. There are several "check points" in the cycle of adult cells at which cells can readily be made to pause as is used commonly in somatic cell transfer, (see below). However, cells in early embryos lack most of those check points. In order to obtain cells in G1 phase it was necessary to cause their arrest at the time of cell division by including an inhibitor of the cytoskeleton, such as nocodazole. Once released from the presence of the drug such cells tend to pass through G1 phase in a comparatively synchronised group.

The proportion of embryos reconstructed according to these procedures that developed to become viable offspring decreased as nuclei were taken from later stages of development of the donor embryo. The decline is observed most clearly in an experiment in the mouse (Cheong et al., 1993). When nuclei were transferred from 2-, 4- or 8-cell stages the proportions of reconstructed and activated eggs that became young at term were 22, 14 and 8%, respectively. A similar effect had been described previously after nuclear transfer in amphibians. The effect was assumed to reflect progressive changes in organisation of the nucleus and chromatin that became more complex and more difficult to reverse.

Differences between species were already apparent in the response to this method of nuclear transfer. Most strikingly, offspring were obtained from the blastocyst stage of sheep (Smith and Wilmut, 1989) and cattle (Bondioli, 1993), but not later than the 8-cell stage in mice when using this approach to nuclear transfer at that time. This response was associated with a comparable difference between species in the stage of development at which transcription from the embryonic genome assumes control of development (Thompson, 1996). In the earliest stages of development proteins and RNA produced in the oocyte before ovulation control development of the embryo. At a species specific stage of development transcription from the embryonic genome is initiated and RNA from the oocyte is lost. This critical event occurs during the two-cell stage in mice, but in 8-16 cell stage in sheep and cattle embryos. After nuclear transfer from a stage of development later than this point it is necessary for the mechanisms that regulate the onset of transcription to be reset. It was pointed out that in the mouse there is less time for this to occur. In addition, it might be assumed that at any stage more changes in chromatin structure would have taken place in those species in which embryonic activation occurred earlier.

Nuclear transfer from somatic cells

It is a matter of historic record that the first use of donor nuclei induced to become quiescent was associated with the production of the first offspring from differentiated cells (Campbell et al., 1996b) and later adult somatic cells (Wilmut et al., 1997). Since then it has become clear that offspring from somatic cells can also be obtained following transfer of nuclei in G1 phase of the active cell cycle (Wells et al., 2003). An accurate comparison of the two approaches of G0 or G1 phases remains to be described.

In the first experiments the donor cells were induced to exit the cycle and become quiescent by reducing the concentration of serum in the culture medium to 0.5% (Campbell et al., 1996b). More recently a similar effect has also been shown after increasing the density of cells in the culture (Gao et al., 2003). In this latter case the increase in development to term that followed

the use of cells at higher density was not associated in any change in the phase of the cell cycle of the embryo stem cells. These results suggests that beneficial differences in chromatin structure can be induced during culture of donor cells in a variety of ways, but these are not understood.

Introducing a delay between nuclear transfer and activation of the reconstructed embryo is a second modification to the cloning procedure that we now know confers benefits in some circumstances. The response to the period of delay varies with species in that it is essential for normal development to term in the mouse (Wakayama et al., 1998), has a beneficial effect in cattle (Wells et al., 1999), but has no effect in sheep (Campbell et al., 1996b). The effect in the mouse is apparently dependent on the presence of cytochalasin during delay, and this intriguing result should be confirmed. The mechanism of this beneficial effect is not understood, although various suggestions have been made, including that remodelling continues during the delay and that intra-cellular structures that are lost during quiescence are re-assembled during the delay.

Offspring have been obtained following transfer of nuclei from somatic cells of adult tissues of seven species; sheep (Wilmut et al., 1997), cow (Wells et al., 1999), goat (Keefer et al., 2001), pig (Polejaeva et al., 2000), mouse (Wakayama et al., 1998), rabbit (Chesne et al., 2002) and cat (Shin et al., 2002) and from mule fetal cells (Woods et al., 2003). However, the same experienced teams have failed to obtain offspring from several other teams, despite considerable research effort. These include rat, dog and rhesus monkey. This finding is assumed to reveal important differences between species in developmental biology, response to nuclear transfer or enabling techniques such as those for oocyte recovery or embryo culture and transfer.

Offspring have been produced from a great variety of different donor cell types (a summary prepared in August 2002 is available on http://www.roslin.ac.uk/public/webtablesGR.pdf). However, not all attempts have been successful. Specifically, offspring were not obtained following transfer from differentiated mouse neural populations nor from Sertoli cells of adult mice (Wakayama et al., 1998). By contrast, offspring were obtained from early stages of neural differentiation (Yamazaki et al., 2001) and from Sertoli cells of juvenile mice (Ogura et al., 2000). Overall there is an impression that as cells differentiate nuclear transfer is less successful extending to mammals the conclusion from early amphibian experiments.

Although nuclear transfer according to this procedure has been repeatable and is in regular use in many different labs around the world, it is still inefficient. Between 1 and 5% of reconstructed embryos survive to become viable offspring regardless of species, donor cell type, details of the procedure or laboratory (Wilmut et al., 2002). This inefficiency is the cumulative result of failures at all stages of development from reconstruction to adulthood. The precise pattern of loss probably varies with species and cell type, but there are very few direct comparisons on which to make such a judgement. In comparing the development of embryos transferred at the blastocyst stage it certainly seems that a greater proportion of those derived from murine ES cells develop to term than is the case for blastocysts derived from other mouse cells (Rideout et al., 2000) . It is suggested that after transfer of nuclei from embryo stem cells early development is particularly limited because it is not possible to synchronise embryo stem cells in G0 or G1 phases of the cell cycle. By contrast, it is suggested that as embryo stem cells have differentiated very little their nuclei are re-programmed more readily allowing a greater proportion of later embryos to develop to term. It would be interesting to know the proportion of embryos that develop to term after transfer of nuclei from an adult stem cell population. In many cases they can be synchronised in G0 or G1 phase and it might be expected that they have differentiated only a little further than embryo stem cells.

A considerable number of causes of loss have been described. In some cases they appear very similar to causes of mortality of neonates produced by natural mating, but others are not reported in these circumstances as reviewed recently (S Rhind et al., Nature Biotechnology. In Press). A cloned lamb produced at Roslin Institute hyperventilated all of the time. When no treatment was found to correct the condition the animal was euthanased at 12 days of age. Detailed histopathology revealed massive hypertrophy of smooth muscles around arterioles in the lung. Apparently this restricted blood flow and prevented adequate gaseous exchange. This observation has an important practical implication. It seems very unlikely that it could have been detected in utero by any means. Immune insufficiency has been reported by several laboratories and is another abnormality with the same important limitation (Renard et al., 1999).

Factors limiting the development of cloned embryos

It is likely that the very high incidence and unusual causes of death both before and after birth reflect the influence of genes whose expression is abnormal and whose effect can be lethal (Wilmut et al., 2002). There is a great deal of evidence of inappropriate gene expression in cloned embryos, foetuses and offspring (Daniels et al., 2000); (Humpherys et al., 2002; Humpherys et al., 2001);. In some cases enabling techniques, such as those for embryo culture, induce the changes. Several conclusions can be drawn from these studies. 1) Patterns of gene expression vary between embryos produced by the same nuclear transfer procedure in one experiment (Bortvin et al., 2003), 2) gene expression sometimes varies from one cell to another in an embryo (Boiani et al., 2002), 3) that the donor cell type influences gene expression (Humpherys et al., 2002), 4) many genes are sometimes affected. We have no way of knowing, but it is quite possible that every gene is affected, but that frequency of error varies from one gene to another and that the consequences of inappropriate expression are different. Despite the observations of inappropriate gene expression (see below) extensive re-programming must occur for development to occur through organogenesis. Chromatin in a donor cell will be organised to support gene expression that is appropriate for that specific tissue. The mechanisms involved include localisation of specific chromosomal regions in regions of active transcription, stable inhibition of expression of some genes, but expression of others. Extensive re-modelling of the nucleus and chromatin must occur if the nucleus of a differentiated cell is able to control embryo and fetal development, including for example that for the regulation of such fundamentally important genes as the *hox I* family of genes which are critical for establishment of many aspects of the body plan.

Unusually large offspring are sometimes born if ruminant embryos are cultured for just a few days in the presence of serum (Walker et al., 1996). These changes are associated with inappropriate expression of imprinted genes that are known to regulate foetal growth and development. In sheep, expression of the IGF2 receptor gene was shown to be reduced in organs from lambs that were unusually large at the time of autopsy late in gestation (Young et al., 2001). By contrast, expression of IGF2 itself was not changed. Rather than acting to signal the presence of the growth factor to the cell the IGF2 receptor is believed to remove and destroy IGF2. In these circumstances the peripheral concentration of IGF2 would be expected to be higher and acting to increase growth. The change in gene expression was related to loss of methylation in regions of the gene known to regulate gene function in most, but not all foetuses that were studied. This difference suggests that other, as yet unknown, genes may also contribute to the unusual foetal growth in some animals. Observations are being made now to discover if expression of this group of imprinted genes is perturbed in foetuses developing from cloned embryos.

Some research concentrated upon expression of imprinted genes because the phenotype of some abnormal cloned animals was very reminiscent of that seen after abnormal expression of one of this group of genes. Imprinted genes are unusual in that a mechanism exists to ensure that only one copy of the gene is expressed (Jaenisch, 1997). By a mechanism that is not completely understood changes are introduced during gametogenesis that ensure that some genes are not expressed. Many of the genes are concerned with the regulation of foetal growth. Molecular techniques have been used in mice to examine the effect of either over expression of the gene or loss of expression of either copy. Either of these circumstances may occur if there are errors in the imprinting mechanisms. There is now detailed confirmation that imprinted genes are sometimes expressed inappropriately, with the most extensive set of observations being in the mouse. However, in one study it was found unexpectedly that only one copy of the gene was expressed in the cloned offspring (Inoue et al., 2002). That is the "imprint" was retained. Rather the change must have been due to the other mechanisms that regulate the level of functioning of the gene, by mechanisms that are common to other, non-imprinted, genes.

Experiments in mice have revealed the variation in gene expression between embryos at the blastocyst stage and within embryos. Expression of the gene Oct4 is known to be essential for normal pre-implantation development in the mouse . Embryos were studied at the blastocyst stage after transfer of nuclei either from ovarian cumulus cells or from embryo stem cells (Bortvin et al., 2003). Whereas Oct4 is expressed in embryo stem cells, it is not in cumulus cells. Microarray analysis has demonstrated that expression of several genes is associated with that of Oct4. All embryos expressed a housekeeping gene, but there were differences in expression of Oct4 and the other genes. At the blastocyst stage expression of Oct4 was observed in all embryos produced from embryo stem cells, but not of those from cumulus cells. This may reflect the fact that the gene is expressed in embryo stem cells so that there is no need for change in the functioning of the gene. By contrast, among those embryos derived from cumulus cells there was variation in

expression of Oct4 and the related genes. Two of the genes studied are situated close together on the same chromosome, but on opposite strands. The fact that in some cases only one of these two genes was expressed suggests that there may be quite local variations in the mechanisms that regulate gene expression.

Extensive differences in gene expression were apparent in placental and liver tissue from cloned mice at term (Humpherys et al., 2002). The mice were derived either from fresh cumulus cells or from cultured embryo stem cells. In comparison to control mice, expression of some 4,000 genes was abnormal in the placenta of cloned animals. Most changes were common to mice derived from both cumulus cells and embryo stem cells, but some were unique to one or other cell type. There were fewer errors in liver tissue, and some of the perturbations were in genes that were not changed in placental tissue.

Future opportunities to improve efficiency

Some improvements in both speed and efficiency in production of cloned embryos are still being obtained by comparatively minor changes to the methods (Oback et al., 2003; Peura, 2003), but it seems very likely that radically new approaches will be required in order to improve the efficiency of nuclear transfer. These may include intervention to assist in the process of reprogramming the transferred nucleus, either by pre-treatment of the donor cells before transfer, treatment of the reconstructed embryo or both. At present relatively little is known of the molecular mechanisms involved in reprogramming in mammals after either fertilisation or nuclear transfer. Failure of reprogramming may reflect inadequate quantities of effective molecules or the absence of the necessary activities. It is important to remember that evolution has never acted to produce oocytes able to reprogramme somatic cell nuclei. No doubt empirical studies are underway in several labs using crude extracts of oocyte and embryo stem cells.

Assessment of cloned animals

There is now a considerable body of literature describing the abnormalities that have been observed in cloned animals, but most of these are in the mouse. By contrast, it seems that some groups involved in livestock cloning have been rather more reticent in reporting the limitations of the procedure. Many people would feel that with the present procedures the unusually high incidence of late pregnancy loss, the occasional difficult birth and the increased frequency of post-natal death make large scale application of the procedure inappropriate on welfare grounds alone. However, in special circumstances when the objective cannot be achieved in any other way nuclear transfer may be ethically acceptable and commercially rewarding.

Large-scale application may be appropriate once the efficiency has been increased and the frequency of abnormalities at term greatly reduced. At this point it will also be essential to assess the performance and health of cloned animals extensively in order to be able to provide potential customers with all of the necessary information. In early experiments when nuclei were transferred from blastomeres of early embryos birth weight of cloned calves was far more variable than expected (Wilson et al., 1995). Indeed it was more variable than in groups of full sibs produced by embryo transfer. This unexpected observation probably reflected the same epigenetic effects that now lead to the unusual pattern of fetal and calf death after transfer of nuclei from somatic cells. As the method of nuclear transfer improves we should expect such variability to reduce, but it will be important to confirm that expectation.

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TABLE : 1 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: AUSTRIA

A.E.T.E 2002

Data collected by
Dr. Karl Bauer

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	84	B / A=	18.5
	Embryos collected	**B**	1525	C / A=	10.5
	Embryos transferable	**C**	883	C / B=	57.9%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	48		
Total i*n vitro* embryos		**F**	48	=(D+E)	
Total number of transferable embryos		**G**	931	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	154		
In vivo	Frozen	**I**	622		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	776	H+I+J+K=	
Number of frozen stored embryos		**M**	26		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	80.1%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	0
Number of calves born from *in vitro* embryos	0
Total	0

* data not available

TABLE : 2 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: BELGIUM

A.E.T.E 2002

Data collected by
Dr. Jean-François Beckers

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1016	B / A=	6.86
	Embryos collected	**B**	6967	C / A=	4.74
	Embryos transferable	**C**	4816	C / B=	69.1%
In vitro *(OPU)*	Nb of oocyte donors		50		
	Nb of OPU sessions		142		
	Nb of transferable embryos	**D**	416		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	38		
Total *in vitro* embryos		**F**	454	=(D+E)	
Total number of transferable embryos		**G**	5270	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1165		
In vivo	Frozen	**I**	2975		
In vitro	Fresh	**J**	38		
In vitro	Frozen	**K**	3		
Total embryos transferred		**L**	4181	H+I+J+K=	
Number of frozen stored embryos		**M**	2290		
% of *in vitro* embryos transferred		**N**	0.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	71.2%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	0
Number of calves born from *in vitro* embryos	121
Total	121

* data not available

TABLE : 3 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: **CROATIA**

A.E.T.E 2002

Data collected by
Dr. Jak_a Petri_/Iva Getz

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	16	B / A=	3.18
	Embryos collected	**B**	51	C / A=	2.75
	Embryos transferable	**C**	44	C / B=	86.3%
In vitro	Nb of oocyte donors		18		
(OPU)	Nb of OPU sessions		82		
	Nb of transferable embryos	**D**	162		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	102		
Total *in vitro* embryos		**F**	264	=(D+E)	
Total number of transferable embryos		**G**	308	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1		
In vivo	Frozen	**I**	0		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	1	H+I+J+K=	
Number of frozen stored embryos		**M**	42		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	0	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	0
Number of calves born from *in vitro* embryos	0
Total	0

* data not available

TABLE : 4 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: CZECH REPUBLIC *A.E.T.E 2002*

Data collected by
Dr. Jirina Petelikova

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1321	B / A=	9.2
	Embryos collected	**B**	12187	C / A=	4.5
	Embryos transferable	**C**	5945	C / B=	48.8%
In vitro *(OPU)*	Nb of oocyte donors		2		
	Nb of OPU sessions		5		
	Nb of transferable embryos	**D**	-		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	159		
Total *in vitro* embryos		**F**	159	=(D+E)	
Total number of transferable embryos		**G**	6104	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3267		
In vivo	Frozen	**I**	2541		
In vitro	Fresh	**J**	50		
In vitro	Frozen	**K**	43		
Total embryos transferred		**L**	5901	H+I+J+K=	
Number of frozen stored embryos		**M**	2590		
% of *in vitro* embryos transferred		**N**	1.6%	(J+K) / L=	
% of frozen embryos transferred		**O**	43.8%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 5 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: DENMARK

A.E.T.E 2002

Data collected by
Dr. Henrik Callesen

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	850	B / A=	10.21
	Embryos collected	**B**	8683	C / A=	6.50
	Embryos transferable	**C**	5529	C / B=	63.7%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	5529	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2255		
In vivo	Frozen	**I**	2247		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	4502	H+I+J+K=	
Number of frozen stored embryos		**M**	2935		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	49.9	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	1796
Number of calves born from *in vitro* embryos	0
Total	1796

* data not available

TABLE : 6 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: ESTONIA *A.E.T.E 2002*

Data collected by
Dr. Y. Jaakma

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	0	B / A=
	Embryos collected	**B**	0	C / A=
	Embryos transferable	**C**	0	C / B=
In vitro	Nb of oocyte donors		0	
(OPU)	Nb of OPU sessions		0	
	Nb of transferable embryos	**D**	0	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0	
Total i*n vitro* embryos		**F**	0	=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	0	
In vivo	Frozen	**I**	93	
In vitro	Fresh	**J**	0	
In vitro	Frozen	**K**	0	
Total embryos transferred		**L**	93	H+I+J+K=
Number of frozen stored embryos		**M**	0	
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=
% of frozen embryos transferred		**O**	100%	(I+K) / L= %

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	23
Number of calves born from *in vitro* embryos	
Total	23

* data not available

TABLE : 7 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: FINLAND

A.E.T.E 2002

Data collected by
Dr. Marja Mikkola

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	441	B / A=	9.37
	Embryos collected	**B**	4134	C / A=	6.38
	Embryos transferable	**C**	2812	C / B=	68.0%
In vitro *(OPU)*	Nb of oocyte donors		7		
	Nb of OPU sessions		24		
	Nb of transferable embryos	**D**	7		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	4		
Total i*n vitro* embryos		**F**	11	=(D+E)	
Total number of transferable embryos		**G**	2823	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	977		
In vivo	Frozen	**I**	1113		
In vitro	Fresh	**J**	8		
In vitro	Frozen	**K**	1		
Total embryos transferred		**L**	2099	H+I+J+K=	
Number of frozen stored embryos		**M**	1286		
% of *in vitro* embryos transferred		**N**	0.4%	(J+K) / L=	
% of frozen embryos transferred		**O**	53.1%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 8 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: FRANCE

A.E.T.E 2002

Data collected by
Dr. Bernard Guérin

Total number of approved E.T. teams in the country	31
Number of teams providing data	28

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	6797	B / A=	9.71
	Embryos collected	**B**	66031	C / A=	5.55
	Embryos transferable	**C**	37725	C / B=	57.1%
In vitro	Nb of oocyte donors		85		
(OPU)	Nb of OPU sessions		144		
	Nb of transferable embryos	**D**	298		
In Vitro	Nb of transferable embryos	**E**	*		
(Slaughtered donors)					
Total *in vitro* embryos		**F**	298	=(D+E)	
Total number of transferable embryos		**G**	38023	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	17562		
In vivo	Frozen	**I**	15158		
In vitro	Fresh	**J**	212		
In vitro	Frozen	**K**	9		
Total embryos transferred		**L**	32941	H+I+J+K=	
Number of frozen stored embryos		**M**	12562		
% of *in vitro* embryos transferred		**N**	0.7%	(J+K) / L=	
% of frozen embryos transferred		**O**	46.0%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 9 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: GERMANY

A.E.T.E 2002

Data collected by
Dr Karin Clauss

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2567	B / A=	9.22
	Embryos collected	**B**	23693	C / A=	5.49
	Embryos transferable	**C**	14099	C / B=	59.5%
In vitro	Nb of oocyte donors		*		
(OPU)	Nb of OPU sessions		1227		
	Nb of transferable embryos	**D**	2894		
In Vitro	Nb of transferable embryos	**E**	*		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	2894	=(D+E)	
Total number of transferable embryos		**G**	16993	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5831		
In vivo	Frozen	**I**	4310		
In vitro	Fresh	**J**	1374		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	11515	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**	11.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	37.4%	(I+K) / L=	%

Number of E.T. calves born (2001)

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

* data not available

TABLE : 10 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: GREECE

A.E.T.E 2002
Data collected by
Dr. E. Vainas

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	19	B / A=	4.84
	Embryos collected	**B**	92	C / A=	3.34
	Embryos transferable	**C**	64	C / B=	69.6%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions				
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	64	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	52		
In vivo	Frozen	**I**	8		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	60	H+I+J+K=	
Number of frozen stored embryos		**M**	12		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	13.3%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	21
Number of calves born from *in vitro* embryos	0
Total	21

* data not available

TABLE : 11 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: HUNGARY

A.E.T.E 2002

Data collected by
Dr. Laszlo Solti

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	128	B / A=	7.36
	Embryos collected	**B**	942	C / A=	3.85
	Embryos transferable	**C**	493	C / B=	52.3%
In vitro	Nb of oocyte donors		*		
(OPU)	Nb of OPU sessions		*		
	Nb of transferable embryos	**D**	*		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	575		
Total *in vitro* embryos		**F**	575	=(D+E)	
Total number of transferable embryos		**G**	1068	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	184		
In vivo	Frozen	**I**	488		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	672	H+I+J+K=	
Number of frozen stored embryos		**M**	477		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	72.6%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	230
Number of calves born from *in vitro* embryos	-
Total	230

* data not available

TABLE : 12 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: IRELAND

A.E.T.E 2002

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**	258	B / A=	8.60
	Embryos collected	**B**	2219	C / A=	5.04
	Embryos transferable	**C**	1300	C / B=	58.6%
In vitro	Nb of oocyte donors		*		
(OPU)	Nb of OPU sessions		*		
	Nb of transferable embryos	**D**	*		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	*		
Total *in vitro* embryos		**F**	*	=(D+E)	
Total number of transferable embryos		**G**	1300	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	462		
In vivo	Frozen	**I**	654		
In vitro	Fresh	**J**	*		
In vitro	Frozen	**K**	*		
Total embryos transferred		**L**	1116	H+I+J+K=	
Number of frozen stored embryos		**M**	838		
% of *in vitro* embryos transferred		**N**	-	(J+K) / L=	
% of frozen embryos transferred		**O**	58.6%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 13 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: ITALY

A.E.T.E 2002
Data collected by
Dr. Francesco Brun

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1078	B / A=	14.31
	Embryos collected	**B**	15428	C / A=	7.03
	Embryos transferable	**C**	7576	C / B=	49.1%
In vitro *(OPU)*	Nb of oocyte donors		173		
	Nb of OPU sessions		750		
	Nb of transferable embryos	**D**	1601		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	2373		
Total i*n vitro* embryos		**F**	3974	=(D+E)	
Total number of transferable embryos		**G**	11550	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3278		
In vivo	Frozen	**I**	3226		
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	4236		
Total embryos transferred		**L**	10840	H+I+J+K=	
Number of frozen stored embryos		**M**	7920		
% of *in vitro* embryos transferred		**N**	40.0%	(J+K) / L=	
% of frozen embryos transferred		**O**	68.8%	(I+K) / L=	%

Number of E.T. calves born (2001)

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

* data not available

TABLE : 14 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: LITHUANIA *A.E.T.E 2002*

Data collected by Dr. Jonas Kutra

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	4	B / A=	9.75
	Embryos collected	**B**	39	C / A=	1.75
	Embryos transferable	**C**	7	C / B=	17.9%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	7	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	0		
In vivo	Frozen	**I**	0		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	0	H+I+J+K=	
Number of frozen stored embryos		**M**	34		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	0	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	0
Number of calves born from *in vitro* embryos	0
Total	0

* data not available

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: (REPUBLIC) MOLDAVIA *A.E.T.E 2002*

Data collected by
Dr. Gregorie Darie

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	64	B / A=	3.70
	Embryos collected	**B**	237	C / A=	0.28
	Embryos transferable	**C**	18	C / B=	7.5%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro	Nb of transferable embryos	**E**	0		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	18	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	164		
In vivo	Frozen	**I**	18		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	182	H+I+J+K=	
Number of frozen stored embryos		**M**	23		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	9.8%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	31
Number of calves born from *in vitro* embryos	0
Total	31

* data not available

TABLE : 16 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: (The) NETHERLANDS *A.E.T.E 2002*

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**	2971	B / A=	6.22
	Embryos collected	**B**	18492	C / A=	5.06
	Embryos transferable	**C**	15030	C / B=	81.3%
In vitro	Nb of oocyte donors		208		
(OPU)	Nb of OPU sessions		2226		
	Nb of transferable embryos	**D**	2073		
In Vitro	Nb of transferable embryos	**E**	25		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	2098	=(D+E)	
Total number of transferable embryos		**G**	17128	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3811		
In vivo	Frozen	**I**	11563		
In vitro	Fresh	**J**	1165		
In vitro	Frozen	**K**	753		
Total embryos transferred		**L**	17292	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**	11.1%	(J+K) / L=	
% of frozen embryos transferred		**O**	71.2%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 17 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: NORWAY

A.E.T.E 2002

Data collected by
Dr. Elisabeth Kommisrüd

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	56	B / A=	6.95
	Embryos collected	**B**	389	C / A=	3.93
	Embryos transferable	**C**	220	C / B=	56.5
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro	Nb of transferable embryos	**E**	0		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	0	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	42		
In vivo	Frozen	**I**	175		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	217	H+I+J+K=	
Number of frozen stored embryos		**M**	153		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	80.6%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	120
Number of calves born from *in vitro* embryos	
Total	120

* data not available

TABLE : 18 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: POLAND

A.E.T.E 2002

Data collected by
Dr. Jedrzej Jaskowski

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	72	B / A=	6.90
	Embryos collected	**B**	497	C / A=	4.01
	Embryos transferable	**C**	289	C / B=	58.1%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	23		
Total i*n vitro* embryos		**F**	23	=(D+E)	
Total number of transferable embryos		**G**	312	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	167		
In vivo	Frozen	**I**	178		
In vitro	Fresh	**J**	18		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	363	H+I+J+K=	
Number of frozen stored embryos		**M**	98		
% of *in vitro* embryos transferred		**N**	4.95%	(J+K) / L=	
% of frozen embryos transferred		**O**	49.0%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 19 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: PORTUGAL ***A.E.T.E 2002***

Data collected by
Dr. Joao Nestor das Chagas e Silva

Total number of approved E.T. teams in the country	7
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	54	B / A=	8.2
	Embryos collected	**B**	442	C / A=	6.2
	Embryos transferable	**C**	336	C / B=	76.0%
In vitro	Nb of oocyte donors		-		
(OPU)	Nb of OPU sessions		-		
	Nb of transferable embryos	**D**	-		
In Vitro	Nb of transferable embryos	**E**	-		
(Slaughtered donors)					
Total *in vitro* embryos		**F**	-	=(D+E)	
Total number of transferable embryos		**G**	336	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	105		
In vivo	Frozen	**I**	116		
In vitro	Fresh	**J**	-		
In vitro	Frozen	**K**	-		
Total embryos transferred		**L**	221	H+I+J+K=	
Number of frozen stored embryos		**M**	105		
% of *in vitro* embryos transferred		**N**	-	(J+K) / L=	
% of frozen embryos transferred		**O**	52.5%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	68
Number of calves born from *in vitro* embryos	0
Total	68

* data not available

TABLE : 20 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: ROMANIA

A.E.T.E 2002

Data collected by
Dr. Stela Zamfirescu

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	27	B / A=	7.74
	Embryos collected	**B**	209	C / A=	7.18
	Embryos transferable	**C**	194	C / B=	92.8%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	194	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	64		
In vivo	Frozen	**I**	116		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	180	H+I+J+K=	
Number of frozen stored embryos		**M**	14		
% of *in vitro* embryos transferred		**N**	-	(J+K) / L=	
% of frozen embryos transferred		**O**	64.4%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	63
Number of calves born from *in vitro* embryos	0
Total	63

* data not available

TABLE : 21 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: SLOVAKIA

A.E.T.E 2002
Data collected by
Dr. Peter Cesnak

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	206	B / A=	10.2
	Embryos collected	**B**	2098	C / A=	5.14
	Embryos transferable	**C**	1060	C / B=	50.5%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	1060	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	837		
In vivo	Frozen	**I**	301		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	1138	H+I+J+K=	
Number of frozen stored embryos		**M**	223		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	26.4%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	664
Number of calves born from *in vitro* embryos	0
Total	664

* data not available

TABLE : 22 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: SPAIN

A.E.T.E 2002
Data collected by
Dr. Julio de la Fuente

Total number of approved E.T. teams in the country	11
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	372	B / A=	9.31
	Embryos collected	**B**	3993	C / A=	4.50
	Embryos transferable	**C**	1798	C / B=	45.0%
In vitro	Nb of oocyte donors		29		
(OPU)	Nb of OPU sessions		293		
	Nb of transferable embryos	**D**	175		
In Vitro	Nb of transferable embryos	**E**	-		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	175	=(D+E)	
Total number of transferable embryos		**G**	1973	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	460		
In vivo	Frozen	**I**	880		
In vitro	Fresh	**J**	11		
In vitro	Frozen	**K**	4		
Total embryos transferred		**L**	1355	H+I+J+K=	
Number of frozen stored embryos		**M**	1636		
% of *in vitro* embryos transferred		**N**	1.1%	(J+K) / L=	
% of frozen embryos transferred		**O**	65.2%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	456
Number of calves born from *in vitro* embryos	-
Total	456

* data not available

TABLE : 23 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: SWEDEN

A.E.T.E 2002

Data collected by
Dr. Hans Gustafsson

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	308	B / A=	6.32
	Embryos collected	**B**	1947	C / A=	3.92
	Embryos transferable	**C**	1209	C / B=	62.1%
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**	-	=(D+E)	
Total number of transferable embryos		**G**	1209	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	485		
In vivo	Frozen	**I**	611		
In vitro	Fresh	**J**	-		
In vitro	Frozen	**K**	4		
Total embryos transferred		**L**	1100	H+I+J+K=	
Number of frozen stored embryos		**M**	724		
% of *in vitro* embryos transferred		**N**	0.3	(J+K) / L=	
% of frozen embryos transferred		**O**	55.9%	(I+K) / L=	%

Number of E.T. calves born (2002)

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

* data not available

TABLE : 24 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: SWITZERLAND *A.E.T.E 2002*

Data collected by Dr. Saner Rainer

Total number of approved E.T. teams in the country	5
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	215	B / A=	11.0
	Embryos collected	**B**	2362	C / A=	7.2
	Embryos transferable	**C**	1549	C / B=	65.6%
In vitro	Nb of oocyte donors		30		
(OPU)	Nb of OPU sessions		270		
	Nb of transferable embryos	**D**	111		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	111	=(D+E)	
Total number of transferable embryos		**G**	1660	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	430		
In vivo	Frozen	**I**	1225		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	138		
Total embryos transferred		**L**	1793	H+I+J+K=	
Number of frozen stored embryos		**M**	1236		
% of *in vitro* embryos transferred		**N**	7.7%	(J+K) / L=	
% of frozen embryos transferred		**O**	76.0%	(I+K) / L=	%

Number of E.T. calves born (2002)

Number of calves born from superovulated embryos	295
Number of calves born from *in vitro* embryos	34
Total	329

* data not available

TABLE : 25 EMBRYO TRANSFER ACTIVITY IN 2002

COUNTRY: UNITED KINGDOM ***A.E.T.E 2002***

Data collected by
Dr. Lydia Breslin

Total number of approved E.T. teams in the country	20
Number of teams providing data	10

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	*	B / A=
	Embryos collected	**B**	*	C / A=
	Embryos transferable	**C**	*	C / B=
In vitro *(OPU)*	Nb of oocyte donors		*	
	Nb of OPU sessions		*	
	Nb of transferable embryos	**D**	*	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	*	
Total i*n vitro* embryos		**F**	*	=(D+E)
Total number of transferable embryos		**G**	*	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	*	
In vivo	Frozen	**I**	*	
			*	
In vitro	Fresh	**J**	*	
In vitro	Frozen	**K**	*	
Total embryos transferred		**L**	3638	H+I+J+K=
Number of frozen stored embryos		**M**	3520	
% of *in vitro* embryos transferred		**N**	*	(J+K) / L=
% of frozen embryos transferred		**O**	*	(I+K) / L= %

Number of E.T. calves born (2002)

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

* data not available

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY IN EUROPE IN 2002

I. EMBRYO PRODUCTION

(Data collected from 25 countries)

***In vivo* produced embryos (superovulation)**	
- number of flushed donors	**18,294**
- number of transferable embryos	**102,996**
- mean number per flushed donor	**5.63**
***In vitro* produced embryos:**	
From OPU	
- number of oocyte donors	**602**
- number of OPU sessions	**5,163**
- number of transferable embryos	**7,737**
From slaughterhouse collected ovaries	
- number of transferable embryos	**3,347**
Total *in vitro*	**11,084**
Total number of transferable embryos	**114,080**

(P. Lonergan, AETE Rostock, 2003)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2002

II. EMBRYO TRANSFERS

(Data collected from 25 countries)

From *In* vivo produced embryos -	**Number of embryos transferred** **90,371***
From *In vitro* produced embryos -	**8,167***
Total number of embryos transferred	**102,176**
Proportion of IVF embryos transferred	**8.29 %**
Proportion of frozen embryos transferred	**54.6 %**

* These numbers are underestimated as the data from 1 country were not available and not included

(P. Lonergan, AETE Rostock 2003)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES*
EUROPE year 2002

Species	Embryo Production	Embryo Transfers	Countries
Sheep	**501**	**398**	Czech rep Greece Hungary Portugal Romania Slovakia
Swine	**1383**	**995**	Czech Rep Hungary Romania
Goat	**76**	**53**	Romania
Horse	**115**	**74**	Austria Finland Hungary Italy

***** numbers are underestimated for Europe as only a limited number of countries answered the questionnaire

(P. Lonergan, AETE Rostock, 2003)

INVITED LECTURES

POSSIBILITIES TO PREDICT TIME OF OVULATION IN CATTLE

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Introduction

For conception to occur, insemination must take place at the correct stage of the cow's oestrus cycle. The chance of fertilisation highly depends on the interval from insemination to ovulation. When cows are inseminated too early chances are that the aged sperm is not capable to fertilise the ovum anymore (Hawk, 1987). When insemination takes place too late chances are that because of the ageing of the egg fertilisation and formation of a viable embryo is not possible anymore (Hunter and Greve, 1997). Indications exist that in practice, an enormous variability exists in the timing of insemination relative to ovulation with subsequent negative consequences for pregnancy rate. The causes of variation in the timing of insemination relative to ovulation include variation in oestrus detection strategy and accuracy, in moment of ovulation after detection of oestrus and in the timing of insemination after detection of oestrus.

In pigs, the optimal time for insemination lies between 0 and 24 h before ovulation (reviewed by Kemp and Soede, 1997). Not much is known about fertilisation rates of cows at various insemination to ovulation intervals. Studies from the 1940's showed that conception rates in cattle differed when cows were bred during different stages of oestrus (Trimberger and Davis, 1943; Barrett and Casida, 1946). This research indicates that there are sub-optimal and optimal times for insemination and that these are probably dependent on the timing of ovulation. The best conception rates (varying from 73% to 86%) were obtained when insemination took place at 6 to 24 hours before ovulation compared with earlier or later inseminations when conception rates of 25 to 57% were observed (Trimberger, 1948). Hall et al. (1959) concluded from their experiment with 51 animals that the best insemination time was 17-12 hours before ovulation; the conception rate in those animals was 55%. In these experiments time of ovulation was assessed by frequent rectal palpation of the ovaries. However, it seems likely that repeated palpation will affect the time of ovulation (Aschbacher et al., 1956; Wishart, 1972). Surprisingly, since then not much information has become available assessing the optimal time of insemination relative to ovulation in cycling dairy cattle. Based on research that was performed in the 1970's, it was assumed that in cattle, insemination should take place between 12 and 18 h before ovulation to get good fertilisation results (reviewed by Hunter, 1994). However, in that research it was assumed that ovulation takes place at a rather stable 12 h after the end of oestrus. From recent research (see later), it is now known that the moment of ovulation in relation with oestrus is far more variable. In recent years research has been done to study effects of different insemination times relative to oestrus characteristics on conception rate (Grove et al., 1996; Dransfield et al., 1998; Xu et al., 1998; Dalton et al. 2001) but time of ovulation was not assessed in these studies. To determine the best time for insemination relative to the time of ovulation there is a need for practical predictors of ovulation time and information on fertilisation rates at various insemination-ovulation intervals. This paper will be restricted to prediction of ovulation. For a parameter to be useful as a predictor for time of ovulation it should have a high accuracy at identifying the appropriate physiologic or behavioural events (that occur at least 12h before ovulation) that correlate highly with ovulation in a high proportion of the animals. It should have a small variation within and between animals, and the measurements should be easy to carry out, repeatable and preferably be automated. An overview will be given about possibilities to predict the time of ovulation according to behavioural oestrus signs, hormone-profiles, body temperature, conductivity of the vaginal mucus and activity-readings. This overview will consist of data found in the literature and two experiments that we have conducted to study possible predictors of time of ovulation.

Possibilities to predict ovulation time

Experimental designs

We conducted two experiments with lactating Holstein-Friesian cows in which observations for behavioural oestrus were carried out every three hours for 30 minutes according to the method of van Eerdenburg et al. (1996). Each time an animal showed an oestrus sign, the assigned number of points was recorded (Table 1).

Table 1. Scoring scale for observed oestrus signs[1]

Oestrus sign	Points
Cajoling	3
Restlessness[2]	5
Sniffing the vulva of another cow	10
Mounted but not standing	10
Resting with chin on the back of another cow	15
(attempt) Mounting other cows	35
(attempt) Mounting head side of other cows	45
Standing heat	100

[1] Each time a sign is observed, the assigned number of points is recorded (modified after Van Eerdenburg, et al., 1996)
[2] Can be recorded only once during an observation period

If the sum of points exceeded 95 during consecutive observation periods, the animal was considered to be in oestrus. Onset of oestrus was defined as the first observation period in which the animal showed oestrus behaviour minus 1.5 hours. End of oestrus was defined as the last observation period in which the animal showed oestrus behaviour plus 1.5 hours. In the first experiment data were collected of 14 cows in which oestrus was synchronised in two consecutive cycles with a norgestomet-oestradiol treatment (Crestar ear-implant, Intervet, Boxmeer, the Netherlands). Blood samples were taken every 1_ hours for LH and oestradiol profiles. Time of ovulation was assessed by rectal ultrasound without manipulation the ovaries every three hours. In the second experiment 95 ovulations were recorded (by ultrasound every three hours) in 67 animals. The animals were fitted with pedometers that recorded the number of steps every two hours (prototype, Nedap, The Netherlands). Of these animals rectal temperature was taken 3-hourly (n=24) and measurements of vaginal conductivity were taken 6-hourly (n=22) from animals with induced luteal regression (2 ml i.m., Prosolvin, Intervet, Boxmeer, The Netherlands). In the other cycles (n=71) no treatment was used to induce luteal regression.

Oestrus behaviour

The behaviour of a cow changes during oestrus influenced by the oestrogens produced by the preovulatory follicle. The most clear sign of oestrus behaviour is the standing heat but this is expressed by < 50% of the cows (van Eerdenburg et al., 1996; 2002; Lyimo et al., 2000; Heres et al., 2000). Attempting to mount other cows is seen in 80% of the cows in oestrus and 54% of the cows in oestrus are mounted but will not stand (van Vliet et al., 1996). Other behavioural signs that are seen more frequent during oestrus are sniffing the vagina of another cow (occurred twice as often during oestrus as between oestrusses) and resting with chin on another cow (occurred four times as often during oestrus as between oestrusses (van Eerdenburg et al., 1996)). From these experiments it is clear that a large variation between animals exists in expression of behaviour. Different experiments have been conducted that studied the interval between onset or end of the behavioural oestrus (defined by first or last standing heat) and the time of ovulation (Table 2).

Table 2. Overview of intervals between onset or end of oestrus and time of ovulation found in different studies.

Interval to ovulation (range) (h)	n	Assessment of ovulation time (interval)	Assessment of oestrus (i.e. standing heat)	Breed	Reference

(interval)					
from onset of oestrus					
27.8 (16-38)	12 5	?	?	Various	Gerasimova, 1940
(20.5-27.0)	3	?	?	Guernsey	Fietta et al., 1968
32.8	13	Palpation (2h)	Visual (continuously)	Beef cows	Randel et al., 1973
31.3 ± 0.6 (se) (26->36)	21	Laparoscopy (once)	Visual (12h)	Beef heifers	Christenson et al., 1975
26.0 ± 4.0 (sd) (21.25-29.0)	4	Laparoscopy (continuously)	Visual (4h)	Holstein	Bernard et al., 1983
24.0/30.0 ± 3.2 (se) (pluri-/biparous)	8	Ultrasound (2h)	Visual (?)	Holstein	Rajamahendra n et al., 1989
27.6 ± 5.4 (sd) (16-40)	93	Ultrasound (2h)	Mounting detector (continuously)	Holstein	Walker et al., 1996
37.7 ± 11.0 (sd) (18-60)	30	Ultrasound (12h)	Visual (?)	Nguni	Augusto et al., 1997
26.6 ± 0.44 (se) (17.6-34.6)	42	Ultrasound (2h)	Visual (continuously)	Nelore	Pinheiro et al., 1998
24.9 ± 3.9 (se)	23	Ultrasound (24h)	Mounting detector (continuously)	Holstein	Lopez et al., 2002
from end of oestrus					
10.5 (3-18)	13 2	Palpation (2h)	Visual (2h)	Ayshire, Guernsey, Jersey, Holstein	Trimberger, 1948
11.1	50	Palpation (4h)	Visual (8h)	Holstein	Aschbacher et al., 1956
12.4	51	Palpation (6h)	Visual (6h)	Holstein, Jersey, Red Sindhi-Holstein	Hall, et al., 1959
9.2 ± 1.3 (se) (6-14)	6	Palpation (2h)	Visual (2h)	Friesian	Wishart et al., 1972

Several authors have looked at the timing of ovulation in relation to the onset of oestrus (see Table 2). Walker et al. (1996) found an interval of first mount (detected by a pressure-sensing system attached to the sacrum region of the cow) until ovulation of 27.6 ± 5.4 (sd) with a range of 16-40h. They assessed time of ovulation by ultrasound every 2h. Lopez et al. (2002) used the same pressure sensing system and found an interval of first mount until ovulation of 24.9 ± 3.9h (se). They assessed time of ovulation once daily by ultrasound. Augusto et al. (1997) found even a more variable interval of 37.7 ± 11.0h (sd) (18-60h) between onset of oestrus and time of ovulation in Nguni-cows. The intervals between onset of oestrus (according to the scale of van Eerdenburg et al., 1996) and time of ovulation in our experiment are in a similar range compared with those studies (we found 30.2h (range: 19-40h) and 31.0h (range: 18.5-48.5h) for exp. 1 and 2 respectively).

Intervals between end of oestrus (standing heat) and time of ovulation range between 3 to 18h with an average of 9.2 to 12.4h (Trimberger, 1948; Aschbacher et al. 1956; Hall et al., 1959; Wishart et al., 1972). Time of ovulation was assessed by rectal palpation every 2, 4 or 6h and oestrus observations were conducted every 2, 6 or 8h. As stated before, rectal palpation is not the ideal method to assess time of ovulation because the preovulatory follicle can rupture during palpation. On the other hand frequent rectal ultrasound does not appear to affect ovulation time (Rajamahendran et al., 1989; Roelofs et al., 2001, unpublished results). In our experiments we

found a larger interval between end of oestrus (according to the scale of van Eerdenburg et al., 1996) and time of ovulation (assessed by rectal ultrasound) of 16.7h in exp.1 and 18.8h in exp.2. Also the variation was larger and ranged from 9.5h to 33.5h.

In a study of van Eerdenburg et al. (2002) it was shown that the intensity of oestrus behaviour was related to the time of ovulation; cows that showed more intensive oestrus behaviour ovulated earlier after the first scanning than cows that showed less intensive behaviour ($r=0.31$, $P=0.01$). They looked at the behaviour twice daily and assessed time of ovulation once a day by ultrasound, starting immediately after AI. According to these experiments, a more accurate monitoring of oestrus expression could lead to a more accurate prediction of ovulation time. However, in practice visual oestrus detection is carried out far less intensive then was done in these experiments. It is hard to assess actual onset or end of oestrus and therefore use visual observations as prediction of time of ovulation. Even when visual observation is carried out every three hours, quite a large variability is found in the timing of ovulation relative to the onset or end of oestrus. It will be time consuming and difficult to automate. A pressure sensing system (assessing mounting attempts) can not be used as automated predictor for time of ovulation, because only cows that show standing heat are detected. Many animals will be missed because sometimes only half of the animals that are in heat will stand to be mounted (Lyimo et al., 2000). So because of the intensity of the labour or the low proportion of animals detected, behavioural oestrus signs do not seem to be useful as predictor of time of ovulation.

Hormone-profiles

Time of ovulation is related to hormone-profiles, especially the preovulatory LH-surge. Bernard et al. (1983) found an interval of onset of the LH-surge to ovulation of 27.3 ± 1.6h (sd) and the end of the LH-surge to ovulation was 17.5 ± 1.5h. Other studies indicate that the peak in the LH-surge occurs approximately 25 hours before ovulation. The data from our experiment agrees with these findings. The peak of the LH-surge occurred 25.3h before ovulation (range: 21.5-27.5; see Figure 1.A). The LH-surge therefore is a reliable predictor for time of ovulation but in practice it is not feasible to determine the LH-peak rapidly enough to predict time of ovulation. Not much research has been done regarding the relation between levels of oestradiol and timing of ovulation. Mosher et al. (1990) found an interval of the estradiol-peak (in plasma) and time of ovulation (assessed by 4-h laparoscopy) in six heifers of 22.30 ± 3.92h (sd). Lopez et al. (2002) found larger intervals between oestradiol-peaks and ovulation (assessed by once daily ultrasound). They measured oestradiol in plasma and milk samples in 23 synchronised heifers and found mean intervals from highest measured plasma and milk oestradiol until ovulation of 30.7 ± 6.3 and 46.7 ± 5.3h, respectively. A large individual variation was found in the time that the highest oestradiol concentration was detected in relation to the onset of oestrus. Our results resemble the data found by Mosher et al. (1990); we found the highest plasma levels of oestradiol on average 23.9h before ovulation, but the range was quite large (17.5-32h; see Figure 1.B).

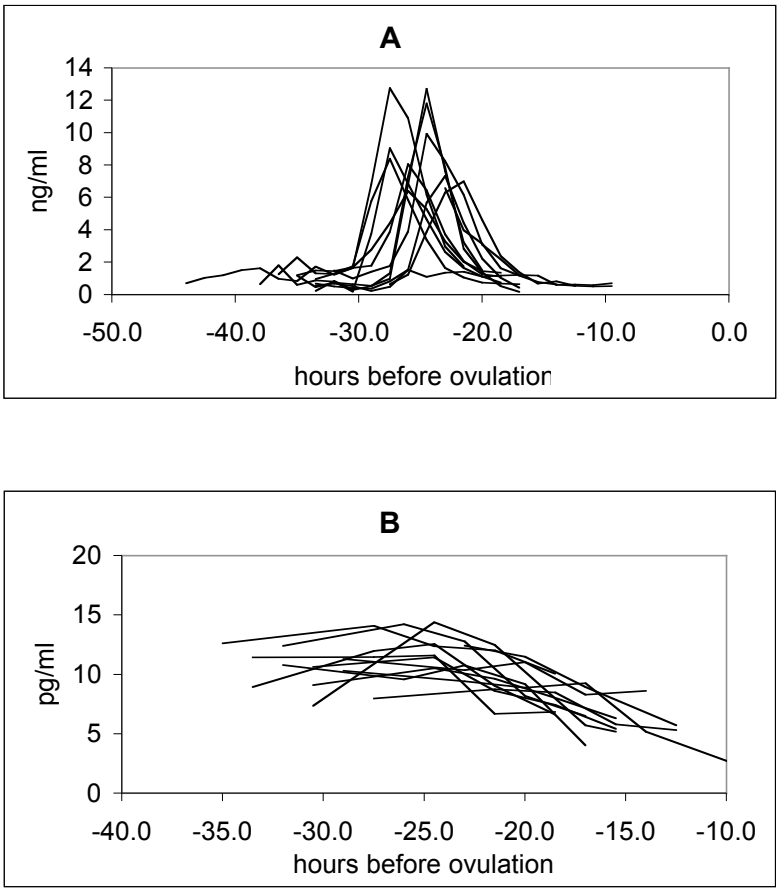

Figure 1. A. LH-profiles of 12 individual cows relative to time of ovulation (Roelofs, unpublished results). B. Oestradiol-profiles of 12 individual cows relative to time of ovulation (Roelofs, unpublished results)

The even larger interval found by Lopez et al. (2002) could be caused by the method of assessment of ovulation. In their experiment they performed ultrasound once daily to assess time of ovulation, Mosher et al. (1990) examined the ovaries every 4h using laparoscopy. Because of the large variation between animals and the difficulty in practice to assess oestradiol-levels it is not suitable as predictor of time of ovulation on the farm.

Vaginal mucus conductivity/resistance

Several studies have shown that vaginal mucus conductivity/resistance changes during oestrus (reviewed by Rorie et al., 2002). Electrical resistance changes in the tissues and secretions of the reproductive tract during the oestrus cycle of a cow. Electrical resistance is lowest during oestrus because of changes in cell density, fluid volume and electrolyte content of the bovine vulva (Ezov et al., 1990). Leidl et al. (1976) found evidence that the resistance of vaginal mucus is affected by oestrogen. Several studies have shown that the lowest resistance coincides with the LH-surge, the interval between lowest resistance and time of ovulation was between 32 and 24h (Schams et al., 1972; Leidl et al., 1976; Schams et al., 1977; Aboul et al., 1983). It is very difficult to quantitatively compare the different studies because different measure units are used. The resistance dropped from an average of 48 Ohm between oestrusses to an average of 30 Ohm during oestrus (Schams et al., 1972). Smith et al. (1989) found an average vagina conductivity value of 3.64 MS/cm during dioestrus compared with 6.29 MS/cm during oestrus. In our experiment we did not find a significant increase in conductivity around oestrus and ovulation. The variation was very high within animals from one measurement to another as well as between

animals. Other studies also found variation both among and within cows, resulting in undesirable high rates of false positives and false negatives (Elving et al., 1983; Lehrer et al., 1995). Also the high labour requirement greatly limit the practicality of this approach.

Temperature

During the oestrus cycle significant changes in body temperature occur (reviewed by Firk et al., 2002). During oestrus the temperature rises. This may be caused by the higher activity during the oestrus, but the mechanism behind the rise is not yet clear. Although this parameter has been related to oestrus-characteristics, only little is known about the relation of this parameter with time of ovulation. In seven heifers a rather variable interval between the peak in vaginal temperature and ovulation (assessed by 4-hourly laparoscopy) of 21.14 ± 6.07h (sd) has been found (range: 16-33h; Mosher et al., 1990). From their experiment they concluded that since ovulation occurs within a consistent interval from the onset of a temperature spike, the onset of a temperature spike might be as good a predictor of ovulation as the LH peak. Rajamahendran et al. (1989) demonstrated in a study with eight animals that the rise in vaginal temperature was a reliable measure of the time of ovulation and the time of the LH-surge. The peak of the vaginal temperature occurred in heifers 22.0 ± 3.5h (se) and in cows 27.0 ± 3.5h (se) before ovulation. The difference in temperature in these studies is about 0.5°C. In our study we did not find a significant change in rectal temperature around oestrus and ovulation. The variation in temperature was very high within animals from one measurement to another as well as between animals. In our study we measured rectal temperature, most other studies use vaginal temperature. Rajamahendran et al. (1989) found a high correlation between vaginal and rectal temperature, but the rise in temperature before ovulation was only significant in the vaginal temperature measurements. Several authors dispute the usefulness of vaginal temperature measurements as an oestrus detection method and therefore as predictor of time of ovulation (Lewis and Newman (1984), Boyd (1984), Grunert (1994) in Firk et al., 2002). They dispute this because body temperature is not a specific indicator for incidence of oestrus but also changes because of inflammatory reactions and variation in environmental temperature. Also because the range in the interval between the rise in temperature to ovulation is quite large and the labour requirements, the temperature is not a practicably useful tool as predictor of ovulation.

Activity

In recent years, several attempts have been made to automate oestrus detection, including use of a pedometer (Maatje et al., 1997). In Figure 3, an activity profile of an individual animal is given. Only a few attempts have been made to relate automated parameters to ovulation time (Walker et al., 1996; Lopez, 2002). In the study of Maatje et al. (1997) time of ovulation was not assessed but different intervals between onset of oestrus according to pedometer readings and time of insemination were used to assess conception rate. They found a strong relation between time of insemination after increase in activity and conception rate. The conception rate was more than 83% when insemination took place 6 to 17h after increase in activity and declined rapidly when insemination took place outside this interval. This strong relation between time of insemination and conception rate assumes that the increase in activity has a good relation with time of ovulation. In our study the mean interval between the beginning of the increase in activity according to pedometer readings and time of ovulation was 29.0h with a range of 14-41h. The peak in the activity occurred 24.7h before ovulation with a range of 11-38h. 82% of the animals showed an increase in activity. Indications exist that activity-levels and the extent of the increase in activity during oestrus vary between farms (L. Roosenschoon, personal communication).

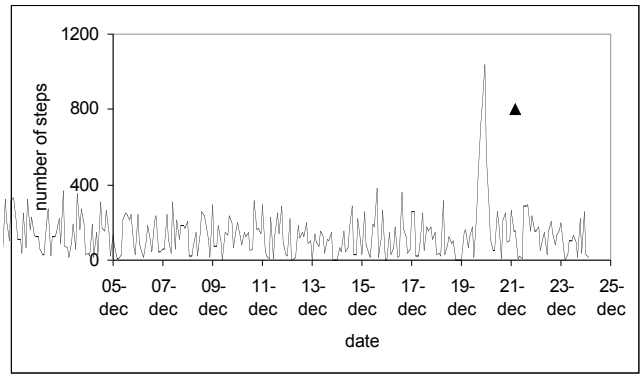

Figure 3. The activity profile of an individual animal, the black triangle indicates the time of ovulation.

So further research has to be done to study the variation between farms. However, in our study the interval between the increase in activity and time of ovulation has a high accuracy in a high proportion of the animals. It has a small variation within and between animals, and the measurements are easy to carry out, repeatable and it is an automated procedure. So pedometer readings seem to be a practical and reliable predictor for time of ovulation.

Conclusion

The peak in the LH-surge and to a lesser extent the decrease in oestradiol are reliable predictors for the time of ovulation, however in practice these parameters are difficult to measure. The behavioural aspects of oestrus show a large variation with the timing of ovulation and the difficulty with these parameters is that intensive observations are needed to assess them. When first standing heat is considered as the onset of oestrus, a lot of oestrusses will be missed because many animals do not show this behaviour. Measurements of vaginal mucus conductivity/resistance or temperature are very labour-intensive and difficult to measure reliably. The increase in activity measured by automated pedometer readings might be a reliable and in practice applicable predictor for the time of ovulation because the measurements are easy to carry out and most of the animals show an increase in activity before ovulation. It is unclear how activity-levels and the increase in activity during oestrus vary between farms. Further research has to be done to study farm differences and test the applicability of pedometer readings as predictor of ovulation.

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IN VITRO PRODUCTION OF EMBRYOS IN FARM ANIMALS

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Introduction

In the past progress in cellular and molecular embryology in farm animals has been difficult due to the limited availability of suitable experimental material at an acceptable cost. Gametes and embryos were obtained in vivo usually from superovulated donors and collected at the required developmental stage. Besides cost, animal welfare concerns have more and more limited the use of animals for experiments. For these and other many reasons the in vitro techniques for the production of mammalian embryos have received great attention and support in the last ten-fifteen years. In vitro production of embryos (IVPE) in farm animals generally includes in vitro oocyte maturation, in vitro fertilisation and the development of the fertilised oocyte to the blastocyst stage. Each of these developmental steps has to be completed successfully if the embryos obtained is expected to establish a viable pregnancy that will deliver a normal offspring. IVPE is a long and complicated procedure that can be compromised at any developmental stage both for biological intrinsic reasons or for technical reasons related to the in vitro environment. These detrimental effects usually become evident later than they occur and this makes it more difficult to address them. Although screening of gene expression is being developed for single embryos, from a practical point of view the assessment of embryo quality is based on subjective morphological criteria. This makes quality control of IVPE techniques rather difficult and the only objective (with the limit imposed by recipient-related factors) criteria is still pregnancy rate after transfer.

The IVPE technology is well established in the bovine and it is increasingly used in practice. In other ruminants the technology is potentially viable, however economical constraints limit its application for breeding. In all other domestic animals it is still at a developmental stage and further refinements are required.

Another emerging application of IVPE is by means of cell nuclear replacement (cloning) alone or in combination with transgenesis. In this case the only difference with conventional IVPE is the absence of fertilisation to generate the embryos while the oocyte maturation and embryo culture steps are the same.

In this paper we will review IVPE in the bovine, species in which it is well established and used in practice, underlining the relevant practical aspects with no ambition to review all the literature on the subject. A brief review of the state of the art in other farm animals will be presented including buffalo, horse, pig, sheep and goat.

Bovine

For IVPE immature oocytes collected from the ovaries of donors of various age and physiological status are used (19). Reliable procedures allow maturation and fertilisation of bovine oocytes in vitro and several culture protocols can be used to grow them for about a week up to the stage suitable for transfer or freezing. In vitro embryo production was developed initially as a research tool and was applied to rescue follicular oocytes of slaughtered donors. In cattle, besides this use, IVPE has become important for the production of embryos from live donors as an alternative to or integrated with MOET because of the advantages and flexibility that it offers (15).

IVPE consists in the completion of three biological steps that are now relatively well established in cattle: oocyte in vitro maturation, in vitro fertilization and embryo culture (51).

In vitro maturation

Oocytes for in vitro maturation can be collected from different types of donors and by different methods as it will be described later. Oocytes are very sensitive to temperature shocks so

it is important to monitor carefully the collection procedure as temperature fluctuations can easily occur. During collection the oocytes are maintained in Dulbecco's PBS or in TCM199 (hepes buffered). The maturation conditions used by the vast majority of the laboratories involve the use of TCM 199 supplemented with 10% fetal calf serum (FCS) and gonadotropins (FSH, LH) in 5% CO2 in air at 38.5 °C. After 20-24 h of incubation the oocytes complete maturation with the extrusion of the first polar body and are ready to be fertilized. If the donors are located far from the main laboratory, maturation can be completed during transport from the site of collection to the laboratory in test tubes in portable battery powered incubators. Under optimal conditions over 90% of the oocytes reach metaphase II. Before fertilisation the cumulus cells are partially removed to leave few corona cell layers surrounding the oocyte.

In vitro fertilization

Frozen semen is always used for in vitro fertilization and a Percoll based separation system is the most common method for isolating the motile sperm fraction after thawing (19). Although other systems can be used (swim-up, simple centrifugation) separation through a Percoll gradient offers the consistency, flexibility and reliability that are required in a commercial setting, where new sires are required on a weekly basis by the clients. Two media are generally used for in vitro fertilization (IVF): a TALP-based medium or a SOF-based medium both without glucose and with varying concentration of heparin. The concentration of spermatozoa that is needed for each bull, in order to achieve maximum fertilization with minimal polyspermy, is determined empirically by performing IVF tests with different sperm concentrations. The fertilized oocytes are fixed 18-20 h after co-incubation with the sperm and the chromatin configuration is analyzed following lacmoid staining. The results of this test indicate the optimal sperm concentration for each bull. IVF is completed overnight after 18-20 h of co-incubation of sperm and eggs. At this time the oocytes are completely denuded of the remaining cumulus cells and spermatozoa and are transferred to a culture system suitable for embryo development.

Amongst the factors affecting the success of IVF the bull has an important role to play. Some bulls perform better than others and a small proportion (<5%) performs really poorly. A second factor affecting IVF success rate is the cumulus oocyte complex. In this respect it is important to standardize the procedures for oocytes preparation before IVF, in order to obtain a homogeneous population of oocytes with a cumulus cells mass as similar as possible and consistent with previous replicates. In fact the amount of cumulus is positively related to the sperm concentration required; oocytes with fewer cumulus cells are more susceptible to polyspermy while those with a large cumulus cell mass are less likely to be fertilized.

Embryo culture

The culture step is the development of the fertilized oocyte to the blastocyst stage. Several protocols have been developed and applied. They include various coculture and cell-free systems and also the in vivo culture procedure in the surrogate sheep oviduct (12, 15,). The latter is the system that yields embryos of quality comparable to MOET embryos especially if they have to be frozen-thawed before transfer. This procedure requires specific surgical expertise and adequate facilities but it is justified by the quality of the embryos produced. It is used mainly in research settings and less in commercial settings with the exception of our own laboratory in which most of the commercial IVP embryos are cultured in the sheep oviduct. With this method the embryos are usually allowed to cleave in vitro and 48-72 h post IVF they are transferred in the sheep oviduct. In the case of small numbers of cleaved embryos from different donors they are embedded into agar chips, since this procedure allows the simultaneous transfer of the embryos of a number of donors to the same oviduct (the different number of embryos in each agar chip is the key to identify different batches of embryos at collection). However, from a practical and economical point of view, the simplest option is to continue the culture in vitro following IVF. At present, in vitro culture (IVC) of early cleavage stage bovine embryos is an area that is still the subject of many investigations. Years ago initial success was obtained with the use of coculture either with oviductal cells (11) or with feeder layers of different cell types such as BRL (27) or Vero cells (6). However, this system requires the management of feeder cells that are subjected to biological variations, especially in the case of oviduct cells. As such they are not of practical use in large scale IVP. More recently the use of semi-defined media in the absence of feeder cells with low or

no serum added and with low oxygen tension has replaced almost entirely the use of coculture. The SOF aa BSA medium (48, 22) is the most popular but some labs also use medium CR1 (44). Embryo development in vitro is usually assessed on D +6 by looking at the embryos that undergo compaction. This is the first differentiation event taking place during development and the embryos that show a clear process of compaction are those more likely to develop normally (31). The evaluation and selection of embryos for transfer or freezing is conducted on D+7. By this time normally developing embryos should have reached at least the early blastocyst stage, the majority being at the blastocyst stage and some at the expanded blastocyst stage. A number of embryos will reach these stages on D+8 or even on D+9. These embryos are considered of lower quality and are used in a commercial setting only for fresh transfer because results after freezing are poor (27).

Mass production of embryos

Large numbers of embryos can be produced from the ovaries of slaughtered donors when the female parental origin is not a must. In this case, the ovaries from donors of the same breed are pooled and processed as a batch. This procedure significantly simplifies all the steps involved in production, identification and freezing and, as a consequence, embryos are produced at low cost and can be commercialised at very competitive pricing. This type of production is used for premium beef breeds for the commercial production of beef calves from dairy herds ("beef from dairy") and it is well developed in countries like Italy or Japan where the beef industry relies on high premium local beef breeds. However, mass production can also be used to produce dairy embryos of average genetics for developing countries. Embryos produced in this way are generally frozen for direct transfer to simplify the transfer procedure.

Genetic recovery

When a donor of high genetic value is slaughtered for various reasons (terminal illness, infertility, age, etc.) her ovaries can be collected, processed separately from those of other donors and the oocytes matured and fertilized with the sire required by the client. This procedure, often defined "genetic recovery", allows the production embryos of known parentage (33). The expected results in terms of embryo production are related to the reasons for the slaughter. The outcome is usually poor (1-2 embryos per donor) for so-called "terminal" donors. This definition includes animals that are in critical general conditions, such as immediate post calving problems, acute mastitis, displaced abomasus, progressed foot or leg injuries etc.. In the case of healthy donors that are slaughtered because of infertility, end-of-career or for the eradication of infectious diseases, the outcome is much better with an average production of 6 or more embryos per donor (32). We have used this technology in the past to save the genetics of herds infected with leucosis, brucellosis and tuberculosis. More recently, with current sanitary threats, we have been involved in the salvage of the genetics of herds stamped out because one animal tested positive to the rapid BSE test after slaughter. Current policy in the European Union is to test at slaughter all animals over 24 month of age. When a case is confirmed the farmer has two options: destroy all the animals of the herd or only aged-matched peers (from one year younger to one year older than the positive animal). The Health Authority compensates the farmer for his losses. In the region of Lombardy, in the north of Italy, the veterinary authority allows farmers to collect the ovaries from the best donors providing that the resulting IVP embryos are frozen until all donors are destroyed and the BSE testing is completed. The safety of these procedure derives from the work of Wrathall and collaborators (52) who have demonstrated that BSE is not transmitted through embryo transfer and on the fact that, so far, a second positive animal has never been found in the same herd. In table 1 we report the outcome of such an operation in one herd ranked in the top 100 herds in Italy. The difficulty of this operation, that might to some extent compromises the results, is the high number of donors that had to be processed in one day. In the cases we have treated, because they were a confined slaughtering, the procedure has been concentrated in the shortest time possible. In the case summarized in Table 1 all the cows (66 animals) were slaughtered on one day and a week later the same occurred for the heifers (91 animals). This concentration of donors creates an overload of work in the IVF lab especially in the days on which the oocytes are collected and fertilised and finally on the day of embryo evaluation and

freezing. Moreover, it requires a strict and careful data collection procedure to register all the relevant information for each donor/batch of oocytes/embryos. From the point of view of the farmer this operation allows the rescue of the genetics of his herd at a cost certainly lower (at least for top herds) than acquiring new genetics on the market (18).

Ovum Pick Up

The most flexible and repeatable technique to produce embryos from any given live donor is offered by the technique of ovum pick up (OPU) or ultrasound guided follicular aspiration (15, 27, 4). A scanner with an adequate endovaginal (or adapted for the vaginal use) sector probe with a guided needle is required to perform this procedure. The needle is connected to a test tube and to a vacuum pump to aspirate the follicular fluid and the oocyte contained in it. A scanner with good resolution and with a probe of at least 6 MHz is used to visualize follicles down to 2-3 mm in size and also to view the needle during follicle aspiration. This procedure can be performed either on-farm or in an IVP residential centre. The donor is confined in a crush, mildly sedated and given an epidural anaesthesia just before collection. OPU has virtually no drawbacks for the donor and can even have a therapeutic effect in some infertile donors affected by ovarian cystic syndrome or similar pathologies that compromise reproductive function. Virtually any female starting from 6 months of age up to the third month of pregnancy and also soon after calving (2-3 weeks), is a suitable donor (27). This makes OPU a very flexible technique that, unlike MOET, does not interfere with the normal reproduction and production cycles of the donor. OPU can be performed sporadically or on a regular basis such as two times a week for many weeks or months. The twice-a-week protocol is the one that yields the maximum number of competent oocytes in a given period of time (26, 21, 24). Another advantage of OPU is that it is not necessary to treat the donor with gonadotropins (some workers however use this treatment) with the inevitable side effects. This is a very important advantage especially for young heifers in which gonadotropin-stimulation can cause mammary oedema and ovarian cystic syndrome, and for show cows where repeated superovulation can cause relaxation of the udder ligament. Many of the infertile cows that we treat at our centre are infertile as a consequence of repeated superovulation. Therefore it is desirable to avoid further gonadotropin-treatment of these animals, if a normal reproductive career has to be restored. A final advantage is the possibility of using over a short time, or even on the same collection (when many oocytes are recovered), more sires to achieve in a short time several different dam-sire combinations. The drawbacks of OPU are higher cost compared to MOET and the requirement of specialized laboratory equipment to perform all the steps of embryo production. In Table 2 are shown the results of the OPU work carried out in our laboratory over the years 1997-2001 (18). We obtain about 2.5 freezable/transferable embryos per cow and about 1.4 for heifers.

Embryo transfer and offspring

Pregnancy rates with IVP embryos can be very variable. This relates to the quality of the embryos as affected by the culture procedure, media used and the subjective evaluation and selection of embryos before freezing and/or transfer. Pregnancy rate is usually acceptable with fresh transfers but becomes more variable amongst different laboratories with frozen embryos. For successful freezing the culture system used to develop the embryos is very critical. A survival rate equal to in vivo produced embryos can be obtained when IVM-IVF embryos are cultured in the sheep oviduct (15). Embryos grown in vitro in SOF-BSA, that undergo a clear compaction step on Day 5 and/or 6, blastulate on Day 7 and are classified as grade 1 (according to the IETS manual),survive well to freezing and thawing. By contrast, culture in vitro in the presence of serum reduces the cryotolerance because of lipid accumulation. In this case vitrification could offer an alternative but it is not yet used in practice (49). Once pregnancies are established there is a moderate increase in the losses in the first trimester that can reach 10 - 12% (15). Reports in the literature indicate that problems may arise at calving. There are reports of extended gestation, dystocia, large calves, increased perinatal mortality, etc, that all together have been termed the Large Offspring Syndrome (53, 39, 13, 29). Most of the calves described in these reports were the result of coculture with granulosa or Buffalo rat liver cells and/or high serum or high BSA and were from a few transfers conducted mainly under uncontrolled conditions by research laboratories. In commercial programmes, where conditions are more controlled, using either the in vivo culture in

the sheep oviduct or the SOF system, without high level of serum or BSA, over 95% of the pregnancies are normal and the incidence of LOS is reduced (15, 39, 50, 45). Because the use of IVP is mainly for intensive breeding of the newest genetics, the bulls used are those with little or imprecise information about calving ease, and this can be another factor contributing to the calving problems.

Buffalo

Most of the work done on buffalo has been with slaughterhouse ovaries (34). Recently ovum pick up has been extended to this species as well (3, 23). In our laboratory we have worked with lactating and repeat breeder non lactating females using exactly the same procedure that we use on cattle both for OPU and for embryo production; interestingly we observed that the buffalo embryos are12 to 24 h more advanced than the bovine counterpart developing in parallel. Five donors were subjected to OPU for several weeks and the number of oocytes recovered remained constant throughout (unpublished data). The results we obtained were 4.5 oocytes per OPU resulting at the end of the production in 0.7 embryos per OPU, this demonstrate that OPU is even more competitive in buffaloes than in cattle when compared to superovulation. Nine frozen thawed embryos were transferred to synchronised recipient heifers and resulted in the birth of 3 buffalo calves (17). Buffalo frozen semen has been the main source of variation and the different bulls used for in vitro fertilisation showed a high degree of variation with an average cleavage rate of only around 40%. More recently the cleavage rate has been higher suggesting that the quality of the frozen semen has improved in general and fertility is less dependent on individual bulls. With this degree of efficiency OPU can be seriously considered an efficient tool for genetic improvement of buffalo.

Horse

Oocyte recovery from mares is complicated by anatomical and physiological differences compared to large ruminants (see 28 as review). The collection of immature oocytes is performed as in cattle however it is necessary to repeatedly flush the follicle with medium to increase the chances to recover the oocyte. The recovery rate from live donors is quite low and might range from 18 to 35% with an average of 2.4 oocytes per OPU (16). More successful is the recovery of oocytes from the preovulatory follicle just before ovulation (40) or with slaughterhouse ovaries. In vitro matured oocytes transferred to the oviduct of inseminated recipients can develop to blastocyst at very high rate (54). The procedures for in vitro maturation are identical to cattle, with the difference that compact cumulus oocyte complexes take a few hour more to reach metaphase II (about 26-28h). In vitro fertilisation is not efficient and only a limited number of foals have been obtained after this procedure (40).

Intracytoplasmic sperm injection (ICSI) looks to be the answer to the IVF problem in horse (9, 29). ICSI of in vivo matured oocytes transferred back to the oviduct of recipient mares has resulted in the birth of foals (36). In our laboratory we have performed ICSI with slaughterhouse oocytes matured in vitro and achieved cleavage (74%) and blastocyst (24%) rates similar to those of cattle in vitro system (14). The embryos obtained were transferred after freezing and thawing resulting in the birth of live offspring. The developmental competence of oocytes with an expanded cumulus at the time of collection was only about a third of that of oocytes with a compact cumulus at recovery. Culture requirements of horse oocytes look similar to those of ruminants in fact after cleavage they were embedded into agar chips and transferred to sheep oviduct for in vivo culture. The use of this technology in mares is foreseen as a therapy of infertility rather then a system to increase the number of offspring from a given mare.

Sheep and goats

Ovaries of slaughtered animals have been used as source of oocytes. On average two cumulus oocyte complexes per ovary (8) can be collected by means of follicular aspiration. The collection of oocytes from live ewes and does can be performed either laparoscopically (2, 46, 47) or surgically by laparotomy (42). In does it has been described also transvaginal ultrasound guided follicular aspiration (25). In small ruminants most authors use a stimulation protocol with gonadotrophin to increase the size of the follicles and thus facilitate the puncture. If endoscopical

or surgical recoveries are repeated several times on the same donor they can cause adhesions that might compromise subsequent fertility of the donors. Large numbers of oocytes (30-60) can be collected per session from few weeks old lambs (10, 43). In adult donors the number of oocytes collected per OPU is similar to that of cattle and it can be in the range of 6-8 both for sheep and goats. In vitro maturation is performed in presence of cysteamine that can increase blastocyst yield (7). Estrus serum, heat inactivated, is used for in vitro fertilisation to capacitate the spermatozoa. Culture conditions are remarkably similar to the bovine. The efficiency of the embryo production system is also comparable to that of cattle and each OPU session in adult donors can result in the production of 1 to 3 transferable embryos. The lambing rate of fresh OPU produced embryos in the sheep has been 41% (43).

Pig

Because of the prolificacy of this species the use of IVPE for breeding purposes is of lesser importance, except for the introduction or movement of genetics in herds with particular health status. The pig is becoming an important species for biomedical applications both as a model for human disease or as a potential source of organs for xenotransplantation. In this latter case, IVPE is combined with transgenesis and cloning (37, 38).

The major limitation of IVPE in pig has been the quality of oocytes obtained by in vitro maturation associated with polyspermic penetration. (1, 5). Nuclear maturation is achieved normally at high rates but cytoplasmatic maturation is considered to be still deficient because of insufficient block to polyspermy and poor male pronuclear formation. The main source of oocytes is the ovaries of prepuberal gilts and follicles over 3mm in size contain oocytes competent for maturation. Maturation is usually completed in 36-42h in medium supplemented with glutathione. Fertilised can occur in different media being the mTBM the most appropriate (35). Attempts to reduce polyspermy have been made adding somatic cells during fertilisation. For development to blastocyst stage NCSU 23 medium (41) is the largely used medium with rate of blastocyst formation in the range of 30%. Improvements have been achieved by using NCSU 23 with low glucose for the first 72 h and increasing it later (20, 1). Another limitation for establishing a pregnancy is the high number of foetuses needed to maintain the pregnancy. Therefore it is necessary to transfer at least 30 embryos per recipient. Usually the survival rate of the transferred embryos ranges from 20 to 30% (1).

Conclusions

In vitro embryo production is well established and used on a routine basis only in bovine. In all other domestic species the technology is less used or there are still technical problems that need to be addressed. The cost of these procedures is higher than conventional superovulation and embryo transfer therefore its use it is justified by the value of the animals produced. This is the reason why there is a niche market in the cattle breeding industry that requires such techniques and it is prepared to pay for it. In all other farm animals these conditions do not exist with the exception of the horse where old mares or subfertile stallions would benefit from assisted reproduction but the technology is still in its infancy for this species. A different situation exists for the use of farm animals for biomedical application, such as genetically engineered animals that produce proteins of pharmaceutical value or that can provide immunocompatible organs. In this latter case, the investments required to develop such technologies would be largely covered by the requests of the potential market derived from their application.

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Table 1. Genetic recovery from culled donors of one BSE infected herd.

	no. donors	no. oocytes (per donor)	no. cleaved (% of oocytes)	no. freezable embryos (% of oocytes) (per donor)	no. transferable embryos (% of oocytes) (per donor)
Cows	66	1413 (21.4)	970 (68.6)	206 (14.6) (3.1)	308 (21.8) (4.7)
Heifers	91	1831 (20.1)	1148 (62.7)	187 (10.2) (2.1)	285 (15.6) (3.1)
Total	157	3244	2118	393	593

Table 2. Embryo production by OPU at LTR in the period 1997-2001. (ref 18)

	no. OPUs	no. oocytes (per OPU)	no. cleaved (per OPU)	cleavage %	no. freezable embryos (per OPU)	no. transferable embryos (per OPU)
Cows	2641	26532 (10.0)	18822 (7.1)	70.9	5945 (2.3)	7403 (2.8)
Heifers	2143	16786 (7.8)	11231 (5.2)	67.0	2593 (1.2)	3284 (1.5)
Total	4784	43318	30053		8538	10687

STRESS AND REPRODUCTION

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Take home messages

- It is important to identify the incidence of major stressors in each group of animals - these will vary from farm to farm, and between ET teams.
- Some every-day events are stressful.
- Lameness and bad calvings have significant effects on fertility.
- Mastitis is also painful and has a major economic impact.
- Financial considerations will dictate emphasis but animal welfare is just as important.

Do we really know how to deal with animals efficiently?

Are we sure that we appreciate all the consequences of those everyday management decisions that we make in the name of domestication of any animal species? What does it matter how we feed our animals? What does it matter which male has the best index for ease of parturition, udder conformation or foot angle? What conditions are like under-foot in our fields, walk-ways and buildings? When and how we trim animals' feet? When and how we disrupt social groups?

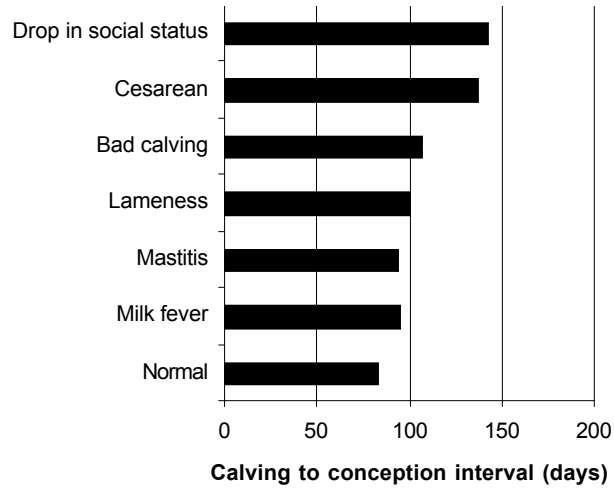
Bringing it to a personal level, do you know how many of the animals you work with have a condition score of less than 2 (on a scale of 1-5) three weeks before the start of an ET programme or the breeding period? Do you know the exact proportion of animals on your ET schedules that have had mastitis or milk fever? Precisely how many were lame, and how lame they were, and when? How many had to have assistance at parturition last year? How many had a dirty uterus after parturition? And what does it matter anyway, when ET teams are making a living and farmers are selling more meat and milk than ever before?

Table 1. Average annual incidence (per 100 cows) of clinical conditions in dairy cattle in UK and consequent lost profit (data from Esslemont et al, 2000)

Problem	**UK**			***Europe***
	% Incidence	Cases per cow	£ per cow	€ per cow
Retained membranes	4	1.0	298	420
Milk fever	8	1.0	220	308
Assistance at calving	9	1.0	5	7
Dirty uterus	15	1.4	166	232
Lameness	17	1.4	273	382
Mastitis	21	1.6	218	305
Oestrus not observed	34	1.4	18	25

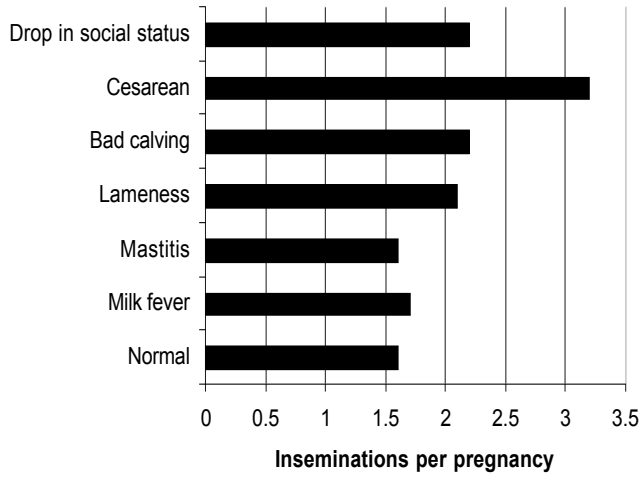
Several studies have attempted to answer the above questions for dairy cattle, and the results are alarming. Data are presented in Table 1 and to hit home, the economic consequences per case are also high-lighted – but remember these data ignore the impact on the cows themselves, i.e. the welfare of the animals.

Clinical events affect fertility



Each of the clinical conditions in Table 1 compromises the welfare of cows as well as having knock-on effects on fertility. The latter has been established by comparing fertility data of normal cows with those of herd-mates suffering from these distressing conditions. Examples of the results from these studies are shown in Figure 1. Remember all the animals had been treated for these conditions, either by the farmer or by a veterinarian.

Figure 1. Influence of treated clinical conditions in UK dairy cows on calving to conception and inseminations per pregnancy compared to normal herd-mates



As might be expected, the severity of each condition also has an effect…

With milk fever, the more severe the case (and hence the worse the stress), the greater the effect on fertility (Parker 1992). On average cows that succumb to milk fever take an extra 10 days to get pregnant; cows that totally collapse with milk fever (downer cows) take 15 days – if they live!

Milk fever or difficult calvings are also known to delay uterine involution. These effects, along with suppression of the immune system due to stress, will predispose cows to uterine infection (endometritis). Infections within the uterus have direct effects on ovarian follicular growth. Postpartum ovarian activity normally begins in the ovary on the opposite side to the previously pregnant horn. The presence of a purulent vaginal discharge decreases the number of animals resuming ovarian activity on that contra-lateral ovary and cows with purulent mucus take an extra 15 days to conceive (Sheldon et al, 2000).

Cows with a difficult/bad calving take an extra 8 days to resume ovarian activity, are more prone to subsequent abnormal cycles, and thus take 23 days longer to conceive than normal herd-mates (Elliott 1987). Our recent studies have also shown that cows with difficult calvings have ovarian follicles approximately 2.5 mm smaller at the start of the breeding period i.e., 35-50 days postpartum (Clarke, Smith and Dobson, *pers comm*). If things get worse during the calving and a caesarean operation is required, the consequences for fertility are dire. Many farmers do not rebreed these animals (culling is an important component of fertility indices) but if the cows are rebred within 50 days after calving, an extra 40 days are required to achieve conception (Lee 1994).

During the nine weeks before diagnosis of lameness, pregnancy rates are low (31%). The severity of lameness can be scored to estimate the extent of stress (number of days lame x [severity score + structure score]; see Collick et al (1989) for details). Compared with normal herd-mates, cows with a high score take 100 days longer to become pregnant, have lower first insemination pregnancy rates (41% versus 56%), require 0.5 more inseminations per pregnancy, and are three times more likely to be culled (22% versus 7%).

Mastitis is also a painful event and subclinical mastitis is routinely detected in dairy cows by an increase in somatic cell counts (SCC). In the UK, a tank bulk milk count of >400,000 cells/ml for three consecutive months means that the farmer can not sell that milk. Even if only one of the five monthly recordings after calving exceeds this limit, 7 days are lost on the calving to pregnancy interval; if all five recordings are too high, 12.5 days are lost (Hendry 1996).

Even social changes affect fertility

Rearrangement of animal groups causes problems, especially in restricted environments such as housing. If one dominant animal is placed within a new group of other animals, the individual somatic cell count of several of the animals (as well as the bulk milk count) will be elevated. Furthermore, changes in the hierarchy of an established group have profound effects on reproduction. We have monitored 3 groups of approximately 50 cows on 3 different commercial dairy farms during the breeding period. On the first day of observation, submissive and dominant behaviour was recorded and the cows were placed in a 'pecking-order'. The degree of lameness was also scored on a 0 - 5 point scale, with 5.0 representing a very lame animal. One month later, the behavioural observations were repeated. A total of 45 animals dropped 5 or more places down the order, and a further 45 animals had risen in hierarchy position. Those animals that did **not** change position maintained adequate fertility, even if they were at the bottom of the pile. However, those that declined in social status had decreased reproduction indices and milk yield, whereas SCCs and lameness scores increased. What was cause, and what was effect? For example, were these indices altered because of the increased lameness, or did animals succumb to lameness because they had become more stressed? More work is required in this area.

Table 2. Summary of fertility and milk production data for 45 pairs of cows that either increased or decreased in social status during the breeding period in three commercial dairy farms (reproduced with permission from Dobson and Smith, 2000).

	Change in social status	
	Increase	Decrease
Calving to conception (days)	97	143
Inseminations per conception	1.6	2.2
Milk yield (kg/day)	+0.58	-1.03
Somatic cell count ('000/ml)	-18	+371
Difference in lameness score	0.21	+0.54

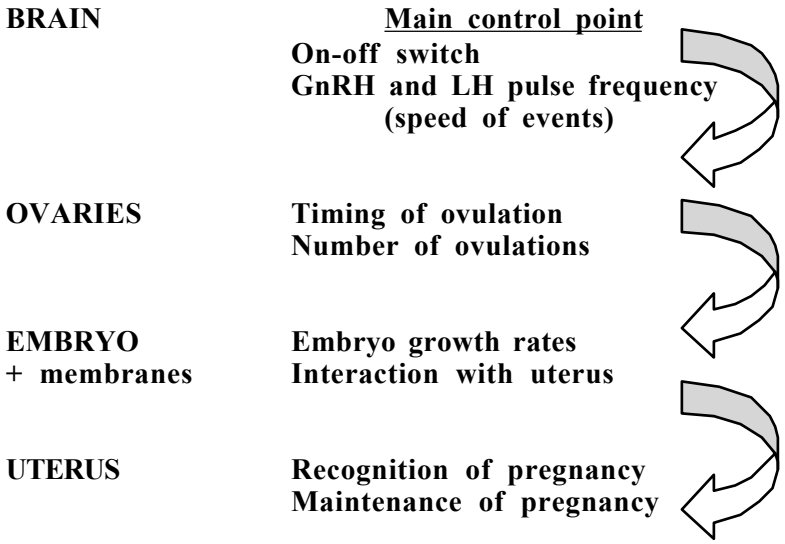
How do you measure stress?

What is stress? Many will define it as a situation during which cortisol secretion is increased. That only shows that an animal has responded to a short-term change in its environment - a reaction that is good for survival. We prefer a definition in terms of revealing an inability to acheive genetic potential, whether that be for milk production, weight gain or, of interest here, fertility. If the stressor is so bad that an animal fails to cope, eventually even such an important function as passing genes on to the next generation (reproduction) will suffer. So, if we can associate stressful/painful events with a reduction in reproductive efficiency, surely this is a good measure of stress? Reproduction is not the only worthwhile index to measure. Any other essential physiological function could be used, e.g., efficiency of food conversion, respiratory quotients, or neural transmission. However, as a reproductive physiologist, it is of interest to examine exactly how stressors can interact with the reproductive system.

The physiology of stress-reproduction interactions

Briefly, there are four principle sites at which stress can influence reproduction (Figure 2). The **brain** including the hypothalamus is the main control centre; it has the major power to maintain the reproductive system. Either to have it switched off completely or, if allowed to function, to determine the speed at which things happen, for example, the frequency of GnRH and thus LH pulses emerging from the brain to will dictate the rate of ovarian follicular growth. There is control at the **ovaries** over the timing of ovulation as well as the final number of follicles that ovulate. The **conceptus** (embryo + plus membranes) must grow at optimum rates and interact with the uterine lining, and finally the **uterus** plays a part in the maternal recognition of pregnancy, the maintenance of the embryo and fetus as well as during uterine refurbishment in the postpartum period.

Figure 2. Possible sites for 'stress' to affect reproductive efficiency

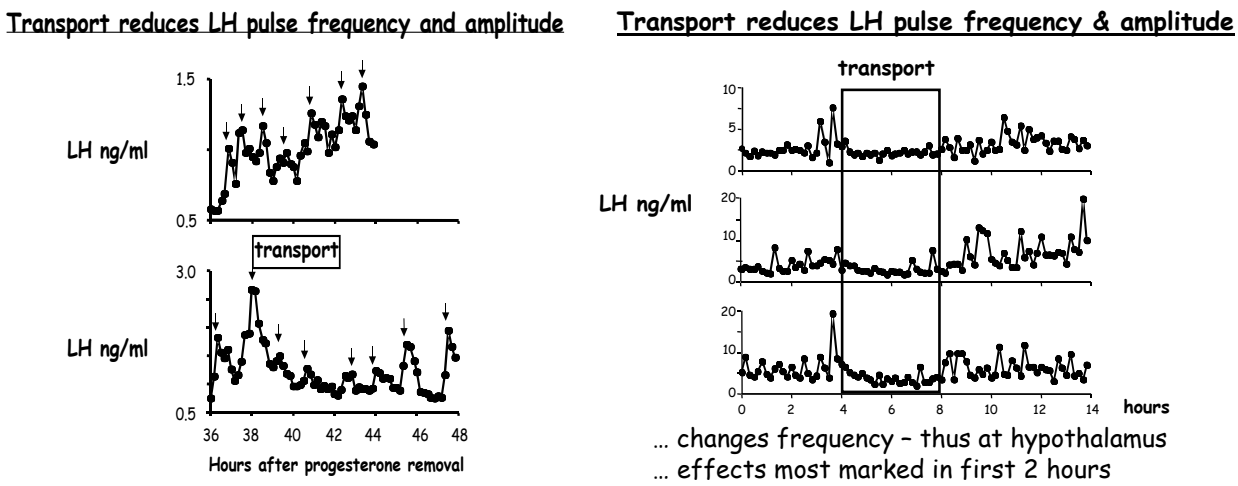


Postpartum refurbishment

Stressors can be either chronic or acute. While many field observations have gathered data concerning effects of chronic stressors, most of the investigations into the basic mechanisms involved in stress-reproduction interactions have centred on studying acute stressors because they can be easily defined and controlled. Acute interactions between the hypothalamus-pituitary-adrenal gland (stress) axis and the hypothalamus-pituitary-ovarian (reproduction) axis have recently been reviewed (Dobson et al 2000a; Dobson et al 2003).

Figure 3. Effect of transport on LH pulsatility in (a) follicular phase ewes, or (b) ovariectomised ewes.

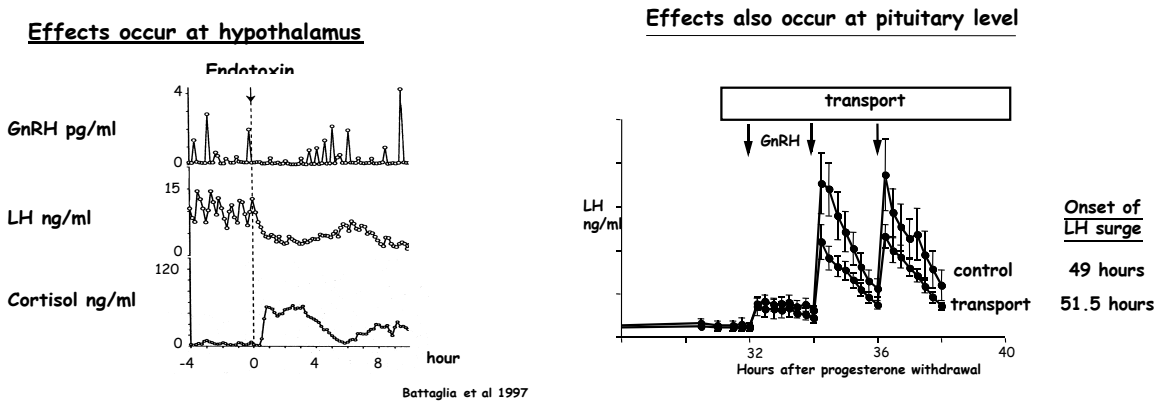
(a)



Briefly, the most important effect is exerted via disturbance of brain/hypothalamus function. Normal pulsatile patterns of GnRH release (and consequently frequency and amplitude of LH pulses secreted from the pituitary) are reduced by exposure to acute stressors such as transport or high-dose insulin administration (Figures 3 and 4). This results in abnormal ovarian function and hence delay or abolition of the LH surge. Thus, stressors disrupt the correct functioning of each part of the hypothalamus-pituitary-ovarian axis.

Figure 4. Effect of (a) endotoxin at hypothalamus level, and (b) transport at pituitary level, in follicular phase ewes.

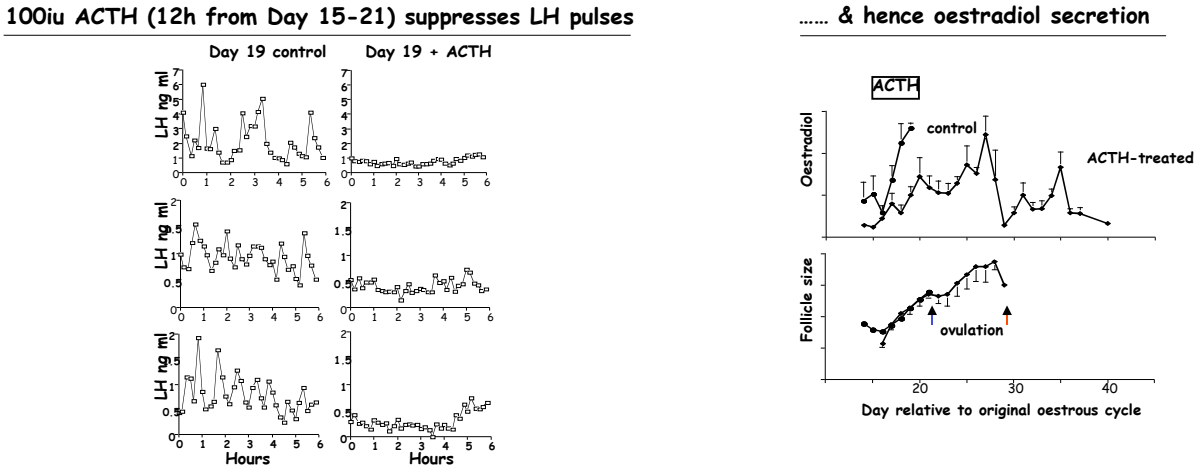
(a) **(b)**



Chronic administration of exogenous ACTH also results in similar effects of acute stressors (Figure 5). The pulsatile pattern of LH is disrupted, oestradiol secretion is lower than normal, the LH surge does not occur at the right time, and ovulation is delayed if it occurs at all (Dobson et al 2000b). The lower oestradiol concentrations could compromise the expression of oestrous behaviour. Similar effects have been observed during exposure to a chronic stressor such as spontaneous Gram-negative mastitis 15-28 days after calving (Huszenicza et al 1998). These cows resume progesterone cyclicity later than healthy animals (48 ± 17 versus 31 ± 12 days) and the duration of basal progesterone values around oestrus is longer (14.7 ± 7.4 versus 9.1 ± 2.2 days).

Figure 5. Effects of ACTH treatment on (a) LH pulsatility, and (b) ovarian structure and function in dairy cows.

(a) **(b)**



What is the evidence for stress effects during ET procedures?

While most commercial embryo teams strive to maintain high pregnancy rates, there is very little evidence in the public domain to identify specific susceptible parts of the technique. Considering embryo donors, transport for 15 to 60 min every 12 h for four days reduced the number of ovulations in response to a superovulation regime involving 5 mg FSH twice daily for four days (15.4 ± 1.7 versus 20.4 ± 2.1; Edwards et al 1987). As far as recipients are concerned, it is worth noting that animals subjected to a five mile walk over a hill two days after oestrus-synchronized insemination were less fertile than non-stressed synchronized herd-mates (81% versus 96% overall pregnancy rate; from Lowman et al 1994). The precise time of embryo loss was not determined but the effects of the post-oestrus stress might persist until the usual time for embryo transfer.

The rest of the limited evidence from ET that is available centres mainly on the effects of heat stress. The intensity of oestrus and superovulatory responses are poorer in cows subjected to heat stress (>33^0 C; Hansen et al 2001). Interestingly, there appears to be genetic variation in this effect and it is also apparent from results following IVM (in vitro maturation; Hansen et al 2001). Thus embryos produced by heat-stressed donor cows are less viable indicating that it might be preferable to collect embryos in cooler months and freeze them for later use (Ealy et al 1993). Indeed, this alternative biotechnological strategy was advocated after transfer of embryos collected from nonheat-stressed superovulated donors significantly increased pregnancy rates compared to AI of heat stressed cows, ie, frozen embryos could be used to overcome summer subfertility (Drost et al 1999). However, the stage at which embryos are lost (Days 7 to 14 or Days 21 to 42) appears to be controversial (Ambrose et al 1999). From measurements of pregnancy specific protein B (PSPB), the later stages are more likely as heat-stressed animals did

eventually produce PSPB indicating that conception had occurred but there was a heat-induced delay in trophoblastic function followed by embryonic death (Vasques et al 1995).

Many ET teams already advise clients to reduce stress in donor and recipient animals. Emphasis is laid on correct nutrition; animals with poor reproductive histories are avoided; and, if possible, several normal postpartum cycles are observed before collecting oocytes/embryos. However, the effects of other insidious stressors could be alleviated.

Evidence strongly suggests that changes in groupings of animals create a stress on both the new animals in the group and on those in the existing group. However, the optimum size of a group of dairy cows has not been determined. Small groups may be better to minimize the effects of stress. Estimates of maximum group sizes for recognition of individual herd members range from 50 to about 100 animals (Hemsworth et al 1995). Commercial ET units that house their own animals (donors and recipients) should appreciate these aspects. Similarly, owner clients could be advised accordingly to achieve maximum pregnancy rates.

There is some evidence to show that human-animal interactions influence stress-responses in cattle (reviewed by Hemsworth et al 1995). Stockmanship affects the incidence of lameness (Chesterton et al 189; Clackson and Ward 1991), and handling of heifers at calving can affect subsequent cortisol secretion, behaviour and longevity (Hemsworth et al 1995). If the personality of the stockman is reflected in these aspects – can the effect be extended to fertility? Furthermore, it is worth noting that, when observing people working with cows, human annoyance reached high levels just before a mealtime, and fatigue reaches its peak when one third of the job remains to be done, irrespective of the size of the job. Displacement activities, such as head scratching, yelling and cursing, also peak at this point, and the quality of decision-making drops (Albright and Arave 1997). This suggests that management of stress in the human resource of ET teams would also be beneficial!

…and the stressful consequences of embryo transfer?

The consequences of stressful calving have already been presented above. Twining induced by multiple ovulation and embryo transfer results in 26% of calves from twin-bearing cows requiring assistance at birth, and 13% calves do not survive (compared to 0% and 0%, respectively for calves from single-bearing cows; reviewed by Hemsworth et al 1995). While legislation has been enacted in several countries to reduce these effects, and the large calf syndrome is being eliminated, these problems provoked by ET must still be monitored.

Conclusion

It is clear from the foregoing evidence that stressors affect fertility. From the perspective of ET teams that are supposed to be promoting enhanced fertility, any inefficiency reflected in poor success rates deserves serious attention. One measure of the importance of stress can be expressed in financial terms but animal welfare is equally, or even more, important. Exposure of an individual to avoidable stress must compromise that animal's welfare, whether application of biotechnology is involved or not. The fact that stressors can be deleterious to such a major aspect of an animals existence as reproduction, emphasizes that stress is really very important and should be minimized whenever possible. It is incumbent on all ET teams to examine their own procedures and their own results in order to reduce stress and improve the welfare of all the animals utilized during their activities.

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CURRENT DEVELOPMENTS IN SPERM SORTING IN FARM ANIMALS

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Introduction

Efficient control of sex selection in farm animals has been a goal which has been sought after for millennia, but until the recognition of the nature of chromosomal determination of sex, attempts to skew the sex ratio were largely based on superstition. Once the underlying mechanism of sex determination was known (Guyer, 1918), a plethora of theories and experiments were carried out (Parkes, 1926). These include the use of alcohol (Danforth, 1926; Crew, 1925), separation of X- and Y- sperm by swim-down through an albumin gradient (Ericsson et al., 1973), surface antigenic differences (Hoppe and Koo, 1984; Hendriksen, 1999; Howes et al. 1997; Blecher, 1999), free flow electrophoresis (Kiddy and Hafs, 1971; Mohri et al. 1987, Kaneko et al. 1984), thin layer countercurrent distribution (Cartwright et al., 1993) and centrifugal countercurrent distribution (Ollero, 2000). This list is not exhaustive but to date no procedure except for that of flow cytometric analysis and sorting has resulted in the production of populations of viable sperm of a purity which might be of commercial interest and indeed a paper by Gledhill (1988) indicated that at that time no putative separation method had been successful.

The use of flow cytometry/cell sorting allows for the separation of viable X- and Y-chromosome bearing sperm (X- and Y- sperm) such that accurate predetermination of sex can be done with repeatability and precision. The first demonstration of this approach in farm animals was demonstrated in the pig (Johnson, 1991) followed by cattle (Cran et al., 1993), sheep (Catt et al., 1996; Cran et al., 1997) and the horse (Buchanan et al., 2000) and represented the culmination of developmental work largely carried out in the USA and Europe. The technique is based on measuring differences in DNA content between X- and Y- chromosome bearing sperm. Several reviews on the subject have been written over the past decade (Johnson, 1992; Johnson et al, 1996; Cran and Johnson, 1996; Garner 2001; Seidel and Garner, 2002; Seidel, 2003).

The ability to sort sperm into pure populations of X- and Y- sperm in cattle has obvious commercial attractions for both dairy and beef farmers. It is perhaps not surprising that the development of a reliable, accurate and rapid method of separating bovine sperm has attracted the attention of a variety of commercial entities. In addition, the efficient delivery of sexed sperm would also greatly impact the pig, sheep and horse industries. Johnson (1989) demonstrated the ability to separate viable sperm into X- and Y- chromosome bearing populations and the production of normal offspring. The United States Department of Agriculture (USDA) obtained a patent (US 5,135,759) on the flow cytometric sorting technique and then subsequently licensed a UK company, Mastercalf Ltd., which was engaged in the commercial development of IVF in cattle. They combined sperm sorting with IVF to produce pre-sexed embryos (Cran et al. 1993,1995). The sorting process, however, was too slow for practical use with routine AI. This had to wait the introduction of high-speed cell sorters, particularly that of the MoFlo®SX which was capable of sorting some ten fold faster than previous instruments. A collaboration between the USDA and Colorado State University demonstrated that, using high speed sorting, sufficient viable sperm could be collected for use in low dose routine AI in cattle (Seidel et al., 1997). This observation led to the establishment of XY, Inc. in 1996. and the following year, XY, Inc. acquired Mastercalf Ltd. The remit of XY, Inc. is to develop sperm sorting in farm and other species, including those which are endangered such that it is sufficiently robust to be transferred to other organisations. This paper describes the procedures used to sex sperm, field trials, particularly in the bovine, and a brief resume of progress in the horse and sheep.

Principles of Sperm Sorting by Flow Cytometry

The only reliable difference between X- and Y- sperm that can be utilised for their separation is the difference in total chromosomal DNA content due to the difference in the size of the X and Y chromosomes. DNA difference varies with by species (Table 1) and for farm animals is in the range of 3.6% to 4.2%. Although size differences between X- and Y- sperm heads can be detected microscopically (van Munster et al., 1999 a,b) attempts to utilise this observation combined with flow cytometry, have not proved successful in obtaining an acceptable purity of sorted sperm (van Munster, 2002). In the current method for sorting sperm it is therefore, necessary to stain sperm DNA with a vital dye which binds stoichiometrically with minimal chromosomal damage.

Table 1. Percentage DNA difference between X- and Y- sperm of different species

Species	DNA Difference (%)
Bull	3.8
Ram	4.2
Boar	3.6
Stallion	3.7
Man	2.8
Chinchilla	7.5

The dye must pass through various sperm membranes without markedly interfering with function, in particular, fertilising capacity and it is desirable to have as little variation between samples from individual bulls and from different bulls although the latter is more difficult to control.. These are demanding parameters and due to cellular variation it is unlikely that they can all be easily met. Hoechst 33342 (2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2',5'-bi-1H-benzimidazole_3 HCl) is the only dye to date which meets these requirements. The dye permeates cell membranes and binds selectively to A-T bases by hydrogen bonding, van der Waals forces and electrostatic interactions. Unlike many other DNA binding dyes, it is not intercalative which minimizes DNA damage.

The difference in DNA between X- and Y- sperm is small compared to the difference in fluorescence emission from most other cell types examined by flow cytometry. It is therefore necessary that parameters such as staining, optics, laser alignment, fluidic turbulence are optimised. Sperm are, of course, in most cases, paddle shaped and because of this, accurate measurement of total DNA of X- and Y- sperm is only possible when the laser strikes the sperm surface perpendicular to the plane of the flat side of the head. In a random stream of sperm flowing through a flow cytometer some 10% of the sperm population is properly orientated. This can be increased to 25% - 30% by changing the shape of the end of the needle from which the sperm exit to a bevel. This results in a sort speed of 800 to 1000 sperm per second of both X- and Y- sperm. Work carried out by Rens et al. (1998, 1999) on the internal geometry of the nozzle from which the sperm exit increased orientation to ~ 70%. This development has been further refined by XY, Inc. and the orientating ceramic nozzle tip (part of the Cytonozzle™, see Fig 1B) is an integral part of all MoFlo® SX sperm sorters. This improvement together with changes to the design of the sorter electronics have increased sort rates up to between 3500 to > 5000 sperm/sex/sec. Further information regarding the function of the MoFlo SX sperm sorter may be found in the review by Seidel and Garner (2002).

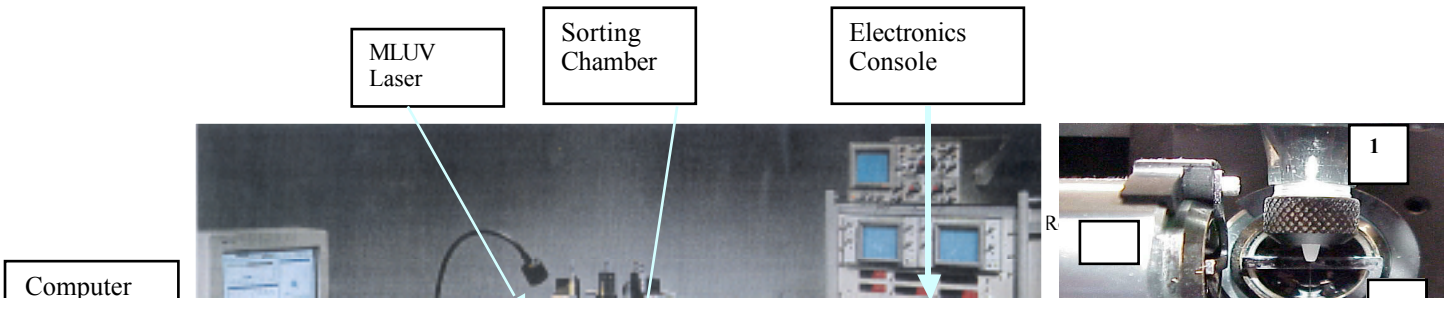

Fig 1 MoFlo® SX Sperm Sorter. The figure on the left (A) shows the general set up of the sorter. The UV laser light is directed into the sorting chamber by means of mirrors and illuminates sperm exiting from the orientating ceramic nozzle shown top right (B) (1) and fluorescence emitted from the sperm is collected by forward (2) and side (3) fluorescence detectors. The signal is amplified and displayed on the computer console where sorting decisions are made and acted upon via the electronics console. The stream is broken into droplets which may be charged positively or negatively dependent on whether they contain an X- or Y- sperm or left uncharged (C). The sperm enriched drops pass between charged plates (C) and X- and Y- populations collected. Droplets containing sperm with signal characteristics lying outside the region of the population being selected, those not containing a sperm or containing a sperm of the correct sex but in an inappropriate orientation or position within the droplet go to waste (white stream).

When considering the use of sperm sorting in a commercial context several factors relating specifically to sorting are important as opposed to those relating to fertility and financial return.

1. By judicious setting of the sort gates (sort gates are regions drawn by the operator around the X- and Y- populations shown in the graphic plots, e.g. R1 in Fig 2A would sort all the orientated population) it is possible to obtain any purity > 50%. However, if resolution of the populations is poor, the gates will be set towards the edge of the population with a concomitantly slower sort rate. It is an infrequent occurrence that the sperm in an ejaculate can not be stained to give two discernible populations. When this does occur, it is likely that the ejaculate would be rejected for other reasons such as low concentration. Obtaining good resolution is dependent on many factors such as staining medium composition, laser function and maintenance and alignment of the flow cytometer.

2. It is clearly desirable to be able to sort as rapidly as possible as this not only allows the most efficient use of the sorter but minimises stressful exposure to sperm. Maximum sort rate of viable sperm would occur if all the sperm in a sample were live, if all the sperm were orientated with regard to the forward fluorescence detector and if all drops contained a sperm which could be identified and sorted. As is shown in Fig 2 the addition of a food dye to the staining solution results in a quenching of fluorescence of those sperm with damage plasma membranes. Thus the population of sperm which is being sorted contains only live sperm.

3. The purity (sex ratio) of the sorted sperm sample is of paramount importance and it is essential to be able to monitor this before straws of sorted semen are distributed. In practice, purity of sort as shown by the sex of calves is about 90% of the desired sex and both X- and Y-sperm have been used in field trials. The sex of the frozen sexed semen can be quantified after thawing by restaining with Hoechst 33342, and removal of the tails, such that only the nuclei are examined, which give better resolution and orientation than intact sperm. Nuclei are slowly analysed for the percentage of X- and Y- sperm (see Fig. 2C).

4 The DNA difference between X- and Y- sperm is small and thus a small shift due to, for example, turbulence in the fluid surrounding the sperm can cause a shift in the relative position of that part of the population which is being sorted thus resulting in a decrease in purity. To obviate this, it has been necessary to have a sorting technician be present for the period of the sort to correct any fluctuations. This has been very demanding of technician time and has been an obstacle to the introduction of multisorter facilities. A collaboration between XY, Inc. and DakoCytomation has resulted in the development of Cytrack™ which allows for small movements in the populations to be automatically tracked and corrected.

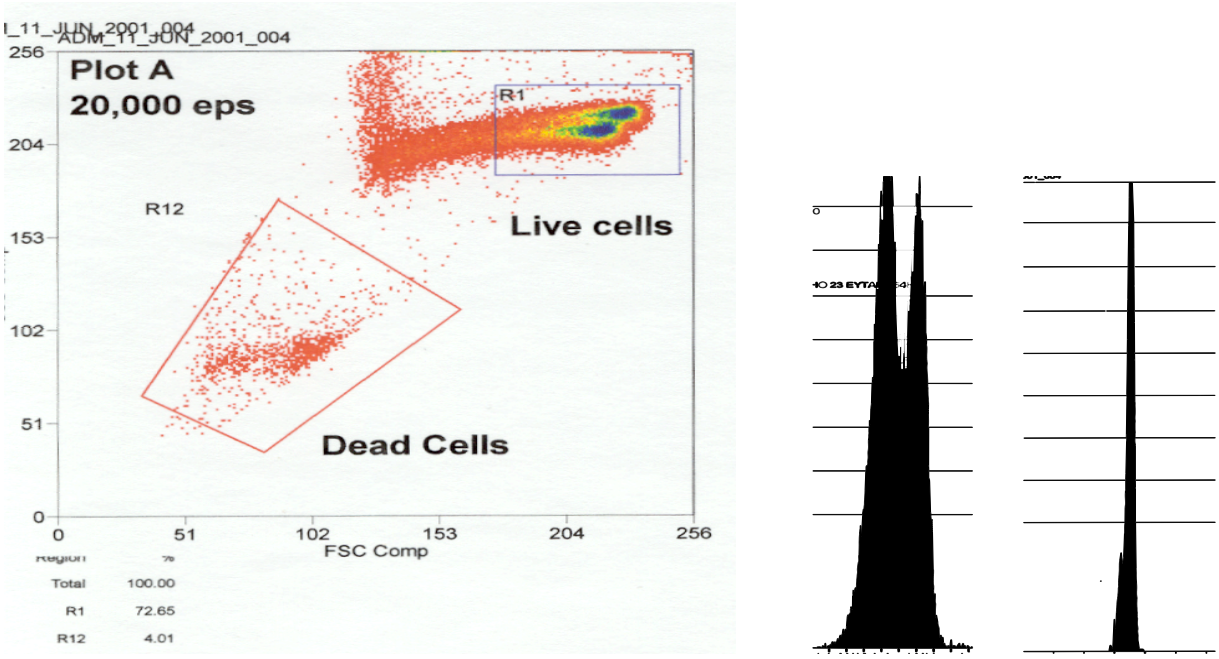

Figure 2. Plots of fluorescent emission from Hoechst stained bull sperm. (A) Printout from the computer screen during sorting. The sperm were passing through the sorter at 20,000 sperm per sec and X- and Y- sperm were sorted at 4400 and 5000 per sec respectively. The population in the lower left have taken up food dye and are considered to be non-viable. The population in the upper right represents live sperm and the region R1 is that proportion which is orientated correctly for accurate analysis. The further sperm are to the right of the histogram (high concentration shown in blue) the greater the number orientated at right angles to the laser beam. In this case the percentage of live sperm being used for sorting is 72.7%. Note the demarcation of the live sperm into an upper (predominantly X- sperm) and lower populations (Y-sperm). (B) A histogram showing X and Y sperm populations. (C) Reanalysis of sorted sperm for DNA. X-sorted sperm were sonicated to remove the tails, re-stained with Hoechst 33342 and slowly passed through the flow cyotometer. Note the shoulder on the left of the histogram representing minor contamination with Y-sperm. The purity of this sample was 93%.

In Figure 2 the region R1 depicts the part of the total sperm population which is correctly orientated and can be accurately measured for DNA and be sorted. This represents in this case 73% of the live population. Some 30% of the live sperm can not therefore be sorted. Reducing the rate at which sperm pass through the sorter increases the degree of orientation somewhat while decreasing sort rate. Seidel and Garner (2003) have indicated, the theoretical sorting rate limit to is ~10000sperm/sec. In practice such a high rate may not be routinely achievable and novel changes to instrument design are likely to be necessary.

Field Trials

Currently, the maximum rate at which sperm can be routinely sorted over extended periods, assuming excellent staining characteristics and a well-functioning sorter is some 5000cells/sex/sec

or 18 X 10^6 cell/hr. Losses during processing amount to some 15%, thus ~15 X 10^6 sperm per hour are available for freezing. Thus it is clearly not practical to use standard doses with sexed semen. Furthermore, sexed sperm undergo a series of challenges during sorting which may impair their function. Experiments have been carried out to examine the effect of the sorting protocols on in vitro sperm function, ability to withstand freezing and fertilising capacity. These are beyond the scope of this paper and have been dealt with elsewhere (Schenk et al., 1999; Garner et al. 2001; Guthrie et al., 2002; Lu et al 1999; Seidel et al. 2003; Suh and Schenk 2003.). In all species it is necessary to employ low dose insemination. The focus of much effort has been directed in establishing the size of the dose, the site and method of deposition, developing protocols to maximise fertilising capacity and the timing of insemination.

Cattle

Due to the long established successful global AI industry in cattle, most attention has been focussed on this species. The first field trial carried out by XY, Inc. in 1997 compared pregnancy rates following insemination in heifers with 3 X 10^5 non-frozen sexed sperm into heifers with 3 X 10^5 non-sexed unfrozen sperm and 15.6 X 10^6 frozen sperm (Table 2). The pregnancy rate from the sexed sperm was 80% that

Table 2. Result of liquid sexed sperm trial

Treatment	No Heifers Bred	No Pregnant Dy 31-34 (%)	No Pregnant Dy 64-67 (%)	No Female Calves Born (%)
Sexed Sperm	45	20 (44)	19 (42)	18 (95)[a]
Low Dose	28	15 (54)	15 (54)	8 (53)[b]
Control	29	16 (55)	15 (52)	11 (73)[b]
Frozen Control				

[a,b] Least Squares means without common superscripts differ (P<.02)

of the unsexed control although statistically there was no difference between the treatments. The efficacy of the sorting procedure was demonstrated by a 95% sex bias. The fertility of the sexed heifers has been demonstrated by their subsequent insemination with sexed sperm and the production of normal calves.

The capacity to freeze sexed sperm was an important step towards the potential commercialisation of the technology. The capacity to freeze sorted sperm is bull dependent and a minimum post thaw motility of 35% is accepted and the discard rate seldom exceeds 10% of all samples processed. A trial comparing frozen sexed with unfrozen sexed sperm was reported in 1999 (Table 3, Seidel et al. 1999). The sex bias was confirmed and there was a reduction in

Table 3. Comparison of liquid and frozen sexed sperm.

Treatment	No heifers	No pregnant Dy 64-67 (%)	No Female Calves Born (%)
Sexed,	37	11 (30)[a]	11 (100)[a]
liquid18°C	35	18 (51)[a,b]	17 (94)[a]
Sexed Frozen	37	27 (73)[b]	16 (59)[b]
Frozen Control			

[a,b]Least square means without common superscripts differ (P<.04) From Seidel et al. 1999

pregnancy rate to some 70% of controls. As a consequence of this trial all subsequent trials have used frozen sexed sperm. The data from a series of trials which were carried out between 1997 and 2000 inseminating 1000 heifers with sexed sperm in 6 herds and 22 beef and dairy bulls of

unknown fertility of various breeds was reported by Seidel et al. (1999). The trial compared the effect of inseminating doses ranging from 1.5 to 3.0 X 10^6 sperm either into the uterine body or bilaterally into the horns. The pregnancy rate for the sexed groups was 47% compared to 68% for the 20 X 10^6 controls. There was a slight benefit inseminating into the uterine horn but this appeared to be dependent on the skill of the technician. There was no benefit of inseminating with the higher dose rate. This has been observed in subsequent trials in which a dose as high as 6 X 10^6 was not shown to have a beneficial effect.

Large-scale field trials have subsequently been undertaken under field conditions with inseminators with varying degrees of skill and on farms with greatly varying management practices. All inseminations were into the uterine body at either 2 or 3 X 10^6 sperm.

Table 4. Sexed field trial

Treatment	No of heifers inseminated	No pregnant (%)
Frozen Sexed	2236	614 (44.6)
Frozen Control	811	505 (62.3)

These trials were carried out with numerous bulls on many farms on each of which there generally were small numbers of inseminations (~10 - ~80). There was considerable variation in pregnancy rates between bulls and between farms. It is interesting to note that the outcome of these trials is similar to that described by Seidel et al (1999) above.

AI management practices and bull fertility will clearly be key factors determining the wide scale implementation of sperm sexing in the commercial sector. The implementation of strict criteria governing how sexed sperm are used will be mandatory as will prior testing of bulls for use in sexing programmes. Large differences in fertility have been observed between bulls when low doses of unsexed sperm are used (Den Daas et al. 1998). It is not unreasonable to expect that a similar or greater impact will be found with sexed sperm. It will most likely be necessary either to check bulls for fertility at a standard dose before release of sexed sperm or to establish a fertility dose response curve for particularly desirable bulls. It seems clear that those bulls which will be most successful for use in sexing will be those which have desirable genetic traits coupled with high fertility. While fertility of low doses of sexed sperm is between 70% and 90% that of standard doses of non-sexed sperm it seems reasonable to expect that this difference will narrow.

Current studies have shown that lowering the pressure within the flow cytometer is beneficial. In a heterospermic assay comparing sperm sorted using 30 psi with sperm sorted using 50 psi it was found that 81% of heifers inseminated were pregnant from sperm sorted at the lower pressure (Seidel et al., 2003). In addition, Suh and Schenk (2003) observed that a lower pressure had a beneficial impact on motility and Campos-Chillon and de la Torre (2003) observed that both cleavage and blastocyst formation rates in IVF were higher at the lower pressure. Field trials are underway to determine whether there is an impact on fertility

There are several applications other than AI which could benefit from the use of sexed sperm. Perhaps the most obvious of these is embryo transfer in dairy or beef systems. At present sexing of embryos is achieved by analysis of embryos using sex specific DNA primer amplification with PCR. While the procedure can accurately determine sex it is time consuming and if only one sex is wanted, half the embryos are discarded and, particularly with frozen biopsied embryos, the pregnancy rates can be low. Following a superovulation regime, Paranace et al. (2003) inseminated 26 cows and 14 heifers at 0h, 12h, and 24h after onset of heat, with 10 X 10^6 sexed sperm at each time point. Control animals were inseminated with conventional semen. They obtained a mean of 4.2+/-3.0, 5.5+/-4.6 and 5.4+/-4.4 viable embryos for the sexed semen placed in cows, heifers and control semen respectively. There was no difference between the groups. However, it should be noted that the control and sexed inseminations were not always from the same sire or within the same trial. Schenk et al. (in press) have taken a different approach,

preferring to inseminate at a fixed time, this being 70-72h after injection of PGF2_, with either 20 X 10^6 or 2 X 10^6 sexed sperm. These treatments were compared with similarly inseminated 40 x 10^6 unsexed sperm. A total of 98 heifers were inseminated and a mean of 2.0, 1.3 and 3.1 transferable embryos were obtained for 20 X 10^6, 2 X 10^6 sexed sperm and 40 X 10^6 sperm respectively. While there was a decrease in the number of embryos produced with 20 million sexed sperm, assuming a 50:50 ratio with the unsexed treatment, there is a net gain of embryos of the desired sex with the higher sexed dose.

At the time of writing there are 25 MoFlos® SX sperm sorters in operation in 8 countries in 5 continents. Two licensees of XY, Inc. have been granted commercial status. Many thousands of offspring have been born predominantly cattle, but also sheep, pigs, horses, rabbits, elk. For reasons outlined above it is unlikely that the sexing procedure is has an effect on calf normalcy. In addition, no gross abnormalities have been reported which can be attributed to the sexing procedure and Catt et al. (1997) did not find that exposure to Hoechst 33342 or to UV laser for the periods involved during sorting caused an increase in endogenous nicks in the DNA. Further, Seidel et al. (2002) who examined progeny after either IVF or AI of sexed sperm in a blind trial found that the sexed offspring were similar in respect of gestation length, birth weight, neonatal deaths and deaths up to weaning. The only significant effects were that male calves were heavier at birth than female. It would appear that there are no detrimental affects of sperm sorting on the offspring. Nevertheless it would be desirable to have a very large data set to be completely certain that there is no small increase in abnormalities.

Horses

The conventional AI dose in the horse has been reported to be 500 X 10^6 sperm (Pickett and Voss, 1975) which is 20 times that often used in the cattle. Reducing the sperm number inseminated by the proportion now used in cattle (~10% of the control) would take an unrealistically long time to sort for production purposes as it would require 50 X 10^6 sperm to be sorted for each dose. Thus, conventional low dose insemination as applicable in cattle is not a practical option in horses. However work by Morris et al. (2000) opened the way for the use of sexed sperm in this species. Using transcervical videoendoscopic hysteroscopy they were able to deposit small volumes of semen at the uterotubal junction. Doses of 10 (n=10), 5 (n=8), 1.0 (n=25), 0.5 (n=14), 0.1 (n=11) and 0.01 (n=10) X 10^6 sperm obtained conception rates of 60%, 75%, 64%, 29 %, 22% and 10% respectively. These are impressive results since acceptable pregnancy results are obtained with as low as 0.2% of the standard dose.

Buchanan et al (2000) obtained a 40% pregnancy rate following insemination of 25 X 10^6 sorted sperm into the tip of the uterine horn ipsilateral to the preovulatory follicle. All the foals were of the expected sex. Lindsey et al. (2002a, 2002b) using endoscopic insemination in a manner similar to that which was employed by Morris et al (2000) has managed to obtained pregnancies with both fresh and frozen sorted sperm with doses as low as 5 X 10^6 sperm. As might be expected when small numbers of animals are used the results are variable ranging from 13% to well over 50%, conception rates and the data is not significantly different from control animals but much higher numbers would be required to confirm this.

Sheep

The cervix of the ewe does not dilate during oestrus. As a result, transcervical insemination is not possible without a high degree of manipulation. Although Halbert et al. (1980) reported acceptable conception rates by this route, others did not. The most common approach to AI in this species is by intrauterine insemination into the uterine horns or at the uterine tubal junction ,which involves a rapid laparoscopic identification of the uterus and speedy injection of the semen via a fine pipette. Conception rates of 50% - 80% with standard semen have been reported (Maxwell and Hewitt, 1986).

Cran et al (1997) inseminated ewes by laparoscopy at the utero-tubal junction with 10 X10^4 fresh sorted sperm and with controls consisting of similar numbers of unsorted sperm. The pregnancy rate for the sorted sperm was 8% and that of the controls was 16%. All the offspring were of the expected sex. Hollinshead et al. (2002) inseminated 4 X 10^6 sorted frozen-thawed sperm into either the utero-tubal junction or into the uterus. Controls comprised 140 X 10^6 unsorted sperm. Conception rate for the sorted treatment was 20% and 54% for the control.

The sex bias was 97%. Maxwell et al. (2003) have subsequently investigated the effect of dose and the timing of insemination with respect to the onset of ovulation. Doses of 5, 10, 20 and 40 X 10^6 sorted frozen-thawed sperm gave conception rates of 33.3, 57.1, 34.5 and 69.6 respectively while control animals which had not been injected with GnRH received 50 X 10^6 unsorted frozen-thawed sperm had a rate of 53.8%. With a sufficiently high dose it is thus possible to obtain a pregnancy rate similar to that of the control animals. However, at a sort rate of 5000 sperm/sec some 40 min would be required to sort one dose.

Summary

Flow cytometry/sorting is an effective means of producing populations of highly purified X- or Y- chromosome bearing sperm. The technology is reliable and has been replicated at several locations. Low dose insemination with sexed sperm results in the production of healthy calves although pregnancy rates are lower than for comparable controls. Each species presents different challenges with respect to sperm handling and although progeny have been born in cattle, horses, sheep and pigs, the only current commercial reality is in respect of cattle. It is likely that within two or three years commercial activity will take place in the horse industry while considerable development is still required for pigs and sheep.

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CURRENT PERSPECTIVES OF SPERM SORTING IN DOMESTIC FARM ANIMALS

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It is not longer only a demand to shift the sex of the offspring generation to the one or other side. After modification of high-speed flowcytometry, a technology is now available that allows to identify X-and Y-chromosome bearing spermatozoa, and from our experience, to select about 15 million spermatozoa per hour with high purity of least 94%. Almost all mammalian spermatozoa can be sex-sorted, if the protocol for preparation is adapted carefully. In the bovine, commercialisation has already begun and sexed semen from several bulls is available for farmers in Europe. Also for the equine and porcine species major improvements have been made for the technology in the recent months and the onset commercial use is foreseeable.

However, it might still be questionable, whether this technique will be introduced into breeding programmes on a larger scale. It will mainly depend on the economical benefit for the farmers. A very valuable analysis of the economic background for sorting was recently reported by Seidel (2003). From that survey the break-even point is dependent firstly on the fertility situation of the herd and secondly on the worth of the desired sex more than the least valuable sex. At least in Germany a decrease in fertility for high yielding milk herds has to be recognized after normal AI. This has to be considered, when decisions are made to introduce the sorting technology into our market. From many studies mainly in cattle, a reduction of 10 to 15% in fertility can be expected for sexed semen in comparison to fertility after AI with normal frozen/thawed semen (Seidel and Johnson 1999).

Reasons for a reduced fertility of flowcytometrically-sexed semen arise from technical limitations and biological reasons. Both areas are objectives of intensive research. Sorting for the determining factors of spermatozoa is based on the relative differentiation of their DNA content. Therefore, cells are stained with a Bisbenzimid (Hoechst 33342). At least a 60-minute co-incubation period at 37-38°C is necessary for the complete labelling of the sperm DNA. In this period, spermatozoa loose a certain amount of energy by stimulated motility reducing their life span. Although motility after sorting looks almost equal to non-sorted spermatozoa, it drops much faster in a thermo-resistance test. Similar decrease of motility may occur also after insemination in the female genital tract and make it necessary to inseminate closer to the time of ovulation. Fortunately, stain concentrations required for labelling X- and Y-bearing chromosome sperm for sorting, induced no phenotypical abnormalities among the newborn piglets (Vazquez et al. 2002).

In addition, high dilution effects caused by the sheath fluid have been reported to effect the post-sort viability of spermatozoa. It is a major reason for premature death if the cells are deposited in the female tract distant from the site of fertilization (Maxwell and Johnson 1999). High dilution effects may also change the response of the female immunological system in the uterus against the sperm surface. The sperm adhesin AWN has been shown to cover freshly ejaculated spermatozoa and it is normally removed from the sperm surface on its way to the utero-tubal junction. It has to be discussed whether substances like adhesins are removed by high dilution and mechanic forces in the capillary tubes of the flowcytometer. A negative effect on functionality of spermatozoa was indicated recently for other sperm adhesins like PSP-I/PSP-II and the heparin-binding spermadhesins (Centurion et al. 2003). Especially in porcine spermatozoa, it was indicated employing the FITC/PNA Lectin binding tests that spermatozoa were more advanced in their capacitation status. This causes high rates of polyspermic fertilization in the IVP system. Normal monospermic fertilization was only possible if the number of spermatozoa used for IVF was reduced to a fifty of the normal concentration. Maxwell et al. (1999) indicated that sperm capacitation associated with flow cytometric sorting could be reduced by the inclusion of seminal plasma in the collection medium. We hypothesise that sorted spermatozoa may induce different immunological pattern in the female genital tract limiting the sperm number reaching the oviduct.

High pressure in capillary tubing of 50 psi has an effect on the sperm quality and the subsequent fertilisation rates. Usually 40 psi seem now to be an acceptable pressure that spermatozoa can take without major losses of their fertilizing capacity and allows to produce a

stable droplet stream at high speed. Based on the morphological investigations directly after sorting, sperm membranes seem to maintain their integrity. However, in combination with other membrane stressing techniques like freezing, it is obvious that the cell stability is reduced. Some information is available that the long unsaturated fatty acid chains of the acrosomal and the plasma membrane disappear. It needs to be elucidated whether Omega III fatty acids are able stabilise membranes if added to a normal diet formulation.

Beside the high pressure in the tubing of the flowcytometer, another source for mechanical stress is caused by the fast speed of the sperm cells when they touch the surface of the collection tube. Therefore, collection tubes need to be pre-filled with a medium that at least partly protects against mechanical stress. TEST-extender supplemented with 2 to 20% highly purified egg yolk and 1% seminal plasma seem to be a reasonable fluid to soften the diving of the spermatozoa into the collection medium. Another positive effect of egg yolk components is the stabilisation of the membranes, presumably by a fast recharge of the electric potential. Especially the mid piece seems to be sensible to micro-electric changes affecting the ATP synthesis. Disturbed ATP synthesis contributes to a reduced motion activity.

Finally, a major source for the reduced fertilizing capacity of sorted spermatozoa could be the combination of the dye and the high energy Laser light. It is known that embryos exposed to such combination are not able to develop further. Luckily, the exposure time of spermatozoa is extremely short. So far, from all existing offspring produced from flowcytometrically sex sorted spermatozoa, no clinical abnormalities have been reported, even if the offspring were produced for several generations from sex-selected parents. Guthrie et al. (2002) tested two different laser power outputs of 25 and 125 mW and found no detrimental effects of the Laser used in a high speed flowcytometer on embryo development.

Beside the improvement to aviod these stress factors, the efficiency of the sorting technology offers some space for improvements. Other than in somatic cells, spermatozoa need to be oriented in a rectangular position of the flat side in front of the Laser beam. One of the major improvements for high speed sorting were the invention made by Rens et al. (1998) when they developed a nozzle assembly that pre-orientates the sperm cell by the shape of the injection unit in combination with the pre-formed hydrodynamic shape of the core stream. However, when sorting for high purity, about 40% of the sortable spermatozoa are lost at this site of the sorting process. A temporary immobilization of the sperm cells might help to increase the number of spermatozoa being included into the sort decisions.

So far, data transfer from the hardware computing sort unit to the computer and backwards is limited to the bus transmission speed. Therefore, not all droplets with sortable spermatozoa are included into the sorting process. Improvement of computer technologies will help to get more spermatozoa being recognized. Other hardware modifications are also possible for this purpose.

An important prerequite to sort spermatozoa is their full coverage within a droplet. If head or tail are outside the droplet, orientation and/or identification of the DNA content may fail. Sort speed is not dependent on the absolute pressure of the system, as it works with pressure differentials. Important is the frequency of the vibrating nozzle assembly forming the droplet stream. The higher the frequency, the smaller the droplets are. In other words, with the given diameter of the ceramic nozzle, the frequency cannot be increased further. The physical limitations due to the cell size have been reached. In relation to this fact electronic and optical recognition failures (abortion rate and coincidence rate) affect the sorted sperm output.

One consequence is that further improvements of the technical prerequites to maximize the production of sexed spermatozoa are necessary and possible. They will come as the technology is further distributed for commercial use.

The second consequence is however, that it is unlikely to make use of all spermatozoa of an ejaculate. Accordingly, other biotechniques are required. A good example is the pig. In normal AI, sows receive at least one billion spermatozoa twice or three times per oestrus cycle. Many of the spermatozoa get lost on their way to the oviduct and potentially only a couple of hundred reach the site of fertilization. Others are lost by reflux, immunological digestion, end up in uterine crypts or do not pass the utero-tubal junction due to a strict selection process by the epithelial cells at the UTJ and the distal part of the oviductal isthmus.

Alternatives to employ sex-sorted semen are the in vitro production of embryos using IVF (Rath et al. 1997 1999, and Aberydeera et al. 1998), or ICSI (Probst und Rath 2003). However, resulting embryos need to be transferred preferably in an early developmental stage. In a study with non-sorted semen, Krüger et al. (2000) showed that it is possible to reduce the amount of

inseminated spermatozoa by a factor of 100, if the volume is reduced in a similar range and the semen is introduced surgically close to the utero-tubal junction. Several studies concentrated on the development of an insemination device that can be introduced non-surgically into the tip of the uterine horn (Wolken et al 2001; Martinez et al. 2001). In our opinion, so far only the AI instrument developed by the group from Murcia (Spain) fulfils the requirements for such a device. In order to inseminate as many sows as possible with their AI instrument, the Spanish scientists used porcine semen after processing it through a flowcytometer, but without activation of the sorting device and obtained reasonable fertility rates and litter sizes. First piglets of the desired sex were produced recently in studies by Rath et al. (2003) and Grossfeld et al.(2003) employing the Spanish AI device with 50 million (70% motility) and 2 ml of volume. From 12 sows four became pregnant and delivered 30 piglets, out of which 29 had the predicted sex. Further research is necessary to improve the results. One key question is how to improve the oestrus diagnostic in the porcine as inseminations need to be performed as close as possible to ovulation.

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SHORT COMMUNICATIONS

EFFECT OF THE OPEN PULLED STRAW VITRIFICATION ON THE CYTOLOGY OF IN VITRO-MATURED PREPUBERTAL AND ADULT BOVINE OOCYTES

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The survival and developmental capacity of bovine oocytes after cryopreservation are greatly impaired, possibly due to organelle damage caused by freezing procedures. The aim of this study was to examine the distribution of chromosomes, microtubules, and microfilaments in calf and cow in vitro matured oocytes after exposure to cryoprotective agents (CPA) or vitrification by the Open Pulled Straw (OPS) method (Vajta et al., 1998, Mol Reprod Dev 51:53-58). Oocytes were matured in vitro for 22 h (Rizos et al., 2001, Theriogenology 56:1-6) and divided into 3 treatments: 1) control; 2) exposed to CPA and 3) vitrified with 20% ethylene glycol + 20% dimethyl sulfoxide and 0.5 M sucrose by the OPS technology. After warming and an additional 2 h of culture, oocytes were fixed and evaluated using specific fluorescent probes before visualization using a confocal microscope. Changes observed in oocytes during CPA exposure and vitrification included chromosome dispersal or decondensation, microtubule depolymerization or disorganization and altered or lack of spindle structure. Diffuse or discontinuous cytoskeletal actin staining was considered abnormal and no staining was classified as missing.

Table 1. Effects of CPA exposure and vitrification by OPS on spindle morphology (chromosome arrangement and microtubule distribution) of IVM cow and calf oocytes

	Cow (%)				Calf (%)			
	n	Normal	Abnormal	Missing	n	Normal	Abnormal	Missing
Control	33	29 (87,8) [a]	2 (6,1)	2 (6,1)	29	24 (82,8) [a]	4 (13,8)	1 (3,4)
CPA control	39	20 (51,3) [bc]	16 (41,0)	3 (7,7)	30	19 (63,3) [ad]	8 (26,7)	3 (10,0)
OPS	30	12 (40,0) [bc]	17 (56,7)	1 (3,3)	34	12 (35,3) [bd]	14 (41,2)	8 (23,5)

[a, b]: Values in a column with different letters are significantly different (P<0,05).
[c, d]: Values in a rows with different letters are significantly different (P<0,05).

Table 2. Effects of CPA exposure and vitrification by OPS on the actin band of IVM calf and cow oocytes

	Cow (%)				Calf (%)			
	n	Normal	Abnormal	Missing	n	Normal	Abnormal	Missing
Control	33	30 (90,9) [a]	0 (0)	3 (9,1)	29	28 (96,5) [a]	1 (3,5)	0 (0)
CPA control	39	31 (79,4) [ab]	4 (10,3)	4 (10,3)	30	24 (80.0) [ab]	4 (13,3)	2 (6,7)
OPS	30	21 (70,0) [b]	4 (13,3)	5 (16,7)	34	24 (70,6) [b]	4 (11,8)	6 (17,6)

[a, b]: Values in a column with different letters are significantly different (P<0,05).

After vitrification or CPA exposure, significantly greater percentages of calf and cow oocytes exhibiting an alteration of spindle morphology were observed when compared to control group. The spindle structure of calf IVM oocytes was significantly more sensitive to OPS vitrification. When oocytes were vitrified by OPS an increase of oocytes with discontinuity or absence of cytoskeletal actin staining was observed when compared to the control group. Cryoprotectant exposure did not differ significantly from control and no differences were observed between calf and cows oocytes in terms of normal distribution of actin microfilaments. These results indicate that the OPS procedure causes irreversible alterations in multiple cytological components of calf and cows oocytes.

Notes

INCREASE OF EMBRYO DEVELOPMENTAL RATE IN VITRO BY SELECTION OF BOVINE OOCYTES BEFORE IVM USING A STAINING TEST: PRELIMINARY RESULTS

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Follicular oocytes recovered from ovaries of slaughtered cattle are commonly used to study in vitro maturation and fertilization and in vitro production of embryos. The relatively low level of efficiency achieved using in vitro embryo production, manifested by the frequent failure of up to 60 % of immature oocytes to reach the blastocyst stage, is almost certainly related to the quality of the oocyte at the beginning of maturation. The aim of the present study was to increase the output of blastocysts after IVM/IVF by using a staining test with brilliant cresyl blue. Brilliant cresyl blue stain determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD) which plays a critical role in cell growth.

Bovine ovaries were obtained from a slaughterhouse and cumulus oocyte complexes (COCs) were recovered by slicing the surface of the ovary. Oocytes with a compact cumulus investment were selected, and split into 3 groups: 1) control, 2) negative control - COCs kept in PBS for 90 minutes (COCs of both groups were not stained before culture), and 3) stained with brilliant cresyl blue for 90 minutes. After that time the COCs were divided into stained (BCB +) and unstained (BCB -). All COCs were cultured for 24 h at 38.5°C under 5% CO_2 in 100 % humidified air in culture medium TCM 199 containing 10% (v/v) FCS and EGF. After IVM, oocytes were fertilized in vitro (IVF) by cryoconserved bovine semen. At 24 h after insemination, the presumptive zygotes were denuded and transferred to Menezo B_2 medium. Twenty-four h after placement in culture, the cleaved embryos were transferred to a BOEC culture for 6 days.

Table 1. Influence of brilliant cresyl blue staining in development of bovine oocytes in vitro

Groups	No. COCs	Cleavage rate Day 2 p.IVF (mean ± SEM)	Blastocyst rate Day 8 p.IVF (mean ± SEM)
Control	73	65.9 ± 4.4	22.9 ± 6.7
Negative control	76	82.1 ± 1.5	20.3 ± 5.5
BCB +	66	79.7 ± 8.0	33.8 ± 2.6 [a]
BCB -	64	80.5 ± 3.7	4.3 ± 2.2 [b]

($P<0.05$)

There were no significant differences between the groups in both the maturation rate and the first cleavage. Differences were obtained at the end of the in vitro procedure. Further investigations are required to increase the number of oocytes, and to substantiate the results obtained.

These preliminary results show that the staining of bovine cumulus oocyte complexes before in vitro maturation could be used to increase the number of developmentally competent oocytes, and to have a marker for oocyte quality as well for techniques like cloning.

Notes

EXPRESSION OF DIFFERENT GENES DURING EARLY EMBRYONIC DEVELOPMENT IN THE HORSE

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It was the aim of the present study to investigate the expression of specific genes involved in the regulation of embryonic development in the horse. As blastocyst formation is considered a critical step during early pregnancy, genes suggested to be involved in the formation of the blastocoele were chosen. These are mainly Na-K-ATPase and members of the aquaporin family.

Embryos were collected from mares inseminated with fresh semen from a fertile stallion at 48h-intervals until ovulation. Mares of group 1 were untreated, mares of group 2 were treated with a vaginal sponge containing 1,55g progesterone from day 1 until day 10 after ovulation. Embryos were recovered by uterine flushing with phosphate buffered saline on day 10 after ovulation. Embryos of good morphological quality (n=10) were frozen at –80 °C until analysis. Mean diameter of embryos from untreated mares was 4.1 ± 1.0 mm (n=6), from progesterone-treated mares was 4.9 ± 0.7 mm (n=4; n.s.). Qualitative PCR on the following enzymes was performed: α_1Na-K-ATPase, β_1Na-K-ATPase, aquaporin-5, prostaglandin-E-synthase. All enzymes were expressed in equine embryos 10 days after ovulation. Quantitative PCR was performed to determine relative gene expression of the enzymes in comparison to 2 housekeeping genes known to be constantly expressed in embryonic tissue (β-actin and GAPDH). Relative gene expression of aquaporin-5, a substance considered responsible for active water transport, was significantly correlated with the size of the embryo ($r=0.71$, $p<0.01$). In embryos from untreated and progesterone-treated mares, there was no difference in the relative expression of α_1Na-K-ATPase, β_1Na-K-ATPase and aquaporin-5. However, prostaglandin-E-synthase which is involved in the synthesis of prostaglandin E, the substance responsible for oviductal transport of the equine embryo, was significantly lower in embryos from progesterone-treated mares ($p<0.05$).

The study is the first to describe the expression of a member of the aquaporin family in equine embryos. The correlation of aquaporin-5 relative gene expression with embryo size suggests an involvement in embryonic blastocoele formation. Maternal factors such as plasma progesterone concentration might affect the expression of genes important for early embryonic life and thus influence embryonic viability.

Notes

ZONA-FREE SOMATIC CELL NUCLEAR TRANSFER IN CATTLE - FIRST HMC CALF IN EUROPE

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Nuclear transfer has mainly relied on micromanipulation-assisted enucleation followed by injection of one donor cell into the perivitelline space, thus requiring sophisticated and expensive micromanipulation equipment and considerable skills from the operator. We tried to address this problem by using the 'zona-free' method of somatic cell nuclear transfer as described by Vajta et. al. (Handmade Cloning, HMC; Biol Reprod 2003, 68:105-113). The process involved in vitro maturation of bovine oocytes, removal of the zona pellucida by pronase, manual bisection of the oocytes, selection of cytoplasts by Hoechst staining, and two-step fusion of one somatic cell with two cytoplasts. The fused reconstructed embryos were then activated by calcium ionophore and dimethylaminopurine and subsequently cultured in the well-of-the-wells (WOW) system.

In the last year, we experimented with a total of 4,674 cumulus-oocyte-complexes using three different sources of somatic cells as nuclei donors, i.e. either 7^{th} or 11^{th} passage fibroblasts from an elite high performing bull's ear skin or granulosa cells from cow. The overall average efficiency of formation of the reconstructed embryos was 78% with a 57% average cleavage rate. Of the cleaved embryos, 45% progressed up to the 8-cell stage and beyond with an overall blastocyst rate of just 2% of the cleaved products and 5% of the > 8-cell embryos. An average of only 26% of the 8-cell stage embryos was able to progress towards further developmental stages. No significant differences were observed between the three groups of somatic cell donors in terms of cleavage rate, number of > 8 cell stage embryos, or the blastocyst rates.

Out of a total of 23 blastocysts recovered, 18 blastocysts (produced using bull's fibroblasts as nuclei donors) were transferred to 18 synchronized recipients resulting in two pregnancies. One pregnancy was lost around day 21 while the other progressed to full term with the birth of a male calf that was apparently healthy and normal, but overweight (i.e. approx. 73 kg). Unfortunately, the calf expired around 50 h after birth, and the preliminary post mortem pathological findings indicate a pulmonary infection and liver cirrhosis. The DNA fingerprinting for the determination of the genetic identity of the calf has confirmed the success of the cloning procedure.

The successful cloning of a male calf from an elite high performing bull for the first time with the HMC technique in Europe indicates the successful adoption and establishment of this technique in our laboratory, although much remains to be done to improve the blastocyst rate. The birth of the cloned calf can also be regarded as an indication of the success of the zona-free technique to produce viable embryos. The significant failure of 74% of the 8-cell stage embryos to convert to blastocysts necessitates further investigations of the 8-cell stage cloned embryos. It is presumed that improvement in activation and culture regimes and a better understanding of the mechanisms of epigenetic reprogramming in the future would help improve the overall success rate of the somatic cell nuclear transfer technology.

Notes

APOPTOSIS OF IN VITRO CULTURED GOAT SOMATIC CELLS EXAMINED WITH YO PRO-1 AND PROPIDIUM IODIDE

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An important factor governing developmental rates of cloned embryos reconstructed by nuclear transfer is the G0/1 phase of the cell cycle in which nuclei donors should exist prior to nuclear transfer. To induce quiescent status of nuclei serum starvation of cultures is used. However, it is known that for certain cell types serum starvation may induce apoptosis leading to degeneration and death of cultured cells.

The aim of this study was to examine the influence of serum starvation on the level of apoptosis in in vitro cultured goat somatic cells. The granulosa and cumulus cells as well as fibroblasts originating from ear tissue were used in the study. TCM199 (Sigma) and/or DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) were used as growth medium for optimal culture. Cells were subsequently regrown and passaged at least 3 times more, then harvested from flasks using Non-Enzymatic Cell Dissociation Reagent (ICN Inc., USA) and used for analysis. Serum-starved cells were cultured in medium containing 0.5% FCS for 5 days. The number of live, necrotic and early apoptotic cells was examined after staining with YO PRO 1 and propidium iodide fluorochromes (Molecular Probes) by fluorescent microscopy and flow cytometry.

Serum starvation for 5 days significantly increased the number of both apoptotic and necrotic cells of granulosa and necrotic cells of cumulus but had no effect on fibroblast cells (Table 1). The results suggest that cell cycle synchronization via serum starvation in some cell types may significantly increase the number of early apoptotic cells. The use of such apoptotic cells, still morphologically normal, may affect the developmental rates of cloned embryos.

Table 1. Live, necrotic and apoptotic cells in granulosa, cumulus and fibroblast cells cultured in optimal conditions or starved for 5 days (t test, * $P<0.001$ for respective pairs)

	Optimal culture			Starved (5 days)		
	Live (%)	Necrotic (%)	Apoptotic (%)	Live (%)	Necrotic (%)	Apoptotic (%)
granulosa cells (n=15)						
Mean	57.77*	24.55*	17.49*	39.53*	32.07*	28.21*
SD	4.70	4.82	3.12	13.04	6.88	8.24
cumulus cells (n=10)						
Mean	40.22	38.04*	20.57	23.29	56.08*	20.44
SD	8.30	10.64	6.09	3.15	5.23	3.93
fibroblasts (n=15)						
Mean	45.13	39.24	15.13	44.17	39.70	14.92
SD	11.13	10.99	5.91	10.73	8.87	5.08

Notes

PARAFFIN OIL IN IN VITRO EMBRYO PRODUCTION: A ROUTINE COMPONENT, A SUDDEN TOXIC AGENT

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Trouble-shooting is often needed in routine systems for in vitro embryo production. Many factors may be responsible for compromised results, including water, chemicals, gases and toxic fumes, temperature, light, etc. The oil used for covering the media is known to be a potential toxicity risk. The toxic effects are related to the source of oil and storage conditions, but the mechanisms are not clearly understood (Van Soom et al., 2001, Reprod Dom Anim 36:169-176).

In our in vitro system (Holm et al., 1999; Theriogenology 52:683-700) a slow decrease in blastocysts rates was experienced over a period of 6 months. Among other factors, the currently used paraffin oil was tested. Three sources of oil, stored at the same conditions in the lab, were compared: paraffin oil batch 221 (currently in use, 6 months old; Merck), paraffin oil batch 238 (3 months old; Merck) and mineral oil embryo tested lot 21K0038 (2 years old; Sigma). Bovine oocytes obtained from abattoir-derived ovaries were matured, fertilized (Day 0) and cultured following the routine protocols, using the same oil for all three phases. Blastocyst rates were determined on Days 7, 8 and 9. Data was analyzed by Genmod-Procedure (SAS Institute Inc.). Results of 4 replicates are presented in Table 1.

Table 1. Blastocysts rates (mean ± SEM) on Day 7, Day 8 and Day 9 under, when matured, fertilized and cultured using different oil sources.

Oil source	No. of Oocytes	Blastocysts/oocytes (%)		
		Day 7	Day 8	Day 9
Paraffin oil 221	305	11±4 ^{a,x}	8±4 ^{a,x}	4±2 ^{a,y}
Paraffin oil 238	218	26±2 ^{b,x}	19±3 ^{b,xy}	15±2 ^{b,y}
Mineral oil	210	39±4 ^{c,x}	42±9 ^{c,x}	37±18 ^{c,x}

^{a,b,c} Differences within columns ($P<0.01$); ^{x,y,z} Differences within rows ($P<0.05$)

The results show higher rates of blastocyst under mineral oil, which were stable from Day 7 until Day 9. Furthermore, the blastocysts in the mineral oil group had superior morphology when evaluated under the stereomicroscope, with clearer and brighter cytoplasm compared with the ones cultured under both paraffin oil batches.

Based on these results, and as paraffin oil has been successfully used for several years in our lab, we presume that besides storage conditions, batch and age of the oil, unknown factors may act differently according to the source of oil. Hence, we suggest that that oil should be always considered and investigated as a potential cause of toxicity, acting either in a slight and almost not detectable way, or resulting in a drastic collapse in the in vitro production system, independent of its previous performance.

Notes

ALTERATION OF REPRODUCTIVE HORMONE LEVELS IN PREGNANT SOWS INDUCED BY REPEATED ACTH APPLICATION AND ITS POSSIBLE INFLUENCE ON PRE – AND POSTNATAL HORMONE SECRETION OF PIGLETS

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Prenatal stress has been seen as a reason for reproductive failure in pig offspring mostly originating from or mediated through altered maternal functions. Experiments were conducted in pregnant gilts (n = 22) to characterize the effects of elevated maternal glucocorticoids on the secretion of reproductive hormones (LH, progesterone, estrone sulfate) during the 1st (EXP I) and 2nd trimester of pregnancy (EXP II), and on glucocorticoid concentrations in umbilical blood vessels of fetuses (EXP III). Furthermore, the pituitary function of newborn piglets of EXP II was checked by a LH-RH challenge test. Transiently elevated cortisol release was repeatedly achieved by application of 100 IU ACTH (Synacthen Depot®) 6 times every second day beginning either on Day 28 (EXP I) or Day 49 (EXP II) of pregnancy.

In sows, LH concentrations were at low basal level (0.1 – 0.2 ng/ml) but with pulsatory release pattern both during the 1st and 2nd trimester of pregnancy. The number of LH pulses/6 h (LSM ± SE) of saline-treated controls increased with ongoing pregnancy (1.4 ± 0.1 in EXP I vs. 2.0 ± 0.2 in EXP II). After ACTH treatment, the number of LH pulses remained unchanged (1.3 ± 0.2 in EXP I and 1.4 ± 0.2 in EXP II). However, differences ($p < 0.05$) were obtained comparing the LH pulse number of ACTH and saline-treated sows during the 2nd trimester of pregnancy. Moreover, areas under the curve (AUC) of each LH pulse and of LH over baseline were significantly reduced by treatment. Levels of progesterone increased ($p < 0.05$) for 150 to 170 min after each ACTH application both in EXP I and EXP II.

In fetuses recovered 3 h after ACTH (EXP III), the plasma cortisol concentrations were significantly increased in the umbilical vein (53.4 ± 4.6 vs. 27.2 ± 4.4 ng/ml) and artery (55.8 ± 3.4 vs. 38.4 ± 3.5 ng/ml), and in the periphery (27.5 ± 1.8 vs. 15.7 ± 1.8 ng/ml) compared to controls. Plasma ACTH concentrations, however, did not differ in fetuses of both treatment groups.

Postnatal LH-RH challenge tests (1st and 28th day p.p.) induced LH surges in piglets both of ACTH- and saline-treated sows but did not differ between groups (1st day: 7.0 ± 2.1 vs. 7.8 ± 1.5 ng/ml; 28th day: 10.5 ± 3.8 vs. 13.5 ± 5.2 ng/ml). However, basal LH of piglets whose mothers were submitted to ACTH was lower on Day 1 (1.1 ± 0.4 vs. 3.0 ± 0.1 ng/ml, $p < 0.05$) but higher on Day 28 (1.2 ± 0.6 vs. 0.3 ± 0.1 ng/ml, $p < 0.05$).

Thus, chronic intermittent ACTH administration is able to influence the release pattern of maternal reproductive hormones. However, these findings demonstrate that these effects are dependent on the stage of pregnancy. Furthermore, it was shown that maternal cortisol can cross the placenta during gestation and thus affect maternal-fetal interactions and, as a result, reproductive function of offspring.

Notes

BIRTH OF LIVE OFFSPRING FROM CULTURED NUCLEAR TRANSFERRED EMBRYOS IN GOATS

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This experiment aimed at comparing in vivo development until birth of goat embryos following somatic nuclear transfer (NT) and transfer to recipients either at an early stage (4-cell) or after in vitro culture up to the blastocyst stage.

Donor nuclei were obtained from cells isolated from a 44-day-old caprine foetus. A foetal fibroblast cell line was frozen, stored and cultured over passage 4. *In vivo* matured oocytes recovered from superovulated goats were used as recipient cytoplasts. NT embryos were produced as previously described (Chesné et al., AETE, 2002, p.152).

In group 1, 4-cell stage NT embryos selected at 35 h were surgically transferred into the oviduct of recipients with a glass pipette (5 to 15 per recipient). In group 2, compacted morulae and blastocysts selected at Day 7 were transferred by coelioscopy into the uterine horn of recipients (3 to 5/recipient).

Plasma progesterone (pg) levels were measured on Day 21, pregnancies were confirmed by ultrasound scanning on Days 35, 45, 55 and maintained until kidding as presented in Table 1.

Table 1. In vivo development of goat embryos after somatic NT according to the embryo developmental stage at transfer

	Group 1 (%) 4-cell stage	Group 2 (%) Blastocysts
No of recipients	17	40
Positive pg test at day 21	8 / 17 (47.1)[a]	32 / 40 (80.0)[b]
Pregnant at day 35	5 / 17 (29.4)	22 / 40 (55.0)
Pregnant at day 45	3 / 17 (17.6)	16 / 40 (40.0)
Pregnant at day 55	1 / 17 (5.9)	13 / 40 (32.5)
Kidding	1 / 17 (5.9)	9 / 40 (22.5)
Number of kids born	1	10
Efficiency (No. of kids / embryo)	0.7% (1 / 144)[c]	6.2% (10/160)[d]

[a, b, c, d:] Values differ significantly ($P<0.05$)

In both groups, most pregnancy losses occurred before day 55 but some late foetal losses after two months were also observed. In group 1, the low pregnancy rate could be due to a deficient selection, too high a number of NT embryos per recipient and the inadequate method used for transfer at such early stages. The kid born in group 1 was overweight (6.0 kg) and considered as a case of large offspring syndrome. Individual birth weights in group 2 were 4.4, 4.4, 4.1, 3.9, 3.9, 3.7, 3.4, 3.2, 2.6, and 2.0 kg as in our normal breeding conditions. Survival one week after kidding was 0/1 in group 1 and 7/10 in group 2.

Notes

IS CLEAVAGE STAGE RETARDATION OF RABBIT OVA, UNDERGOING GENE MICROINJECTION, RELATED TO EMBRYO APOPTOSIS?

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Embryo manipulation in vitro, as used for transgenic animal production, reduces the blastocyst yield due to cell fragmentation and cleavage stage arrest, but the exact causes of this are not clear. Most fragmented or cleavage arrested human embryos show signs of apoptosis (Hardy, 1999, Rev Reprod 4:125-134). Frequency of apoptosis may serve as an indicator of embryo quality (Byrne et al., 1999, J Reprod Fertil 117:97-105). The aim of our work was to determine whether the decreased developmental rate of microinjected ova is due to apoptosis in subsequent embryos. We detected apoptosis (TUNEL assay) in blastocysts derived either from intact or gene-microinjected eggs, as well as in cleavage-stage arrested embryos after microinjection (Mi). In total, 503 pronuclear stage rabbit eggs (19-20 hpc) were gene-microinjected (Gene-Mi) into male pronucleus; 50 saline-microinjected eggs (Saline-Mi) and 278 intact eggs (Non-Mi) were used as controls. All groups were cultured either without or with EGF (20 or 200 ng/ml) in the culture medium up to 94-96 hpc. The resulting blastocysts and arrested embryos were divided, formalin-fixed, and stained for TUNEL and with propidium iodide. In total, 188 embryos were analysed using confocal scanning microscope. Among blastocysts, the Gene-Mi group had a higher TUNEL-index and a lower cell number than Non-Mi group, but there was no difference in the average TUNEL+ cell number per embryo between these groups (Table 1). EGF in the culture medium either diminished (at 200 ng/ml) or completely eliminated (at 20 ng/ml) an unfavourable influence of Mi on TUNEL-index and cell number. A comparison of Saline-Mi and Gene-Mi groups shows the deleterious effect of Mi on the embryo may be due to mechanical damages during the procedure, and not due to toxicity of the DNA solution. Cleavage-retarded embryos, 91% of which had fragmentation, exhibited a substantially lower cell number and a higher TUNEL-index when compared with blastocysts of all groups (Table 1).

Table 1. Influence of Mi and EGF on embryo apoptosis and total cell number

Experimental groups	Parameters		
	TUNEL-index, * %	TUNEL cells* per embryo	Embryo cell number†
Non-Mi (Intact ova)	6 ± 0.63 [a]	6.5 ± 1.76 [a,d]	132 ± 3.9 [a]
Saline-Mi	13.8 ± 1.8 [b]	8.1 ± 1.14 [a]	78.6 ± 10.2 [b]
Gene-Mi	13.26 ± 1.23 [b,c]	9.8 ± 1.26 [a]	94 ± 7.1 [b,c]
Gene-Mi + EGF 20	3.7 ± 0.5 [d]	3.6 ± 0.67 [b,c]	124.5 ± 7 [a]
Gene-Mi + EGF 200	6.25 ± 1.05 [a]	7.7 ±1.6 [a]	127.2 ± 7.8 [a]
Cleavage retarded embryos	18.8 ± 1.64 [e]	5 ± 0.75 [c,d]	33.4 ± 5.3 [d]

*- One-way ANOVA ($p < 0.01$); † - Kruskal-Wallis ANOVA ($p < 0.05$)

The results demonstrate that the increased TUNEL-index in embryos derived from microinjection, is a result of a decreased cell number due to blastomere fragmentation. This suggests that apoptosis is one, but not the sole, cause of decreased embryo viability.

Notes

ISOLATION AND CULTURE OF MULTIPOTENT BOVINE BONE MARROW MESENCHYMAL STEM CELLS

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Recent studies have determined that stem cells exist in most tissues. In particular a population of bone marrow cells capable of differentiating in multiple lineages has been detected. In humans, but also in other species such as mouse, rat and cat, these cells can proliferate in vitro for many passages displaying a stable fibroblast-like phenotype and they can be differentiated not only to classical mesenchymal derivatives such as bone, fat, cartilage and muscle, but also to other lineages such as oval hepatocyte and neurons.

Furthermore, in the mouse, a particular population of these cells, if injected into an early blastocyst, can contribute to most somatic tissue. All these characteristics make bone marrow cells a potential source of cells useful for therapy, transgenesis and cloning.

In this preliminary study we report the isolation, culture, characterization and differentiation of mesenchymal stem cells from bovine bone marrow.

Bone marrow cells were aspirated from the iliac crest of aone-month calf, immediately after slaughter, using an 11 g bone marrow biopsy/aspiration needle. Twenty ml syringes containing 3 ml of aspiration medium (TCM199/DMEM) with 5000 IU of heparin were used and about 15 ml of bone marrow were removed. After multiple PBS washings, the cell suspension was loaded onto 75%, 60%, 30% Percoll gradient and centrifuged at 1100g for 30 min. Cells were collected from the 30-60% interface and washed; residual erythrocytes were removed resuspending the cell pellet for 1 min in 1 ml of a solution 0.83% NH_4Cl in Tris HCl 10 mM, then 9 ml of culture medium were added and the suspension was washed three times. Following counting, cells were plated at 200,000 cells/cm^2 and cultured in M199/DMEM + 10% FBS. Medium was replaced at 24 and 72 hours (in order to remove hematopoietic cells) and then twice weekly. In 10 days these fibroblastic cells grew as colonies and were subcultured at 5000 cells/cm^2 for at least 10 passages. Expression of protamine2, a germinal tissue marker, was detected by RT-PCR in undifferentiated cells from passage 2.

When exposed to osteogenic medium, containing Dexamethasone 100 nM, Ascorbic Acid 0.25 mM, bglycerolphosphate 10 mM the cells changed their morphology from spindle-shaped to cuboidal. At 8-10 days of culture the cells were positive to alkaline phosphatase staining and from second-third week they formed aggregates Von Kossa staining positive.

Furthermore, undifferentiated cells from passage 2 were treated with 3 mM 5-azacytidine, a demethylating agent, for 24 h and then maintained in culture medium for at least 2 weeks. After this time, about 20% of cells changed their shape becoming round, few became bi- or multi-nucleated and some cells exhibited visible lipid vacuoles identified by Red Oil staining.

Therefore we conclude that we have isolated from bovine bone marrow a population of cells that have multilineage differentiation potential.

Notes

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN BOVINE EMBRYOS USING SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION

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Recent studies by our group have revealed that the post fertilization culture environment of bovine embryos influences the quality of the resulting blastocysts (Rizos et al., 2002, Mol Reprod Dev 61:234-248). In general, the quality of embryos produced in vitro is inferior to that of those produced in vivo. The analysis of differences in mRNA expression between in vivo and in vitro produced embryos may identify the molecular basis behind this problem.

The objective of the present study was to identify differentially expressed genes in bovine Day 7 blastocysts derived from either in vivo or in vitro culture treatments. Zygotes produced by in vitro maturation and fertilization were randomly divided into 2 groups and were cultured for 6 days either in vivo, in the ewe oviduct, or in vitro, in synthetic oviduct fluid medium. Day 7 blastocysts recovered from both systems were snap frozen and stored at -80°C until use. mRNA was extracted from 5 pools of 10 blastocysts from each culture group using the Dynabead mRNA Direct™ Kit (Dynal, Norway). Linear amplification of the mRNA was carried out using the T7 promotor for in vitro transcription (MessageAmp™ aRNA Kit, Ambion, Tx, USA, # 1750) and the amplified antisense RNA was subsequently reverse transcribed. PCR cDNA products enriched for differentially expressed genes in each treatment group were generated using suppressive subtractive hybridisation (PCR select cDNA subtraction kit, Clontech, CA, USA #K1804-1). Following subtraction, the differentially expressed amplicons were subcloned and sequenced. The sequences were submitted to the non-redundant and expressed sequence tag (EST) databases at the National Centre for Biotechnology Information (NCBI) using the BLAST algorithm. To date, 20 amplicons in the in vivo cultured group encoded 9 genes and 5 ESTs and 31 amplicons in the in vitro cultured group encoded 12 genes and 16 ESTs. Interestingly, Ferritin, a gene involved in iron storage and intracellular iron distribution, the null mutation of which is lethal in early mouse embryos (Ferreira et al., 2000, J Biol Chem 275:3021-3024), was upregulated in the in vivo cultured embryos and HSP 10 (Heat Shock Protein) was upregulated in in vitro cultured embryos, consistent with previous studies (Wrenzycki et al., 1998, J Reprod Fertil 112:387-398). Studies are ongoing to verify the differential expression of these genes in unsubtracted replicate populations of embryos from the two groups.

In conclusion, we have identified a number of genes that appear to be differentially expressed in bovine blastocysts produced in vivo and in vitro which may be linked to blastocyst quality.

Notes

VITRIFICATION OF SHEEP BLASTOCYSTS : THE CHOICE OF PROTOCOL DOES NOT APPEAR TO AFFECT THE PREGNANCY AND LAMBING RATE

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The aim of this paper was to compare the viability of in vitro produced (IVP) and in vivo derived (IVD) ovine embryos cryopreserved with different vitrification protocols. IVP blastocysts were produced by in-vitro maturation, fertilization and culture (IVM/IVF/IVC) of oocytes from slaughtered ewes while IVD embryos were collected from superovulated and inseminated animals. Embryos were cryopreserved after exposure at room temperature either for 5 min in 10% glycerol (G), then for 5 min in 10% G + 20% ethylene glycol (EG), then for 30 sec. in 25% G + 25% EG (Glycerol group), or for 3 min in 10% EG + 10% dimethyl sulphoxide (DMSO), then for 30 sec in 20% EG + 20% DMSO + 0.3 M Sucrose (DMSO group). One group of in-vitro produced embryos was vitrified similarly to the DMSO group, but with 0.75 M Sucrose in the vitrification solution (DMSO 0.75 group). Glycerol group embryos were then loaded into French straws or OPS (Open Pulled Straws) while the DMSO group embryos were all loaded into OPS. At warming, the embryos were placed either into a 0.5, 0.250, or 0.125 M Sucrose solution for 3 min each (three-step dilution) or directly into a 0.5 M Sucrose solution (one-step dilution). The DMSO 0.75 group was warmed directly by plunging the embryos into a TCM-199 + 20% serum in absence of sucrose (direct dilution). Embryos from all groups were transferred in pairs into synchronised recipient ewes and allowed to go to term. The lambing rate within each group, IVP and IVD embryos, indicated that there was no statistical difference among the vitrification protocols. In conclusion, thew choice of protocol does not appear to influence the results; these could be affected by the quality of the embryo.

Table 1. Re-expansion and viability of embryos in vitro produced and cryopreserved with five different vitrification protocols.

	Protocol	Cryoprotectant	Device	N° of dilution step at warming	Re-expansion (%)	Blastocysts transferred	Pregnancy (40 d) (%)	Lambs born /Vitrified embryos (%)	Lambs born /Transferred embryos (%)
In vitro	A	G+EG	French straws	3 (sucrose)	46/68 (67.6)	46	12/24 (50.0)	10/68 (14.7)	10/46 (21.7)
	B	G+EG	French straws	1 (sucrose)	-	42	12/21 (57.1)	10/42 (23.8)	-
	C	G+EG	OPS	1 (sucrose)	-	58	15/29 (51.7)	13/58 (22.4)	-
	D	DMSO+EG+ 0.3 M sucrose	OPS	1 (sucrose)	-	36	9/18 (50.0)	7/36 (19.4)	-
	E	DMSO+EG+ 0.75 M sucrose	OPS	1 (20% FCS)	-	21	5/10 (50.0)	5/21 (23.8)	
In vivo	F	G+EG	French straws	3 (sucrose)	52/62 (83.8)	52	19/27 (70.3)	39/62 (62.9)	39/52 (75.0)
	G	G+EG	OPS	3 (sucrose)	40/50 (80.0)	40	14/20 (70.0)	30/50 (60.0)	30/40 (75.0)
	H	G+EG	OPS	1 (sucrose)	32/42 (76.1)	32	12/16 (75.0)	24/42 (57.1)	24/32 (75.1)
	I	DMSO+EG+ 0.3 M sucrose	OPS	1(sucrose)	-	43	15/21 (71.4)	26/43 (60.1)	

Notes

LOW RESPONDERS IN CATTLE OPU-IVF?

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In human IVF, the concept of low responders (LR) is well known and generally defined as women having a poor response to gonadotropins in a previous induction cycle. It is a common clinical problem in human IVF practice (Keay et al., 1997 Brit J Obst Gyn 104:521-527). There is no uniform definition, but many authors have used a cutoff based on the number of mature follicles at the time of hCG injection. The concept is used to try new treatments on women considered LR. Moreover, attempts are made to select these women proactively (various tests). The aim of this retrospective study was to examine the usability of the LR concept in bovine OPU-IVF.

The results are derived from a database of 575 OPU sessions on 114 animals. All animals were treated (every other week) with FSH (40% of the dose used to superovulate an adult animal, 4 equal a.m.-p.m. injections spread over 2 days, the last one 48 h before OPU). The zygotes were cultured in SOF-serum. The developmental % with this culture system was affected by numbers of cumulus oocyte complexes (COC) put in culture per session: 1-4 COC: 13%[a], 5-8 COC: 28% [b], 9-12 COC: 30%[b], 13-16 COC: 35%[b], ([ab]$p<0.05$, chi square).

From these data, our "low response cutoff" for COC numbers was 4. For the mean recovery rate was 79% (7179 COC/9057 follicles), the cutoff for follicle numbers was defined at 5 (5*0.8=4). As a cutoff for follicle numbers without taking into account a cutoff for COC numbers would be meaningless, we defined (1) a low response session for follicle numbers as a session with ≤ 5 follicles : follicle LR; (2) a low response session for COC numbers as a session with ≤ 4 COC : COC LR; anmd (3) a genuine low responder animal as one which in ≥ 1 session had ≤ 5 follicles and ≤ 4 COC.

Analysis of the database revealed 52/575 follicle LR (9%) and 63/575 COC LR (11%) sessions. Detailed analysis of the low response data revealed three kinds of low responses: follicle LR without COC LR (more COC than punctured follicles: observation problem), follicle LR and COC LR (follicle numbers too low and COC numbers too low: genuine low response problem), COC LR without follicle LR (low number of COC despite a number of follicles of more than 5: recovery rate problem) (Table 1).

Table 1: Detailed analysis of the results of the low response sessions: frequency of occurence.

	Follicle LR	COC LR	Follicle LR	COC LR
Observation problem	+	-	16/52 OPU (31%)	
Genuine low response problem	+	+	36/52 OPU (69%)	36/63 OPU (57%)
Recovery rate problem	-	+		27/63 OPU (43%)

Twenty-seven of 114 animals (24%) were categorised as genuine low responders. They underwent 212 OPU sessions (212/575 = 37% of all OPU sessions). They suffered LR in 17% (36/212) of their OPU sessions vs 6% (36/575) in the total population.

In conclusion, low response in our study concerned an observation problem, a recovery rate problem and a genuine low response problem. The genuine low response problem occured in 24% of anima1s in 1 OPU out of 6 (17%). Given this high frequency, the LR concept deserves further investigation.

Notes

QUANTIFICATION OF LIPIDS IN SINGLE BOVINE AND PIG OOCYTES USING A FLUORESCENT PROBE, NILE RED

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Lipid content is a determining factor of oocyte and embryo quality (Abe et al., Mol Reprod Dev 2002; 61: 57-66; Kim et al., Reproduction 2001; 122: 131-8). However, the current techniques of quantification of lipids in cells cannot be applied to single oocytes or embryos. The aim of this work was to develop and test a sensitive and accurate technique enabling the assessment of lipid content in single oocytes or embryos. A fluorescent dye, Nile Red, that stains specifically intracellular lipid droplets (Greenspan and Fowler, J Histochem Cytochem 1985; 33: 833-6) was used. Fluorescence was quantified using a photometer connected to a microscope.

Immature bovine and porcine oocytes were denuded and fixed (2% glutaraldehyde, 2% formaldehyde) for 24 h. Then oocytes were stained with Nile Red (10 µg/ml dissolved in a solution of NaCl 0.9%; PVP 1 mg/ml in water) for various times at room temperature. The dye fluoresces only when located in the lipid droplets. A new Nile Red solution was prepared for each replicate. Oocytes in 25 µl of the dye solution were then allocated individually to wells of a 386 well microplate. The fluorescence of the whole oocyte was evaluated with a fluorescent inverted microscope (Leitz; Excitation: 400-500nm and Emission: 515LP) at a 100-fold magnification, was amplified with a photomultiplier and quantified with a photometer. Each oocyte was evaluated in duplicate. The results were expressed in arbitrary units of fluorescence. Lipid droplets were also visualised in the oocytes.

An incubation of at least 10 h in the 10 µg/ml Nile Red solution was necessary to obtain a stable signal. Using this protocol, we observed that the fluorescence remained stable for several days, even after repeated measurements, but the oocytes have to be kept in the staining solution. The use of a filter on the UV light was necessary to avoid bleaching. The fluorescence was restricted to lipid droplets and no fluorescence was observed in the cytosol or in the nuclear compartments. In the absence of standards, the technique only allowed for a relative evaluation of lipid content. In order to verify the technique, we compared the lipid content of bovine and pig oocytes (n=30). Using our technique, we confirmed that pig oocytes contained 2.2-fold more lipids in droplets than bovine oocytes (mean ± SEM in arbitrary units of fluorescence: bovine oocytes: 233 ± 11; pig oocytes: 513 ± 17; ANOVA 1, $p<0.01$). This is in agreement with the study of McEvoy et al. (J Reprod Fertil 2000; 118:163-70) who observed a 2.5-fold increase in lipid content between the two species using gas chromatographic analysis on pools of 1000 oocytes.

In conclusion, our new technique enables the relative quantification of the lipid content in lipid drops of single oocytes. Similar data were also obtained with single embryos. Using Nile Red staining, it is also possible to combine, on the same sample, the quantification of lipids and the evaluation of the size and distribution of lipid droplets within oocytes and embryonic cells.

Notes

INFLUENCE OF FOLLICLE SIZE ON THE QUALITY OF BUFFALO CUMULUS OOCYTE COMPLEXES AND CONCENTRATION OF STEROIDS IN FOLLICULAR FLUID

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The aim of the present study was to investigate the effect of follicle size on the morphology of buffalo cumulus oocyte complexes (COCs), chromatin configuration, level of apoptosis in cumulus cells, and concentration of progesterone and oestradiol-17β in follicular fluids of different follicle sizes. Visible follicles were carefully isolated and classified according to their size into 3 categories: < 3 mm (2 to 3 mm), 3 to 5 mm and > 5 mm (5 to 8 mm). A total of 295 COCs were recovered from these three follicle categories; COCs were classified according to number and compactness of cumulus layers. Only COCs with compact cumulus were used for IVM, which were mostly obtained from follicles < 3 and 3 to 5 mm (56-57% and 57.1%, respectively) in comparison to 34.3% from large follicles.

The chromatin configuration in the oocytes at the time of recovery derived from < 3 mm and 3 to 5 mm follicles was arrested at the germinal vesicle stage (60% and 70.8%, respectively). The highest proportion of oocytes in meiotic resumption was found in oocytes obtained from larger follicles (77.8% vs. 40 and 29.2% from < 3mm and 3 to 5 mm diameter follicles, respectively).

Cumulus morphology, chromatin configuration at the time of recovery and the follicle size were correlated. In contrast, no significant differences were found in the maturation capacity (metaphase II) of COCs derived from follicles with different diameters.

Apoptosis of cumulus at time of recovery decreased with increasing follicle size, but the differences were not significant (0.9, 0.6, 0.2%, respectively). After 24 h of IVM, an increase in apoptosis was observed in cumulus cells from all follicle sizes, and a significant difference ($P < 0.05$) was observed in cumulus cells from 3 to 5 mm follicles compared to < 3 and > 5 mm follicles (24.4 vs. 4.7 and 3.5%, respectively).

The concentration of progesterone in buffalo follicular fluid was not influenced by the follicle size, while the concentration of oestradiol-17β was significantly higher in follicles from 3 to 5 mm and > 5 mm (782 ± 67 ng/ml and 788 ± 67 ng/ml, respectively) diameter in comparison to follicular fluid from follicles < 3 mm (682 ± 67 ng/ml) diameter.

Notes

OUTCOME OF PREGNANCIES AFTER TRANSFER OF BOVINE IVP EMBRYOS CULTURED IN THE SERUM FREE SEQUENTIAL MEDIA, ISM_1/ISM_2

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The sequential media ISM_1 / ISM_2 (MediCult, Copenhagen), have been successfully used to improve blastocyst rate in humans after IVF and replace coculture. In a previous study, we evaluated the use of this semi-defined sequential media for the culture of bovine IVM-IVF derived embryos in the absence of serum addition. Culture in ISM_1/ISM_2 yielded 49% blastocysts of good quality as assessed by their cell number and did not significantly modify the composition of fatty acids in blastocysts compared to controls (Heyman et al., 2002, Theriogenology 57:671). The aim of the present study was to assess the potential for full term development of such IVP bovine blastocysts grown on ISM_1/ISM_2 and detect any possible effects on offspring.

IVF zygotes were cultured in 30 µl microdrops of ISM_1 for 3 days then in ISM_2 for 4 days at 39°C in a 5% O_2-CO_2 atmosphere. Blastocysts selected on Day 7 were transferred to synchronous Day 7 recipient heifers (Charolais or crossbred, one embryo per recipient). Comparison was made with control Day 7 blastocysts developed in a co-culture system using Vero cells and B2 medium supplemented with 2.5% FCS (Menck et al., 1997, Reprod Nutr Dev 37:141-150). All transfers were performed on the same experimental farm by the same technician. After transfer, plasma progesterone tests were performed on Day 21 on recipients of both groups and then pregnancy was assessed by repeated scanning of the fetus on Days 35, 50, 70, 90 and followed up to calving. Birth weight and characteristics of calves born were recorded after natural calving.

After transfer, 12 of the 25 blastocysts from the ISM group developed into live calves (48%) which is very similar to the 49% (25/51) calving rate achieved from blastocysts of the control co-culture group (Table 1).

Table 1: Evolution of pregnancies after transfer of blastocysts developed in ISM or co-culture

Group	Recipients	Pg test Day 21	Confirmed Pregnancy Rate				
			Day 35	Day 50	Day 70	Day 90	Calvings
ISM embryos	25	17/25 68.0%	12/25 48.0%	12/25 48.0%	12/25 48.0%	12/25 48.0%	12/25 48.0%
Control embryos	51	32/51 62.7%	27/51 52.9%	25/51 49.0%	25/51 49.0%	25/51 49.0%	25/51 49.0%

Calves born from blastocysts cultured in ISM had a mean birth weight of 45.17± 4.95 kg which was not different from that of the control group (45.71±7.50 kg). Among the calves born, no case of Large Offspring Syndrome (LOS) was observed and mean pregnancy length was 280.8 and 282.3 days for ISM and control groups, respectively.

The use of ISM_1/ISM_2 sequential culture system for developing bovine blastocysts offers an interesting alternative to co-culture, thus avoiding any potential risk related to the use of cell layers and serum. From this limited number of transfers, we did not observe any fetal loss and all the calves born survived over one month (12/12, 100%) in the ISM group compared to 80% (20/25) in the control co-culture group. We are currently extending the use of ISM serum free media for culture of bovine NT embryos.

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Notes

CULTURE OF BOVINE EMBRYOS WITH DIFFERENT CONCENTRATIONS OF L-LACTATE, DL-LACTATE, PYRUVATE AND B VITAMINS

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Lactate and pyruvate are important components of culture media. However, as weak acids, they may decrease intracellular pH (pHi). Use of DL-lactate in culture media can reduce pH_i, because, although only L-lactate can be metabolized, both isomers affect pH. The aim of this study was to compare effects of different concentrations of L-lactate and DL-lactate and effects of B vitamins on embryo development. Bovine oocytes (N=3,520), aspirated from abattoir-derived ovaries, were matured for 23 h in a chemically-defined medium similar to SOF, with 10 mM L-lactate, 1 mM alanyl-glutamine, 2 mM fructose, 5 mg/ml FAF-BSA (CDM-M), in 5% CO_2 in air, at 38.5°C, and then fertilized for 18h in CDM plus 2 _g/ml heparin and 2 mM caffeine (CDM-F). During culture in 5% O_2/5% CO_2/90% N_2 in CDM-1 (nonessential amino acids, 10 _M EDTA and 0.5 mM fructose) for 2.5 days and CDM-2 (CDM-1 plus essential amino acids, no EDTA and 2 mM fructose) for 4 more days, we tested L-lactate, DL-lactate and pyruvate at different doses, making up four treatments: DL10: 10 mM DL-lactate and 0.5 mM pyruvate during the entire culture period; L10: 10 mM L-lactate and 0.5 mM pyruvate; L10P2: 10 mM L-lactate and 0.5 mM pyruvate in CDM-1 and 0.2 mM pyruvate in CDM-2; and L5: 5 mM L-lactate and 0.5 mM pyruvate. In CDM-2, we divided all treatments in two groups, with 1x or no vitamin B mix (100x BME vitamin solution, Sigma). At days 6.5 and 7.5 after IVF, the stage (4= morula ... 8=hatched blastocyst), quality (1=excellent ... 3=poor), and darkness (1=light… 4=dark) of the embryos were evaluated; at day 7.5, blastocysts were fixed and stained to count cells. Data were analyzed by factorial ANOVA, with factors lactate (DL10, L10, L10P2 and L5) and vitamin B (0, 1x). The experiment was replicated 11 times with semen of 4 bulls, using 3, 3, 3, and 2 times for each bull. At day 6.5, the stage of embryos cultured in DL10 averaged 4.96 ± 0.13, and their quality and lightness were 2.04 ± 0.12 and 2.29 ± 0.13, respectively, while the stage of embryos cultured in media L10 averaged 5.48 ± 0.12 and their quality and lightness were 1.64 ± 0.10 and 1.83 ± 0.11, respectively ($P<0.05$). The compact morula/blastocyst rate per oocyte was lower with DL10 than with L10P2 (18.4% vs 25.7%, respectively; $P<0.05$). At day 7.5 of in vitro culture, the percentage of blastocysts was lower with 10 mM of DL-lactate than 10 mM of L-lactate (16.9% vs 24.9%; $P<0.05$). There was no effect of B vitamins on embryonic development ($P>0.1$). There was no difference ($P>0.1$) among treatments in numbers of cells/blastocyst (avg. 113 cells). In this study, L-lactate improved embryonic development and quality compared to DL-lactate.

Table 1. Main effect least-squares means ± pooled standard errors for treatments.

	Day 6.5				Day 7.5
	Stage	Quality	Lightness	Compact morulae or blastocysts %	Blastocysts %
DL10	4.96±0.13 a	2.04±0.12 a	2.29±0.13 a	18.4±0.02 a	16.9±0.02 a
L10	5.48±0.12 b	1.64±0.10 b	1.83±0.11 b	24.7±0.02 a,b	24.9±0.02 b
L10P2	5.31±012 a,b	1.68±0.11 a,b	1.82±0.12 b	25.7±0.02 b	21.9±0.02 a,b
L5	5.31±0.12 a,b	1.87±0.11 a,b	1.96±0.12 a,b	24.6±0.02 a,b	22.9±0.02 a,b
Vit B Yes	5.32±0.09	1.82±0.08	2.04±0.09	24.0±0.01	21.9±0.01
Vit B No	5.20±0.09	1.80±0.08	1.91±0.08	22.7±0.01	21.5±0.01

a,b: Values within columns without common superscripts differ ($P<0.05$).

Notes

EMBRYO TRANSFER STATISTICS DURING THE LAST 6 YEARS IN POLAND

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Commercial ET activity in Poland began in 1986. From the start 15 embryo transfer teams were involved; at present only 9 remain. Activity has been precisely monitored for six years. During this period the cows used for superovulation (SOV) in Poland were mainly of the Black-White or Holstein breed. Only 35.9 % of donor cows were of other breeds (Limousin, Charolais, Simmental). In the last 4 years there has been a rise in the percentage of heifers amongst the donor animals. All cows were superovulated with FSH – especially Ovagen (Biogen), which is the only officially registered product in Poland. Earlier the SOV was carried out with Folltropin, Stimufol and others products.

A decrease in the number of superovulated and flushed cows was observed during 1997 – 2001. In 1997, 736 cows were superovulated, whereas in the years 1998-2001, respectively, 390, 257, 183, 169 were used. In 2002, only 72 cows were superovulated, 10 times lower than 6 years ago. From all donors cows flushed in 1997-2002, respectively, 4507, 2671, 1481, 885, 890, 497 total ova and embryos were recovered. The mean number of transferable embryos was 3.4 to 5 per cow. The total number of all transferred embryos was 2688, 1617, 1449, 700, 594, 345, respectively, during 1997-2002. In the same years the number of embryos transferred fresh (FR) was 1127, 672, 433, 229, 220 and 167. The mean conception rate (CR) after FR embryos was 47 – 52.4%. The first embryos frozen with ethylene glycol were transferred in Poland in 1997. In that year, from all transferred embryos, 769 (28.6%) were transferred by direct transfer (DT). The number of imported DT embryos was 202. The percentage of direct transferred embryos has increased over the years. For example in 1998-2002 they were respectively 37.8, 37.4, 43.6, 44.9 and 51.5% embryos transferred using DT. The CR after direct transferred embryos was relatively high (43 – 47.9%). No differences in respect to CR were observed between imported and native DT embryos. The number of glycerol-frozen (GLY) embryos was 792, 333, 477, 222 and 107, respectively, during 1997-2001. In 2002, no embryos frozen in GLY were transferred. There was a large difference in CR after transfer of GLY embryos (34.9 – 60.1%). No commercially in vitro produced embryos were transferred in Poland in this period. However, during 2000–2002, 32 in vitro produced fresh or frozen embryos were transferred for scientific purposes. The first sexed DT embryos (24) were imported from Holland and transferred in 2000.

In conclusion, reduction of the cattle population in Poland and unfavourable circumstances on the cattle breeding market are contributing to the decrease of modern biotechnology methods in reproduction.

Notes

THE INFLUENCE OF INCUBATION TEMPERATURE DURING LIPOFECTION ON MOTILITY OF BOAR SPERMATOZOA

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At present, transgenesis can be achieved by the use of various methods e.g. DNA microinjection, embryonic stem cell transfection or retroviral vectors. The idea of using a spermatozoa for introducing exogenous DNA into an oocyte became a very promising and simple tool. While the binding of DNA molecules to spermatozoa have now a solid experimental base, there is limited data about successful introduction of exogenous DNA into the spermatozoa. It requires the use of several complicated steps. It would be very interesting to find a simple and effective method of introduction of exogenous DNA into spermatozoa. One of the most effective and simple method of cell transfection is lipofection. In the present experiment we investigated the toxic effect of DNA-lipofection reagent solution on an boar sperm cells. We also investigated the influence of incubation temperature during lipofection on sperm cell motility. Five ml each of diluted and plasma-free spermatozoa at a concentration of: (A) 80 x 10^6/ml and (B) 60 x 10^6/ml were incubated with pCMV-FutpA/PolyFect Transfection Reagent (Qiagen) solution for 4-5 h at room temperature or at 37^0C. Subsequently, the motility of transfected spermatozoa were at the level of: (A) 20% progressive and 40% total motility, (B) 10% and 60% respectively. Within sperm cell incubated at 37^0C noticed motility were: (A) 30% progressive and 60% total, (B) 20% and 40% respectively. Our results indicated that DNA-lipofection solution has no toxic effect on boar sperm cell.

Notes

THE EFFECT OF MODIFICATION OF TRANSFER PROCEDURE IN PRODUCTION OF TRANSGENIC PIGS FOR XENOTRANSPLANTATION

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Currently the major limiting factor to organ transplantation is the increasing shortage of suitable donor organs. One solution to this problem would be the use of animal organs – xenotransplantation. Most interest is now being directed towards the use of the pig as a donor of organs for humans. Pigs are relatively easy to genetically modify but the effectiveness of the process is still not satisfactory. There are several methods which could be used to produce genetically modified animals e.g. cloning, embryonic stem cell transfection or retroviral vectors. Considering all the advantages and disadvantages, DNA microinjection into the pronuclei of fertilized eggs today is the best performing technique.

To perform genetic modification in pigs via DNA microinjection it is still necessary to increase its effectiveness. This can be achieved by improvement of vectors used and also by modifications of the procedure.

In our experiment we modified the transplantation procedure. pCMV-FutpA vector was microinjected into one of the visualized pronuclei of a pig zygote. From 35 donor pigs, 561 eggs were obtained and 438 were microinjected. Surgical transfer of transformed zygotes was modified by the introduction of the whole pool of zygotes (20-25 per recipient) into one of the oviducts only instead of the routine transplantation into both oviducts. In effect surgery time was shortened, manipulations on the oviducts were reduced and increase of pregnancy rate was observed: 7 from 17 recipients became pregnant (44%). Pregnancy rates obtained previously, where transplantations were done into both oviducts were around 36%.

Notes

THE EFFECTS OF KETOPROFEN ON THE GROWTH OF OVULATORY FOLLICLE AND OVULATION IN DAIRY COWS

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Studies have confirmed that nonsteroidal anti-inflammatory drugs (NSAIDs) are increasingly used for the treatment of food-producing animals, particularly cattle. Ketoprofen is a propionic acid NSAID with a strong anti-inflammatory property. Final growth of the ovulatory follicle and ovulation are regarded as two classic examples of physiologic inflammatory reactions. The present experiment was designed to determine the effects of ketoprofen on the growth of the ovulatory follicle and ovulation in dairy cows. The oestrous cycles of 8 reproductively sound non-milking cows (345 ± 37 kg LW) were synchronised using 2 injections of $PGF_{2\alpha}$ (30 mg im, Lutalyse, Upjohn) 14 days apart. In the control group, cows were administered a 0.9% saline solution daily from Day 8 (Day -3) of the subsequent cycle at 24 h intervals over 4 days. The presence of a dominant follicle (≥ 9mm) on Days 7 and 8 of the oestrous cycle was confirmed by a serial ultrasonography. After an oestrous cycle rest, cows were daily given the recommended therapeutic dose (3 mg/kg, im) of ketoprofen (Ketofen 100, Merieux/Webster, Australia) initiated on Day 8 (Day -3) of the synchronised oestrous cycle at 24 h intervals over 4 days. All cows received an im injection of $PGF_{2\alpha}$ (30 mg, Lutalyse) either 6 h prior to the first treatment of saline solution or 6 h prior to the first injection of Ketoprofen. Ultrasonography of the ovaries was performed daily from the day before commencing (Day -4) experimental treatments until Day 2 after induced oestrus (Day 2) to monitor growth of the ovulatory follicle and ovulation and then on Day 10 after oestrus (Day 10) to determine the presence and the size of the corpus luteum. Serum progesterone concentration was also determined on the day of oestrus (Day 0) to ensure the regression of the CL. Results are shown in Table 1. Data were analysed using repeated measures ANOVA and a Wilcoxon signed rank test where appropriate.

Table 1. The effects of administration of ketoprofen on the growth of ovulatory follicle and ovulation in eight cows (mean ± SEM).

	Saline	Ketoprofen
Diameter of DF (mm) on Day 8 (Day -3)	9.5 ± 0.6	9.3 ± 0.3
Daily growth rate of OF (mm/day)	1.0 ± 0.1[a]	0.5 ± 0.4[b]
Diameter of OF (mm) on day of oestrus (Day 0)	13.8 ± 1.3[a]	10.9 ± 0.4[b]
No. of standing heat observed per h	9.9 ± 1.7[c]	4.4 ± 1.3[d]
CL size (mm) on Day 10 after oestrus	14.8 ± 0.9[a]	9.2 ± 0.6[b]

DF=Dominant Follicle(≥9mm); OF=Ovulatory Follicle. [a,b] ($p<0.05$); [c,d] ($p=0.07$).

By 48 h after standing oestrus, ovulation had taken place in 7 of 8 saline-treated cows; however when the cows were treated with ketoprofen only 3 cows had ovulated. These results demonstrate that administration of ketoprofen during the pre-and periovulatory period in dairy cows may impair final growth of ovulatory follicle leading to a disturbance in the normal process of ovulation.

Notes

FOLLICULAR DEVELOPMENT, CORPUS LUTEUM FUNCTION AND FERTILITY AFTER USAGE OF A SHORT-TERM PROGESTERONE TREATMENT AT METOESTROUS AND DIOESTROUS PHASES IN DAIRY COWS

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A factor influencing pregnancy rate in cattle is the presence or absence of a functional corpus luteum (CL) at the time of initiating an oestrus synchronisation treatment. Two experiments were designed to determine: 1) the pregnancy rate following the application of a short-term usage of an intravaginal progesterone implant (CIDR) in combination with oestradiol benzoate (E-17β), in the presence or absence of a CL in dairy cows; and 2) the effects of the short-term CIDR treatment and E-17β in combination, given at metoestrous and dioestrous phases on follicular development and CL function in dairy cows. In Exp. I, 88 cyclic lactating Holstein cows with a normal previous calving history (3-6 yr old) were used. After a gynacological examination, cows were divided into 2 groups (with CL; n=52 and without CL; n=36). All cows were treated with a CIDR (1.9 g progesterone; InterAg, NZ) plus an im injection of 5 mg oestradiol benzoate (AbuReihan, Iran). Six days later, cows were intramuscularly administered a luteolytic dose of $PGF_{2\alpha}$ (30 mg, Upjohn, Belgium) and on Day 7, all CIDRs were removed. Then, cows observed in heat were artificially inseminated (AI) using high quality frozen-thawed semen. Pregnancy diagnosis was performed 50 days after AI by rectal palpation. In Exp. II, 20 cyclic dairy cows, mainly Holstein (3-6 yr old) with a history of normal calving were selected and randomly allocated, with stratification by age, to one of three groups: untreated control group (n=5) and two treatment groups in which cows, as in Exp. I, received a CIDR plus an im administration of 5 mg E-17β on Day 3 (n=8) or Day 9 (n=7) after a synchronised oestrus (day 0 = oestrus). Six days later, cows were intramuscularly administered a luteolytic dose of $PGF_{2\alpha}$ (30 mg Lutalyse) and on Day 7, all CIDRs were removed. Ovarian ultrasonography was performed daily from Day 0 until removal of CIDR implants and continued to day of ovulation and then on day 10 after ovulation. The ovaries of the control cows were also scanned during a complete oestrous cycle. Blood samples were collected on Day 10 after ovulation to determine plasma progesterone concentrations. Data were analysed statistically using a χ^2 test (Exp. I) and a Duncan's one way ANOVA and a t-test (Exp. II) where appropriate. Pregnancy rate was higher in cows treated with a 7-day CIDR and E-17β in combination, in the presence of a CL than in cows receiving the same combination treatment in the absence of a CL (32/52; 61% vs 17/36; 47%; P<0.1), respectively. Results of Exp. II are shown in the table below:

	Control (n = 5)	Day 3 (n = 8)	Day 9 (n = 7)
Diameter of Ovulatory Follicle (mm)	18.0 ± 1.0^{a}	16.2 ± 1.2^{b}	19.5 ± 1.2^{ac}
Interval, E-17β to Wave Emergence (days)	-	4.5 ± 0.5^{a}	4.1 ± 0.4^{a}
Diameter of CL 10 Days After Ovulation (mm)	17.2 ± 2.5^{a}	21.0 ± 3.1^{b}	23.0 ± 2.9^{c}
P_4 Conc. 10 Days After Ovulation (ng/ml)	6.0 ± 0.9^{a}	7.4 ± 1.2^{a}	9.5 ± 2.2^{b}

P_4=Progesterone

a,b,c; values with different superscript within a row are different (P<0.05).

It can be concluded that reduction in the size of the ovulatory follicle results in a reduced size of the CL and lower circulating progesterone concentrations on Day 10 after ovulation. This may contribute to the decreased pregnancy rate when a short-term progesterone treatment is applied in the absence of a mature CL for oestrus synchronisation in dairy cows.

Notes

DEVELOPMENTAL COMPETENCE OF OOCYTES ORIGINATED FROM EARLY ANTRAL OVARIAN FOLLICLES IN CATTLE

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It has been known that the mammalian ovary contains a huge number of small oocytes, of which only a small proportion grows to final size, matures, and ovulates. Since >99.9% of ovarian follicles undergo atresia, it would be of great practical benefit if these follicles, destined to become atretic, could be rescued by a long-term in vitro culture, thus offering a large pool of oocytes for IVM and IVF.

In the present study we compared the growth, survival and the maturational competence of bovine oocytes originating from early antral follicles and cultured in vitro: (1) in intact follicles (IF) and (2) as cumulus-oocyte-complexes with pieces of parietal granulosa cells (COCGs).

Early antral ovarian follicles, 0.4 to 0.7 mm in diameter, were isolated from bovine ovaries collected after slaughter. Both COCGs and IF were embedded in collagen gels and cultured in TCM 199 with 10% fetal calf serum and 4 mM hypoxathine, respectively, for 7 and 14 days. The diameters of the IF were measured every 3 days. After the growth culture, the gels and follicles were ruptured with fine forceps and the COCs were recovered. One group of COCs was denuded and fixed to evaluate chromatin configuration; the second group of COCs showing normal morphology was matured in TCM 199 supplemented with 20% oestrous cow serum for 24 h and than also fixed and evaluated for chromatin configuration.

The isolated COCGs (n=534) started to form antral follicle-like structures after 24 h of culture (18.7% ± 3.9%). As the culture time was prolonged, the proportion of these structures increased to 56.9% ± 6.3% on Day 2 to 3 of culture and slightly decreased on Day 7 (40.6% ± 5.5%).

After being in growth culture for 7 days the oocytes were recovered from the newly created follicle-like structure. At this time 27.0% of the oocytes were at the GV-stage, 40.4% had undergone GVBD (and reached mainly early diakinesis), 1.5% were mature and 19.8% degenerated. The remainder of the oocytes were either without chromatin structures (7.5%) or were activated (3.8%). After a subsequent 24 h of IVM of COCs with normal morphology 50.3% of oocytes underwent GVBD, and 23.0% were mature (i.e. reached telophase I or metaphase II with polar body).

The isolated follicles (n=142) increased their diameter during in vitro culture as has been previously shown (K_tska et al., Theriogenology 2000; 54:247-260). After 14 days of culture 43.5% of the oocytes enclosed in IF remained at the GV stage, 26.1% had undergone GVBD, 19.6% had degenerated chromatin, 10.9% were without detectable chromatin and no mature oocytes were observed. After 24 h of IVM culture, 49.0% of the oocytes were still in GV-stage, 30.2% had undergone GVBD and only 10.4% reached metaphase II. Moreover, some oocytes had degenerated (5.2%) while in others no chromatin structures (5.2%) were found.

The results show that isolated bovine COCs with pieces of parietal granulosa create follicle-like structures in culture and that their oocytes can achieve meiotic competence and mature at a 2-fold higher rate than oocytes cultured in intact follicles.

Notes

EFFECT OF HEPARIN TREATMENT OF FRESH AND FROZEN-THAWED SPERM ON IN VITRO AND IN VIVO DEVELOPMENT OF GOAT IVP EMBRYOS

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Investigations on in vitro embryo production in goats have been developed over the past decade. However, in comparison with other domestic species, especially cattle, those studies have been the subject of few reports despite its usefulness for both basic research and commercial application. The objective of this study was to compare the possibility of IVP in goats using immature follicular oocytes that, after in vitro maturation (IVM), were fertilized with fresh or frozen sperm of a single buck capacitated in the presence or absence of heparin.

Immature oocytes, recovered from slaughtered ovaries, after 27 h of IVM in modified TCM 199, were fertilized in vitro with fresh or frozen-thawed sperm of a single buck. Semen preparation was carried out according to the modified method for capacitation of bull spermatozoa using 45 min incubation in heparin solution (50 _g/ml of sperm suspension) before spermatozoa were added to oocytes in TALP-IVF. Control sperm, non-treated with heparin, was incubated with the same volume of TALP medium alone. After IVF, the zygotes were cultured for 24 h in medium B_2 (INRA) yielding, respectively, 62.2% (120/193) and 79.4% (85/107) cleaved embryos following IVF with fresh, heparin-treated and control sperm versus 51.7% (148/286) and 38.8% (69/178) cleaved embryos following IVF with frozen-thawed, heparin-treated and control sperm. The resulting embryos were co-cultured with goat oviduct epithelial cells for another 8 days, i.e. up to the hatched blastocyst stage. Of the cultured embryos, 26.7% (16/60), 48.2% (41/85), 37.7% (29/77) and 34.8% (24/69) reached the blastocyst stage and 43.7% (7/16), 70.7% (29/41), 48.3% (14/29) and 62.5% (15/24) of blastocysts hatched, respectively for fresh heparin-treated, fresh control, frozen-thawed heparin-treated and frozen-thawed control sperm, respectively. Irrespective of semen, no differences were observed in blastocyst quality both in vitro (comparisons of cell number in Day 9 hatched blastocysts) and in vivo (development to term after embryo transfer to synchronized recipients). Overall, 64 embryos obtained after IVF with fresh and frozen-thawed sperm were surgically transferred to 11 synchronized recipients. Results are presented in Table 1.

Table 1. Comparison of in vivo development of goat embryos resulting from IVF with fresh and frozen-thawed sperm of a single buck

Sperm type	Recipients N (embryos)	Pregnancy diagnosis (%) at Day 35		Kidding Day 70	Embryo survival N (%)
Fresh	6 (34)	5 (83.3)	4 (66.7)	4 (66.7)	7 (20.6)
Frozen-thawed	5 (30)	4 (80.0)	3 (60.0)	3 (60.0)	6 (20.0)

We conclude that heparin treatment of caprine sperm significantly improves cleavage rate following IVF with frozen-thawed sperm. However, heparin treatment of fresh caprine sperm negatively affected embryo developmental competence. Our IVP system allows the obtention of embryos with full developmental competence using both fresh and frozen-thawed sperm.

Notes

GLUCOSE TRANSPORTER EXPRESSION IN BOVINE EXPANDED BLASTOCYSTS CULTURED UNTIL DAY 7 POST OVULATION AFTER COLLECTION AT DIFFERENT TIMES OF IN VIVO DEVELOPMENT

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Glucose transport is critical for mammalian blastocyst formation and subsequent development. Differences in the mRNA expression of glucose transporter genes (Glut-1, Glut-3, Glut-4) between in vitro cultured and in vivo developed bovine preimplantation embryos have been reported (Knijn et al., 2002, Reproduction 124:365-375; Lazzari et al., 2002, Biol Reprod 67:767-775). Recently, the presence of a novel, insulin-regulated glucose transporter Glut-8, has been demonstrated in mice and obviously affects embryo survival (Pinto et al., 2002, Biol Reprod 66:1729-1733). The maternal-to-embryonic transition period (MET) is crucial for normal progression of development. In this study we investigated whether passing the MET in vitro or in vivo influences the expression of mRNA of Glut-1, 3, 4 and 8 in single expanded blastocysts. Embryos were collected from 41 cows, superovulated with FSH, at 3 time points post ovulation (po): 45 h po, before MET (45 h group), at 100 h po, after MET (100 h group) and at Day 7 po (in vivo group). The embryos collected at 45 and 100 h po were further cultured in synthetic oviductal fluid (SOF) medium until Day 7 po. A control group of morulae and blastocysts was obtained from abattoir oocytes after IVM/IVF and IVC in SOF medium (in vitro group). The developmental stage of the embryos was determined with stereomicroscopy. For the analysis of mRNA, a semi-quantitative RT-PCR was performed using rabbit globin mRNA as an external standard. Poly(A)RNA was prepared using a Dynabead oligo-dT mRNA purification kit (Dynal A.S., Oslo, Norway). PCR products were separated on 2% agarose gels and stained with ethidium bromide. The gene transcripts were quantified by digital imaging and the relative abundances were calculated. At least 10 replicates were performed for each gene in each group. Data were analyzed by ANOVA followed by multiple pairwise comparisons using the Tukey test.

The relative abundance of Glut-3, 4 and 8 transcripts was significantly different between completely in vitro cultured and entirely in vivo developed blastocysts (Fig.1). The 45 h in-vivo group and the 100 h in-vivo groups were not different from the in-vitro group but were also not different from the in-vivo group, with the exception of Glut-3 transcripts that were significantly higher expressed in the 100 h in-vivo group compared to the in vivo group. This could be due to the change of environment at 100 h po because Glut-3 is supposed to be a sensitive marker for embryo environment (Lazzari et al., 2002). In conclusion, in vitro production affects expression of mRNA of Glut-3, 4 and 8 in bovine expanded blastocysts. However, no influence of passing the MET in vitro or in vivo could be observed.

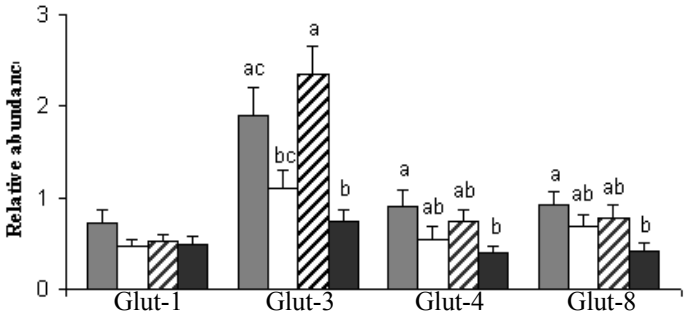

Fig 1. Relative abundance of Glut-1, 3, 4 and 8 transcripts (values shown as mean ± sem) in expanded blastocysts after complete culture in vitro (grey bars), embryos obtained at 45 h (open bars) and 100 h po (striped bars), from FSH-stimulated cows and cultured in vitro until day 7 and expanded blastocysts from FSH-stimulated cows, developed entirely in vivo (black bars). Bars with different superscripts differ significantly (a:b:c, $P \leq 0.05$).

Notes

EFFECT OF SOMATOTROPIN ON DEVELOPMENT CAPACITY OF IN VITRO MATURED BOVINE OOCYTE FROM ANIMALS OF DIFFERENT AGE

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The development potential of bovine oocytes has been related to many factors: the morphology of cumulus-oocyte complexes (COC), origin of COC, culture system etc. Recently we showed that the addition of recombinant bovine somatotropin (rbGH-"Monsanto") during maturation of bovine oocytes decreased level of pyknotic cells in cumulus (Kuzmina et al.,1999).

The objective of the present study was to compare the developmental potential of in vitro matured bovine oocytes depending on animal age and culture system. We used COC from cows (5-6 lactations), heifers (1.5-2 years) and calves (1-2-months old). Three systems were used for culture of COC: TCM 199 (Sigma) with 20% steer serum (control); TCM 199 with 20% steer serum and 5 ng/ml rbGH; TCM 199 with 20% steer serum, 5 ng/ml rbGH and 1×10^6 granulosa cells.

A total of 1552 COC were cultured. There was no difference in the rate of oocyte maturation or in percentages of oocytes with chromosome degeneration depending on culture system and age of the animals. rbGH supplemented to maturation medium in the presence of granulosa cells enhanced the proportion of cleaved embryos in all groups of experiments. In accordance with these results, bovine follicle granulosa cells appear to be involved in the realization of the effect of somatotropin on cytoplasmic maturation thus influencing the in vitro developmental competence of oocyte. The results of the present study demonstrate a beneficial effect of adding rbGH and follicle somatic cells (granulosa cells) during in vitro maturation of oocytes from prepubertal animals on subsequent embryo development to the blastocyst stage after IVF.

Table 1. Influence of bovine somatotropin on developmental capacity of in vitro matured bovine oocytes obtained from animals of different ages.

Age of animal	Culture system	In vitro maturation		Embryo development		
		No. of oocytes	Matured % (n)	No. of oocytes	Cleaved % (n)	Mor/Bla., D 7 % (n)
calves	control	60	86.7 (52)	98	32.7 (32)[a]	18.4 (18)[l]
(1-2	1	71	85.9 (61)	91	48.4 (44)[b]	30.8 (28)
months)	2	75	86.7 (65)	87	58.6 (51)[c]	41.4 (36)[k]
cows	control	77	87.0 (67)	101	46.5 (47)[d]	27.7 (28)
(5-6	1	80	87.5 (70)	99	58.5 (58)	35.4 (35)
lactations)	2	75	86.7 (65)	98	64.3 (63)[e]	39.7 (39)
heifers	control	78	84.6 (66)	104	46.1 (48)[f]	29.8 (31)
(1.5-2	1	81	86.4 (70)	101	54.5 (55)	35.6 (36)
years)	2	77	85.7 (66)	99	61.6 (61)[g]	40.4 (40)

[a,b] $P<0.05$; [a,c] $P<0.001$; [d,e] $P<0.05$; [f,g] $P<0.05$, [f,k] $P<0.01$; Chi^2 - test

Notes

THE INFLUENCE OF STAINING WITH NILE RED AND HOECHST ON SUBSEQUENT DEVELOPMENTAL CAPACITIES OF BOVINE ZYGOTES IN VITRO.

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The use of morphological characteristics for the assessment of oocyte and embryo quality as a sole parameter has been under considerable pressure over the past few years. Pregnancies obtained with blastocysts that were initially graded as poor quality on a morphological basis have been reported. In addition, several reports illustrate the variability among assessors when it comes to morphological embryo quality assessment. Therefore, there is a need for more objective non-invasive techniques to evaluate quality of oocytes, zygotes and embryos. A possible contribution to quality assessment can be expected from the use of 'in life' supravital staining techniques such as the DNA specific Hoechst and Nile Red that can be used for the assessment of the lipid content in oocytes and embryos. However, little is known about the possible toxic influences of the use of those supravital dyes on embryo developmental capacity. The aim of this study was to assess the consequences of the use of fluorescent supravital staining with Hoechst and Nile Red and the impact of fluorescent light on the developmental capacity of bovine zygotes grown in vitro.

Ovaries were collected at the slaughterhouse and follicles with a diameter between 2 and 8 mm were punctured. Grade I cumulus oocyte complexes (COC) were matured in maturation medium (TCM199, 20% fetal calf serum, glutamine 0.4 mM, pyruvate 0.2 mM and gentamycin 50 _g/ml) for 24 h at 39°C in a 5% CO_2 atmosphere. A total of 400 COCs with an intact, non-expanded cumulus was used (2 replicates). In vitro fertilization took place using a standard fertilization protocol (Van Soom *et al.*, 1996). Following fertilization, 4 groups of presumptive zygotes were selected. One group (Group A) served as a negative control group, being processed for culture in a routine in vitro embryo production set up using SOF medium. Groups B and C were transferred to prepared solutions of the supravital fluorescent staining agents Nile Red (10_g/ml NaCl 0.9%) and Hoechst (10 _l/ml NaCl 0.9%) respectively. The final group (D) was placed in 2.5 ml NaCl 0.9% to serve as a positive control group, in the absence of staining agents. Wells containing groups B, C and D were kept on a stage-warmer for 10 minutes in the absence of light. Subsequently, all zygotes were recovered from the staining solutions and the PBS and transferred to washing medium. All wells were exposed to UV light during 20 seconds and examined using the corresponding filters. Zygotes in the positive control group (D) were examined for 10 seconds with both filters. Finally, all groups of zygotes were processed in a routine in vitro embryo production unit (see above) in SOF medium while their in vitro growth and development was assessed after 48 h, with special attention to the percentage of cleaved embryos. The number of blastocysts in the different groups was recorded on Day 7. Results are shown in Table 1.

Table 1. The percentage of cleaved zygotes (48 h) and developing blastocyst (Day 7) following exposure to fluorescent supravital staining agents Hoechst and Nile Red.

Treatment	Cleavage at 48 h (%)	Blastocysts at Day 7 (%)
Negative control	69[a]	26[a]
Positive control (UV-light)	63[a,b]	26[a]
Nile Red (UV-light)	50[b]	2[b]
Hoechst (UV-light)	56[b]	2[b]

Percentages with a different superscript are significantly different ($P < 0.05$)

Results clearly indicate that staining of bovine zygotes with Nile Red or Hoechst has a detrimental effect on first cleavage and blastocyst production. UV-light exposure, without staining apparently has no negative impact. We can conclude that it is not possible to use zygotes that were stained with this concentration of Nile Red and Hoechst, for subsequent embryo culture. Further research is needed to investigate the effect of other concentrations of the dye.

Notes

A COMPARISON OF THE LIPID CONTENT OF IMMATURE AND MATURE BOVINE OOCYTES AND OF MORULAE AFTER STAINING WITH NILE RED

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Previous studies have extensively investigated the effect of in vitro culture media on the lipid contents of bovine oocytes and embryos. However, little is known about the morphological aspects of the ooplasm of immature oocytes in relation to their lipid content. Recently we have developed a method to visualize and quantify the differences in lipid content in a single oocyte or embryo by means of a fluorescent dye and a photometer. The aim of this study was to use this method to investigate if immature oocytes with a dark ooplasm contain more lipids than oocytes with a pale ooplasm and to compare the lipid content of mature oocytes and morulae (Day 6) generated either in serum-supplemented or in serum-free media during in vitro maturation or in vitro culture, respectively.

Ovaries were collected at the slaughterhouse and follicles (2-8 mm in diameter) were punctured. Only cumulus oocyte complexes (COC) with an intact, non-expanded cumulus investment were used. After washing, COC were vortexed (12 min) to remove the cumulus cells. The cumulus free, immature oocytes were then divided into three groups according to the appearance of their ooplasm (binocular stereomicroscope, 40x): dark, intermediate and pale oocytes. New COC were matured for 24 h at 39°C (5% CO_2) in either serum-supplemented (TCM199, 20% FCS) or serum-free (TCM199, EGF 10ng/ml) maturation medium. Following maturation, some of the COC were vortexed (5 min) to remove the cumulus cells. The remaining serum-matured COC were fertilized following a routine in vitro fertilisation for embryo production purposes. After vortexing (2 min), zygotes were cultured for 5 days in groups of 25 in 50 _l culture drops of SOF with (5% FCS) or without (0.3% BSA) FCS under mineral oil (5% CO_2, 5% O_2, 90% N_2). Only grade I morulae (D6) of both groups were used for further analysis. The immature oocytes (dark, intermediate and pale), the mature oocytes (matured either in the presence or the absence of FCS) and the morulae (cultured in SOF with or without FCS) were fixed in 2% paraformaldehyde and 2% glutaraldehyde in PBS (4°C, >24h). Oocytes and morulae were then stained overnight in Nile Red (10 _g/ml NaCl 0.9%), a fluorescent dye for the detection of intracellular lipid droplets by fluorescence microscopy. Oocytes and morulae were put individually in 25 _l drops of the Nile Red solution in a well of a 384-well plate. A photometer attached to a fluorescence microscope was used to measure the emitted fluorescence from the whole oocyte or embryo (excitation wave length: 500 nm; emission wavelength: 582 nm). A magnification of 100x was used, focused on the entire zona pellucida.

Fluorescence is proportional to the lipid content of droplets in the oocytes or morulae. Dark oocytes emitted a significantly higher amount of fluorescent light and thus contain more lipids than intermediate and pale oocytes ($P < 0.05$). Oocytes matured in the presence of serum did not have a higher lipid content than oocytes matured without serum, partially due to the large variation between oocytes in both groups. This finding is in contrast with the results of Kim *et al*. (Reproduction 2001; 122:131-138). Morulae (Day 6 post insemination) that were cultured in SOF with FCS, contained significantly more lipid droplets than morulae cultured in serum-free conditions. Our findings confirm the results of Abe *et al*. (Mol Reprod Dev 1999; 61:57-66) and Ferguson and Leese (J Reprod Fertil 1999; 37:189-219). This lipid accumulation, caused by the added serum, is suggested to be responsible for the reduced cryotolerance of embryos grown in the presence of serum.

In conclusion, this study illustrates that Nile Red staining followed by the quantification of the emitted fluorescent light with a photometer is suitable for the visualization and comparison of the intracellular lipid contents in single bovine oocytes or embryos. Our study furthermore confirms that dark immature oocytes contain more lipid than oocytes with a pale apprearance of the ooplasm and that the addition of serum to the maturation medium does not cause an increase of the lipid content. Morulae cultured in the presence of serum do accumulate lipid droplets and hence contain more lipids than embryos cultured in serum-free conditions.

Notes

ASSESSMENT OF NUMBER OF OOCYTES RETRIEVED FROM INDIVIDUAL COWS CAN BE BASED ON FEW COLLECTIONS

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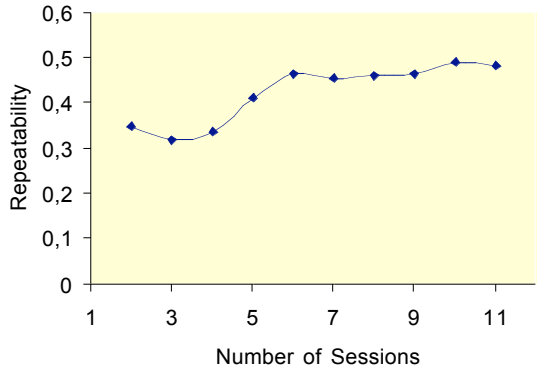
Donor cows used for Ovum-Pick-Up vary considerably in the number of oocytes recovered. However, there seems to be a certain oocyte number that is characteristic for each donor cow. This number has previously been suggested as something that can be predicted after rather few collections (Kruip et al., 1994, Proc. Eur. Conf. Embryo Techn. & Genet. Engin. in Cattle & Sheep, Kraków, Poland,1:117-126), but no more detailed statistical evaluation have since been presented.

The objective of this study was to estimate the degree of repeatability of ultrasound-guided follicular aspiration (ovum pick up, OPU) and from that calculate the number of aspirations (OPU sessions) needed to describe the individual cow's response.

On one private dairy farm, 49 heifers and cows of two breeds (Red and White Danish or Holstein-Friesian) were subjected to twice weekly transvaginal follicular aspiration performed by the same operator and technician over a total of 74 sessions. All animals were subjected to a pre-collection training session followed by 9 to 11 aspirations. A total of 1666 oocytes were recovered, with an average of 4.6 ± 3.2 oocytes recovered per session per animal.

The number of oocytes was log-e transformed, in order to obtain an approximate normal distribution, and were subsequently analysed statistically using a mixed linear model (Mixed Procedure, SAS Institute Inc.) including breed, parity and session as systematic (fixed) factors. The random part of the model contained animal within breed and parity, and date of collection. The repeatability was estimated from the random variance components as the ratio of animal to total random variance. The mean standard error of prediction for animal was estimated for increasing number of oocyte collection sessions.

A 1.5 fold higher number of oocytes were collected from the Red and White Danish as compared to the Holstein-Friesian animals ($P<0.05$). Parity and session number did not affect oocyte number. The repeatability increased from about 0.3 to 0.5 with increasing number of collections (Figure 1). The standard error of prediction of individual animal oocyte retrieval decreased from 0.33 at a single collection, asymptotically towards 0.18 with advancing number of collections (Figure 2).



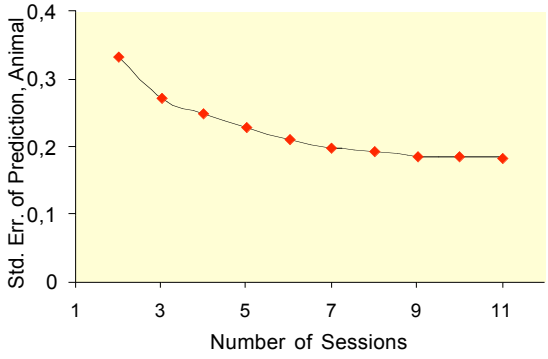


Figure 1 (left): Effect of number of collections (sessions) on the repeatability.
Figure 2 (right): Effect of repeated collections (sessions) on the standard error of prediction.

It is concluded, that oocyte retrieval is a repeatable trait in cows and heifers, and because the standard error of prediction decreases only a little when more than four collections are performed, a small number of collections are sufficient for accurate assessment of individuals for this trait.

Notes

OESTRUS BEHAVIOR AND OVULATION RATE OF WHITE MORADA NOVA SHEEP USED IN A MOET PROGRAM

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Sheep explored in Northeastern Brazil represents an important economical source for farmers, being explored mainly for the meat and skin production. Thus, several breeds of sheep raised in Northeastern of Brazil have been used in crossbreeding programs with sheep of specialized breeds in order to increase meat production. However, local breeds of sheep such as White Morada Nova have been characterized as endangered. Therefore, it is necessary to maintain and increase the number of White Morada Nova sheep and the best method to multiply sheep at a faster rate is through Multiple Ovulation and Embryo Transfer (MOET). However, it is necessary to know the oestrus behavior and ovulatory activity of White Morada Nova prior to the application of MOET in this local sheep breed. Thus, the aim of this study was to evaluate the oestrus behavior and ovulation rate in White Morada Nova ewes. The experiment was carried out in the Laboratory of Physiology and Control of Reproduction, in Fortaleza, which is located at 3°43'47'' South and 38°30'37'' West. Five White Morada Nova ewes aged (mean ± SD) 2.4 ± 1.3 years and weighing (mean ± SD) 28.2 ± 5.9 kg at the start of study were used. They were kept in a straw-bedded pen throughout the study. The ewes received elephant grass (Pennisetum purpureum) ad libitum and were supplemented with a commercial concentrate with 18% crude protein. They had free access to water and to a mineral salt. During two months, oestrus behavior of ewes was monitored twice daily (8:00 a.m. and 4:00 p.m.) using a ram with an apron. Immobilization of the female when mounted by the male was considered to be a sign of occurrence of estrus. The occurrence and the length of oestrus were recorded for each ewe. Seven days after estrus, the ewes were submitted to laparoscopy in order to record the ovulation rate. It was observed 100.0% (5/5) of ewes in oestrus and the mean (± SD) oestrus length was 36.0 ± 12.0 h. In addition, all oestrus behavior was followed by at least one ovulation. The mean (± SD) ovulation rate was 1.6 ± 0.6. From the results of this assay it can be concluded that White Morada Nova ewes exhibit estrus associated with ovulation similar to other sheep breeds raised in Northeastern of Brazil. Thus, sheep-breeding programs in Brazil under intensive management could benefit from more extensive use of White Morada Nova sheep due to normal oestrus and ovulatory activities. However, additional studies that evaluate other steps of MOET procedures to be applied in this local sheep breed are necessary.

Notes

INFLUENCE OF MILK PARAMETERS ON EMBRYO PRODUCTION FOLLOWING SUPEROVULATION IN THE MONTBELIARD BREED

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The aim of this study was to investigate in lactating Montbeliard donor cows the influence of milk parameters on embryo production after superovulation. Embryo production results following 309 flushes performed between January 1999 and February 2002 were studied. To avoid bias on milk parameters interpretation, cows were selected to be collected during their 2nd to 7th lactation and before 150 days after calving. All cows were superovulated with a total dose of 500 µg of pFSH (8 injections i.m. during 4 days ; decreasing doses ; STIMUFOL ND, Merial, Fr) together with a prostaglandin injection at time of the 5th FSH injection. AI were performed 12 and 24 hrs after standing oestrus. Embryos were collected on Day 7 and their viability estimated according to IETS morphological criteria. Milk production Fat (FC) and protein (PC) contents were recorded at all milk controls preceding embryo collection. Other factors, such as paternal origin of the donor, rank of lactation, time interval between calving and flushing and sire of AI were recorded together with herd factors such as housing and feeding type. The influence of the potential variation factors was assessed by ANOVA (SAS, MIXED procedure). Results are presented as means ± s.d..

The mean numbers of total, transferable, grade 1, degenerated embryos and unfertilized oocytes were respectively of 9.3±6.2, 5.6±5.0, 2.8±3.8, 2.4±3.0 and 1.3±2.7. On the 309 cows, 192 were collected after the third control postpartum. Protein and fat contents decreased between the first and the 2nd control (realized respectively 22.5 ±10 and 54 ± 12 days after calving). High PC at the 2nd and/or at the last control before collection and shorter calving to collection intervals were associated with better embryo production results (P<0,05 ; Table 1). The proportion of collection without any viable embryo was lower when FC was close or lower than PC (FC-PC ≤ 2 : 7,1% vs FC-PC > 2 : 16.3% ; P=0.05). It has been reported that PC is related to energy supply, whereas FC is influenced by type of feeding, and decreases with high quantity of concentrates. It can be hypothesized that donor cows with high milk PC before collection and/or in which PC and FC are clos to each other are more likely to be fed with high energy levels. This could stimulate embryo production and quality during the first months of lactation.

Table 1 : Mean numbers of total (TOT) and viable (VIA) embryos collected according to calving to collection interval and protein content ad last control before collection

Calving to collection interval (days)	Protein content at last control				TOTAL	
	≤ 32 g/kg		>32 g/kg			
	TOT	*VIA*	TOT	*VIA*	TOT	*VIA*
≤ 90	9.0±6.1	*5.3±4.3*	12.2±7.1	*7.9±5.4*	9.9±6.5[a]	*6.0±4.8[c]*
> 90	8.5±4.3	*5.0±4.2*	8.9±7.6	*5.8±6.3*	8.7±5.8[b]	*5.3±5.1[d]*
TOTAL	8.8±5.4[a]	*5.1±4.2[c]*	10.3±7.6[b]	*6.6±6.0[d]*	a vs b, c vs d, P<0,05	

These results underline the relationship between energy status and embryo production results in lactating cows. Protein content at the last control before collection was related to embryo production results. Taking into account simultaneously the fat content may help to predict the response of donor cows. However, the high variability of fat content from one control to another may limit the usefulness of this measurement.

Notes

SEX DETERMINATION OF OVINE EMBRYOS (*Ovis aries*) BY DUPLEX POLYMERASE CHAIN REACTION.

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We developed a duplex polymerase chain reaction (PCR)-based method for accurate sex determination of preimplantation or frozen ovine embryos.

Ovine oocytes collected from slaughtered animals were matured, fertilized and cultured in vitro for 7 days. On Day 7, embryos were evaluated and submitted for biopsy. The 5-10 biopsied cells were immediately transferred into PCR-tubes containing lysis buffer (PCR buffer 1X, $MgCl_2$ 1.5 mM and proteinase K 0.15 μg/μl). The PCR utilizes two different sets of primers: the first pair recognizes bovine Y chromosome-specific sequences (100% homology with the correspondant sequences of ovine Y chromosome) that are amplified in males only; the second pair recognizes the bovine specific satellite sequence, but we could utilize it in the ovine embryos because the conservation of DNA satellite regions among mammalian genomes allows the use of heterologous primers in closely related species, such as cattle and sheep. Furthermore, we sequenced the corresponding band, finding 100% homology with the bovine specific satellite sequence.

The trial was carried out in two steps: in the first one, 146 samples of somatic cells (59 composed of oviductal sheep cells used as female samples and 75 lamb fibroblasts used as male samples), were divided in to 3 groups for each sex according to the number of cells (2, 5, 30, respectively). These were amplified, with 91.8% efficiency. The second step was carried out on 21 embryos at the morula or blastocyst stage. The biopsied cells (from 5 to 10) were immediately lysed and amplified as described above, with 100% efficiency. The 21 embryos were transferred to 11 recipient ewes and 8 lambs were born. The sex, as determined by PCR, corresponded to the anatomical sex in 7/8 cases (87.5% accuracy), showing that the assay is sufficiently accurate.

Notes

STUDY OF CYTOPLASMIC MATURATION OF BOVINE OOCYTES: INVESTIGATION OF REGULATION OF TRANSLATION DURING MEIOTIC MATURATION

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The objective of this study was to investigate the cytoplasmic processes of oocyte maturation. This approach can contribute to an improvement of in vitro maturation systems and can be important to obtain better yields and quality of in vitro produced embryos.

From previous studies it is known that gene expression during meiotic maturation (transition from prophase I to metaphase II) of bovine oocytes is regulated mainly at the level of translation. The rate limiting step in overall protein synthesis is the initiation phase. Therefore, the activity (phosphorylation state) of translational initiation factor eIF4E and the potential repressor of eIF4E function, the binding protein 4E-BP1, as well as signalling cascades for their phosphorylation were analysed. EIF4E is part of the factor eIF4F which enhances cap-dependent translation by stimulating the binding of the small ribosomal subunit to the mRNA. 4E-BP1 prevents the formation of the eIF4F and therefore inhibits translation.

Bovine oocytes were maturated for different times (0, 6, 10, 14, 20, 24 hrs) and analysed for the phosphorylation status of specific M-phase kinases (MAPK and cdc2K) and the kinase Akt by SDS-PAGE and Western-Blotting or by an in vitro kinase assay. The phosphorylation of eIF4E and 4E-BP1 was investigated by isoelectric focusing and Western-Blotting. The influence of the inhibition of cdc2K or MAPK pathways on eIF4E phosphorylation was elucidated by specific inhibitors PD 098059 and Butyrolactone I (BL I). The abundance of c-mos mRNA was analysed by RT-PCR.

The results show that eIF4E phosphorylation occurred strictly parallel with the activation of MAPK and cdc2K. It started at the time of GVBD and in M II all factors were fully phosphorylated. Maturation in the presence of BL I or PD 098059 showed that MAPK pathway rather than cdc2K is responsible for eIF4E phosphorylation. It seams that during meiotic maturation of bovine oocytes the activated c-Mos not only can upregulate MAPK via MEK but probably also through another still unknown kinase.

On the other hand, first results indicate that the kinases, Akt and cdc2K, are involved in phosphorylating 4E-BP1 on different sites. It can be noticed that the interplay between this three kinase pathways is responsible for the precise regulation of cap-dependent translation.

In conclusion, our results indicate that phosphorylation of eIF4E at the time of GVBD through the MAPK pathway is responsible for enhanced translation rates at this time of development. Furthermore, the results indicate that 4E-BP1 can be involved in repression of translation in metaphase II. The analysis of the sites of 4E-BP1, which are phosphorylated by Akt or cdc2K pathways and the precise timing, remains to be investigated.

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Notes

LONG TERM EFFECT OF MOUSE EMBRYO IN VITRO CULTURE IN THE PRESENCE OF SERUM ON IMPRINTING, DEVELOPMENT AND BEHAVIOUR

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The long-term developmental and behavioural consequences of mammalian embryo culture are unknown. In order to better understand this relationship, epigenetic modifications at blastocyst stage, foetal development, growth and behaviour of adult mice were evaluated after inducing suboptimal in vitro embryo culture conditions with an excess of foetal calf serum. One-cell embryos obtained from superovulated B6CBAF1 female mice were cultured for 4 days in KSOM in the presence of 10% fetal calf serum (Group A), or in presence of 1 g/L BSA (Control Group). Messenger RNA was isolated from embryos reaching blastocyst stage (95% in group A and 35% in the Control Group) and the expression of the imprinted genes H19, IGFII, Mest/PegI, and Grb10/Meg1 was measured by quantitative Real Time PCR. Embryos reaching this developmental stage were also transferred to pseudopregnant CD1 females. Embryo transfer generated 43 group A-derived mice (24 males and 19 females), and 35 control group-derived mice (13 males and 12 females). Both groups were submitted to the following developmental and behavioural tests: somatometric, preweaning developmental test (Fox battery), neuromotor developmental profile (neurologic, sensory, and motor test), locomotion activity on week 20 and 65 (open field), and exploratory/anxiety behaviour (elevated plus maze, and open field). At two years old, animals were sacrificed and were histologically analyzed. The mRNA expression of imprinted genes was significantly affected in blastocysts cultured in the presence of serum. Two of the 8 measures of preweaning development and some specific measures in neuromotor development, such as walking activity, were observed later in Group A, indicating a delayed psychomotor development and a hypoactive behaviour. Thirty-four weeks after birth, postnatal weight gain of Group A female mice was significantly higher than control counterparts. No significant differences were observed between groups in the elevated plus maze test or in the open field test at 20 weeks of age. In the open field test, however, motor activity of 64-week-old females from Group A was higher than in control females and males produced in the presence of serum. We found that mice derived from cultured embryos with FCS exhibit specific anxiety alterations and display deficiencies in spatial memory. These data suggest that culture of preimplantation mouse embryos in presence of serum influences the regulation of growth-related imprinted genes, delays the appearance of some developmental milestones, and leads to aberrant growth and behaviour. Our results indicate that long-term programming of postnatal development, growth and physiology can be induced irreversibly during the preimplantation period of development by in vitro culture in the presence of serum.

Notes

MOUSE ICSI WITH FROZEN-THAWED SPERM: IMPACT OF SPERM FREEZING PROCEDURE AND SPERM DONOR STRAIN

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Normal mouse offspring can be obtained from oocytes injected with frozen-thawed spermatozoa without cryoprotection. However, embryo development can be affected by the sperm freezing procedure and gamete donor strain. In this study, we observed that direct contact of mouse spermatozoa with liquid nitrogen does not affect the ability to activate injected oocytes but severely restricts subsequent embryo development. Tris-EDTA buffer and M2 medium were also shown to be better sperm freezing extenders than DPBS. In addition, differences in embryo development after ICSI with frozen-thawed spermatozoa were observed between hybrid sperm donor strains. In order to test the effect of sperm contact with liquid nitrogen, M2-diluted frozen-thawed C57CBA sperm heads with and without direct contact with liquid nitrogen, were injected into C57CBA oocytes and in vitro embryo development compared. In all cases, statistical analysis was done with SigmaStat (Jandel Scientific, San Rafael, CA) Software package. In both cases, most oocytes (89 and 94%) were activated and fertilized normally having extruded a second polar body 2-3 h after injection and formed two pronuclei 3-4 h later regardless of the type of sperm contact with liquid nitrogen. Developmental progress to 2-cell, 4-cell, morula/early blastocyst and expanded blastocyst stages was, however, significantly compromised when fertilization was performed with sperm heads that directly contacted liquid nitrogen (18% vs. 35% surviving oocytes reached expanded blastocyst stage).

While testing the effect of the sperm freezing media, B6D2F1 oocytes were injected with B6D2F1 sperm cryopreserved in DPBS, Tris-EDTA DNA microinjection buffer or M2 medium. The 58% and 53% embryo development to expanded blastocyst stage obtained, respectively, with sperm cryopreserved in Tris-EDTA and M2 medium, were both significantly higher than the 36% obtained with sperm cryopreserved in DPBS.

Finally, the effect of oocyte and/or sperm donor strain was evaluated by comparing in vitro embryo development obtained with the gametes of two hybrid mouse strains, C57CBAF1 and B6D2F1, in a two by two factorial design. For this experiment sperm samples were frozen in Tris-EDTA buffer. In our experimental results we could not see an oocyte donor strain effect on the efficiency of the ICSI technique employed. They were rather influenced by the type of fertilizing spermatozoa. While only 35% of the C57CBA and 30% of the B6D2F1 oocytes fertilized with frozen-thawed C57CBA spermatozoa developed to the expanded blastocyst stage, 56% and 54% were the respective percentages when the fertilizing male gametes were frozen-thawed B6D2F1 spermatozoa. A possible homogametic developmental advantage (either C57CBA or B6D2F1) over a heterogametic one was also not visible during in vitro embryo development.

Notes

THE EFFECT OF HARVESTING TECHNIQUE ON EFFICIENCY OF OOCYTE COLLECTION AND THEIR MEIOTIC COMPETENCE IN DROMEDARY CAMELS

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The purpose of this investigation was to develop an efficient method for harvesting oocytes from dromedary camel ovaries and to study its effect on their subsequent maturation in vitro. Ovaries were collected from an abattoir and transported to the laboratory in 0.9% NaCl solution at 35 to 39°C. Oocytes/follicles were collected within 4-5 h after ovarian collection in PBS with 2% fetal calf serum, using three different harvesting techniques. Oocytes were collected by aspirating the follicular contents with an 18-G needle attached to a disposable syringe (Method I, n = 163 ovaries) or to a constant aspirating pressure (100 mm Hg) applied by a vacuum pump (Cook Aust, Brisbane, Australia) which was connected through a PVC tubing to a 50-ml plastic Falcon tube (Method II, n = 117 ovaries). Individual follicles were excised from ovaries with the help of scissors and bone curvets and the follicles were dissected with two needles while observing under a stereomicroscope (Method III, n = 117). Degenerated oocytes were discarded and the cumulus enclosed oocytes (CEOs) and partially or completely denuded oocytes (CDOs) were washed in maturation medium of TCM 199 supplemented with 0.33 mM pyruvate, 10 _g/ml FSH and LH, 1 _g/ml oestradiol and 10% oestrus camel serum. Oocytes were then transferred to 4-well dishes containing 0.4 ml/well of maturation medium and incubated at 38°C and 5% CO_2 in air for 40 h. At the end of maturation period, oocytes were removed denuded of cumulus cells mechanically as well as by treatment with 1 mg/ml hyaluronidase and the proportion of oocytes at the metaphase II (MII) stage was determined.

The recovery rate of oocytes was higher ($P < 0.01$) with Method III compared with Method I and II (482/513 (94%) vs 341/1041 (31%) and 249/807 (33%), respectively). A higher proportion of oocytes collected with Method I or II were either completely or partially denuded compared with Method III (31, 15% vs 1%). The proportion of viable oocytes after IVM was 266 (78%), 145 (60%) and 334 (70%) and those achieving MII stage was 103 (39%), 72 (50 %) and 154 (49%) of the viable oocytes collected by Method I, II and III, respectively. The number of oocytes/ovary attaining maturation (MII-stage) was higher when the oocytes were harvested by the follicle dissection method compared with aspiration with syringe or constant vacuum pump (1.31, vs 0.63, 0.62).

It can be concluded that a higher proportion of CEOs may be recovered by follicle dissection compared to follicle aspiration using either a needle attached to a syringe or a vacuum pressure pump. The higher recovery rate, with a comparable proportion of viable and matured oocytes, results in an overall increase in the number of matured (MII) oocytes/ovary using follicle dissection compared with aspiration procedures.

Notes

DEVELOPMENTAL COMPETENCE OF OOCYTES FROM HOLSTEIN-FRIESIAN NULLIPAROUS HEIFERS AND LACTATING COWS IN THE EARLY POST PARTUM PERIOD

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Increased genetic capability for milk production coupled with changes in nutritional management and larger herd size have been associated with the decline in fertility of lactating cows (Butler, 1998, J Dairy Sci, 81:2533-2539). Nutritional requirements shift abruptly after parturition as milk production rapidly increases and the ensuing negative energy balance lasts 4-10 weeks, which may coincide with the period when the animal is being rebred.

The aim of this study was to compare nulliparous Holstein-Friesian heifers and cows in the early post partum period in terms of the number and size of follicles available for aspiration, the morphology of the oocytes recovered and their subsequent developmental competence after in vitro maturation, fertilization and culture.

Post pubertal Holstein-Friesian heifers (n=10) and lactating cows (n=10) were used. All animals were kept indoors for the duration of the experiment. Heifers were fed ad lib grass silage, supplemented with 50 kg of beet pulp nuts per tonne of silage at ensiling. Cows were fed with 8 kg concentrates and ad lib silage (50% maize silage, supplemented with 3 kg maize protein balancer per head at feeding and 50% grass silage).

Oocytes were collected by ovum pick-up (OPU) twice weekly for 5 weeks (for the cows, beginning on Day 43±17 post partum, Mean±SD). The recovered oocytes were graded (Grade 1 to 4) in terms of their surrounding cumulus cells and the appearance of the cytoplasm. Grade 1-2 oocytes were matured in vitro and then fertilized using frozen-thawed bull semen and subsequently cultured up to Day 8 in synthetic oviduct fluid supplemented with 10 % fetal calf serum.

The follicle size distribution was similar between the groups (Table 1). The number of follicles punctured, the number of oocytes recovered and the number of Grade 1-2 oocytes per animal were significantly lower in cows than in heifers (Table 1). However, the number of Grade 1-2 oocytes as a percentage of total oocytes was similar between the two groups (66.7% for heifers and 67.8% for cows). There was no difference in the cleavage rate or blastocyst yield between the groups (50.6% vs 45.7% and 6.9% vs 12.4% for cows and heifers, respectively).

In conclusion, these results indicate that during the early post partum period, lactating Holstein-Friesian cows submitted to OPU have fewer follicles available for aspiration and therefore yield less good quality oocytes than non-lactating heifers; however, the developmental competence of the recovered oocytes does not differ between the groups.

Table 1. Size distribution of follicles punctured by OPU and number and grade of oocytes recovered per animal.

Group	2-4 mm n (%)	5-10 mm n (%)	>10 mm n (%)	Follicles punctured/animal n ± SE	Oocytes recovered/animal n ± SE	Grade 1-2 oocytes n ± SE	(%)[1]
Heifers	583 (56.1)	422 (40.6)	35 (3.4)	10.4 ± 0.6[a]	4.5 ± 0.2[a]	3.0 ± 0.2[a]	(66.7)
Cows	384 (52.7)	279 (42.3)	33 (5.0)	7.6 ± 0.4[b]	2.8 ± 0.1[b]	1.9 ± 0.1[b]	(67.8)

[1] % of Grade 1-2 oocytes out of the total number of oocytes recovered.
[a,b]Values in the same column with different superscript differ significantly (P<0.01).

Notes

COMPARISON OF THREE METHODS FOR PARTHENOGENETIC ACTIVATION OF BOVINE OOCYTES AND THEIR USE IN A SOMATIC CELL CLONING PROGRAM

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In the process of nuclear transfer, oocyte activation is a crucial step. Somatic cells enter interphase immediately after mitosis, in contrast to oocytes, which are arrested at metaphase of the second meiotic division. Rescue from this state is achieved by activation of the oocyte. Best results are obtained when the activation agents invoke a rise in intracellular calcium, followed by a transient inhibition of the general phosphorylation status of intracellular proteins, or even better, a specific inhibition of the synthesis or phosphorylation of proteins directly involved in the cell cycle.

In this work, we compared the parthenogenetic activation of bovine oocytes using protocols that involve the combination of a calcium riser agent, with phosphorylation or protein synthesis inhibitors. Bovine oocytes were obtained from ovaries of slaughtered cows and in vitro matured. Activation was performed by one of the following treatments: 1) incubation for 5 min in 5 µM ionomycin, followed by 2 h incubation in 1.9 mM 6-dimethyl aminopurine (6-DMAP), 2) incubation for 5 min in 7% absolute ethanol, followed by 5 h incubation in 10 µg/ml of cycloheximide (CHX) and 3) a single direct electric current pulse (2.0 Kv/cm for 30 µsec) followed by 5 h incubation in 10 µg/ml CHX. For somatic cell cloning, we used either method 2 or 3. When an electric pulse was used as calcium trigger, cytoplast were fused to somatic cells and activated with the same pulse. For nuclear transfer, (NT) we used skin fibroblast from an adult elite Cuban Siboney cow. All embryos were cultured in CR1aa supplemented with 0.3% bovine serum albumin, essentially fatty acid free, 2% fetal calf serum and 12.5 µm of ß-mercapthoethanol in at atmosphere of 5% CO_2 in air at 39^0C.

The percentage cleavage and blastocyst rate for each activation protocol was: protocol 1) 45.7±8.23 and 0, protocol 2) 83.4±10.02 and 6.07±1.34, protocol 3) 39.8±11.56 and 13.6±3.67. The data are the means and standard deviation of at least 5 replicates and the initial number of oocytes employed in the experiments was 319, 659 and 118 for protocols 1, 2 and 3 respectively. We further compared the efficiency of producing cloned blastocysts using protocols 2 and 3. The use of ethanol in combination with CHX yielded 9.14%±2.81 of transferable blastocysts out of 164 reconstructed and fused couplets. For electric pulse and CHX protocol, we obtained 19.3% ±5.23 respectively out of 114 fused couplets.

From our data, we concluded that, at least in our hands, the use of a protein phosphorylation inhibitor such as 6-DMAP was not effective for the induction of blastocyst development in parthenogenetically activated oocytes, while CHX either in combination with ethanol or electric pulse promoted higher rate of blastocysts development in both parthenogenetically activated and cloned embryos. The efficiency of nuclear transfer was lower than previously observed for this cell line. This is due to only high-quality oocytes selected from the ovaries of slaughtered cows, whereas in these experiments all available oocytes were matured and all oocytes extruding a polar body were enucleated and used for NT experiments, or parthenogenetically activated. The intrinsic quality of the oocytes, before maturation could also account for the variability observed in the cleavage rate among experimental groups.

Notes

COMPARISON OF TRANSRECTAL ULTRASOUND SCANNING, PROGESTERONE AND PREGNANCY-ASSOCIATED GLYCOPROTEIN ASSAYS IN PLASMA SAMPLES FOR EARLY PREGNANCY DIAGNOSIS IN THE GOAT

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Clinical methods and hormonal assays are available to diagnose pregnancy in goats at around 3 weeks after breeding. Real-time transrectal ultrasound scanning (US) provides an efficient tool for early pregnancy diagnosis in goats from approximately 23 d after mating (Martínez *et al.*, Theriogenology 1998;49:1555-65). Serum or milk progesterone (P4) levels have been widely used to detect pregnancy in goats at 21-22 d post breeding (Corteel et al., Proc. 3th Int Conf Goat Prod, Tucson, Arizona, USA 1982:584-601). The first report using specific antisera for detecting pregnancy-associated glycoproteins (PAG) allowed an accurate discrimination between pregnant and non-pregnant goats from 21 d after breeding (González *et al.*, Theriogenology 1999;52:717-25). Several studies have been realized to evaluate in separate each of these methods. Nevertheless, due to differences such as goat breed, age, etc., it is difficult to establish a suitable comparison. The aim of the present work was to compare the accuracy of the pregnancy diagnosis by transrectal US, P4 assay and PAG detection at the same time and in the same animals.

Dairy Canary goats (n=143) were synchronized with an 11-day fluorogestone-acetate (FGA) intravaginal sponge followed by $PGF_{2\alpha}$ and eCG 2 days before FGA withdrawal. Blood samples were collected at Days 20, 22, 24 and 26 after mating to determine by P4 RIA (Menchaca *et al.*, Theriogenology 2002;57:1411-19) and PAG (González *et al.*, 1999) concentrations. Transrectal US (Aloka SSD-500, 7.5 MHz) examinations were performed at the same time, and the goats were considered as pregnants when the embryo heartbeat could be detected (Martínez *et al.* 1998). To confirm the pregnancy status, transabdominal US (Aloka SSD-500, 5 MHz) was performed on Day 42.

There were 79 pregnant and 64 non-pregnant goats which was confirmed by parturition or abortion. Table 1 shows the results of the 3 methods of pregnancy diagnosis.

Table 1. Sensitivity, specificity and accuracy of each pregnancy diagnosis method.

Days after breeding	Sensitivity (%)			Specificity (%)			Accuracy (%)		
	US	P4	PAG	US	P4	PAG	US	P4	PAG
20	11.4	--	53.2	100	--	100	55.7	--	76.6
22	44.3	100	94.9	100	65.6	100	72.2	82.8	97.5
24	78.5	--	97.5	100	--	100	89.3	--	98.8
26	98.7	--	100	100	--	100	99.4	--	100

US=transrectal ultrasound scanning P4=progesterone PAG=pregnancy-associated glycoprotein

Transrectal US scanning and the determination of PAG concentrations, provide a very accurate pregnancy diagnosis at 26 and 24 days after breeding, respectively; on the contrary, P4 assay in plasma samples performed on Day 22 after breeding was accurate to detect pregnant animals, but not for detecting the non-pregnant ones.

Notes

VITRIFICATION OF *IN VITRO* MATURED PIG OOCYTES: ULTRASTRUCTURAL STUDY OF THE DISTRIBUTION OF CHROMOSOMES, MICROTUBULES AND ACTIN MICROFILAMENTS

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Experiments were carried out to evaluate the viability and ultra-structural changes of pig oocytes cryopreserved in Open Pulled Straws (OPS). Oocytes that had been allowed to mature (as described by Romar et al., 2003, Theriogenology 59:975-86) for 42 h were divided in two treatment groups and one control. Group 1, COCs were treated with Cytochalasin B and vitrified by stepwise exposure to EG (5, 10, 15, 20, 25, 30 and 40% for 5, 2, 2, 2, 1, 0.5 and 0.5 min, respectively), loaded into OPS, direct plunging in liquid nitrogen, rewarming for 3 s in air and removal of the cryoprotectant by exposure to a graded series of sucrose solutions (0.75, 0.5 and 0.25M for 5, 2 and 2 min, respectively). Group 2, COCs were exposed to Cytochalasin B and cryoprotectant agents (CPA) without any cooling. After treatment, the oocytes were cultured in *in vitro* maturation medium for an additional 2 h, fixed and evaluated using specific fluorescent probes before visualization using a confocal microscopy. Spindle structure was regarded as abnormal when there was partial or total disorganization or complete lack of microtubules. The chromosomal organization was abnormal when it showed chromosome dispersal, decondensation or missing. Abnormal distribution of actin microfilaments included disrupted, irregular or absence of cytoskeletal actin staining.

Table 1. Effects of CPA exposure and vitrification on spindle morphology (chromosome arrangement and microtubule distribution) of IVM pig oocytes

		Normal	Spindle morphology		Chromosomes		
	n	Spindle	Disorganized	Missing	Dispersed	Decondensed	Missing
Control	25	21 (84,0) [a]	1 (4,0)	2 (8,0)	1 (4,0)	0 (0)	3 (12,0)
CPA control	24	10 (41,7) [b]	8 (33.3)	6 (25,0)	6 (25,0)	1 (4,2)	7 (29,1)
OPS	18	5(27.8) [c]	7 (38.9)	5 (27.7)	6 (33,3)	0 (0)	7 (38,9)

Values in parentheses are percentages.
[a, b, c]: Values in a column with different letters are significantly different (P<0,05).

Table 2. Effects of CPA exposure and vitrification on the actin band of IVM pig oocytes

	N	Normal	Disrupted	Diffuse	Missing
Control	25	18 (72,0) [a]	7 (28,0)	0 (0)	0 (0)
CPA control	24	10 (41,7) [b]	9 (37,5)	3 (12,5)	2 (8,3)
OPS	18	6 (33.3) [b]	9 (50,0)	1 (5,6)	2(11,1)

Values in parentheses are percentages.
[a, b]: Values in a column with different letters are significantly different (P<0,05).

Freezing and CPA exposure had a significant deleterious effect on all cellular components examined. Statistically significant differences were observed on both microtubule and chromosome configuration of vitrified and CPA oocytes compared with control group. Freezing and CPA exposure had also a dramatic effect on cytoskeletal actin. The percentages of oocytes with normal actin was significantly lower after vitrification and CPA exposure compared to control oocytes. These results show that the protocol followed had a deleterious effect on the organization of the meiotic spindle and actin cytoskeletal of IVM matured pig oocytes.

Notes

FOLLICULAR DINAMICS, IN VIVO AND IN VITRO PRODUCTION OF WATER BUFFALO EMBRYOS IN THE CONDITIONS OF CUBA

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The buffalo population in Cuba has grown from 2,984 imported animals during the late eighties to more than 30,000 at present. Both water and swamp buffalo are currently used in the country, mainly for milk production, although the former is more suitable because of its better milk yield and reproductive performance. This population increase occurred despite the fact that only natural mating has been used for their reproduction and that no genetic selection is being carried out. The goal of this work was to apply modern technologies that can impact on the genetic improvement of the water buffalo at the expense of using swamp females as surrogate mothers for embryos.

We studied the follicular dynamics of 16 estrus cycles for each of 4 heifers with a body condition score of 4 in the 5-point scale used in the country. Daily transrectal ultrasound examination was conducted using an Aloka SSD-500 ultrasound device coupled to a convex 5 MHz transducer. Selected females n= 4 between 6 and 7 years old, were superovulated with 40 mg of FSH (Folltropin, Vetrepharm Inc, Ontario, Canada) administered in either a single subcutaneous dose, or in the traditional decreasing scheme as used in cattle superovulation. Superovulatory treatments began at the beginning of the second ovulatory wave. For in vitro maturation and fertilization (IVM/IVF), ovaries from 12 slaughtered buffalo cows were collected and their follicles punctured. The cumulus-oocyte complexes were in vitro matured for 22 h in TCM 199 supplemented with FSH, LH, beta estradiol and 10% fetal calf serum in at atmosphere of 5% CO_2 in air at 39^0C. Oocytes were in vitro fertilized with frozen/thawed semen at a concentration of 1 x 10^6 sperm/ml. Twenty-four h after IVF, embryos were cultured in CR1aa supplemented with 10% fetal calf serum under the same conditions.

We detected mainly two waves of follicular growth during the oestrous cycle of buffalo heifers, 67.8% of the analyzed estrus showed two waves of follicle growth, while the remaining 31.8% showed three waves. The beginning of the second wave ocurred in average 2.5 days later in two-wave cycles when compared to three-wave cycles ($P<0.05$) and the beginning of the third wave was scored at day 17 after ovulation. The results of follicular dynamics provided us with a tool for designing superovulatory regimes for buffalo.

An average of 9.5 embryos/female (range from 8 to 11) were obtained after superovulation treatment with a single dose of FSH injection, while 8.7 (average from 7 to 10) from the traditional protocol. The average of transferable embryos (M + B) per donor was 3.09 and 2.8 per female for the respective treatments. No significant different were found, but the single dose was more suitable from the practical print of view (less stress, easier handling and lower cost).

We also established an IVM/IVF protocol for water buffalo based on our experience with cattle oocytes. A total of 38 oocytes was colleted from 24 ovaries (1.6 per ovary), 9 (23.7%) cleaved and 3 (7.8%) reached blastocyst stage after 8d of in vitro culture. At the moment of writing this abstract, three of these embryos were transferred to naturally cycling swamp buffalo females. In conclusion, we set up a series of methodologies that could have an impact on a program for genetic improvement of buffalo livestock of Cuba.

Notes

VIROSOMES TRANSFER FOREIGN GENES INTO BOVINE SPERMATOZOA

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Objectives: Apart from pronuclear microinjection of transgenes the use of sperm cells as vehicles for foreign DNA during artificial fertilization is the objective of many efforts. Several approaches have been published to generate sperm cells bearing transgenes. Viruses are the most effective agents to transfer genetic material into living cells. Here we report on experiments designed to exploit the fusion potential of reconstituted influenza virus envelopes (virosomes) for the transfer of foreign DNA into bovine spermatozoa.

Methods: We developed a protocol for the preparation of virosomes bearing influenza X-31 hemagglutinin (HA) inserted into lipid bilayers composed of exogenous and virus lipids. In previous experiments, we optimized the ratio of exogenous sphingomyelin, phosphatidylcholine, cholesterol and phosphatidylethanolamine for virosome reconstitution, fusion extent and sperm cell vitality after fusion (Markgraf et al., Cloning 2001, 3,1: 11-21). A reporter gene plasmid coding for enhanced green fluorescent protein (pEGFP) was enclosed during virosome reconstitution. Virosomes were applied to bovine sperm cells after cryopreservation. Fusion was triggered in selected motile sperm cells by a transient drop of the pH to 5.0.

Results: The virosomes were characterized morphologically, biochemically and functionally. Electronmicroscopic studies showed closed multilayer vesicles with an inner diameter around 100 nm. Protein analyses revealed that influenza HA was reconstituted in the lipid layers to high yields. Quantitative PCR analysis after DNAse treatment demonstrated that up to 30% of the applied pEGFP was located inside the virosomes. After incubation of fluorescence labelled virosomes with cryopreserved "swim up" bull spermatozoa we assessed the extent of fusion by fluorescence microscopy. The results show that about 70-80% of the treated sperm cells remained vital and were fused with virosomes. Quantitative PCR analysis of sperm suspension after DNAse and neuraminidase treatment demonstrated a few hundred pEGFP incorporated per cell. In addition the transfer of genetic material from virosomes into sperm cells was shown using fluorescein-tagged pEGFP.

Conclusions: Spermatozoa fused with influenza X-31 virosomes carry exogenous DNA and remain vital, offering the basis for creating transgenic farm animals by in vitro fertilization or artificial insemination.

Notes

IN VITRO DEVELOPMENTAL POTENTIAL OF PORCINE EMBRYOS RECONSTRUCTED WITH CUMULUS CELL NUCLEI: EFFECT OF CULTURE CONDITIONS

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In the study the effect of in vitro culture conditions on the developmental potential of nuclear transfer (NT) porcine embryos was investigated. Cumulus cells surrounding in vitro matured porcine oocytes were the source of donor nuclei and Metaphase II oocytes were the source of recipient cytoplasm in somatic cloning procedure. Maternal chromosomes were removed by chemically assisted microsurgical technique (Yin et al., 2002, Biol. Reprod. 67: 442-6). Matured oocytes exhibiting the first polar body were incubated in medium supplemented with demecolcine (DMCC) and sucrose for 1 h. Then, oocytes with protruding plasma membrane were transferred into medium containing DMCC and cytochalasin B (CB) and membrane protrusion was aspirated into enucleation pipette. Reconstruction of embryos was done by direct microinjection of cumulus cell karyoplasts into a cytoplasm of previously enucleated oocytes. After 1 h incubation in medium with DMCC, reconstituted oocytes were artificially activated by application of three successive DC pulses of 1.0 kV cm^{-1} for 80 _sec and incubated in medium supplemented with CB for 2 h. The activated NT oocytes were cultured in humidified atmosphere of 5% CO_2 in air at 38.5ºC in a 50 _L drop of NCSU-23/BSA followed of NCSU-23/FBS medium covered with mineral oil (Group I, standard) or in co-culture system either with cumulus cells (Group II) or with oviductal epithelial cells (Group III) in 500 _L of the same medium.

A total of 342 enucleated oocytes were injected with cumulus cell karyoplasts. After activation, 312 reconstituted oocytes (91.23%) were in vitro cultured for 7 to 8 days in three different physicochemical culture conditions (129, 79 and 104, in Group I, II and II, respectively). The proportion of reconstructed embryos that cleaved was approximate in several experimental groups (68.9%, 89/129; 56.0%, 44/79, and 68.0%, 71/104, in Group I, II and III, respectively). Development to morula stage was not different in three culture conditions (29.2%, 26/89; 34.1%, 15/44; 26.8%, 19/71, in Group I, II and III, respectively). Blastocyst development rate was similar in all groups (4.5%, 4/89; 6.8%, 3/44 and 7.0%, 5/71 in Group I, II and III, respectively) and was not statistically different among treatment groups ($P>0.05$; analysis by chi-square test).

In conclusion, the in vitro culture conditions used for reconstituted porcine embryos had no significant effect on developmental competences of porcine NT embryos and although the percentage of obtained morulae was relatively high in all experimental groups, blastocyst formation rate decreased considerably. However, blastocyst percentage tended to be higher in the co-culture system groups than in the group without somatic cell feeder layer, although the difference was not statistically significant.

Notes

MITOCHONDRIAL DNA IN OOCYTES FROM COWS SELECTED FOR IN VITRO BLASTOCYST PRODUCTION

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Cows which are "good" or "bad" blastocyst producers using OPU and IVF have been identified by Tamassia *et al.* (17th A.E.T.E. 2001, Lyon - France, p 174.). Later, it was demonstrated with the same animals that the oocyte ATP contents varied significantly among them and that females with blastocyst rates above 25% had significantly more ATP before than after in vitro maturation (Tamassia *et al.* 2002, 18th A.E.T.E., Rolduc – Netherlands, p 232). Mitochondria are the cell's "powerhouse", supplying the energy necessary for embryonic development. Their concentration in the oocyte is associated with embryo development in humans (Reynier *et al.*, 2001, Mol Hum Reprod 7:425-429) while, polymorphisms in the mtDNA control region (CR) affect the quantity of transferable cloned (NT) embryos (Bruggerhoff *et al.*, 2002, Biol Reprod 66:367-373) and the calving rate in beef cows (Sutarno *et al.*, 2002, Theriogenology 57:1603-1610). The objective of this study is to analyse the mtDNA-CR and measure the quantity of mtDNA in oocytes from cows previously selected for IVP.

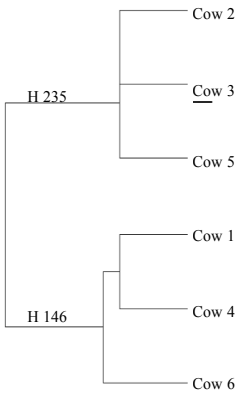
Oocytes were collected twice a week for 12 non-consecutive weeks from the six selected animals and frozen denuded in PBS for mtDNA quantification. Oocyte DNA was extracted with the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) and quantification was performed using Real time PCR and the LyghtCycler-Faststart DNA master SYBR Green 1 kit (Roche Diagnostics, Mannheim, Germany). Primer positions are DC3 (5977-5999) and RC1 (6166-6146) according to the Cambridge reference system. DNA from white blood cells was used for sequencing of the mtDNA-CR. Primers and PCR conditions for the mtDNA-CR sequencing are described in Bruggerhoof *et al.* (2002). Amplified CR fragment was sequenced and compared between animals.

Sequencing the mtDNA-CR enabled the creation of an evolutionary tree with two major haplogroups (see Figure): H146 and H235. Retrospective analysis of blastocyst production shows that haplogroup H235 produced significantly more blastocyst than H146 (36.7 ± 34.0 % vs. 22.3 ± 27.5 % respectively). The mean quantity of mtDNA per oocytes varied significantly among animals (see Table) however, the best and worse blastocyst producer had similar quantity of mtDNA. Haplogroup had no effect on mtDNA copy number per oocyte (374 758 ± 272 329, n = 149 and 391 582 ± 249 704, n = 141 for H146 and H235 respectively). Nevertheless, an important intra-animal variation is observed in mtDNA (CV). The data show independence between mtDNA copies per oocyte and blastocyst production. At this point, it is only possible to speculate the relationship between this large variation in mtDNA copy per oocyte and the large variation observed in embryo production. Further studies shall enable us to verify the association between these two parameters.

MtDNA contents and blastocyst production; animals are ranked by haplogroup and blastocyst rate

ID	N	mtDNA Mean ± SD	CV (%)	mtDNA Haplogroup	Mean Blast. Rate (%) [2]
Cow 5	41	311 607 ^{y} ± 216 484	69.5	H-235	50.4 ^{k}
Cow 2	64	361 087 ^{y} ± 238 824	66.1	H-235	31.7 ^{lm}
Cow 3	36	536 878 ^{z} ± 249 547	46.5	H-235	26.4 ^{lmn}
Cow 1	48	391 171 ^{yz} ± 298 318	76.3	H-146	34.3 ^{kl}
Cow 6	44	416 320 ^{yz} ± 311 246	74.8	H-146	19.3 ^{mn}
Cow 4	57	328 853 ^{y} ± 207 829	63.2	H-146	12.4 ^{n}

Values in the same column with the same subscripts are not different (p>0.05)
[2] Reference Tamassia *et al.*



MITOCHONDRIAL AGGREGATION PATTERNS AND ACTIVITY IN PORCINE OOCYTES AND APOPTOSIS IN SURROUNDING CUMULUS CELLS DEPENDS ON THE STAGE OF PREOVULATORY MATURATION

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In this study, we evaluated the distribution and oxidative activity of mitochondria in ex vivo preovulatory porcine oocytes using the fluorescence probe MitoTracker CMTM Ros Orange. Cumulus-oocyte complexes were classified according to cumulus morphology and time from hCG administration. The meiotic configuration of the oocytes and the degree of apoptosis in the surrounding cumulus cells were also evaluated.

Oestrus was synchronized in 45 crossbred Landrace gilts by feeding altrenogest for 15 days and administering 1000 IU PMSG on Day 16. The LH peak was simulated by treatment with 500 IU hCG, given 80 h after PMSG. Endoscopic oocyte recovery was carried out 2 h before or 10, 22 or 34 h after hCG administration. Altogether 454 COCs were aspirated from follicles with a diameter of more than 5 mm. Cumulus morphology in the majority of COCs recovered 2 h before and 10 h after hCG was compact (60.4 and 52.7 %, respectively; $P<0.05$). At 22 h after hCG, COC morphology changed significantly from 10 h dramatically: 74 % of COCs had an expanded cumulus ($P<0.01$). At 34 h after hCG, 100% of recovered COCs had an expanded cumulus. The percentage of oocytes with a mature meiotic configuration differed among COC morphologies and increased as the interval after hCG administration increased ($P<0.05$).

The type of mitochondrial distribution in the oocytes (n=336) changed from homogeneous to heterogeneous as the interval after hCG administration increased ($P<0.01$) and was associated with the cumulus morphology. Representative mitochondrial distributions were found as follow: - 2 h: fine homogeneous in compact and dispersed COCs; 10 h: granulated homogeneous in compact and dispersed COCs; 22 h: granulated homogeneous in expanded COCs; and 34 h: granulated heterogeneous and clustered heterogeneous in expanded COCs ($P<0.01$).

The oxidative activity of mitochondria measured by fluorescence intensity (Em: 570 nm) per oocyte after Mitotracker CMTM Ros Orange labelling increased in the oocyte as the interval post hCG increased ($P<0.01$) and depended on the type of mitochondrial distribution. Lowest oxidative activity of mitochondria was found in oocytes with fine homogeneous distribution (253.1 ± 9.4 μA). The oxidative activity increased (334.4 ± 10.3 μA) in oocytes with granulated homogeneous distribution of mitochondria, and reached highest level in oocytes with granulated heterogeneous (400.9 ± 13.0 μA) and heterogeneous clustered distribution (492.8 ± 13.9 μA) ($P<0.01$). Concerning mitochondrial activity in oocytes apoptosis in surrounding cumulus cells increased in a time depending manner during preovulatory maturation in vivo ($P<0.01$).

These results indicate there is a relationship between meiotic progression, cumulus expansion and mitochondrial redistribution and their oxidative activity during final preovulatory maturation in pig oocytes. It seems to be that increased levels of mitochondrial activity in oocytes are related to increased levels of apoptosis in surrounding cumulus cells, and might be caused by the mitochondria.

Notes

ASSESSMENT OF A NEW UTERO TUBAL JUNCTION INSEMINATION DEVICE IN THE WATER BUFFALO UNDER FIELD CONDITIONS

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A new artificial insemination device (Ghent device) for utero-tubal junction (UTJ) insemination has been developed at the University of Ghent (Belgium). To assess the efficacy of the Ghent device, 67 buffalo cows (Bubalus bubalis) were inseminated in a field trial. Two different insemination methods were used: 1) insemination with the conventional insemination device in the uterine body, and 2) insemination with the Ghent device near the utero-tubal junction ipsi-lateral to the site of ovulation. Artificial insemination was performed twice at 72 and 96 h after administration of prostaglandins to buffalo cows with a functional corpus luteum. Conventional inseminations were performed with full ($16 - 20 \times 10^6$) and half insemination doses of frozen-thawed semen, while UTJ-inseminations were performed with full, half and quarter insemination doses.

Table 1: Conception rate (CR) after insemination with full (18×10^6), half (9×10^6) and quarter (4.5×10^6) insemination doses, with the conventional insemination device into the uterine body (CD body), and with the Ghent device near the utero-tubal junction (GD UTJ).

	CD body			GD UTJ		
Insemination dose	No. of AIs	Pregnant	CR	No. of AIs	Pregnant	CR
18×10^6	15	8	53%	12	6	50%
9×10^6	19	8	42%	11	5	45%
4.5×10^6	-	-		10	5	50%

When inseminations were performed with the conventional insemination device, halving the insemination dose resulted in a decrease of conception rates from 53% to 42%. However, no difference in conception rates was observed when UTJ-insemination was performed with a full or quarter standard insemination dose (50%).

Although more inseminations are required to confirm our findings, this field trial demonstrates that UTJ-inseminations in the buffalo can be performed with only one quarter of the standard insemination dose without a reduction in conception rates.

Notes

A COMPARISON OF THE DEVELOPMENT OF BOVINE EMBRYOS CLONED FROM FIBROBLASTS OF TWO DIFFERENT GENETIC ORIGINS

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Cloning by nuclear transfer (NT) has allowed the production of animals from a variety of adult and fetal somatic cell types either among the same breed or different breeds of cattle. However, the poor efficiency in full term development of cloned embryos makes it difficult to get relevant information on the potential of a donor cell line with respect to the genotype of the donor animal within the same breed of cattle. Here we report a comparison of the success rate of somatic cell cloning using adult skin fibroblasts from two Holstein females of high genetic merit.

Primary cultures of adult bovine fibroblasts were established from ear skin biopsies of 1 year old heifers and the cell lines derived from these primary cultures were used as sources of donor cells for NT between passage 3 to 13. Donor fibroblasts to be used for NT were grown to confluence. Recipient oocytes were matured in vitro and enucleated at 20-22 h post maturation (hpm). Embryos were reconstructed by fusion with donor cells at 23-24 hpm. Reconstructed embryos were activated in 10µg/ml cycloheximide and 5µg/ml cytochalasin B for 5 h after fusion, then cocultured on Vero cells for 7 days in microdrops of B2 medium supplemented with 2.5% FCS. Grade 1 and 2 embryos were selected in a total of 9 replicate experiments for each of the 2 cell lines, and transferred into synchronous recipients (1 or 2 blastocyst/recipient). The pregnancies were followed by ultrasonography.

The reconstructed embryos cleaved at the same rate in both groups of donor cells and in vitro development to morula and blastocyst stage was not significantly different ($p>0.05$; Chi-square) (Table 1). However, pregnancy rates after transfer of NT blastocyst were lower from pregnancy initiation (Day 21) and up to term in cows carrying OV029 clones ($p<0.001$). Only 2 calves were obtained from 127 blastocysts transferred in this group while 15 calves were born from 101 transferred blastocysts of the OV5538 genotype (Table2).

Table 1. In vitro development of embryos cloned from two genotypes of donor fibroblasts

Donor Genotype	Reconstructed embryos	Fused (%)	Cleaved (%) of fused	Morula (%) of fused	Blastocysts (%) of fused
OV029	445	352 (79.1)	252 (71.6)	140 (39.8)	127 (36.1)
OV5538	418	322 (77.0)	226 (70.2)	145 (45.0)	138 (42.9)

Table 2. Rates of pregnancies after transfer of cloned blastocysts from different donor genotype

Donor Genotype	Embryo Transferred	Recipients	Pg test D21	Confirmed Pregnancy D 35	D70	D90	Calving
OV029	127	85	29/85 (34.1%)[a]	9/85 (10.5%)[a]	8/85 (9.4%)[a]	8/85 (9.4%)[a]	2/85 (2.3%)[a]
OV5538	101	60	46/60 (76.7%)[b]	34/60 (56.7%)[b]	27/60 (45.0%)[b]	22/60 (36.7%)[b]	15/60 (25.0%)[b]

[a, b] Percentages within columns differ ($p<0.001$)

These results may indicate that different genotypes within the same breed have no effect on in vitro development of NT embryos, but a very high incidence on further in vivo development. However, further experiments are necessary to exclude a culture-induced change in the individual cell lines.

Notes

THREE YEARS OF EMBRYO TRANSFER (ET) IN THE MARE

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ET allows the obtention of pregnancies from high genetic, economic or sporting value, aged or very young mares, subjects in competition activity and moreover, to increase the production of a single mare in the same season (1,3,5). The biggest limiting factor at present is the inability to superovulate the mare such that it is possible to recover one or exceptionally two embryos for each cycle (6). Costs and ideological opposition from some breeders associations to admit foals born by ET reduce the application. Our team has performed 51 flushings in three breeding seasons (2001, 2002 and 2003, to May) on 30 donors. For each, one recipient has been synchronized (+1 and –3 days, where Day 0 is the day of ovulation of the donor) with PGF2 (Luprostiol, 11.25 mg/subj. IM) and hCG (4000 UI/ subj. EV). Donors, aged between 3 and 20 years, are randomly spread in different categories (normal/poor fertility, pregnant or repeatedly inseminated without success in the previous season, good/poor endometrial functionality, etc.) All subjects were inseminated with fresh or frozen/thawed semen following an individual clinical criterion, supported by scanner examination of the ovaries. Recipients, aged from 3 to 14 years are maiden or subjects with a previous regular reproductive career. Uterine flushing were performed from Day 7 to Day 8 with PBS at 38°C. A cuffed catheter, 12 mm diameter, was introduced through the cervix and the cuff was filled with 60 ml of PBS. The uterine flushing, during the first season, was performed with larger amount of fluids, (more than 1_ L) which were collected in bottles and decanted or filtered in the lab. The whole procedure was repeated two or three times. To now, only 1L of PBS is utilized for uterine flushing. The recovered fluid was immediately filtered with an EM-Con filter and reutilised for the following washings. The procedure was repeated 4-6 times. During the last recovery a delicate manipulation of the genital tract was performed. The 20 ml remaining aliquot of filtered media was transferred in a Petri dish and observed under a microscope at 12-60X, to locate and classify the embryo that was immediately, non surgically, transferred to the synchronized recipient. The results of the trial are exposed in Table 1.

Table 1.

Season	Flushing	Recovered embryos (%)	Transferred embryos (%)	Pregnancy (D14)(%)	Pregnancy (D28)(%)	Pregnancy (D56)(%)	Parturition (%)
2001	14	7 (50)	6 (85.7)	3 (50)	3 (50)	3 (50)	3 (50)
2002	13	12 (92.3)	12 (100)	10 (83.3)	10 (83.3)	10 (83.3)	10 (83.3)
2003	24	13 (54.16)	13 (100)	12 (92.30)	11 (91.66)	11(100)	-

29 embryos were classified as grade A (excellent or good), 1 as grade B and 2 as poor quality. The reported recovery rates range between 60 and 70% (2). The lower result of our team in 2003 need deeper investigations and commentary on donors status. The pregnancy rate is comparable to data recently reported for surgical transfer (86.5%)(4). This encouraging performance is attributable to the optimal management of the whole trial: management of donors and recipients, practice during the flushing and transfer, handling in laboratory. The small numbers and the heterogeneity of the same one have not allowed statistic analysis (AI timing, fresh versus frozen semen, donor's age and anamnesis, etc). The satisfactory results have encouraged the demands especially for poor fertility donors and further data will become available.

(1) Camillo F. et al., 2002, Theriogenology 58:627-630. (2) Carnevale EM et al., 2000, Theriogenology 54:965-979. (3) Lagneaux D, Duchamp G, 1999, INRA Prod. Anim., 12:344-345. (4) Jasko DJ, 2002, Theriogenology 58:713-715. (5) McKinnon AO, Voss JL, Equine Reproduction, ed Lea & Febiger, 1993, 357. (6) Squires EL et al. 1999, Theriogenology 51:91-104.

Notes

PROGRESS OF FERTILIZATION IN VIVO IN oFSH-SUPEROVULATED HEIFERS WITH DEFINED LH SURGE

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The superovulation (SO) procedure with defined LH surge facilitates the harvesting of oocytes and embryos at fixed times during in vivo maturation, fertilization and early embryonic development. It has been reported previously that such a SO procedure may produce a higher rate of non-fertilized oocytes (van de Leemput et al., Theriogenology 2001, 55:573-592). Therefore, we investigated the state of fertilization of in vivo matured oocytes collected shortly after ovulation in oFSH-SO heifers with a defined LH surge.

Synchronized, cyclic HF heifers (n=6, 16-22 months of age) were treated for SO as described previously (Knijn et al., Reproduction 2002, 124:365-375) using oFSH (Ovagen ICP, Auckland, New Zealand) and prostaglandin (Prosolvin, Intervet International BV, Boxmeer, The Netherlands) while endogenous release of the LH surge was inhibited by a norgestomet ear implant (Crestar, Intervet International BV). At 18-20.5 h after the last FSH dose, GnRH (Receptal, Intervet International BV) was administered resulting in an LH surge 2.5 ± 0.6 (SD) h later (maximum of the LH surge 26.9 ± 5.0 ng/ml). AI was carried out 15-16 h after GnRH and animals were slaughtered at 42 h after GnRH. Genital tracts were recovered and immediately transported to the laboratory. The oviducts were dissected and flushed with PBS (Bio Whittaker Company, Verviers, Belgium) to collect the embryos, which were then fixed in glutaraldehyde for DAPI staining. The number of ovulations was counted to calculate the recovery rate (64% ± 14 embryos collected/ovulations). Blood samples were collected every 3 h from 32 h before GnRH and every hour for 8 h thereafter for RIA of LH.

Since all six animals showed one single, normal LH surge zygotes were collected on average at 39.5 h after the induced LH surge (i.e., in a period of presumably 10 to 16 h after ovulation). All collected embryos (n=60; 10 ± 7.6 embryos per heifer) were at the 1-cell stage. Overall, 82% were fertilized, 12% were not fertilized, 5% were degenerated, and 1 embryo was lost. The zygotes were categorized according to the classification into different stages of the fertilization process as described by Laurincik et al. (Theriogenology 1994, 42:1285-1293; Table 1).

Table 1. Distribution of zygotes according to chronology of pronucleus development at 39.5 h after the LH surge in oFSH-superovulated heifers.

Stage	Description	Percentage (number of zygotes)*
Sperm-head	any stage of sperm-head decondensation	7.1 (n=3)
Migrating	two spherical pronuclei at some distance apart	57.1 (n=24)
Apposed	two pronuclei in close apposition	35.8 (n=15)

*percentage expressed over determined stage (n=42 zygotes); non-determined zygotes n=7

Although the majority (57%) of the zygotes were in the "migrating" stage a substantial proportion were advanced to the apposed stage and a few were retarded. This variation of stage is probably related to the process of multiple ovulations in which oocytes are released in a time span of about 6 hours. The distribution of the stages varied markedly. For example, in one heifer 11 of 15 zygotes were in the migrating stage and in another 9 of 12 in the apposed stage while the interval between LH surge and recovery of embryos was no more than 1 h different. The fertilization rate of 82% is similar to that observed for other SO procedures. However, the progress of fertilization appeared to be delayed. At 35 h after the LH surge the apposed stage has been reported to become the dominant stage (Laurincik et al., Theriogenology 1994, 42). This may be explained by an effect of the norgestomet treatment in our procedure by delaying the time the major proportion of the oocytes is released, or by affecting the oviductal milieu before and during fertilization.

In conclusion, the SO procedure with defined LH surge does not affect fertilization rate but delays the progress of fertilization, and can be used effectively to collect zygotes at specific stages of development.

Notes

THE INFLUENCE OF AN ELECTRIC FIELD ON THE POTENTIAL DEVELOPMENT OF RABBIT EMBRYOS

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The purpose of this study was to test the influence of electric field strength and duration as well as the number of pulses applied at a wide range of values and in several combinations on the fusion, on the developmental rate of rabbit zygote.

Sexually matured female rabbits were superovulated with 100 j.m. PMSG. 72h later just before mating they received one injection of 100 j. m. HCG. 24 h after mating fertilized ova were recoverd from oviducts of the does by flushing in phosphate- buffered saline. Briefly embryos were placed separately beetwen the electrodes and they were treated by electric field with different parameters (number of pulses, strength and duration) Following electrical treatment embryos were cultued in B2 medium suplemented with 10% of FCS to the blastocyst stage. At this stage they were subjected to an cytogenetic examination. We observed that as the pulse duration and strength increased the number of embryos reaching the blastocyst stage slighlty decreased, but we did not find any chromosomal abnormalities.

Also, freshly collected 1- cell embryos were transfered to the pseudopregnant does. The influence of the different parameters of electric field was determined by the implantation rate recorded on day 10 of pregnancy and by the number of live-born young.

The results suggest that as duration and strength of electric pulse increase most of transferred embryos die before implantation.

Notes

RESEARCHES REGARDING THE SUPEROVULATION TREATMENT IN EWES WITH STIMUFOL

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The Stimufol is realized by Beckers and col. (1997), it is a product that has a high purification, containing FSH and LH, in proportion of 80% and respectively 20%. Used by a special protocol in adult ewes, was determinated the increasing of the number and quality of in vivo obtained embryos.

The objective of our researches was to obtain an ovulation rate bigger than 7, reducing to minimum the unovulated follicles and increasing the number of preimplantational embryos, freezing them in liquid nitrogen.

The researches were done on 3-4 years old female ewes, with the same age and physiological status. The Stimufol, in a dose of 250 µg, injected six times in equal doses, the last injection being administrated at 12 hours after finishing the progesteronic treatment. The quality of embryos was established after usual methods.

The results were appreciated through analyzing the ovulation rate, nonovulated follicles and utilizable embryos.

Table 1. The superovulation rate after treatment with Stimufol in different seasons.

Season		Statistics	Anestrous	OR	NF	UE	
	n		days	n	n	n	%
NS	7	$\overline{X} \pm sx$	153.5±67.88	7.55±7.76	1.27±3.35	2.73±2.05	56.05±38.74
		(min.-max.)	(92-241)	(1-26)	(0-11)	(0-6)	(0-100)
ES	11	$\overline{X} \pm sx$	87.29±58.40	7.43±2.81	0.00±0.00	4.14±1.95	81.29±23.98
		(min.-max.)	(36-201)	(5-13)	(0)	(1-6)	(33.33-100)

SN – normal season; CS – extra season; OR – ovulation rate;
NF – nonovulated follicles; UE – utilizable embryos.

The superovulation of 18 ewes with Stimufol was produced 7.43 – 7.55 ovulation rate when was administrated in normal and extra seasons period. The number of utilizable embryos was biggest in extra season period (4.14 vs. 3.83 embryos). It was registered a very big individual ovarian reaction after treatment of Stimufol in the both seasons (1 -26).

Notes

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