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EUROPEAN EMBRYO TRANSFER ASSOCIATION

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21st SCIENTIFIC MEETING

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Professor Jean-Paul Renard

Special Celebration

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Keszthely 9th and 10th September 2005

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C O N T E N T S

Professor Jean-Paul Renard

A.E.T.E. Medalist 2005

Jean-Paul Renard
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Jean-Paul Renard was born in 1945 in Dax, a city located in the south west of France and well known for its rugby team and bullfighting from which he probably inherited his fighting spirit in the numerous activities he was to undertake later.

He spent his undergraduate life in Paris where he attended the National Agricultural Engineer School of Grignon for his higher education in 1965. Being a young 'Ingeneer' in 1969, he had to enter the military service where he served for two years in the frame of Technical Cooperation between France and Laos.

After a couple of years as teacher at the Agricultural School at Rambouillet, he really started his research activity in 1975 in the laboratory of Animal Physiology headed by Charles Thibault at INRA, Jouy en Josas. Under the direction of Dr du Mesnil du Buisson, he became immediately involved in the problems associated with embryo transfer. The main applied objective at that time was to develop non surgical approaches for embryo recovery and transfer in cattle. He studied the control of metabolic activity of mammalian embryo at the beginning of their development to improve in vitro culture. He demonstrated in cattle and sheep the possibility for cultured and/or cooled embryos to develop to term after uterine transfer. He also established there the basic facts about the behaviour of embryonic cells at very low temperature, allowing the establishment of an efficient method for embryo freezing. This work was performed mainly on rabbit and cattle embryos, but Jean-Paul also collaborated with physicians in order to extend the freezing method to the human embryo. This probably had at least two consequences on the future activity of Jean-Paul. First, he became an inescapable partner for the physician interested in human assisted reproductive technologies, even if the first experiments on human embryo freezing resulted in the "explosion" of a very precious material. Second, he realised at that time that he was keeping in the same container embryos from cows, rabbits and humans, which was a situation ethically questionable from many aspects. This information, given to the media, urged the decision to create in France the National Committee for Ethics in Life Science and Health

From 1983 to 1990, he carried on his research at the Pasteur Institute in Paris. By using the DDK strain of mice and nuclear transplantation experiments between hybrid eggs, he observed an incompatibility between the paternal genome and the maternal cytoplasm, which was a demonstration of the involvement of a differential imprinting of parental genomes in embryonic development. Working together with Charles Babinet, he has been the first to demonstrate on the basis of these mutant mice that an abnormal interaction between the fertilized egg cytoplasm and the embryonic genome could alter the first cellular differentiations later in embryonic development. Since that time, Jean-Paul Renard has always kept in his group a research activity dedicated to the study of the regulation of maternal information in the very early stages of embryo development. During this same period of time, he also used the nuclear transfer technique to reconstruct diploid embryos at the 2-cell stage from nuclei of haploid parthenotes in mouse.

He always keeps in mind the interest and the potential consequences of his work for human health both being attentive to the needs of medical research and carefully evaluating the possible applications of his research for human health. In this respect, he has been involved in ethical concerns about reproduction and was an active participant in the National Committee for Ethics in Life Science and Health from 1982 to 1989. He also was a member of the board of administration and of specialized commissions of the National Institute for Medical Research and Health (INSERM).

He became director of research at INRA in 1986 and, in 1990, he was asked to set a research group on developmental biology in a new building devoted to animal biotechnologies in Jouy en Josas. Starting with five collaborators, he orientated his work toward the onset of the transcriptional activity in the mammalian zygote and the related maturation of the chromatin during the cleaving stages of the early embryo. In this context, he implanted his skill in nuclear transfer in his laboratory in order to provide for these fundamental aspects an experimental situation where the relation between the nucleus and the cytoplasm are altered in the zygote. This led to the application of reproductive biotechnologies in domestic species and to the improvement of oocyte preparation for the embryonic cloning in rabbits and cows. It stepped for nuclear transfer technology which came with the somatic cloning. In 1998, a couple of months after Jean-Paul Renard's team obtained the birth of the first cows derived from somatic cells in Europe, and their sudden death after six weeks, he got the conviction that this result was scientifically relevant and he was the first to alert the scientific community about the likely "long term effects" of somatic cell cloning in animals. He could confirm these data on mouse clones, this reinforced his interest in nuclear transfer as a model to characterise the embryonic genome regulation and its disturbance in relation to the long term development. Following his idea that species other than the mouse have great advantages in specific studies and applications, he developed the collaborations with French start-up in order to obtain offspring from somatic nuclear transfer in rabbit and rat for the first time in the world. In the mean time, he progressively increased the number of scientists involved in his team and took the head of a large group including teams from the veterinary school of Maisons-Alfort in 2001. Today he is in charge of a large research group (about 110 people) working on several aspects of animal reproduction, from oocyte maturation and activation to the fetal and postnatal development.

Finally, considering the importance of student education for the future of research in France and Europe he convinces his colleagues to be involved in University training and he himself holds a position of Consultant Professor at the National Agricultural Institute of Paris-Grignon. Jean-Paul Renard is an example of enthusiasm and pugnacity for his close collaborators and for the numerous foreign partners that are involved in common project with him.

Xavier VIGNON

NUCLEAR TRANSFER, EMBRYO TRANSFER AND ROBUSTNESS OF BIOLOGICAL SYSTEMS

RENARD J.P.

INRA, Developmental Biology and Reproduction Unit ,
UMR 1198, 78352, Jouy en Josas, France

Introduction

Domestic animals are complex biological systems. Thousands of years of selective breeding has created a diversity among breeds which constitute a unique resource for understanding the genetic basis of phenotypic variation. Several species of farm animals are today largely artifically reproduced even in remoted areas of the world. Reproductive biotechnologies have contributed to their extensive use as experimental models in applied research. In this paper I provide some arguments supporting the view that beyond their classical use in agriculture and their involvement already in several areas of biomedical research, domestic animals, mainly ruminants and pigs, are emerging as essential models in basic science. Wwe have now entered the postgenoic era requiring systemic approaches to exploit the genomic information already available in public databases (Pennisi 2003). Domestic animals share several developmental and reproductive traits which can be more advantageously exploited than in rodents for understanding the integrated functions of multicomponent biological systems at the whole organism level. They will contribute to the "renaissance of physiology" (Strange, 2005). I will focus my arguments on the latest reproductive biotechnology, cloning, and, according to Article 2 of its statutes, on the main object of the 'Association Europeenne de Transfert Embryonnaire' (AETE), namely, embryo transfer.

Robustness and reproductive biology: a matter of body building

The term "robustness" has been initially used to qualify a statistical estimator that remains relatively insensitive to either small variations affecting a whole set of data used to describe a system, or large variations affecting only few of those data (Launer and Wilkinson, 1979). By extension, the concept of robustness starts to be used in biology to design the property that allows a complex living system to maintain its functions despite external and internal perturbations (Kitano, 2004).

The completion of draft genome sequences for several of farm mammals species lead to a renewing of the methods used for complex-trait analysis (Andersson and Georges, 2004). It offers new oportunuities for the selection of traits that contribute to robustness when animals are exposed to unpredictable environmental and genetic perturbations. Meanwhile systems that are robust face fragility and performance setback as an inherent trade-off (Hartwell et al., 1999). Part of these drawbacks can be minimised using a strictly controlled herd management. This, however, leaves us with a poor knowledge of the basic architecture required for animal robustness.

In farm species such as ruminants or pigs, individual animals or herds have for a longtime been used in common by scientists from different academic backgrounds, not only for more efficient use of costly experimental models but also the study of organs or tissues of economical value and to determine how they interact in health and disease. In other words,

applied perspectives with farm animals have driven specific but already integrated questionning, a first step for a systemic approach of complexity.

Nuclear transfer: kicking out a dogma

The first live animal born following nuclear transfer of an adult somatic nucleus was Dolly the sheep. This result immediately stimulated considerable interest all over the world although it was considered with skepticism for several months by several reputed scientists (Sgaramella and Zinder, 1998). This success was in fact too provocative for those who had spent months, more than a decade ago, trying to reprogram the activity of embryonic nuclei in the academic mouse model, this after a first claim that the transfer of nuclei from the inner cell mass cells of a mouse blastocyst into an ennucleated zygote could result in live-born pups (Illmensee and Hoppe, 1981). The claim turned to be the result of chaotic, if not fraudulent experiments (Newmark, 1984) and the conclusion that the cloning of mammals by simple nuclear transfer was biologically impossible survived over these ruins as a dogma.

Today cloning as a technique is recognized but whether surviving adults can be considered as phenotypically 'normal' remains a matter of debate (Wilmut, 2002). One of the arguments often raised to support this view is that cloning is "inefficient" (Solter, 2000). This renders live adult clones to be considered as the fortunate survivors of a highly perturbing process, namely nuclear transfer. Although the likelihood of a nuclear transfer-derived embryo developing into a healthy adult is only few percent, there is evidence that much higher rates of success can be achieved under some favourable, but still not well understood, combinations of circumstances. We reported for instance on the birth of 15 healthy cloned calves after the transfer of only 60 blastocysts (25% full term development), themselves obtained in vitro at a rate (40%) similar to the one achieved after in vitro fertilisation (Vignon et al, 2003). Similar results have been reported for several other farm animal species in contrast to the mouse where very few labs have succeded in obtaining live offspring from somatic donor cells (Solter, 2000). Moreover, there is also evidence that animals born from adult somatic nuclei can be physiologically of normal appearance, fertile and with a normal lifespan (Beaujean et al., 2005).

These examples provide compelling evidence that nuclear transfer can already be used to test the robustness of an embryo, namely to characterise the range of operational conditions within which a system (the developing embryo) can continue either to operate correctly or…..to fail gracefully! Normality is not precluded by "low efficiency". Rather normality requires a comprehensive analysis of the variance in physiological traits and in gene expression of adult nuclear transfer animals similar to the one initiated with mice derived from natural mating (Pritchard et al, 2001). There are today more than a thousand adult clones in cattle which means that such an analysis has become feasible with farm animals

Altering an old concept: programming

The fact that embryos reconstructed from differentiated somatic adult nuclei can develop into healthy animals challenges the concept of 'program', coined more than 40 years ago by F. Jacob and J. Monod. This concept refers to a genome-contained and time dependent controlled sequence of pre-determined events for protein synthesis (Jacob, 1976). Clearly, nuclear transfer deals with the reversibility of this program and thus the management of time during the development of a complex living organism.

Various types of somatic cells have now been successfully used to generate embryos that can develop apparently normally throughout the preimplantation period of embryonic development. There is also some evidence that heterospecific nuclear transfer can lead to the completion of the blastocyst stage from which pluripotent embryonic cells can apparently be derived in vitro (Chen et al., 2003). These results demonstrate that nuclei of somatic cells can function in highly unpredictable environements. They put into a developmental context the observations made with heterocaryons showing that the fusion of two different somatic cell types results in histone modification and in an initial decondensation of chromatin (Kimura, 2004). For instance, histone H3 which is methylated on its lysine 9 residue in mouse somatic nuclei is rapidly and uniformly demethylated in the pericentromeric heterochromatin regions after nuclear transfer (Nathalie Beaujan, personal comunication). Also, the somatic linker histone H1 molecules are rapidly replaced by oocyte variants within 5 minutes after nuclear transfer (Gao et al., 2004).

These data bring decisive new information: they show that a somatic chromatin can be rapidly reorganised into an embryonic one after nuclear transfer. This considerably extends our perception of nuclear robustness since a wide range of molecular networks such as those that regulate the cell cycle and the signalling pathways controling proliferation and differenciation are active during the development of a reconstructed embryo into a blastocyst. Moreover these observations suggest that the somatic nucleus is structurally "reset to zero" as it is rapidly reorganised into embryonic chromatin. Is it, however, sufficient to consider that the genome-contained and time dependent controlled sequence of pre-determined events for protein synthesis has been "reprogrammed"? Without a rigorous definition, this neologism participates in the murder of scientific language. Reprogramming has recently been defined as ''the molecular dominance of one distinct cell type over another, resulting in the transformation of the pliant nucleus to the dominant type'' (Western and Surani, 2002). This definition is well adapted to the context of nuclear transfer where the introduction of a somatic nucleus (the pliant nucleus) into an oocyte (the dominant type) can result in the birth of live animals. It points to the major role of the oocyte cytoplasm but more importantly sheds light not on the genome itself but on its environment. Controlling the environment would reduce the variations affecting the set of molecular information required by the embryo to initiate its development. This leads to a paradigmatic approach were the first events of embryonic development, oocyte activation for instance, are driven from outisde by combining elegantly biology and microfluidics to become free of the huge variability associated with self-organised processes (Ozil et al., 2004)

Early development in domestic animals: welcome differences between species

In this context, what can embryos from farm animals bring to the understanding of the negative and positive feedback based systems that permit such a robust response? Recent data from the literature provides two types of answers.

First, a multispecies approach offers a means of confirming or not the conclusion raised from the academic mouse model. Comparative studies have to be considered as part of nuclear transfer research because of two main limitations of nuclear transfer: the micromanipulation technique itself which is highly dependent on the skill of the individual researcher and the limited amount of biological material available. This is well exemplified with studies on DNA methylation, one of the best-studied epigenetic modifications in all organisms. Mammalian

somatic cells show elevated DNA methylation levels with low methyltransferase activity whereas gametes are less methylated and sperm are more methylated than oocytes. As a consequence, the genome of the embryo is hypomethylated and subsequently undergoes global de novo methylation (Reik et al., 2001). However, changes in the DNA methylation status of a transferred somatic nucleus differs between species. A rapid and global demethylation of the paternal genome after fertilisation and a remethylation of the embryonic genome in the inner mass cells at the blastocyst stage are observed only in the mouse. In cattle, the demethylation process is less pronounced (Bourc'his et al., 2001) and even less in the rabbit (Chen et al, 2004); in the sheep demethylation still occurs at the blastocyst stage (Young and Beaujean, 2004).

Second, the timing of developmental events that characterise early embryogenesis differs between mammalian species. This is the case for the multi-step process of embryonic transcriptional activation, the first evidence of an embryo nuclear synthetic activity but also the first target for external (for instance in vitro culture) or internal (nuclear remodelling) perturbations. In the mouse this event occurs over a protracted period of time between the S phase of the one cell stage and the G2 phase of the two cell stage whereas in most other mammalian species, including humans, it spans over several cell cycles (Thompson et al., 1998).

Species such as cattle and rabbits, where embryonic genome activation is completed only at the 8/16 cell stages, are probably more adapted than the mouse for analysing how minor alterations to developmental kinetics can affect the robustness of the embryos in terms of transcription initiation. In the same way, the timing of events during the blastocyst stage dramatically differs between species. In rodents, trophoblast and endoderm specific differentiation occur only one day after implantation (Day 4.5 after fertilisation, Rossant, 2004) whereas in ruminants and pigs embryonic and extra-embryonic differentiations are effective more than 10 days before the first signs of implantation (21 dpi in bovine embryos, Guillomot, 1995); during that period the blastocyst has to maintain an active metabolic activity to ensure the dramatic elongation of its epithelial-like trophoblastic tissue (Hue et al., 2001, Maddox-Hyttel et al., 2003).

Nuclear tranfer and embryo physiology: time to tame genomics

The time lapse between differentiation and implantation encountered in ruminants and pigs make these species well adapted models in uncoupling the mechanisms of trophoblast growth and differentiation from those of implantation. These species offer the opportunuity to study robustness in relation to time using a biological system, the growing blastocyst, which acquires a new degree of complexity when functional relationships are established with the surrounding uterine epithelium (the endometrium). Insight into the regulatory networks that govern these mechanisms becomes accessible through the development of comprehensive and species specific genomic tools (Ushizawa et al., 2004, Whitworth et al., 2005).

A first list of genes that are improperly expressed in nuclear transfer cattle early blastocysts has recently been published (Pfister-Genskow et al., 2005). Using a wider and dedicated microarray made from more than 7 000 cDNA sequences expressed in placental and foetal cattle tissues it seems, however, that reprogramming of somatic cattle nuclei into Day 7 blastocysts leads to a globally unchanged picture in comparison with in vivo blastocysts whereas in vitro produced embryos are surprisingly more affected (Smith et al., personal

communication). Recently, in our lab, using an embryo stage-matched approach combined with a hierarchical clustering of genes differentially expressed after hybridisation of extracted messengers to a dedicated 10 000 cDNA array, we observed that it was more the dynamics of gene profiles than abberant specific gene expression that could be used as a signature of the activities of elongating bovine trophoblasts isolated before the onset of implantation (apposition) at 17 days (Hue et al., personal communication). How is this dynamics altered in nuclear transfer derived blastocysts? The answer to this question will provide an insight into the robustness of peri-implantation blastocysts. Such embryos can be investigated in cattle using repeated in vivo recoveries from the same donor, and this is an additional attractive property of the bovine model.

The challenge today with mammalian embryos is more in canalising a deluge of molecular and cellular data that threatens biology. Beyond the importance that has to be given to statistical plans when building experimental protocols for genomic analysis, the choice of the animal model in relation to the question under study now plays a decisive role. Long lasting effects of early environemental perturbations are central to robustness and farm animal models are probably the good choice to replace this question in an in vivo context

Removing the veil off the placenta: an entry into robustness

In vivo analysis of a developing foetus requires embryo transfer and surrogate mothers which considerably increases the cost of experiments. These have to be carefully justified if a systemic analysis of data is to be considered.

Epidemiological studies accumulated during the last 15 years in humans demonstrate an increased incidence of prevalent physiopathologies such as type 2 diabetes and obesity at adult age in children who are small for their gestational age (Barker et al., 1989) . Work in animals, mainly in the ewe, confirm that when undernourished, the fetus adapts to its altered environment by modifying its metabolism and that these modifications become permanent (McMillen and Robinson, 2005). The "thrifty phenotype" characterises such foetuses with a retarded growth. It means that they adapt their metabolism to fulfill the requirements of the vital organs (brain, heart). For instance, the fat stored in the adipocytes will be used at the end of foetal development, when brain requirements are most important, but the body will continue to store this fat after birth as a result of this conditioning. The other organs (liver, kidney, pancreas) are subsequently restricted, which predisposes the individual to adult diseases like dyslipemia, insulin resistance or diabetes (Vickers et al., 2000). Postnatal growth of human foetuses that remained small for their gestational age can be altered by dietary intervention, and there is a critical window for nutritional programming of the growth trajectory during the first nine months after birth (Fewtrell et al., 2001). Nutritional intervention with an "aggressive nutrition" during the first weeks post-partum may be capable of minimizing the interruption of nutrients that occurs at birth. Rapid catch-up growth, however, can be associated with subsequent development of the metabolic syndrome. This is the consequence of the adaptive foetal response which can alter permanently the physiological functions post-partum.

Experimental studies indicate that the relationship between maternal nutrition, foetal growth and adult pathologies can have a very early origin even at the periconceptional period (Bertram et al., 2002). Since nutritional effects affecting adult physiology can be induced very early during embryonic life and prior to placenta formation, these effects are not only due to a foetal/neonatal

adaptive response (as evidenced by uterine environment perturbations during gestation). These long term nutritional effects may be considered as an "adaptive-predictive response" (Hanson and Gluckman, 2005) meaning that they are not immediately beneficial to foetal growth but can be expressed later, depending on environmental conditions. By extension, nutritional factors as well as other factors, like the uterine environment, may affect this response, including those that can have transgenerational effects and therefore an impact on evolution.

Long gestations in farm animals: all but a constraint

The above data add a new dimension to the relationship between robustness and time. They show that the past of a living system (the fertilised embryo) largely affects its future (the resulting offspring and adult) and make us once more recall that individual phenotypic variations have not only a genetic but also a physiological basis. Complex-trait analysis requires more than genome sequences analysis! A variety of new tools for functional genomics including large scale RNAi screening in mammalian cells will likely become a routine experimental approach in the near future as whole genome mammalian siRNA libraries and high-throughput methodologies are developed and refined (Balija et al., 2004). Furthermore, tools for the study of protein-protein interaction mapping are becoming available, although today only in non mammalian models such as Drosiphila (Giot et al., 2003). They will add new complexities to the analysis of biological systems. They will perhaps add too much. The temptation is there to perform system biology without a detailed understanding of the biology and to collect data on the system for the experimentator's own sake. Simplified and adapted models are required to study the long term consequenes of foetal robustness. Rodents, with their short gestation period and the limited access to the foetus, have so far allowed only a restricted approach to be taken. Farm animals appears to be much better alternatives. We are currently developing in the sheep a simplified model of nutritional restriction using a technique of uterine embolisation which can locally reduce the blood flow to the placenta (Pelage et al., 2001). Placental restriction in this species leads to an hypoxemic and hypoglycaemic foetus with increased plasma lactate (McMillen et al., 2003). These foetal perturbations occur despite unmodified arterial pH, a situation similar to what happens in uterine growth retarded babies (Economides et al., 1991). The sheep model makes it possible to induce nutritional perturbations at different stages of pregnancy in a controlled manner. Foetal growth disregulations can be followed in utero using advanced imaging techniques (Doppler, 3D and 4D ultrasonography). Moreover, interventional surgery (uterine and foetal catheterism) can be performed without preventing the pregnancy going to term. By combining in vivo sampling and global transcription profile analysis of embryonic (perimplantation), foetal and placental tissues, an accurate testing of foetal robustness to perturbations of nutritional environment has become an achievable goal. Since defects in the placental development of nuclear transfer embryos are one of the main causes of mortality in sheep (De Sousa et al., 2001) and also in cattle (Chavatte-Palmer et al., 2002), our approach will bridge a systemic analysis of an evolving organ, the placenta (beginning with extraembryonic tissues) to highthroughput molecular analysis.

Profiling placental functions in long lasting gestation models is more than opening a black box. It directly questions the concept of foetal programming with potential practical consequences on pregnancy and afterbirth nutritional management.

Conclusion: robustness and ontogeny of evolving biological systems

Nuclear transfer has now proved to be compatible with the generation of healthy and fertile adult clones, and this in several domestic mammalian species. Although these clones represent only few percent of the reconstructed embryos they demonstrate that the genome-contained and time-dependent controlled sequences of events can be made reversible. This questions the management of time during the development of a complex living system. Cloned embryos can develop to the blastocyst stage at a high rate but developmental arrest frequently occurs during the major morphogenetic event of gastrulation. Strikingly enough, only a few recurrent patterns of abnormalities are then observed rather than the wide and diverse spectrum of phenotypes that would have been expected if epigenetic errors were distributed stochastically. This indicates that reprograming is not an unpredictable process as often frequently considered today: rather the poor ability of cloned embryos to develop into live offspring appears to be largely due to a growth-mediated perturbation of foetal maternal relationships at implantation. These perturbations may affect embryo patterning during the establishment of the body plan when time constraints are important. At later stages, they appear to be directly related to fetal adjustments in response to adverse changes in the biological environment of the developing organism with permanent consequences that are potentially advantageous in foetal life but often confer physiopathologies after birth.

Nuclear transfer not only confirms the importance of epigenetics in the functional plasticity of the genome but also reveals the potential of mammalian embryos to adapt to environmental perturbations throughout the process of development. Cloning and embryo transfer in farm animals are becoming pivotal tools in the analysis of robustness during the ontogeny of biological complex systems. Beyond the ethical requirements for research with large animals, a new challenge is offered to European biotechnology laboratories which have been probably kept too much away from funding resources in this field (Claxton et al. 2004). Brave new Dolly?

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National Statistical Data of Bovine Embryo Transfer Activity in Europe in 2004

TABLE : 1 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **BELGIUM** ***A.E.T.E 2005***

Data collected by
Dr. Beckers Jean-François

Total number of approved E.T. teams in the country	35
Number of teams providing data	7

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1095	B/A=	6.9
	Embryos collected	**B**	7570	C/A=	4.5
	Embryos transferable	**C**	4880	C/B=	64.5%
In vitro	Nb of oocyte donors		112		
(OPU)	Nb of OPU sessions		206		
	Nb of transferable embryos	**D**	442		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	5322	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1850		
In vivo	Frozen	**I**	4950	72.8% frozen	
In vitro	Fresh	**J**	390		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	7190	H+I+J+K=	
Number of frozen stored embryos		**M**	2530		
% of *in vitro* embryos transferred		**N**	5.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	68.8%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 2 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **CZECH REPUBLIC** ***A.E.T.E 2005***

Data collected by
Dr. Pytloun Jaroslav

Total number of approved E.T. teams in the country	9
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1184	B/A=	10.3
	Embryos collected	**B**	12150	C/A=	5.4
	Embryos transferable	**C**	6400	C/B=	52.6%
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**	136		
Total i*n vitro* embryos		**F**	136	=(D+E)	
Total number of transferable embryos		**G**	6536	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3654		
In vivo	Frozen	**I**	2685	42.4% frozen	
In vitro	Fresh	**J**	54		
In vitro	Frozen	**K**	34	38.6% frozen	
Total embryos transferred		**L**	6427	H+I+J+K=	
Number of frozen stored embryos		**M**	2828		
% of *in vitro* embryos transferred		**N**	1.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	42.3%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 3 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **DENMARK** ***A.E.T.E 2005***

Data collected by
Dr. Callesen Henrik

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	579	B/A=	11.0
	Embryos collected	**B**	6365	C/A=	6.9
	Embryos transferable	**C**	3983	C/B=	62.6%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	3983	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2015		
In vivo	Frozen	**I**	1523		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3538	H+I+J+K=	
Number of frozen stored embryos		**M**	1888		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	43.0%	(I+K)/L=	%

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	1856
Number of calves born from *in vitro* embryos	
Total	1856

TABLE : 4 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **ENGLAND** ***A.E.T.E 2005***

Data collected by
Dr. Liddle Alison

Total number of approved E.T. teams in the country	18
Number of teams providing data	7

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**	5000	(fresh + frozen)
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**		H+I+J+K=
Number of frozen stored embryos		**M**	4053	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L= %

Number of E.T. calves born (2004)

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

TABLE : 5 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: ESTONIA ***A.E.T.E 2005***

Data collected by
Dr . Jaakma Ülle

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=	5.3
	Embryos collected	**B**	21	C/A=	1.8
	Embryos transferable	**C**	7	C/B=	33.3%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**		=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	7		
In vivo	Frozen	**I**	10		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**		H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	58.8%	(I+K)/L=	%

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	10
Number of calves born from *in vitro* embryos	
Total	10

TABLE : 6 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **FINLAND** ***A.E.T.E 2005***

Data collected by
Dr. Mikkola Marja

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	550	B/A=	9.2
	Embryos collected	**B**	5042	C/A=	6.0
	Embryos transferable	**C**	3301	C/B=	65.5%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**	40		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	40	=(D+E)	
Total number of transferable embryos		**G**	3341	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1328		
In vivo	Frozen	**I**	1634	55.2% frozen	
In vitro	Fresh	**J**	22		
In vitro	Frozen	**K**	1	4.3% frozen	
Total embryos transferred		**L**	2985	H+I+J+K=	
Number of frozen stored embryos		**M**	1729		
% of *in vitro* embryos transferred		**N**	0.8%	(J+K)/L=	
% of frozen embryos transferred		**O**	54.8%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 7 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **FRANCE** ***A.E.T.E 2005***

Data collected by
Dr. Guérin Bernard

Total number of approved E.T. teams in the country	30
Number of teams providing data	24

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5520	B/A=	9.7
	Embryos collected	**B**	53421	C/A=	5.6
	Embryos transferable	**C**	30841	C/B=	57.7%
In vitro	Nb of oocyte donors		76		
(OPU)	Nb of OPU sessions		291		
	Nb of transferable embryos	**D**	468		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	3		
Total i*n vitro* embryos		**F**	472	=(D+E)	
Total number of transferable embryos		**G**	31312	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	14518		
In vivo	Frozen	**I**	14896	50.6% frozen	
In vitro	Fresh	**J**	184		
In vitro	Frozen	**K**	20	9.8% frozen	
Total embryos transferred		**L**	29618	H+I+J+K=	
Number of frozen stored embryos		**M**	11520		
% of *in vitro* embryos transferred		**N**	0.7%	(J+K)/L=	
% of frozen embryos transferred		**O**	50.4%	(I+K)/L=	%

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	3883*
Number of calves born from *in vitro* embryos	86
Total	3969

* 7142 transferred embryos

TABLE : 8 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **GERMANY** ***A.E.T.E 2005***

Data collected by
Dr. Cramer Hubert

Total number of approved E.T. teams in the country	16
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2806	B/A=	11.5
	Embryos collected	**B**	32193	C/A=	6.6
	Embryos transferable	**C**	18621	C/B=	57.8%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions		1462		
	Nb of transferable embryos	**D**	1598		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	1598	=(D+E)	
Total number of transferable embryos		**G**	20219	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	6754		
In vivo	Frozen	**I**	3768	35.8% frozen	
In vitro	Fresh	**J**	763		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	11285	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	6.8%	(J+K)/L=	
% of frozen embryos transferred		**O**	33.4%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 9 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **GREECE** ***A.E.T.E 2005***

Data collected by
Dr. Samartzi Fonteini

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B=
In vitro	Nb of oocyte donors			
(OPU)	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**		=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	6	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	6	H+I+J+K=
Number of frozen stored embryos		**M**	45	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L= %

Number of E.T. calves born (2004)

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

TABLE : 10 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **HUNGARY** ***A.E.T.E 2005***

Data collected by
Dr. Solti Laszlo

Total number of approved E.T. teams in the country	6
Number of teams providing data	5

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	134	B/A=
	Embryos collected	**B**		C/A= 5.9
	Embryos transferable	**C**	787	C/B=
In vitro	Nb of oocyte donors			
(OPU)	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro	Nb of transferable embryos	**E**		
(Slaughtered donors)				
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**	787	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	384	
In vivo	Frozen	**I**	229	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	613	H+I+J+K=
Number of frozen stored embryos		**M**	174	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	37.4%	(I+K)/L= %

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	331
Number of calves born from *in vitro* embryos	
Total	331

TABLE : 11 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **IRELAND** ***A.E.T.E 2005***

Data collected by
Dr. Lonergan Pat

Total number of approved E.T. teams in the country	6
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	247	B/A=	8.4
	Embryos collected	**B**	2085	C/A=	5.3
	Embryos transferable	**C**	1315	C/B=	63.1%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	1315	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	482		
In vivo	Frozen	**I**	762	61.3% frozen	
In vitro	Fresh	**J**	80		
In vitro	Frozen	**K**	120	60.0% frozen	
Total embryos transferred		**L**	1444	H+I+J+K=	
Number of frozen stored embryos		**M**	832		
% of *in vitro* embryos transferred		**N**	13.9%	(J+K)/L=	
% of frozen embryos transferred		**O**	61.1%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 12 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **ITALY** ***A.E.T.E 2005***

Data collected by
Dr. Brun Francesco

Total number of approved E.T. teams in the country	1021
Number of teams providing data	14227

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1021	B/A=	13.9
	Embryos collected	**B**	14227	C/A=	6.8
	Embryos transferable	**C**	6923	C/B=	48.7%
In vitro	Nb of oocyte donors		141		
(OPU)	Nb of OPU sessions		259		
	Nb of transferable embryos	**D**	629		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	2449		
Total i*n vitro* embryos		**F**	3078	=(D+E)	
Total number of transferable embryos		**G**	10001	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2391		
In vivo	Frozen	**I**	3587	60.0% frozen	
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	2098	95.5% frozen	
Total embryos transferred		**L**	8176	H+I+J+K=	
Number of frozen stored embryos		**M**	6755		
% of *in vitro* embryos transferred		**N**	26.9%	(J+K)/L=	
% of frozen embryos transferred		**O**	69.5%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 13 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **LITHUANIA** ***A.E.T.E 2005***

Data collected by
Dr. Kutra Jonas

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

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

Number of E.T. calves born (2004)

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

TABLE : 14 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **THE NETHERLANDS** ***A.E.T.E 2005***

Data collected by
Dr. Landman Bas

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	3223	B/A=
	Embryos collected	**B**		C/A= 5.9%
	Embryos transferable	**C**	18952	C/B=
In vitro	Nb of oocyte donors		265	
(OPU)	Nb of OPU sessions		2311	
	Nb of transferable embryos	**D**	2266	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	100	
Total i*n vitro* embryos		**F**	2366	=(D+E)
Total number of transferable embryos		**G**	21318	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	4050	
In vivo	Frozen	**I**	10728	72.6% frozen
In vitro	Fresh	**J**	784	
In vitro	Frozen	**K**	904	53.6% frozen
Total embryos transferred		**L**	16466	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**	10.3%	(J+K)/L=
% of frozen embryos transferred		**O**	70.6%	(I+K)/L= %

Number of E.T. calves born (2004)

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

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **NORWAY** ***A.E.T.E 2005***

Data collected by
Dr. Kommisrüd Elisabeth

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	21	B/A=	5.0
	Embryos collected	**B**	105	C/A=	2.7
	Embryos transferable	**C**	57	C/B=	54.3%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	57	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	15		
In vivo	Frozen	**I**	112		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	127	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	88.2%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 16 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **PORTUGAL** ***A.E.T.E 2005***

Data collected by
Dr. das Chagas e Silva Joao Nestor

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	92	B/A=	9.5
	Embryos collected	**B**	876	C/A=	6.0
	Embryos transferable	**C**	552	C/B=	63.0%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	552	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	342		
In vivo	Frozen	**I**	159		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	501	H+I+J+K=	
Number of frozen stored embryos		**M**	251		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	31.7%	(I+K)/L=	%

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	136
Number of calves born from *in vitro* embryos	
Total	136

TABLE : 17 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **ROMANIA** ***A.E.T.E 2005***

Data collected by
Dr. Zamfirescu Stela

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	27	B/A=	8.1
	Embryos collected	**B**	218	C/A=	7.6
	Embryos transferable	**C**	205	C/B=	94.0%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	205	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	76		
In vivo	Frozen	**I**	86		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	162	H+I+J+K=	
Number of frozen stored embryos		**M**	129		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	53.5%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 18 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **RUSSIA** ***A.E.T.E 2005***

Data collected by
Dr. Egiazarian Artour

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	26	B/A=	8.0
	Embryos collected	**B**	208	C/A=	5.0
	Embryos transferable	**C**	130	C/B=	62.5%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	130	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	147		
In vivo	Frozen	**I**	69		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	216	H+I+J+K=	
Number of frozen stored embryos		**M**	1035		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	31.9%	(I+K)/L=	%

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	37
Number of calves born from *in vitro* embryos	
Total	37

TABLE : 19 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **SLOVAKIA** ***A.E.T.E 2005***

Data collected by
Dr. Cesnak Peter

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	12	B/A=	6.6%
	Embryos collected	**B**	79	C/A=	2.5%
	Embryos transferable	**C**	30	C/B=	38.1%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**		=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	11		
In vivo	Frozen	**I**	39		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	50	H+I+J+K=	
Number of frozen stored embryos		**M**	19		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	78.0%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 20 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **SPAIN** ***A.E.T.E 2005***

Data collected by
Dr. De la Fuente Julio

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	335	B/A=	10.6
	Embryos collected	**B**	3561	C/A=	5.0
	Embryos transferable	**C**	1687	C/B=	47.4%
In vitro	Nb of oocyte donors		2		
(OPU)	Nb of OPU sessions		5		
	Nb of transferable embryos	**D**	20		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	8		
Total i*n vitro* embryos		**F**	28	=(D+E)	
Total number of transferable embryos		**G**	1715	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	408		
In vivo	Frozen	**I**	913	69.1% frozen	
In vitro	Fresh	**J**	6		
In vitro	Frozen	**K**	2	25.0% frozen	
Total embryos transferred		**L**	1329	H+I+J+K=	
Number of frozen stored embryos		**M**	1245		
% of *in vitro* embryos transferred		**N**	0.6%	(J+K)/L=	
% of frozen embryos transferred		**O**	68.6%	(I+K)/L=	%

Number of E.T. calves born (2004)

Number of calves born from superovulated embryos	433
Number of calves born from *in vitro* embryos	2
Total	435

TABLE : 21 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: SWEDEN

A.E.T.E 2005

Data collected by
Dr. Gustafsson Hans

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	328	B/A=	6.7
	Embryos collected	**B**	2192	C/A=	4.0
	Embryos transferable	**C**	1300	C/B=	59.3%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	1300	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	376		
In vivo	Frozen	**I**	924		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1300	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	71.1%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

TABLE : 22 EMBRYO TRANSFER ACTIVITY IN 2004

COUNTRY: **SWITZERLAND** ***A.E.T.E 2005***

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**	254	B/A=	11.8
	Embryos collected	**B**	3000	C/A=	7.9
	Embryos transferable	**C**	2018	C/B=	67.3%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	2018	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	494		
In vivo	Frozen	**I**	1169	70.3% frozen	
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	17		
Total embryos transferred		**L**	1680	H+I+J+K=	
Number of frozen stored embryos		**M**	1524		
% of *in vitro* embryos transferred		**N**	1.0%	(J+K)/L=	
% of frozen embryos transferred		**O**	70.6%	(I+K)/L=	%

Number of E.T. calves born (2004)

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

Table : 23 Embryo Transfer Activity in 2004

COUNTRY: **YUGOSLAVIA** ***A.E.T.E 2005***

Data collected by
Dr. Veselinovic Spasoje

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

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

Number of E.T. calves born (2004)

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

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2004

I. EMBRYO PRODUCTION

(Data collected from 23 countries)

In vivo* produced embryos (superovulation)	
- number of flushed donors	17,458
- number of transferable embryos	101,989
- mean number per flushed donor	5.84
***In vitro* produced embryos:**	
From OPU	
- number of OPU sessions	4,534
- number of transferable embryos	5,423
- mean number per session	1.20
From slaughterhouse collected ovaries	
- number of transferable embryos	2,736
Total *in vitro*	8,159
Total number of transferable embryos	110,148

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

(S. Merton, AETE Keszthely, 2005)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2004

II. EMBRYO TRANSFERS

(Data collected from 23 countries)

***In* vivo produced embryos ***	**Number of embryos transferred** 87,551 (39,302 fresh / 48,249 frozen)
***In vitro* produced embryos**	5,579 (2,383 fresh / 3,196 frozen)
Total number of embryos transferred	98,130
Proportion of IVF embryos transferred	5.7%
Proportion of frozen embryos transferred	55.2%

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

(S. Merton, AETE Keszthely, 2005)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2004

Species	Embryo Production	Embryo Transfers	Countries
Sheep	99	70	France Greece Romania
Swine	389	387	Czech Republic Hungary Switzerland
Goat	41	41	Romania
Horse	446	387	Czech Republic Finland France Italy Sweden

(S. Merton, AETE Keszthely, 2005)

INVITED LECTURES

ETHICAL ASPECTS OF ANIMAL CLONING

GJERRIS M.

Danish Centre for Bioethics and Risk Assesment,
The Royal Veterinary and Agricultural University, Denmark
mgj@kvl.dk

Summary:In this article the ethical discussion of animal cloning is analyzed with respect to the two main perspectives in the debate. One perspective is a traditional legislative and scientific evaluation of risks to human health, the environment and animal welfare. This perspective focuses on the ethical questions hat arise from physical risks to individual entities and is shown to leave out a broader an more multi-dimensional perception of the ethical questions involved. These can, however, be identified within a more general perspective that can be found in studies of public perceptions of animal cloning. The different arguments are discussed with the intention of making suggestions to a concept of ethical dialogue that does not end in either a monological information stream or marketing research, but leads to a deeper understanding of the different perspectives. This in turn is discussed as a way of forming a regulative framework for the technology that both respects the plurality of ethical viewpoints in contemporary society and seeks a socially robust solution to the ethical disagreement on animal cloning.

1: Introduction

The biotechnological development that has taken place since the 1970's has had two main consequences. First of all a profound understanding of and ability to manipulate living organisms at the genetic level and secondly a seemingly endlessly debate about the ethical aspects of biotechnology in general and certain applications especially. On of the most debated applications has without a doubt been the cloning technology. Ever since the presentation of the Dorset eve Dolly back in 1997 cloning has been a popular subject in the news media and a controversial issue in the debate on biotechnology.

It is worth noting, however, that the debate on cloning very early on took a turn whereby the debate on the ethical aspects of animal cloning was eclipsed by the debate on the prospect of the technology being used to humans – especially in the media. Furthermore, a lot of nations and international bodies passed laws and signed treaties, protocols etc. explicitly banning research into human cloning. As can be seen from the current controversies about human stem cells this discussion is far from over. But during these often heated discussions it is to some degree forgotten that although the prospect of cloning human beings certainly raises ethical questions, these questions are of a different kind than the questions that animal cloning raises. And that where human cloning is an application of the technology that might be possible one day, the cloning of animals is a reality right here and now.

This does not mean that the issue of animal cloning has not been discussed within an ethical perspective, but it has had the consequence that the discussion never really made it into the public sphere. This development is probably one of the reasons why animal cloning has so far been neglected from a legislative point of view. In all the countries and regions where the

research into animal cloning is taking place the technology today is regulated indirectly through existing regulation on animal welfare and consumer protection (Lassen et al. 2005) and not through legislation specifically addressing animal cloning. Another reason is, of course, that the technology is still rather new. In this respect the legislative system can be compared with the way that a religious tradition adapts to changes: It changes slowly and usually tries to accommodate to changes by reinterpreting the existing tradition rather than changing it. This way of regulating the technology is, however, about to change. Both on national and international levels initiatives have been taken to evaluate the current regulation mechanisms. This sudden interest in animal cloning from the legislative bodies is mostly motivated by two factors

A: There is a growing belief among people working within the political system in EU and the US that the clones are about ready to leave the labs and enter the market place. Although the success-rate in cloning animals remains low (Cloning in Public 2005a) products from cloned animals and their offspring are being developed both within the biomedical and the food industry and they are believed to be marketable in the near future. However, the unclear legislative framework that the industry is facing right now is considered one of the main barriers for the move from lab to market. By getting clear legislation into place, it is thus believed that the way for cloned products will be facilitated (Galli et al. 2004). It should be noted though that with the current success rates it is hard to see that cloned animals can play anything but a peripheral role in e.g. the food industry. And the biomedical applications of cloning as e.g. bioreactors has proven to be much harder to develop than initially expected (Cloning in Public 2005). The current interest in creating specific legislation should perhaps more be seen as a way of paving the way for the investments in further developing the technology that will be necessary to create a cost-efficient tool.

Whether one chooses to interpret the current situation of the technology in terms of marketability one way or the other, there seems to be an understanding between scientists and policy-makers that either new regulation must be introduced or a decision to regulate by existing regulation taken. Two examples should suffice to show the necessity of this: 1: It needs to be decided if the products should be evaluated on the basis of the process that was used to produce them or if only the end-result should be evaluated. This has to do both with the question of labelling the products and with the question of juridical responsibility. 2: It needs to be decided if and to what extent cloned animals and their off-spring pose risks to human health, the environment, the genetic variation etc. so that negative consequences can be avoided or at least anticipated.

B: The perceived leap from research to production, from lab to super-market, will undoubtedly refuel the ethical debates about animal cloning and bring them to the fore in the public debate. From a public perspective it is one thing that a small amount of animals are produced for research purposes in confined laboratories, but quite another if it is suddenly announced that cloned animals will soon be brought to the "public square" in huge numbers. This reaction is quite natural and can be seen in many aspects of human life. The closer something gets to us, the stronger we react. And since anticipating public reactions was something that was not done, when genetically modified plants were introduced to the market place and the consequences of this are well known, it seems prudent to anticipate them this time around.

Both from an industrial perspective and from a societal perspective it makes good sense to figure out ways of regulating the technology. The motivation from the industrial perspective is obvious – to sell the products, some degree of public acceptance is needed and one way of ensuring that is to have a legal framework that can reassure the public that at least some of its worries has been taken into consideration. Things do become complicated when seen from a societal perspective, but for now it is enough to state that in a pluralistic and democratic society it is desirable aim (both morally and pragmatically) to create a socially robust legislative environment.

The debate on the cloning of farm animals was begun in 1997, but it never really took off. But, as argued here, it certainly will in the near future. Whether this new interest is based in realistic expectations to the short-term developments in the marketability of the technology or based in an attempt to strengthen research into the technology with the implicit purpose of facilitating the road from lab to market by showing potential investors that the possible products will be marketable, is very hard to say. The mixture of basic research and industrial research that is so typical for biotechnology has as one of its negative consequences that it is difficult to draw a realistic picture of how close the technology is to the market. What is clear, however, is that animal cloning is being put on the societal agenda again by the initiatives to engage the public in a debate on the ethical and social dimensions of the perceived technological development. The rest of this article will focus on this discussion.

Initially I will briefly discuss the cultural and philosophical context that such a discussion will be embedded in to explain some basic conditions in ethical debates today. An understanding of this context is, in my view, a prerequisite to enter the debate since a failure to consider the current context can only lead to misunderstandings. Secondly I will identify two kinds of ethical perspectives on animal cloning: a typical and traditional legislative and scientific perspective and a more general public perspective and describe the kind of ethical questions that the different perspectives tale into account. Finally I will discuss how the different perspectives can enter into a fruitful dialogue that can inform the legislative process in a qualified way.

2: Ethics in the 21. Century

The American bioethicist Tristram Engelhardt describes the current context for ethical debates as a situation where friends and strangers are living side by side in the same society having to decide together how to regulate it. The point is that friends (in this connection) are other people with whom you share some basic convictions about life, the universe and everything. Together you form a kind of ethical community within which certain core values (authorities) are accepted and the interpretation of these values can be meaningful discussed. Outside this community (but still within the same society) exists other communities with different core values. And since the acceptance of these core values is not based on scientific inquiry into their validity but more on the social, emotional and intellectual context of people accepting them, there is no scientifically rational and/or logical deductible way of figuring out, who has the true/right or best values (Engelhardt 1996).

This does not mean that all core value carry the same “truth”. We judge them by their consequences to our life, by their accordance with our world-view in general, with their ability to guide us when in doubt and several other factors. But although the more extreme and/or weird values are usually discarded this way, several mutually excluding values might exist

within the same society, living within each their ethical community. One very clear example of this is the differences in core values that make some people endorse animal rights while others "only" endorse animal welfare.

This description of the current context of ethical debate is rather broad and non-controversial. Much more controversial are the different answers given to the question: to what extent can dialogue lead to a common understanding and perhaps even sharing of core values between different ethical communities? Or in other words: are there any values that we can agree upon? I will, however, not enter into this debate in this context. Suffice is to say that the gaps experienced in discussions of biotechnology are not easily bridged. What I will be looking for is instead a deeper understanding of the some of the perspectives that exist in the debate on animal cloning; both to gain a clearer understanding of the different viewpoints and to suggest what realistic outcome can be expected from a dialogue between them.

The role of ethics in the debate on animal cloning especially (and biotechnology in general) can accordingly be said to be the role of the mediator that seeks to help different groups to a better understanding of each other's viewpoints. Ethics can thus be seen as a flashlight that can enlighten the dialogue. Any idea that ethics can ultimately solve the disagreements that undoubtedly will persist to exist even after a deeper understanding of the counterpart's arguments have been reached, should, however, be laid aside. Ethics is no longer, and probably never has been, a hammer. If one expects that having a dialogue on the ethical aspects of animal cloning will lead to the forming of a consensus, one is therefore most likely to be disappointed (Gjerris 2004).

Finally it is important to remember that ethics is no different from science in that it is based on a certain methodology. What differs is that where scientists within the same field usually agree upon at least some methodological premises, ethics consist of a variety of methodologies that can differ up to the extent that they are mutually exclusive (again – the differences can be traced back to the kind of ethical community that nourishes a particular kind of ethics). But one thing that almost all kinds of ethics have in common is that the concept of *truth* that they employ is much broader than within the natural sciences. This means that *ethical truth* cannot be established in the same way as *scientific truth* – and hence not proven in the same way. To say that caring constitutes an ethical relationship without which we would not be fully human, is thus an ethical truth that can be demonstrated through different means (examples, stories, analysis of the concept etc), but in no way validated as either a mathematical truth (2+2=4) or an empirical truth (the earth goes around the sun) or a scientific theory (living beings evolve through mutation and adaptation). Thus ethics is by no means irrational or even without rules for valid arguments. But ethics employ different kinds of rationality than the natural sciences and methodologies that may vary widely between the different traditions.

3: Animal cloning and ethics – the legislative and scientific perspective

New technologies are traditionally evaluated by the legislative authorities along certain specific parameters that can be characterized as scientific in the way that they are perceived to be neutral and objective and accessible for scientific research. Thus risk assessments of the possible consequences for human health, the environment and sometimes animal welfare have been the standard procedure for some years now, when e.g. genetically modified plants and animals are evaluated (Hansen et al. 2003). And legislation on how to regulate these entities

are usually based on that such procedures should be carried out before they can be approved by the authorities. This can be seen as a consequence of both the perceived enlightenment of western culture and the perceived right of the individual to choose his or her own ethical and religious values and live by them. It is a basic notion in the western, liberal societies that on a societal level, legislation should be based on scientifically informed data that is seen as accessible to all members of the society, whereas the ethical and religious values that form people's attitudes, as mentioned above, only are truly accessible within an ethical community and therefore should not influence societal legislation (Reinders 2000).

This has to some extent had the consequence that within both the legislative framework and in the scientific world the word ethics has almost been equated with scientific risk assessment. There has been a clear tendency that other issues that from non-scientific perspectives are just as important in an ethical context has been disregarded in the debate as irrational, metaphysical or religiously based
concerns that did not belong in the public discourse on biotechnology and ethics. Why and how this development has happened is a very complex question that I will not seek to answer here. Suffice is to say that the disregardment of other concerns than problems that are to a certain extent at least scientifically accessible have left a huge gap in the dialogue between scientists on the one side and the public on the other.

This is most strikingly seen in the notion of a *knowledge gap* that to some extent still pervades the understanding among scientists and legislators of the real root of the public scepticism towards biotechnology. Logically such an understanding leads to a call for informing the public in the belief that additional information will change the scepticism to optimism about the new possibilities. As I will show later, this notion is not confirmed by research into the connection between knowledge of biotechnology and acceptance of it. I will return to this notion in chapter 4. For now it is only necessary to state that there seems to exist a discrepancy between what is perceived as legitimate ethical concerns to be discussed in a public context within the scientific community and the legislative framework and within the public. In the following these different concerns will be described more closely. It should be noted that this categorization of the arguments that is based on examples from articles by scientists and advisory reports to legislative bodies, is a simplification of the debate that is necessary to stay within the limits of this article, but by no means an oversimplification. The examples have all been chosen because they illustrate something typically found within the literature, not because of their uniqueness.

3.1: Risks to humans

As mentioned above, two themes surface almost permanently when people within the legislative framework and scientists express the ethical problems that have to be taken into account regarding the cloning of animals. A: What are the risks to human health? and B: What are the risks to animal welfare?

It is evident that any technology that presents a risk to human health, either directly through effects on the human body or indirectly through effects on the environment and/or other living beings that then pose a risk to the human body, will have to be ethically evaluated. With regards to animal cloning the most obvious risks are either that the products from cloned animals will have unexpected effects in the human body compared to products from non-

cloned animals or that the cloned animals some how will cause change and damage to natural systems either on a species or ecosystem level.

The first kind of risks are currently under examination and a number of articles discussing the differences between products from cloned and non-cloned animals have been published in recent years (Tian et al., 2005, Norman & Walsh 2004, Takahashi & Ito, 2004, FDA 2003). The evidence so far shows no signs of risks to human health from products derived form cloned animals or their off-spring. Although the evidence so far is limited in size, it is noteworthy that it all points in the same direction. The biological compounds in these products do not deviate from products derived form non-cloned animals and therefore cannot be said to pose additional risks to human health.

The second kinds of risks – risks to the environment at large or to specific species that subsequently may harm humans – are harder to evaluate. Most scenarios concern the influence that escaped cloned animals might have on a natural population. But since most cloned animals will be both highly valuable and easy to contain in closed environment and it is not obvious how a cloned animal might harm a natural species by breeding its genes into it, this risk is often disregarded. It should be noted however that cloned animals that are genetically modified as well, will be able to introduce new genes into a natural species. In these scenarios where animals as mice, fish or pigs may escape from confinement and breed with a wild living population the foreseen changes will have to be described for each situation. Such studies have primarily been done on the consequences of genetically modified fish (salmon for instance) escaping and breeding with the natural population. Although estimates vary such a scenario could pose serious risks both to the affected wild living population and to human welfare (PEW, 2003).

Another risk related to this is the risk of losing genetic diversity within a certain species of animals. If cloning is used as a tool within the traditional breeding scheme and only a few animals with particularly desirable traits end up forming the basis for a whole species or sub-species of e.g. chickens, this group of animals will be more vulnerable to infectious diseases since they will share most of their genetic material. This could cause harm both to human welfare due to economic losses and to animals due to their sufferings (GAEIB, 1997, FAWC, 1998). Most scientists do not, however, believe that such a scenario is realistic, since it is clear from the outset to all involved stakeholders that a breeding programme that does not protect the genetic diversity within a species will be a mistake. On the contrary cloning is often seen as a method to increase genetic diversity by enabling breeders to bring back rare or extinct species (Ryder, 2002).

3.2: Risks to animals

I turn now to the risks relating to animal cloning that concerns the welfare of the cloned animals and their off-spring. First of all it should be noted that the concept of animal welfare itself is a complex concept where opinions about what to take into account differ among scientists (Olsson & Sandøe, 2004). In this article only the most prominent concepts will be discussed.

Discussions of animal welfare are mostly focused on the experiences of the individual animal. This means that not only broader subjects as species and ecosystems falls outside this discussion (except in cases where the way these subjects are treated do harm to an individual

animal) but also that the animal itself is outside the scope of the discussion until it reaches a state where it is capable of having experiences. Gametes, blastocysts, foetuses etc. are not able to experience anything (until a certain stage) and they are therefore only of interest to the degree that the treatment of them affects animals that are able to have experiences.

The second distinction is drawn between what can be called a hedonistic and a perfectionist perspective. The first perspective focus on the individual animal and its subjective experiences of pain and pleasure where as the other also includes questions about what kind of pain and pleasures that the animal experiences. To the first perspective the only thing important is whether the animal in questions feels pain or pleasure, regardless of what causes these experiences whereas the other perspective takes into account whether these experiences can be said to be a natural part of the animal's life, thus entailing an objective understanding of what constitutes a natural (and thereby ethically good) life.

To the hedonistic perspective there is nothing wrong in denying the animal the possibility to follow its instincts as long as this does not affect the welfare of the animal – lead to negative experiences. What is central to hedonism is the presence of pleasant mental states and the absence of unpleasant ones (Appleby & Sandøe, 2002). How these mental states are brought about is not interesting. Rarely is it the case though, that one can deny an animal the possibility to follow its instincts without causing suffering to the animal, but through breeding (either conventional or cloning/GM) changes can be induced in the animal that will make it more fit for the conditions under which it will have to live. And since there are no negative consequences to the individual animal – it will not have painful experiences, such a use of breeding technology is seen as unproblematic. This means that for instance the welfare problems caused by battery cage egg production could be solved through breeding chickens that did not suffer because of these conditions rather than changing the conditions (Rollin 1995).

To the perfectionist such a practice raises questions about what the natural life of a chicken is and what experiences that constitutes such a life. In stead of changing the chicken the perfectionist would look for ways in which to allow the chicken to fulfil its natural potential as much as possible through changes in the production system. And even though these changes may cause new painful experiences to the chicken, these are preferable to the painful experiences in the battery cage or indeed even to a life as battery cage chicken where the pain caused by the frustrated instincts have been lessened or even removed through breeding. Life as free ranging chicken is obviously less protected than life as a battery hen. Disease, feather picking and cannibalism occur frequently within a flock of chickens (Kjær & Sørensen, 2002). But to the perfectionist this is a preferable situation, since the chickens are living much closer to their natural state.[1] This rough picture of the different positions within the animal welfare debate hardly do justice to the participants, but it is only meant as a way of making some distinctions crucial to this article.

What is most noteworthy about the ethical considerations that typically surface within a legislative and scientific perspective regarding the welfare of cloned animals is that they are

[1] One can of course argue that by changing the animal genetically, one creates a new kind of animal that has a different way of living a natural life than its "parents". What is required then is to respect the naturalness of this new animal, which in this could be to put it in a battery cage (Rollin 1995). This way of thinking is however very much at odds with the immediate understanding of the concept of naturalness and we will not discuss it further in this context.

limited to hedonistic concerns. Only the actual pleasant or unpleasant experiences of individual animals are usually taken into account or even mentioned. This means that for instance the extremely low success rate in cloning only attracts ethical interest when it comes to the animals born with different problems. The huge number of fertilized eggs that never develop into a foetus, still less a born animal, that is able of feeling pain, is only seen as a technical problem that limits the usefulness of the technology. And furthermore the discussion of the naturalness of the cloned animal, in the cases where it actually is born and lives, is either not mentioned or dismissed (Galli et al 2004, Faber et al. 2003, Seamark 2003, NAS 2002).

What is mentioned as an ethical problem is the unpleasant experiences that the technology produces both in donor animals and the clones themselves. Depending on the species used different degrees of invasive measures are necessary to insert the cloned embryos in the incubator-animal and, in the case where unfertilized egg-cells are needed from living animal, invasive procedures are necessary too. This, however, is no different from the problems encountered in other breeding technologies such as AI and embryo transfer etc. These problems are therefore seldom discussed but just acknowledged as a necessary evil that must be accepted, if breeding technologies are to be used (NAS 2002) – if they are mentioned at all. Concerning the cloned animals themselves a number of welfare problems are mentioned that can be gathered under the heading *LOS - Large Offspring Syndrome*. These include: placental abnormalities, fetal overgrowth, prolonged gestation, stillbirth, hypoxia, respiratory failure and circulatory problems, lack of post-natal vigour, increased body temperature at birth, malformations in the urogenital tract (hydronephrosis, testicular hypoplasia), malformations in liver and brain, immune dysfunction, lymphoid hypoplasia, anaemia, thymic atrophy and bacterial and viral infections. The reasons for these problems are still poorly understood, but are usually seen as effects of both the manipulation of the egg necessary to produce the cloned embryo and as effects caused by the fact that the fertilized egg is matured in vitro. The last assumption is supported by the fact that most of the described effects can also be seen in other reproductive technologies (Cloning in Public 2005).

These welfare problems for the cloned animals are usually described as non-acceptable both from an ethical and a economic point of view, but almost always seen as problems that can be solved through research and experiments that will lead to a mastering of the technology. The ethical questions raised by animal cloning can thus be solved through technological progress.

A very brief summary of the character of the ethical problems as seen from a scientific and legislative perspective could be the quotation that is used in the preface of the 2002 National Academy Sciences report on animal biotechnology: *What we have before us are some breathtaking opportunities disguised as insoluble problems.*[2] The main focus is on physical risks to humans and animal welfare and these risks are basically seen as technological challenges that, given research and time, will be solved. The view is that the technology itself is acceptable and can be used for human purposes that will justify any remaining animal welfare problems – but these should be diminished as much as possible.

[2] John W. Gardner, 1965, upon appointment as the Secretary of the Department of Health, Education, and Welfare (NAS 2002, p. ix).

4: Animal cloning and ethics – the public perspective

Turning to the public perspectives on ethics and animal cloning I will attempt to draw as general picture of the attitudes as done above by pinpointing some concerns that typically show up in both quantitative and qualitative studies of public attitudes towards cloning of animals.

Several tendencies in these studies are worth mentioning. First of all it is important to notice that to the public there is not a huge, if any, difference between genetically modifying and cloning an animal. This lack of differentiation sometimes causes confusion since people coming from the scientific perspective know about the differences in the applied technology. This can very easily, as mentioned above, lead to the idea that if just the public was more informed, the ethical questions would somehow disappear. The notion of such a knowledge gap is probably behind most calls for more information to the public and is often mentioned as the main reason for the hesitation of the public towards biotechnology when scientists are asked. Several studies suggest, however, that information in itself will not make the technology more acceptable to the public. In repeated quantitative studies on a European scale it has been shown that the level of knowledge within a population does not affect the attitudes towards the technology. Denmark, for instance, has one of the most informed populations in Europe (at least when it comes to biotechnology) but nonetheless one of the most critical towards it (Hansen et al. 2003).

A more fruitful way of interpreting the public lack of interest in the specific technological methods applied could be to realise that the importance of the technology is more paramount to a scientific understanding of the procedures than to an ethical reflection upon them. Roughly speaking it does not matter how the animal was manipulated and which technologies were involved, to raise a series of ethical questions about the notion of using biotechnology on animals at all. It has importance, of course, to the way that the risk analysis of e.g. eating products from cloned animals are carried out,[3] and it has importance if the ethical questions raised are about the problems of creating animals with genes from other species in their genetic material, but if the ethical questions raised concern questions about integrity, naturalness etc. then the actual technology involved may not be that important. This does not mean that a basic knowledge of the technologies involved is not preferable, but sometimes its importance might be overemphasized from the scientific perspective and lead to a monologue of information from the scientific side rather than a dialogue between scientists and the public (Hansen 2003).

Another distinctive tendency is that the reasons for using the technology are very important to the public. Almost all studies show that there is a sharp distinction between biotechnologies used for agricultural purposes and for medical purposes – the latter being much more acceptable. Thus an evaluation of the perceived usefulness of the technology plays an important role in shaping the attitudes of the public. The usefulness of developing new medical treatment is generally perceived to be much higher than the usefulness of increased agricultural production. But also within this clear divide it is visible that the use of biotechnology on animals is perceived as problematic. As a consequence medical applications that involve the use of animals command only low levels of support (Lassen et al. 2005). What

[3] Although the American National Academy of Science to a large degree actually suggest using the same methods applied to products from genetically engineered animals (NAS 2004).

should be learned from this is partly that the concept of usefulness is broader in the public context than within a legislative and scientific context where usually all applications that are economically viable are seen as useful and that even what is acknowledged as useful from a public perspective might still not be supported due to other reasons.

One of these reasons are that although the notions of risks to human health and animal welfare are not absent in the public mind, these concerns are of a more multidimensional kind than found in the more scientific understanding of these notions. Research into risk perception has shown that the idea that what is happening is a clash between "expert" rationality and "lay" irrationality is neither a helpful or accurate understanding of the situation (Hansen et al. 2003). Usefulness and risk are both concepts that are framed by more general understandings of life, the universe and everything. So what is seen as useful or an acceptable risk from a narrow scientific perspective might not look the same from a broader, more multidimensional perspective.

Looking for instance at the risk assessment literature on animal cloning it is clear that it is of a very technical nature and mainly deals with the composition of products derived from cloned animals and/or their off-spring whereas other more complex and social questions as the economic effects cloning might have in the agricultural sector if widely implemented by the breeding industry are not mentioned (Galli et al. 2004) or seen as beyond the reach of scientific research (NAS 2004). And while it is true that the potential influence of large scale cloning of breeding animals on the agricultural sector must be evaluated in a much different way than the influence of a certain animal protein on the human body, there exists nonetheless a risk that cloning might concentrate the economical control of the industry in very few hands. And that is by some people seen as a risk that is just as relevant as the physical one. When people object to a certain technology they may not just be irrational but simply have different values and interest that cannot be addressed by science – at least not in the same way as the physical risks are.

Regarding animal welfare it should also be made clear that although the concept of naturalness does not play any role within a hedonistic perspective, it is nonetheless an important concept within the public anxiety about the cloning technology that can be said to contain a kind of perfectionist perspective in this regard. An illustration of this is the following Eurobarometer survey from 1999 about animal cloning. Participants were asked to assess 12 statements about cloning on a five point scale, where 5 = 'strongly agree' and 1 = 'strongly disagree'. The two most strongly supported statements concerned the perceived unnaturalness of cloning. Thus the average score of the statement "even if animal cloning has advantages, it is basically against nature" and "animal cloning threatens the natural order of things" scored 4.24 and 4.12 respectively. Similarly most agreed (average score 3.85) that animal cloning was simply not necessary, and most disagreed with the statements that animal cloning presents no danger to future generations (average 2.19) and that the risks involved in animal cloning are acceptable (average 2,28) (INRA 2000).

There are many aspects to the concept of naturalness. One of the most important is that it can not be reduced to scientifically quantifiable data. Rejections of the notion of naturalness as expressed in the above statements by either pointing to the fact that monozygotic twins are sort of natural clones or pointing to the unnaturalness of animals produced by other reproductive technologies or pointing to the unnaturalness of domesticated animals in general (Seamark 2003, Rollin 1995) are therefore, although scientifically correct, missing the point.

The debate about animal cloning could even have the opposite effect and make people more aware of, and critical of, other reproductive technologies used within animal breeding (Lassen et al. 2005).

Naturalness is evidently a multi-dimensional concept, like risk and usefulness. In the literature it is usually treated as an independent subcategory called "other moral concerns", unless the unnaturalness is seen as a risk to human health or the environment. This category is often described as concerns that are based on feelings, religion, intuitions, metaphysics or other ways of reflection that seen from a scientific perspective often seem irrational or at least beyond the reach of science (NAS 2002). Thus concepts as "inherent value", "naturalness", "integrity", "playing God" etc. are gathered together and implicitly (sometimes even explicitly) treated as being not only different from the other concerns, but also somehow less subjects of consideration. That this division is mostly a reflection inherent in the legislative and scientific perspective and hardly captures the complexity of the public understanding of the ethical problems is one thing, quite another is that it often leaves aside the concerns about naturalness and integrity as irrational concepts that cannot be discussed. And although it is true that there is very little to say about these concepts from a scientific perspective, this hardly validates the exclusion of them from the discussion. The scientific perspective is but one of the perspectives that it will be necessary to employ to figure out how to understand animal cloning in an ethically responsible way. As the British philosopher Mary Midgley has pointed out, the idea of having different perspectives on the world is not to have them competing for supremacy, but to add different dimensions to our understanding of it (Midgley 2000).

That concepts as naturalness or integrity are foreign to the scientific perspective does not mean that they are irrational or inappropriate subjects of a rational discussion – unless one equates rationality with scientific methodology. Naturalness can thus be understood as a concept that tries to capture the distinction between the familiarity of the animal that is expressed through our understanding of its usefulness to humans and our understanding of it as something alien and independent that cannot be reduced to merely a mean to our ends. Implicit in this distinction is a notion of the amount of control over the animal that we can exercise without violating its naturalness or integrity. In this sense naturalness can be understood as the opposite ideal to a total commodification of the animal as a mere natural resource. Although these notions are hardly scientific in nature they are neither irrational or necessarily build upon elaborate religious or philosophical systems. Rather they are ways of expressing very basic experiences of animals as something more than a biological machine to fulfil our needs.

What is lacking today is not knowledge about the importance that concerns of the e.g. unnaturalness of cloning plays in the public evaluation of the technology (NAS 2002), but a more complex and nuanced explication of such concepts that can feed into the dialogue and prevent the false dichotomy between the "informed" science perspective and the "irrational" public perspective. What is needed is basically a more multidimensional understanding of the concept of rationality that can facilitate a broader dialogue on the ethics of animal cloning in the public sphere.

5: Why have a dialogue?

To call for a dialogue on the ethical aspects of biotechnological applications – in this case animal cloning – is not very original. Since the marketing of and subsequent rejection by consumers of genetically modified food in the 1990´ies it has been the standard solution to the "problem" of the lack of enthusiasm (to put it mildly) in the European public. Calls for and different attempts to promote dialogue between science, industry, legislative authorities and the public have been initiated both on national and regional levels in Europe and the recommendation to promote dialogue is present in almost all official reports (Nielsen et al. 2005).

The notion of having a dialogue about new technologies on a societal base involving different stakeholders is hard to argue against. But one should be very aware that dialogue (as risk, usefulness and naturalness) is a complex concept that changes according to the purpose of the pursued by initiating it. The content of the concept of dialogue can very generally speaking be said to be decided by the reasons for supporting the idea in the first place. There seems to be a continuum from those that support dialogue because it is a way of ensuring the public some sort of democratic or semi-democratic influence on the way that new technologies are supported through research-funding and in the application phase to those who see public participation as a way of legitimizing the technologies in the eyes of the public. In the first case the goal is to live up to some democratic ideals of some sort without influencing the result of the participation whereas in the other case the whole point is to get the technologies accepted.

In the real world the motivation for seeking public participation is seldom a clear-cut case, placing the motivation somewhere in between the two extremes in the continuum. But as a rule of thumb it is fair to say that the closer one is to be motivated by democratic ideals rather than being motivated by interest in having the technologies accepted, the more content of a meaningful kind can be placed in the concept of dialogue. This has most clearly been seen in the above mentioned case of the notion of the knowledge gap. As stated this notion is rooted in the belief that the reason for public rejection of technology is a lack of information. A firm belief in this direction usually leads to a belief that the concept of dialogue can be transformed into a monological information stream that will result in wider acceptance of a given technology.

This extreme seems to be, although stubbornly reoccurring from time to time, about to go extinct. Instead another extreme form of dialogue might be forming; a dialogue that is as monological as the first, but that has the public rather than the scientists doing all the talking. It consists of different stakeholders (especially legislative bodies / industry) believing that the way to have a dialogue is to ask the other person what he wants, and then give it to him. So when facing a technological development where it is not foreseeable how the public will react, the way to use dialogue is to ask the public (typically through quantitative polling) what kind of development they want and then try to bring that development about. This approach basically turns dialogue into marketing research. It can of course, within this understanding of what constitutes a dialogue, always be discussed how high a level of knowledge it would be preferable that the public has on the subject - whether they should just be polled about their top-of-the-head opinion or if they should be offered some kind of chance to actually deliberate the questions in hand. But in the end it will still just be marketing research, done more or less eloquently and sophisticated.

What I would like to suggest is that concept of dialogue implies that there are two or more different opinions about something and that the people holding these opinions are willing to discuss them, holding a small window open in the back of their minds to the possibility that they may be wrong. A dialogue where it is from the outset decided that one of the parties is either uninformed or should, no matter what the arguments, be submitted to due to its political (voters) or economical (consumers) influence, is by all standards not a dialogue but more like either religious proselytizising or, as I said, marketing.

In conclusion it should be remembered that going into dialogue with the public may not only be complicated in respect to A: How one is to identify "the public" that may consist of several ethical communities, B: How to actually find ways in which it is possible to hear what opinions it or they may have and C: What opinions to count as informed/real/serious in the end. It may also be difficult in respect to the answers one give to the question of why we should have a dialogue at all.

The objectives of dialogue should neither be over-stated. What is characteristic of dialogues is that they bring attention to and deeper understanding of the arguments of the counterpart. Ideally a dialogue on animal cloning could thus lead to a deeper understanding of the notion of naturalness and other moral concerns on the side of the policymakers/legislative bodies/industry and scientists, whereas the public might better understand the technologies involved, the realistic prospects of the technology and the positive and negative effects of the technology on human health and animal welfare. But this deeper understanding of the perspective and arguments of the counterpart in the dialogue does not necessarily lead to a consensus. Hopes that a real dialogue on the ethics of animal cloning could lead to a general agreement on how to ethically evaluate the technology will thus be disappointed. What could be the end-result, however, could be a socially robust regulation of the technology where some applications are accepted and others rejected and – most importantly – where even the opponents of the accepted applications would feel that their concerns and worries had been taken seriously. This is, I think, a both worthy and obtainable goal within the kind of democratic societies that exist in the western world today. And it is, furthermore, perhaps the only way to ensure that certain applications of biotechnologies such as animal cloning will eventually be generally accepted as part of the solution to some of the problems that contemporary societies face.

6: Conclusion

In this article I have described some of the major ethical questions that the cloning of animal raises by describing typical concerns within two very distinct perspectives – the scientific and the public. I have shown how the scientific perspective is mainly focused on assessing risks to human health and individual subjectively experienced animal welfare whereas the public perspectives contains concerns that can be described both as more multidimensional and far-reaching, both when it comes to human health and animal welfare but also with regards to concepts as naturalness and integrity. These concepts are then shown not to be necessarily an expression of irrationality or a lack of information but more an expression of a different ethical perspective that is informed by a broader concept of rationality than a typical scientific perspective.

Finally I describe how the societal context of today necessitates ethical dialogue on issues as animal cloning and discuss different notions of dialogue currently initiated to find ways of gaining acceptance of the technology or inform regulation of it. Two common interpretations of the concept of dialogue are rejected. First the notion of a knowledge gap between the scientific perspective and the public that leads to a monological situation where science just informs the unknowing masses. Second the notion of dialogue as a marketing device by which public acceptance is sought by accommodating to its wishes, is rejected. I suggest instead that all parties should enter the dialogue with the intent of seeking a deeper understanding of each other's perspectives thus facilitating a nuanced regulation of the technology where all participants are heard and a socially robust solution to the ethical questions that animal cloning raises, sought.

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FOLLICULAR SELECTION IN FARM ANIMALS: REGULATION MECHANISM OF GRANULOSA CELL APOPTOSIS DURING ATRESIA

MANABE N., MATSUDA-MINEHATA F., GOTO Y., INOUE N., MAEDA A., CHENG Y., LI J.

Research Unit for Animal Life Sciences, Animal Resource Science Center, The University of Tokyo, 3145 Ago, Ibaraki-Iwama 319-0206, Japan.
E-mail: amanabe@mail.ecc.u-tokyo.ac.jp

Summary: More than 99% of follicles undergo a degenerative process known as "atresia", in mammalian ovaries, and only a few follicles ovulate during ovarian follicular development. We have investigated the molecular mechanism of selective follicular atresia in mammalian ovaries, and have reported that follicular selection dominantly depends on granulosa cell apoptosis. However, we have little knowledge of the molecular mechanisms that control apoptotic cell death in granulosa cells during follicle selection. To date, at least five cell death ligand-receptor systems [tumor necrosis factor (TNF)α and receptors, Fas (also called APO-1/CD95) ligand and receptors, TNF-related apoptosis-inducing ligand (TRAIL; also called APO-2) and receptors, APO-3 ligand and receptors, and PFG-5 ligand and receptors] have been reported in granulosa cells of porcine ovaries. Some cell death ligand-receptor systems have "decoy" receptors, which act as inhibitors of cell death ligand-induced apoptosis in granulosa cells. Moreover, we showed that the porcine granulosa cell is a type II apoptotic cell, which has the mitochondrion-dependent apoptosis-signaling pathway. Briefly, the cell death receptor-mediated apoptosis signaling pathway in granulosa cells has been suggested to be as follows. (1) A cell death ligand binds to the extracellular domain of a cell death receptor, which contains an intracellular death domain (DD). (2) The intracellular DD of the cell death receptor interacts with the DD of the adaptor protein (Fas-associated death domain: FADD) through a homophilic DD interaction. (3) FADD activates an initiator caspase (procaspase-8; also called FLICE), which is a bipartite molecule, containing an N-terminal death effector domain (DED) and a C-terminal DD. (4) Procaspase-8 begins auto-proteolytic cleavage and activation. (5) The auto-activated caspase-8 cleaves Bid protein. (6) The truncated Bid releases cytochrome cfrom mitochondrion. (7) Cytochrome c and ATP-dependent oligimerization of apoptotic protease-activating factor-1 (Apaf-1) allows recruitment of procaspase-9 into the apoptosome complex. Activation of procaspase-9 is mediated by means of a conformational change. (8) The activated caspase-9 cleaves downstream effector caspases (caspase-3). (9) Finally, apoptosis is induced. Recently, we found two intracellular inhibitor proteins [cellular FLICE-like inhibitory protein short form ($cFLIP_S$) and long form ($cFLIP_L$)], which were strongly expressed in granulosa cells, and they may act as anti-apoptotic/survival factors. Further *in vivo* and *in vitro* studies will elucidate the largely unknown molecular mechanisms, e. g. which cell death ligand-receptor system is the dominant factor controling the granulosa cell apoptosis of selective follicular atresia in mammalian ovaries. If we could elucidate the molecular mechanism of granulosa cell apoptosis (follicular selection), we could accurately diagnose the healthy ovulating follicles and precisely evaluate the oocyte quality. We hope that the mechanism will be clarified and lead to an integrated understanding of the regulation mechanism.

Key words: *Apoptosis, cell death ligand, cell death receptor, follicular atresia, granulosa cell, porcine ovary.*

Introduction

In mammalian ovaries, during growth and development of the oocytes, which are enclosed and mothered by granulosa cells in follicles, less than 1% of oocytes are selected and the remainder are removed. The total number of ovulations, which is genetically regulated and called the "ovulation rate", is an important parameter of the reproductive efficiency of farm animals, especially of sows. Although the ovulation rate is a major limiting factor in determining the number of offspring born, numerous additional factors act upon the uterus and conceptuses throughout gestation and contribute to the number of healthy fetuses that develop to term. The process of follicular atresia is as follows. (1) Scattered pyknotic nuclei (apoptotic bodies) are seen in the granulosa cells. (2) Detachment of the granulosa layer from the follicular basement membrane, whose structure is not destroyed, is observed. (3) Then, fragmentation of the basal membrane begins. (4) Cell debris and macrophages, which invade from the stromal area of the ovary, are seen in the antrum of the follicle. (5) Finally, the follicle disappears. In granulosa cells of atretic follicles, increase expression of some mRNAs is seen while reduced synthesis of DNA and protein is noted. At the early stage of atresia, endocrine cells of the theca interna layer undergo hypertrophy, the oocyte undergoes meiosis-like changes, followed by oocyte fragmentation, and disruption of the oocyte-cumulus connection. The morphological changes in granulosa cells at the earliest stage of atresia are due to apoptosis, which is also confirmed by a biochemical hallmark of apoptosis, DNA fragmentation multiples of approximately 180 bp. Moreover, there are species-specific differences in the detailed characteristics of granulosa cell apoptosis, for instance, in the localization of apoptotic cells in the granulosa and theca interna layers during atresia. Here, we mainly focus on the follicle selection which is regulated by discriminating atresia of growing follicles, and describe the regulation factors that affect follicular atresia. A brief description of how some factors might regulate atresia or follicular development is given. The present review discusses some recent observations in our laboratory concerning the cell death receptor, decoy receptor and intracellular apoptosis-regulating protein roles in the regulation of follicle selection in porcine ovaries.

Follicular Development and Atresia

During embryogenesis, primordial germ cells migrate from the yolk sac through the dorsal mesentery of the hindgut to the genital ridge. The germ cells undergo extensive proliferation, and somatic cells derived from the mesenchyme of the genital ridge also proliferate. Then the somatic cells, called follicular epithelial cells (granulosa cells), enclose the germ cells to form the primordial follicles. After mitosis (somatic cell division), the first meiotic division begins in the germ cells (primary oocytes). The primary oocyte becomes arrested in the diplotene stage of meiosis, until the surrounding follicle leaves the primordial stage (primordial follicles), and starts to grow to reach ovulation. Approximately 5 million primordial follicles are present in both ovaries 10 days after birth in sows (1.2, 1.1, 4 and 1 million primordial follicles in cows, sheep, women and mice, respectively). During fertile life in sows, at the most 1,600 oocytes (less than 0.14% of total primary oocytes) will ovulate, and all others will disappear. Such a low success rate is considered the result of an important selection process for quality of oocytes. In adult sows, primordial follicles are not stimulated to grow and develop at the same time, and only a small number of primordial follicles begin their development, while the remainder remain quiescent. Initiation of follicular growth involves endocrinological actions, mainly follicular stimulating hormone (FSH), and local modulating factors from granulosa cells and endocrine cells of the theca interna layer and from

the growing oocytes. The early growth stage of primary follicles (follicles with mono-layer follicular epithelial/granulosa cells) and/or secondary follicles (follicles with stratified granulosa cells but without antrum) is characterized by a dramatic increase in proliferation of granulosa cells (rapid increase in number and size). Subsequently, granulosa cells separate from each other resulting in the formation of the follicular antrum (tertiary follicle). In the tertiary follicles, cumulus cells are differentiated from granulosa cells and surround the primary oocyte, which has germinal vesicle (GV). Due to a large increase in proliferation of granulosa cells and an increase in the size of the antrum, growth rates of the tertiary follicles show an exponential curve. In the oocyte (secondary oocyte), meiosis restarts (GV disappearance: called GV breakdown), and the first polar body divides. Finally, selected follicles burst, and the secondary oocytes ovulate. Recent studies in FSH receptor (FSHR) knock out mice and experimental rodents with hypophysectomy showed that FSH is essential for antrum formation in secondary follicles and post-antral follicular development in tertiary follicles, and that FSH acts as an accelerator and a survival factor for the follicles. In gilts, FSH administration induces follicular recruitment and increases the ovulation rate in a dose-dependent manner. Active immunization against inhibin, which is produced by granulosa cells and inhibits FSH excretion, increased the number of ovulations. The number of recruited follicles might depend on blood FSH levels and/or on the number of follicles responsive to FSH present at the time of recruitment. Unfortunately, we have insufficient knowledge of the number of FSH-responsive follicles related to the endocrinological event occurring before recruitment, or what other factors might influence it, and how follicles are accurately selected during follicular growth and development.

Preparatory to describing the cell death ligand, its receptor and intracellular signaling systems in porcine ovaries, here we briefly mention the outline profiles of growth, development and atresia of the follicles. Primary follicles require 84 days to grow to the tertiary stage, and an additional 19 days are necessary to grow up to the ovulatory size, approximately 10 mm in diameter. The process of follicle selection during the tertiary stage growth and development probably takes place continuously from days 13 to 14 of the estrous cycle to ovulation (day 21 of the estrous cycle). During the final maturation stage, the growth rate of tertiary follicles from 3 to 10 mm in diameter is approximately 1.14 mm/day, and the endocrine cells of the theca layers and granulosa cells secrete significant amounts of steroid hormones, peptide hormones, prostaglandins and other physiologically active substances, which participate locally in follicular growth, development and atresia and act as coordinators of the hypothalamic-hypophyseal-ovarian axis. On day 16 of the estrous cycle, approximately 160 to 200 tertiary follicles/ovaries are present, and then 150 to 190 follicles degenerate and disappear from the ovaries through the process of atresia. Atresia may occur at any time during growth and development of follicles, but most follicles disappear before reaching 6 mm in diameter in sows. On day 21 of the estrous cycle, secondary oocytes are ovulated from approximately 10 matured follicles, which are appropriately selected. Within each mammalian species, the ovulation rate is regulated within a relatively narrow range, and changes in rates of follicular atresia may alter the number of ovulated follicles. In pigs, the Meishan sow, a traditional Chinese breed, has a higher ovulation rate and a larger number of piglets than conventional European breeds. On day 16 of the estrous cycle, Meishan sows have more tertiary follicles than Large White sows, and the number of atretic follicles, which disappear from days 16 to 19 of the estrous cycle, is larger in Large White sows than in Meishan sows. Thus, the greater ovulation rate in the Meishan breed is related to differences in both follicular recruitment and atresia. Our preliminary study in Mangalica sows, which are native Hungarian pigs and have a lower ovulation rate and a smaller number of piglets (approximately 5) than

conventional European breeds, showed that the lower ovulation rate in the Mangalica breed is also related to differences in both follicular recruitment and atresia.

Apoptosis in Granulosa Cells

During follicular growth and development in sows, more than 99% of follicles selectively disappear. Follicular atresia is primarily induced by granulosa cell apoptosis, which is characterized biochemically (internucleosomal DNA fragmentation) and morphologically (cell shrinkage, plasma membrane blebbing and formation of apoptic bodies) [6-14, 42-46]. Although considerable progress has been made in understanding the regulation mechanisms of apoptosis in granulosa cells during follicular atresia, the description of the apoptotic pathway in granulosa cells has not been completed. Apoptotic stimuli and intracellular signal transduction pathways involved in granulosa cell apoptosis remain to be determined, and many investigators have been studying which trigger molecules induce granulosa cell apoptosis, and how intercellular apoptotic signals are transmitted in the granulosa cells. Recently, some cell-specific cell death ligands and cell death receptors were found by the researchers working in the fields of immunology and cancer therapy. The major intracellular signaling pathway leading from the cell death ligand binding the cell death receptor to initiation of apoptosis is the caspase cascade system. Each cell death receptor activates a specific caspase cascade within seconds of cell death ligand binding, causing apoptotic cell death within hours. To date, at least five cell death ligand-receptor systems [tumor necrosis factor (TNF)-α and receptors, Fas ligand and receptors, TNF-related apoptosis-inducing ligand (TRAIL) and receptors, APO-3 ligand and receptors, and PFG-5 ligand and receptors] have been reported in granulosa cells of porcine ovaries. Some cell death ligand-receptor systems have "decoy" receptors, which binds with ligand and act as extracellular inhibitors against cell death ligand-induced apoptosis in granulosa cells. Moreover, we showed that the porcine granulosa cell is a type II apoptotic cell, which has the mitochondrion-dependent apoptosis-signaling pathway. The cell death receptor-mediated apoptosis signaling pathway in porcine granulosa cells has been suggested to be as follows. (1) A cell death ligand binds to the extracellular domain of a cell death receptor, which contains an intracellular death domain (DD); (2) the intracellular DD of the cell death receptor interacts with the DD of the adaptor protein (Fas-associated death domain: FADD) through a homophilic DD interaction; (3) FADD activates an initiator caspase (procaspase-8; also called FLICE), which is a bipartite molecule, containing an N-terminal death effector domain (DED) and a C-terminal DD; (4) procaspase-8 begins auto-proteolytic cleavage and activation; the activated caspase-8 cleaves Bid-protein, and the truncated Bid releases cytochrome c from mitochondrion. Cytochrome c and ATP-dependent oligimerization of the apoptotic protease-activating factor-1 (Apaf-1) allows recruitment of caspase-9 into the apoptsome complex. Activation of caspase-9 is mediated by means of conformational change. The activated caspase-9 cleaves downstream effector caspases (caspase-3), and apoptosis is induced. Recently, we found two intracellular inhibitor proteins (cellular FLICE-like inhibitory protein short form and long form: $cFLIP_S$ and $cFLIP_L$, respectively), which were strongly expressed in granulosa cells and may act as anti-apoptotic/survival factors. Thus, porcine granulosa cells have some cell death ligand and receptor systems, however, as described below, their precise roles on the regulation of follicle selection have yet to be determined. Further *in vivo* and *in vitro* studies are necessary to elucidate which cell death ligand and receptor system is dominant in controling the granulosa cell apoptosis.

Interestingly, specific changes in glycoconjugates of cell surface membrane-glycoprotein, which act as regulators for phagocytosis in neighboring granulosa cells in atretic follicles, have been demonstrated in granulosa cells of atretic follicles. Expression of kinase cascades regulating entry into cell death and proliferation was also reported in them. Thus, the degeneration of atretic follicles in mammalian ovaries can be explained by apoptotic cell death of granulosa cells and endocrine cells of the theca interna layer, but the degenerating changes in cumulus cells during follicular atresia had not been investigated in detail. We examined them histologically, cytologically and biochemically and confirmed that apoptosis occurs in granulosa cells, but not cumulus cells or oocytes in tertiary follicles in the early stage of atresia, when no macrophages were detected. DNA fragmentation was visualized on ovarian sections using the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method, and cytological changes were determined by transmission electron microscopy (TEM). In healthy follicles, no apoptotic cells were observed among granulosa or cumulus cells, or the cells in internal or external theca layers, or oocytes. In the early stage of atresia, apoptosis was detected in scattered granulosa cells located on the inner surface of the follicular wall, but not in cumulus cells, oocytes or the cells of internal or external theca layers. Condensed nuclei, a typical apoptotic feature, were seen in scattered granulosa cells, but cumulus cells and oocytes with normal ultrastructure were seen in the same follicle. In the late stage of atresia, granulosa cells scattered on the inner surface of the follicular wall began to undergo apoptosis, but no TUNEL-positive cells were detected among the cumulus cells or oocytes. Finally, in the progressed stage of atresia, many apoptotic cells were located in the area abutting the basement membrane, which was partly broken, and macrophages had invaded the follicular antrum and ingurgitated the apoptotic granulosa cells and cell debris.

Our comparative studies of the progression of granulosa cell apoptosis during follicular atresia revealed that there are species-specific differences in the apoptotic process in granulosa cells. In ovaries of rodents (rats and mice), randomly scattered apoptotic cells were observed in granulosa layers of follicles at the earliest stage. In bovine ovaries, granulosa cells located on the outer surface of the follicular wall appeared to undergo apoptosis at the earliest stage of atresia, followed by neighboring granulosa cells.. In porcine ovaries, however, granulosa cells located on the inner surface of the granulosa layer appeared to undergo apoptosis in the earliest stage of atresia. In the ovaries of these mammalian species, detachment and degeneration of the granulosa layer, fragmentation of basement membrane, apoptotic endocrine cells in the theca interna layers were observed at the progressed stage. No apoptotic cells were observed in the theca externa layers during the early to middle stages of atresia. Thus, we concluded that apoptosis occurring in granulosa cells is an initial symptom of atresia in mammalian ovaries, but the initiation areas of granulosa cell apoptosis are different among the species, indicating local mechanisms of regulation of granulosa cell apoptosis, in particular the apoptotic stimuli induction mechanism may be different among mammalian species. To date, however, we have insufficient information on the induction mechanisms of granulosa cell apoptosis.

Cell Death Ligand and Receptor Systems in Granulosa Cells

Fas Ligand and Fas system

Apoptosis eliminates individual cells when they are no longer needed or have become seriously damaged by viral infection, cancerous transformation, etc. Mammals have evolved a

signaling mechanism that actively directs cells to die by apoptosis. This process, which we refer to as instructive apoptosis, is critical particularly in the immune system. The most studied paradigm for instructive apoptosis is that of the Fas ligand (FasL) and Fas. FasL belongs to a family of proteins that have structural homology to TNF. FasL and TNFα define a subset of TNF-superfamily members that have apoptosis-inducing activity. FasL is predominantly expressed in activated T lymphocytes and natural killer cells and in several immune-privileged tissues and induces apoptosis in target cells through Fas. Fas, a transmembrane glycoprotein, belongs to the TNF/nerve growth factor (NGF) receptor superfamily and mediates apoptosis. Ligation of cell-surface Fas by FasL delivers an apoptotic signal that rapidly commits the cell to apoptotic cell death. *In vitro* studies show that FasL is critical for T-cell apoptosis. Furthermore, in mice or humans carrying spontaneous mutations in the genes encoding FasL or Fas, and in Fas gene knockout mice, accumulated lymphocytes resulting in a massive, lethal enlargement of lymph nodes have been observed. It is considered that the main biological role of the FasL and Fas system is to signal instructive apoptosis during peripheral deletion of lymphocytes. In addition, FasL and Fas-mediated apoptosis contributes to elimination of virus-infected cells and cancer cells by cytotoxic lymphocytes.

Our recent preliminary experiments indicated that constitutive expression of mRNAs of FasL and Fas estimated by conventional and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) methods, and FasL and Fas proteins assessed by Western blotting analysis, were detected in granulosa cells of both healthy and atretic follicles of porcine ovaries. Also, immunohistochemical staining for these proteins showed Fas localized in the cytoplasmic region but not in the cell-surface area in granulosa cells of both healthy and atretic follicles, and RT-PCR data showed constant mRNA expression of DcR3, a decoy receptor for FasL, in follicular cells of porcine ovaries. To date, we have not detected the expression of soluble decoy receptor (FasB), which is strongly expressed in functional luteal cells of murine ovaries and inhibits the induction of apoptosis, in follicular or luteal cells of porcine ovaries. Thus, we presume that the FasL and Fas system does not play a considerable role either in granulosa cell apoptosis during follicular atresia, or in luteal cells during luteolysis in porcine ovaries.

TNFα and TNF-receptor system

TNFα can induce both cell death and cell proliferation and exerts its effects by binding to either TNF receptor (TNFR)-1 or TNFR2. TNFα induces apoptosis in a variety of tumor cells, and the intracellular apoptosis signal pathway mediated by TNFR1 has been suggested to be as follows. (1) First, TNFα binds to the extracellular domain of TNFR1, which contains an intracellular death domain (DD). (2) The intracellular DD of the receptor interacts with the DD of the adaptor protein (TNF receptor-associated death domain protein: TRADD). (3) The DD of TRADD binds with the DD of another adaptor protein (Fas-associated death domain protein: FADD; also called MORT1). (4) FADD activates initiator caspase (procaspase-8). (5) Finally, the caspase cascade is activated for intracellular transduction of the apoptotic signal (named TNFR1-TRADD-FADD-caspase-8 signaling axis). In contrast, when TNFα acts as a survival/anti-apoptotic factor, the intracellular signal pathway mediated by TNFR1 has been suggested to be as follows. (1) TNFα binds to the extracellular domain of TNFR1. (2) The intracellular DD of the receptor interacts with the DD of TRADD. (3) The DD of TRADD binds with the DD of receptor interacting protein (RIP). (4) RIP interacts with TNF receptor-associated factors (TRAF) 2. (5) Then, TRAF2 mediates the physical interaction of the TNFR1-signaling complex with the nuclear factor (NF)-κB-inducing inhibitor of κB kinase

(IKK) and inhibitor of apoptosis proteins (cIAP) 1 (named TNFR1-TRADD-RIP-TRAF2 signaling axis). (6) Consequently expression of survival/anti-apoptotic genes is up-regulated. Thus, TRADD and RIP are key proteins at the point of divergence of cell death and cell proliferation in the TNFR1 signaling process, and TRAF2 is a good indicator of TNFα-dependent cell proliferation. When TNFα binds with TNFR2, which is a non-DD-containing TNF receptor. TNFα acts as a survival/anti-apoptotic and/or proliferating factor. The intracellular signal pathway mediated by TNFR2 has been suggested to be as follows [116-121]. (1) TNFα binds to the extracellular domain of TNFR2. (2) Although TRAF2 cannot bind directly to TNFR1 as described above, TRAF2 can interact directly with TNFR2. Thus, activated TNFR2 interacts with TRAF2. (3) NF-κB is activated. (4) Consequently expression of cell survival, growth and differentiation genes is up-regulated and apoptosis induced by TNFα is prevented. Thus, when TNFα bound TNFR2 interacts with TRAF2, expression of survival/anti-apoptotic/cell proliferation genes is up-regulated [121]. TRAF2 expression is considered to be a good indicator of TNFα-dependent cell proliferation in both TNFR1 and TNFR2 signaling cascades. Our data indicate that TNFα acts as a survival factor in granulosa cells during follicular atresia in porcine ovaries. Although the biological roles of TNFα and its receptor system in ovarian tissues are largely unknown, TNFα and TNFR2 system may dominantly contribute to the selective survival of necessary cells under physiological conditions. Our findings suggest that TNFα and its receptor system, TNFR2, but not TNFR1, dominantly detected in ovarian tissue, play crucial roles in induction of survival/proliferating signals in granulosa cells during follicular growth in porcine ovaries. Further studies are necessary to determine which molecular system dominantly regulates the disappearance of TNFα receptor-associated proteins in granulosa cells in the early stage of atresia, and which intracellular signal transduction pathway dominantly causes granulosa cell survival.

TRAIL and TRAIL-receptor system

Through the screening of DNA databases for expressed sequence tags similar to TNF□, TNF-related apoptosis-inducing ligand (TRAIL), a novel cell death ligand, was identified in 1995. The cDNA sequence of TRAIL is similar to that of FasL, and *in vitro* functions of TRAIL are also like those of FasL and TNFα. TRAIL mRNA is expressed constitutively in many organs/tissues. TRAIL interacts with receptors that belong to the TNFR superfamily. The receptors in the TNFR superfamily have several cysteine-rich domains in their extracellular N-terminal region. The cytoplasmic sequence divides the TNFR superfamily into two subgroups that either possess or lack a DD. The DD-containing receptors (called death receptors: DRs) include TNFR1, Fas, DR3, DR4, DR5 and DR6. The DD couples each receptor to caspase cascades that induce apoptosis or to kinase cascades that turn on proliferating gene expression through NF□B and AP-1. Recently, an interesting subgroup of TNFR-homologues was found and named as "decoy" receptor (DcR). It has extracellular domains similar to active receptors, binds to ligands and acts as an inhibitor, rather than a transducer of apoptotic signaling. To date, the DcRs in the TNFR superfamily include DcR1, DcR2, osteoprotegerin (OPG), DcR3 and PFG-6. DcR1, DcR2 and PFG-6 are cell-membrane proteins, and OPG and DcR3 are secreted/soluble proteins. Four of the novel cellular receptors bind to TRAIL and are structurally related. Each has two extracellular cysteine-rich domains, and shows closer homology to the other TRAIL receptors than to the rest of the TNFR superfamily. TRAIL binds to all of these receptors with equivalent high affinities. DR4 and DR5 have cytoplasmic DDs. DcR1 lacks a cytoplasmic region, and appears to be attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor. DcR1-bearing cells

pretreated with phosphatidylinositol specific phospholipase C (PI-PLC), which cleaves the GPI anchor and leads to removal of DcR1 from the cell surface, showed a marked increase in apoptosis induced by TRAIL, indicating that DcR1 has an inhibitory effect on TRAIL-induced apoptosis. DcR2 is also a cell-surface receptor and has a cytoplasmic DD, but it is two-thirds shorter than a typical DD of active receptors and does not signal apoptosis. The extracellular domains of DcR1 or DcR2 compete with those of DR4 or DR5 for ligand binding. *In vitro* studies show that cell transfection with DcR1 or DcR2 inhibits apoptosis induction by TRAIL. Thus, DcR1 and DcR2 compete with DR4 and DR5 for ligand binding and can act as decoys for DR4 and DR5, preventing TRAIL from inducing apoptosis through DR4 and DR5. Hence, cells that express DcR1 and/or DcR2 at high levels relative to DR4 or DR5 may use the decoys as protection against the cytotoxic action of TRAIL.

Our experiments were performed to determine the physiological roles of TRAIL and its receptor system on granulosa cell apoptosis during follicular atresia in porcine ovaries. Firstly, we histochemically examined the localization of TRAIL and its receptors in porcine ovaries. A marked reduction in the expression of DcR1, which has high affinity for TRAIL, was demonstrated in granulosa cells of atretic follicles, but no marked differences were seen in expression of TRAIL, DR4 or DR5 in granulosa cells between healthy and atretic follicles. No biochemically positive reaction against DcR2 protein and mRNA was detected. Secondly, we examined the changes in expression and localization of mRNA of TRADD, which transmits the death signal from receptors to intracellular components, in granulosa cells during follicular atresia. RT-PCR and *in situ* hybridization analyses revealed increased mRNA expression of TRADD in granulosa cells, demonstrated only in atretic follicles. Finally, to confirm the inhibitory activity of DcR1 in granulosa cells, primary cultured granulosa cells prepared from healthy follicles of porcine ovaries were treated with the enzyme, PI-PLC, to cleave GPI anchor of DcR1 and to remove DcR1 from the cell surface, and then incubated with TRAIL. PI-PLC treatment increased the number of apoptotic cells induced by TRAIL. Our findings indicate that TRAIL and its receptors (DR4, DR5 and DcR1) are involved in induction of apoptosis in granulosa cells during atresia, and that DcR1 plays an inhibitory role in granulosa cell apoptosis, at least in porcine ovaries.

Novel cell death receptor and decoy receptor system

We prepared some monoclonal antibodies against granulosa cells prepared from porcine ovarian follicles, which recognized a novel cell death receptor and decoy receptor as follows. Individual preovulatory tertiary follicles, approximately 3 mm in diameter, were dissected from porcine ovaries under a surgical dissecting microscope. Because the progesterone/estradiol-17β ratio of follicular fluid in each follicle provides a good index of follicular atresia in sows, when the progesterone/estradiol-17β ratio of follicular fluid quantified using (^{125}I)-RIA kits was less than 15, the follicle was classified as a healthy follicle. The granulosa layer was removed from each follicle and incubated in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution containing 10 mM ethylenediaminetetraacetic acid disodium salt and 6.8 mM ethyleneglycol-*bis*-tetraacetic acid for 15 min at 22 C, and then granulosa cells were isolated by gentle pipetting. Female BALB/c mice were immunized intravenously with the isolated granulosa cells. The spleen cells from immunized mice, which produced anti-granulosa cell antibodies, were fused with Sp2/O-Ag14 mouse myeloma cells by the standard hybridization techniques. The hybridoma cells were selected by conventional hypoxanthine-aminopterin-thymidine and hypoxanthine-thymidine method. Then, the hybridoma cells producing antibodies against the granulosa cells were screened by immunofluorescence

staining using frozen sections of porcine ovaries, and antibody class was determined by an ELISA method. Three hybridoma cell lines (PFG-5, PFG-6 and PFG-7 clones) were selected and cloned by limiting dilution. PFG-5, PFG-6 and PFG-7 clones produced IgM, IgG and IgG antibodies to porcine granulosa cell surface components, respectively. To produce monoclonal antibody, 2,6,10,14-tetramethylpentadecane pretreated female BALB/c mice received an intraperitoneal injection of these hybridoma cells, and then ascites were obtained from the mice 2 weeks after the injection. Each antibody was purified using ultrafiltration equipment and preparative high-performance liquid chromatography. To determine the characteristics of each monoclonal antibody, frozen serial sections of porcine ovaries were cut on a cryostat, mounted on 3-aminopropyltriethoxysilane precoated glass slides, and fixed with precooled acetone for 5 min at -80 C, and then they were incubated with each purified monoclonal antibody after preincubation with normal goat serum. The sections were washed, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or IgG antibody, and then examined with a confocal laser scanning microscope. PFG-5 and PFG-7 showed strong FITC-staining on granulosa cells of healthy and atretic follicles. PFG-6 antibody reacted with granulosa cells of healthy follicles. These antibodies did not label the theca interna or externa cells, basement membrane, or ovarian stroma cells in either healthy or atretic follicles. Moreover, they showed neither specific binding to the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung or skeletal muscle. To determine the antigen profiles, we performed conventional Western blotting and two-dimensional (2D)-Western blotting analyses as follows. Homogenized ovarian tissues (granulosa cells, cumulus cells, oocytes and luteal bodies) and other organs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Homogenized samples of cell membrane fractions prepared from isolated granulosa cells of healthy and atretic follicles were separated by 2D-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes, and then the membranes were incubated with each monoclonal antibody. Immunological reaction products were visualized using a commercial staining kit. On conventional Western blotting, PFG-5, PFG-6 and PFG-7 antibodies reacted only with granulosa cells, but not with any other ovarian tissues or organs. On 2D-Western blotting analysis for cell membrane fractions, the two specific spots (molecular weight 42 kD and isoelectric point pI 5.2, and 55 kD, pI 5.9; named PFG-6 and PFG-5 antigens, respectively) of PFG-5 antibody were seen in the samples from healthy follicles, and only one specific spot of PFG-5 antigen was detected in those from atretic follicles. Abundant PFG-6 antigen expression was noted in the granulosa cells of healthy follicles. PFG-6 antibody recognized only PFG-6 antigen, and PFG-7 antibody recognized PFG-5 antigen. The cell-killing activity of PFG-5 antibody, but not PFG-6 and PFG-7 antibodies, was found as follows. Isolated granulosa cells prepared from healthy follicles were cultured in tissue culture medium containing antibody (0.00001 to 1,000 μg/ml of PFG-5, PFG-6 or PFG-7) for 1 to 72 h at 37 C. After incubation, cultured granulosa cells were stained by the TUNEL method using a commercial kit to determine the apoptotic cells, and the nuclei of cultured cells were stained with Hoechst 33258 to observe their morphology. After staining, the cells were examined with a fluorescence microscope. Moreover, DNA samples prepared from cultured granulosa cells were electrophoresed to assess the DNA fragmentation. No TUNEL-positive apoptotic cells were detected in the granulosa cells cultured with vehicle or with PFG-6 or PFG-7 antibodies, while many TUNEL-positive round nuclei and small condensed nuclear fractions (apoptotic bodies; a morphological hallmark of apoptotic cell death) were observed in the cells cultured with more than 0.01 μg/ml PFG-5 antibody for more than 6 h. The PFG-5 antibody-incubated DNA sample displayed a ladder pattern on electrophoresis (biochemical hallmark of apoptosis), while DNA samples of the

granulosa cells cultured with vehicle or with PFG-6 or PFG-7 antibodies displayed no ladder pattern. Interestingly, when the cells were cultured in medium containing both PFG-5 and PFG-6 antibodies (PFG-6 antibody added first), an extremely low dose of PFG-5 antibody (0.0001 μg/ml) could induce apoptosis, indicating that PFG-6 antibody binds to PFG-6 antigen to block the binding activity of PFG-5 antibody to PFG-6 antigen. When the cells were cultured in medium containing both PFG-5 and PFG-7 antibodies (PFG-7 antibody added first), no apoptotic cell was seen, indicating the inhibitory effect of PFG-7 on PFG-5 antibody-inducible apoptosis. Based on our *in vivo* and *in vitro* preliminary experiments, we presume that interleukin-6 may control the expression of these novel receptors and intracellular inhibitors in granulosa cells of porcine ovarian follicles. The molecular weights of well-known apoptosis-mediating receptors, Fas and TNFR1, are 45-46 and 65 kD, respectively, and the molecular weights of the granulosa cell surface antigens, PFG-5 and PFG-6 antigens, are 55 and 42 kD, respectively. Fas was immunohistochemically detected in the granulosa cells and luteal cells of both healthy and atretic follicles in rodent ovaries and in the thymus, liver, heart and lung, but TNFR1 was not detected in ovarian follicles or luteal bodies. Both PFG-5 and PFG-6 antigens were only detected in the granulosa cells but not in luteal cells or other organs. Furthermore, PFG-5 antibody but not PFG-6 or PFG-7 antibodies could induce apoptotic cell death in primary cultured granulosa cells. Interestingly, the apoptosis inducing ability of PFG-5 antibody was heightened by pretreatment with PFG-6 antibody, which was added first into the culture medium. The cultured granulosa cells were not killed by PFG-5 antibody in the presence of PFG-7 antibody, which was added first into the culture medium. Abundant expression of PFG-6 antigen was noted in granulosa cells of healthy follicles, but no expression was demonstrated in granulosa cells of atretic follicles. Based on the biochemical and immunochemical findings, PFG-5 and PFG-6 antigens are different from the known apoptosis-mediating receptors, Fas or TNFR1. We hypothesize that PFG-5 antigen acts as a cell death receptor specifically expressed on the granulosa cells, and that PFG-6 antigen acts as a decoy receptor for PFG-5 antigen inhibiting apoptotic signals through PFG-5 antigen. A cell surface mechanism exists for the regulation of cellular responsiveness to pro-apoptotic stimuli. Cell death receptors transmit an apoptotic signal, and decoy receptors act as modulators of cell death receptors, and both are expressed on the same granulosa cells. It is considered that overexpression of the decoy receptor on the surface of granulosa cells inhibits apoptotic signal induced by the ligand for the apoptosis inducing receptor. Unfortunately, we have no data on the natural ligand for the novel cell death receptor, and the physiological properties of this receptor system are not well understood. These antibodies will require sensitive probes to investigate the unique cell death receptor on the granulosa cell membrane and its natural ligand, to elucidate cell surface mechanisms for the regulation of apoptosis, and to define the intercellular pathway of apoptotic signal transduction in granulosa cells of porcine ovaries. we have demonstrated caspases and related proteins, and their mRNAs are expressed in porcine granulosa cells. Expression levels of Apaf-1, FAD were decreased during follicular atresia, but those of activated caspase-3, caspase-8 and caspase-9 were increased. No changes in expression levels of FasL or Fas during atresia were noted. We believe that these caspase cascade components, which locate downstream of the cell death ligand and receptor system, transduce the intracellular apoptotic signal. However, the detailed mechanisms of signal transduction have not been identified, and further studies are needed to understand the molecular mechanisms responsible for granulosa cell apoptosis.

Intracellular Apoptosis Inhibitory Factors in Granulosa Cells

We recently isolated the porcine cDNA encoding cellular FLICE (procaspase-8)-like inhibitory protein (cFLIP), which inhibits death receptor-mediated apoptosis signal transduction. In porcine organs, two alternative splicing isoforms of cFLIP, porcine cellular FLIP-short form (pcFLIP$_S$, 642 bp and 214-aa) and -long form (pcFLIP$_L$, 1446 bp and 482-aa), were identified in a cDNA library prepared from granulosa cells of porcine ovarian follicles. pcFLIP$_S$ contained 2 tandem DEDs in the N-terminal region and a short C-terminal region. pcFLIP$_L$ contained two tandem DEDs in the N-terminal region and a caspase-like domain in the C-terminal region. High levels of pcFLIP$_S$ mRNA were detected in the colon, heart and lung, and high levels of pcFLIP$_L$ mRNA were detected in the heart and thymus. In the ovaries, both pcFLIP$_S$ and pcFLIP$_L$ mRNAs were highly expressed in granulosa cells of healthy follicles, suggesting that these cFLIPS play important roles in the regulation mechanism of apoptosis in ovarian follicular granulosa cells. We hypothesize that the cell death receptor-mediated apoptosis signaling pathway and intracellular inhibitory mechanism in granulosa cells are as follows. (1) A cell death ligand binds to the extracellular domain of a cell death receptor, which contains an intracellular DD. (2) The intracellular DD of cell death receptor interacts with the DD of the adaptor protein (FADD) through a homophilic DD interaction. (3) FADD activates an initiator caspase (procaspase-8/FLICE), which is a bipartite molecule, containing an N-terminal DED and a C-terminal DD. (4) In healthy follicles, the DED of FADD interacts with the DED of cFLIP$_S$ and/or cFLIP$_L$ to inhibit the apoptosis signal transduction. (5) In atretic follicles, procaspase-8 begins auto-proteolytic cleavage and activation. (6) The auto-activated caspase-8 cleaves Bid protein. (7) The truncated Bid releases cytochrome c from mitochondrion. (8) Cytochrome c and ATP-dependent oligimerization of Apaf-1 protein allows recruitment of procaspase-9 into the apoptsome complex. (9) Activation of procaspase-9 is mediated by means of conformational change. The activated caspase-9 cleaves downstream effector caspases (procaspase-3). (10) finally, apoptosis is induced. cFLIP$_S$ and cFLIP$_L$ may act as anti-apoptotic/survival factors in granulosa cells. Further *in vivo* and *in vitro* studies will elucidate the largely unknown molecular mechanisms (which cell death ligand-receptor system is the dominant factor for controling the granulosa cell apoptosis) of selective follicular atresia in mammalian ovaries.

Conclusions and Future Prospects

Biochemical and genetic studies have unraveled many of the signaling mechanisms that mediate induction of instructive apoptosis by the cell death ligand and cell death receptor system. Recently, genome sequencing has revealed some new cell death ligands, cell death receptors and their specific decoy receptors, which are categorized under the TNF-ligand and receptor superfamilies. It is likely that some novel cell death ligands and cell death receptors use similar intracellular signal inducing pathways to TNF□ and TNFRs and the FasL and Fas system, although the molecular details of each pathway are still unknown. To date, many researchers in the field of immunology and oncology have revealed the unique biological roles of the cell death ligand-receptor systems and the molecular mechanisms that integrate their functions in the immune system, but the biological roles and molecular mechanisms of these ligand-receptor systems in the reproductive system are largely unknown. Apoptosis, an active form of cell suicide, plays a key role in the demise of the majority of mammalian gonadal cells (follicular granulosa and luteal cells in ovaries and germ cells in testes) during reproductive life. In mammalian ovaries, a balance of cell proliferation and apoptosis in granulosa cells is maintained in healthy follicles and any imbalance of the two processes can

lead to atretic change in follicles. Recent studies have indicated the crucial role of the cell death ligand and cell death receptor system as a survival factor and/or apoptotic factor in follicular granulosa cells, which act as inducers of follicular growth and/or atresia. Based on our findings in porcine ovaries, we presume that the TNFα and TNFR2 system, no TNFR1 is detectable, acts as a survival/growth factor in granulosa cells, and that the FasL and Fas system may control luteal cell death during luteolysis but not granulosa cell death during atresia. Also, TRAIL and its receptors (DR4, DR5 and DcR1, but not DcR2), and unknown ligand(s) and receptors (PFG-5 and PFG-6) contribute to selective cell death of granulosa cells during atresia. Furthermore, we believe that intracellular factors, which locate downstream of the cell-surface ligand and receptor complex, modulate cell death signal transduction from the receptors (for example $cFLIP_S$ and $cFLIP_L$), and that intragonadal survival factors in the ovary (estrogens, insulin-like growth factor-I, epidermal growth factor, basic fibroblast growth factor, interleukin-1β, nitrogen monoxide, etc.) and apoptotic factors (androgens, gonadotrophin releasing hormone-like peptide, interleukin-6, etc.) interact with the cell death ligand and cell death receptor systems. Unfortunately, we do not have enough detailed knowledge of the interaction between survival/growth and apoptotic factors and cell death ligand and cell death receptor systems, and such interaction may be important in regulating the induction of the death of ovarian follicles. Since our knowledge of the regulation mechanism of follicular selection is limited, further studies are essential to understand the cellular and molecular mechanisms responsible for follicular selection, in other words, granulosa cell apoptosis. If we could elucidate the molecular mechanism of follicular selection (the regulation mechanism of granulosa cell apoptosis), we could accurately predict/diagnose healthy follicles (ovulating follicles) and precisely evaluate the oocytes. We hope that the mechanism will be clarified and lead to an integrated understanding of the regulation mechanism.

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MARKER-ASSISTED SELECTION IN CATTLE

REINSCH N.

Forschungsbereich Genetik und Biometrie
Forschungsinstitut für die Biologie landwirtschaftlicher Nutztiere, FBN
Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

Summary: Marker-assisted selection is expected to alter selection schemes in dairy cattle. This article is an outline of the fundamental ideas and covers bottom-up and top-down selection, marker-assisted BLUP-evaluation and situations with linkage equilibrium and disequilibrium as well as genotype-assisted selection as a special case.

Quantitative trait loci

Quantitative traits exhibit a continuous variation: within a certain biological range any phenotypic measure may be observed. Typical examples are milk yield, somatic cell count or daily gain. Quantitative traits are usually determined by a large but unknown number of genes, called "quantitative trait loci". The chromosomal position of such QTL can be determined relative to known markers in mapping experiments, provided that different alleles of such a QTL cause sufficiently large phenotypic differences.

There is still considerable variation among animals sharing the some QTL genotype (figure 1). This variation is due to other genes (polygenes) and environmental noise. Despite of this variation the mean phenotype is different for each QTL-genotypic group.

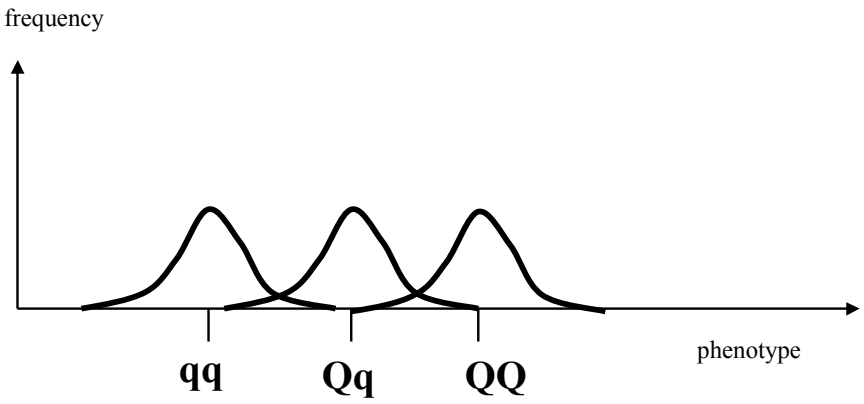

Figure 1: Distribution of phenotypes for different QTL-genotypes.

Marker-assisted genetic evaluation under linkage equilibrium

We consider a situation, where a QTL and one or several linked markers are known. There are QQ-, Q_q - and qq-animals in the population, i.e. there are two alleles at the QTL (Q and q). The QTL genotype itself remains however hidden, since there are only indirect markers without own effect on the phenotype and no gene test is available. Homozygous animals always pass the same allele to all their progeny and therefore the main interest is in the homozygotes for the purpose of selection. In order to detect heterozygous Q_q-animals we analyse paternal half-sib families, which result from artificial insemination in cattle.

The basic principle of "bottom-up" selection is depicted in figure 2. A bull is heterozygous 1, 2 at a marker and its QTL-genotype is unknown a priori. After genotyping the bulls' progeny for the marker all offspring can be attributed to two groups: group I comprises all progeny with 1 as the paternal marker-allele and all members of group II have inherited a paternal 2-allele. By comparing the mean phenotype of both groups we draw conclusions the bulls' QTL-genotype and the linkage phase between alleles at the marker and the QTL. The bull is presumably heterozygous Q_q when both groups differ in their phenotypic mean. The difference is positive because of the higher mean of the first group, then it can be concluded, that in vast majority of cases the first marker-allele is coinherited with the Q-allele (phase I: 1-Q). The opposite is true when a negative difference has been found: marker-allele 2 is coinherited with Q and q is accompanied by marker-allele 1 (phase II: 1-q and 2-Q).

Having assessed the linkage phase between marker- and QTL-alleles those of the bulls' offspring can be selected, which carry the paternal copy of the favourable QTL-allele (Q). Usually it is reasonable to assume that phase I- an phase II-sires are equally frequent in the population, a situation which is known as linkage equilibrium. Consequently a comparison of phenotypic means has to be carried out again for each bull entering the breeding scheme. Therefore the basic principle is to subdivide each paternal half-sib family according to paternal marker-alleles and to "charge" the marker-alleles with information on the associated phenotypic effect by comparing the mean trait values of both subgroups and to figure out which marker-allele represent the unfavourable (Q) and the favourable (q) QTL-allele in the sire's progeny.

The so-called "top-down" approach is a variant, where the paternal half-sib family consists of progeny tested AI-sires. The main difference is, that to each son the average performance of his own progeny is attributed as a "phenotype". Everything else is analogous to the "bottom-up" approach: in figure 3 the offspring of group I-bulls are somewhat darker when compared to offspring of group II-bulls.

The conclusion is, that a Q-allele causing a higher proportion of block coat must be linked to marker-allele 1 of the grandsire. A sufficiently large number of AI-sires descending from each bull-sire is a prerequisite for the "top down" approach. This will be given in larger populations like Holsteins or Simmental, in smaller populations only the "bottom-up" approach may be possible. Both approaches represent the basic sources of information for marker-assisted selection in a situation of linkage equilibrium between QTL and marker. This information can be used for pre-selection of males and females.

Figure 2:

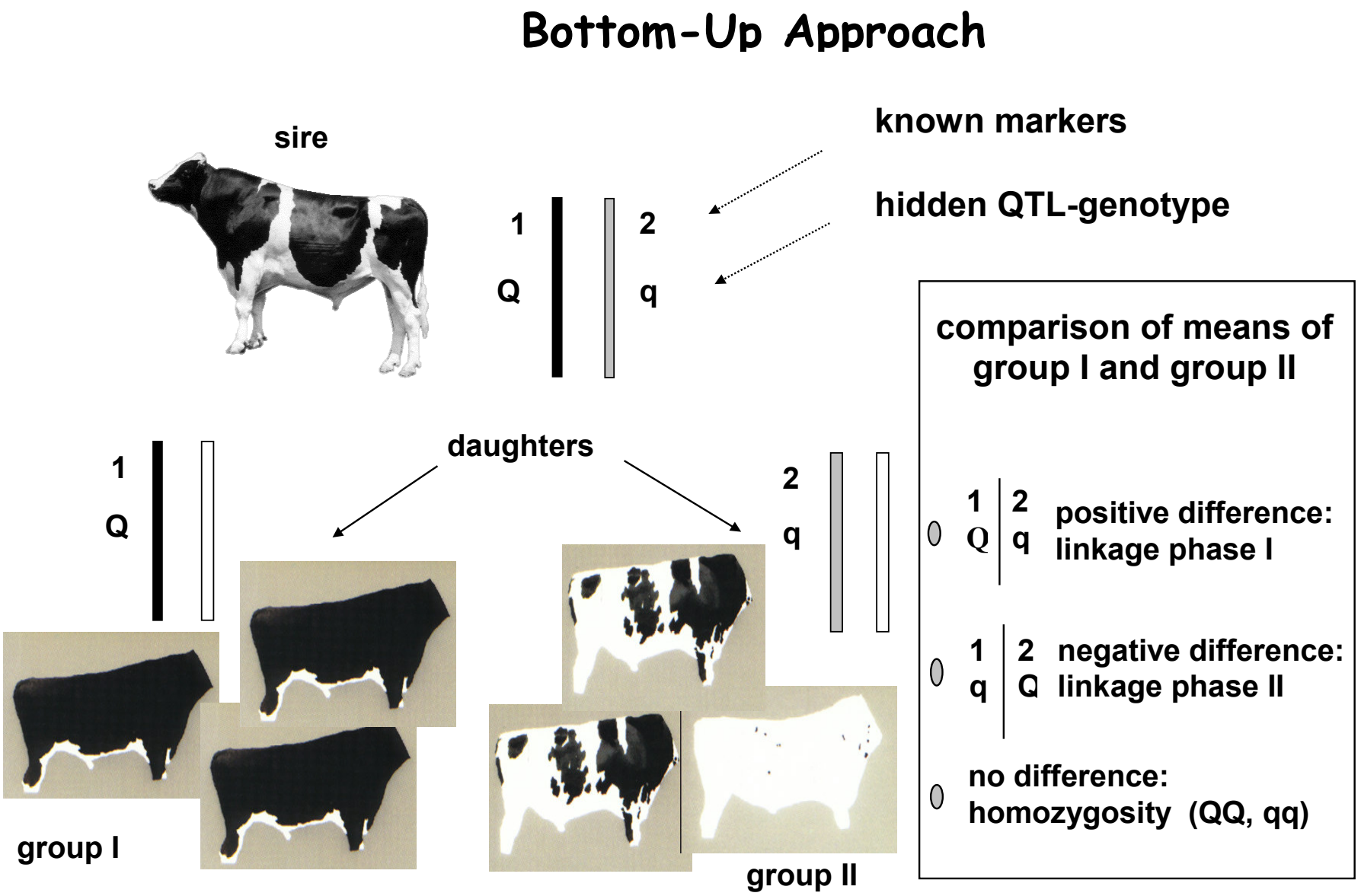
Bottom-Up Approach
sire
known markers
hidden QTL-genotype
1
Q
2
q
daughters
1
Q
2
q
group I
group II
comparison of means of group I and group II
1 2
Q q
positive difference: linkage phase I
1 2
q Q
negative difference: linkage phase II
no difference: homozygosity (QQ, qq)

Figure 3:

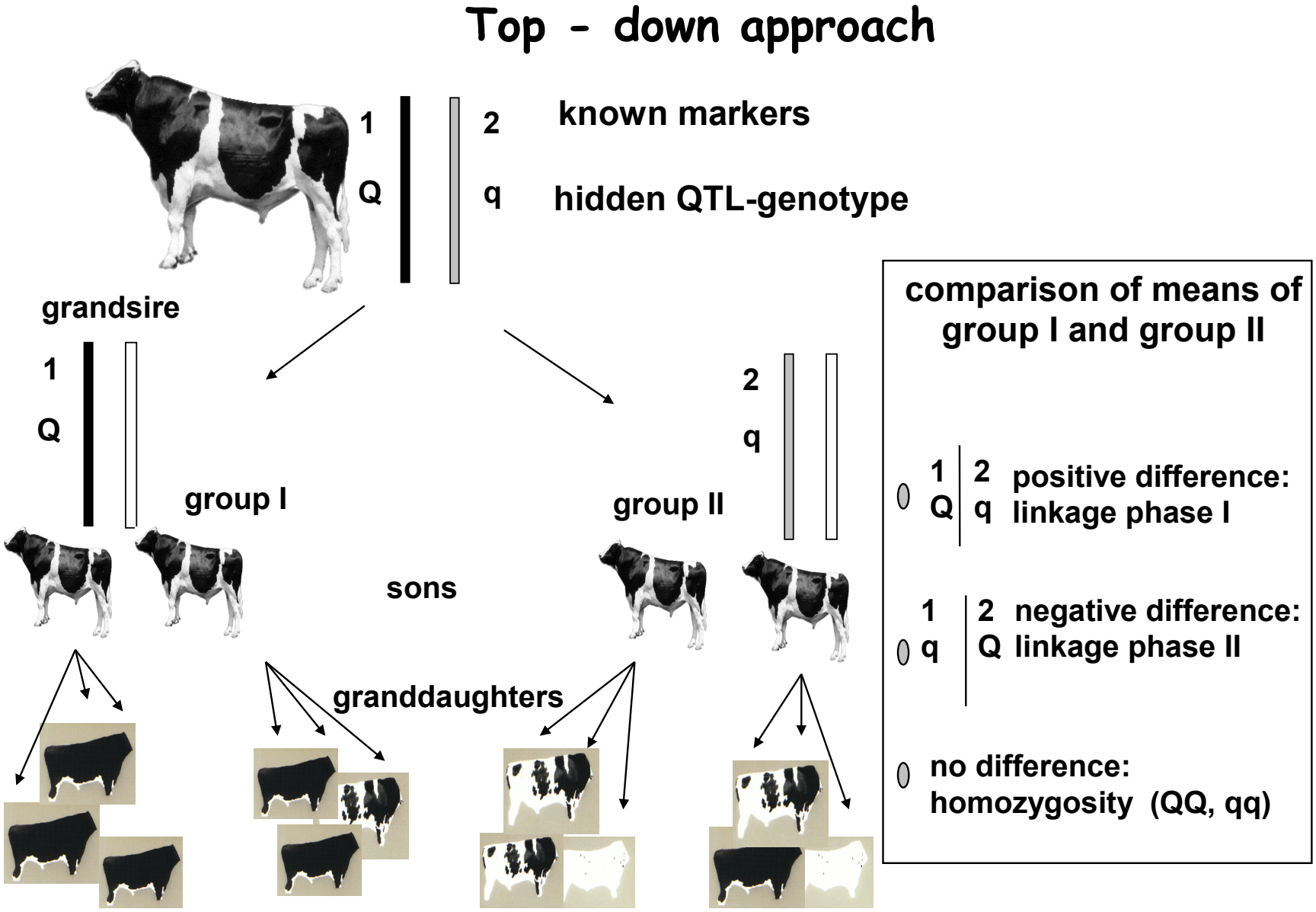
Top - down approach
1
Q
2
q
known markers
hidden QTL-genotype
grandsire
1
Q
2
q
group I
group II
sons
granddaughters
comparison of means of group I and group II
1 2 positive difference:
Q q linkage phase I
1 2 negative difference:
q Q linkage phase II
no difference:
homozygosity (QQ, qq)

Figure 2: Bottom-up selection: a paternal half-sib family is subdivided into two groups. Group I comprises progeny carrying the first paternal marker allele and group II those with the second allele. A comparison of the phenotypic means of both groups gives information about the QTL-genotype of the sire and the linkage-phase between alleles at the marker and the QTL in this particular family. Solid bars are chromosomes derived from the bull, empty bars represent chromosomes derived from dams.

Figure 3: Top-down selection follows the same principle as bottom-up selection. The difference is that progeny-tested bulls are genotyped. An average measure of the progeny's phenotype is attributed to each son and used for the comparison of means between group I and II.

Pre-selection of sons, grandsons and bull dams

Pre-selection of sons can be done by the bottom-up approach and is based on QTL-heterozygosity and a known linkage phase between QTL- and marker-alleles for a particular bull-sire.
Figure 5 shows the life cycle of a typical AI-bull. This life cycle is not altered by marker-assisted selection, the generation interval therefore remains constant. Marker-assisted selection is applied as a first stage of pre-selection of test-bull candidates at a very early age, almost suddenly after birth. At this time there is no information on the candidate's breeding value from progeny at all, only from pedigree.
Pedigree information tends to be similar for test-bull candidates, since the number of bull-sires and bull-dams is limited and both are highly selected. Therefore marker information provides a possibility to differentiate among young test-bull candidates and to capitalise on this information for pre-selection.
Top-down pre-election works in an analogous manner, but pre-selection takes place in grandsons and requires to trace the markers over three generations.

Marker-assisted genetic evaluation under linkage equilibrium

A much more efficient way to perform MAS is by applying in marker-assisted genetic evaluation. "Charging" the markers with information by subdividing paternal half-sib families with respect to their paternally inherited marker alleles remains the basic principle. All information is however continued across families rather than only within and the results are easier interpreted and used by practical breeders.
Figure 4 shows a genome composed from paternal and maternal alleles, which may associate with a positive or negative effect on the phenotypic. An animal with a large proportion of positive alleles is likely to exhibit a high phenotype measure and transmits positive alleles to its progeny in the majority of cases. Such an animal has a high positive breeding value, which can be estimated from phenotypic records and pedigree.
When marker-assisted genetic evaluation (MA-BLUP) is applied, the genome is considered as consisting of two parts: (1) the QTL, which can be traced through the pedigree by using markers and (2) the so-called polygenic part, which represents all other genes with an effect on the phenotype. Therefore three components of the breeding value are estimated for each animal in a MA-BLUP evaluation: an effect for the paternal QTL-allele, an effect for the maternal QTL-allele and polygenic part.
The relevant criterion for selection purposes is the total breeding value for a particular trait, which is just the sum of these three components.

In order to continue information from different half-sib families it is necessary to genotype animals, which provide this necessary genetic link, namely bull-dams.
In figure 6 the bulls Justus und Jockl both have genotyped progeny with records and from these the linkage phase between marker-alleles of both bulls and their QTL-alleles can be desired. The QTL-alleles can be traced with a high probability from Justus and Jockl down to their grandsons. In the example there are three bull-brothers and all of them share the same polygenic breeding value, which does not depend on marker information, but only on phenotypes and pedigree.
The effects of the paternal and maternal QTL-alleles are added to the polygenic component. The first bull-brother gets the highest total breeding value and will therefore preferably chosen for entering the progeny testing scheme. Preselection of young test-bull candidates and young bull-dams is done at the same time as outlined for the top-down and bottom-up approach. Compared to both of these basic kinds of marker-assisted selection the use of MA-BLUP provides some advantages. It is not necessary to decide whether a bull is heterozygous or homozygous at the QTL, estimates of a bull's QTL-alleles show a larger (for heterozygotes) or a smaller (for homozygotes) difference and the best available estimates are contained in the estimate of the total breeding value. A "bad" marker genotype can be compensated by a high polygenic effect, because all components of the total breeding value are continued together to a single value in an optimum manner. Breeding values from MA-BLUP can be compared across families just in the same way as it is done with BLUP-results without markers. In summary top-down and bottom-up information are used in a more effective way.

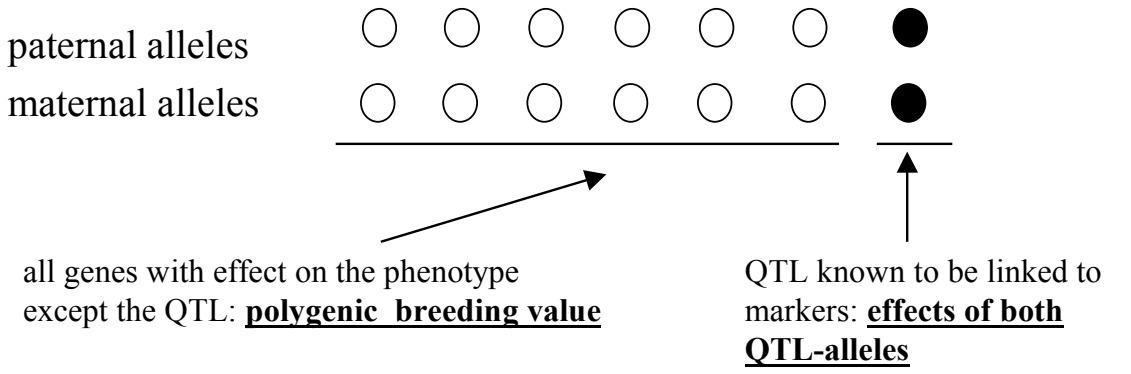

Figure 4: For merker-assisted genetic evaluation the genome is divided into QTL and all other genes. Three respective effects are estimated for each animal: a paternal and a maternal QTL-effect and a polygenic effect. The total breeding value is the sum of these three components.

Implementing MA-BLUP

Practical MA-BLUP implementations rely on results of genetic evaluations without markers. All phenotypic measures are precorrected for fixed effects (management group effects), random permanent environmental effects and half of the mother's breeding value by using estimates from routine genetic evaluation. These precorrected phenotypes are summarised in one phenotypic average for the offspring of each bull, so-called daughter-yield deviation of a bull. Since these daughter-yield deviations are assigned to each bull we get a model with one equation for each bull. Since there are no equations for ungenotyped progeny the total number

of equations remains in a magnitude of thousands or tens of thousands rather than millions even after supplementing with equations for bull dams and selection candidates.

Genotype-assisted selection

Marker-assisted genetic evaluation is significantly easier when a QTL can be explained by a mutation in a single gene. With the help of a gene-test the genotype can directly be assessed in the laboratory. In order to estimate polygenic effects we need mean effects of genotypes and again the total breeding value is the sum of a QTL-component and a polygenic part.
It remains necessary to "charge" the genotypes with phenotypic information, even when a gene-test is available: a sufficient number of animals has to be genotyped in order to estimate the effects of genotypes and polygenic effects with high accuracy. It should be noted that GAS requires almost the same amount of logistics (collection of tissue samples, data base for genotypes, data processing …) as MAS when properly performed by looking on both at the QTL and the polygenic component of the breeding value.

Linkage disequilibrium

Linkage disequilibrium is a more favourable situation for marker-assisted evaluation and selection. When phase I and II (figures 2 and 3) are not equally frequent in the population, but one of them is for more frequent than the other, the population is in disequilibrium. In case of a strong disequilibrium (e.g. a ratio of frequencies of 1:99) the situation is almost equal to a gene test: in almost all families marker-allele 1 would be accompanied by a Q QTL-allele. Then the marker genotype would immediately tell all about the QTL genotype, though with a certain small error rate. The use of markers in linkage equilibrium is in-between MAS under equlibrium and genotype-assisted selection. The efficiency of disequilibrium-MAS and GAS may be very similar when the disequlibrium is sufficiently strong, which can be expected when markers are very close to the QTL. Such markers will be found in fine-mapping experiments. In cases where a QTL is caused by polymorphisms in gene clusters such as the MUC-complex, and no "simple" gene test with few alleles can be developed, linkage- disequilibrium markers may remain the tool of choice for MAS. Since the focus of research has turned from QTL-detection to QTL-fine-mapping it can be expected that more and more linkage-disequilibrium markers become available for breeding purposes

Conclusions

MA-BLUP and marker-assisted selection and can be applied both under linkage eqilibrium and disequilibrium. A gene-test provides a perfect linkage-disequilibrium. In the future selection of bulls will be done in two-steps: marker-assisted preselection followed by progeny testing. As a result of fine-mapping research a growing number of disequilibrium markers and gene tests will become available for application in the first step.

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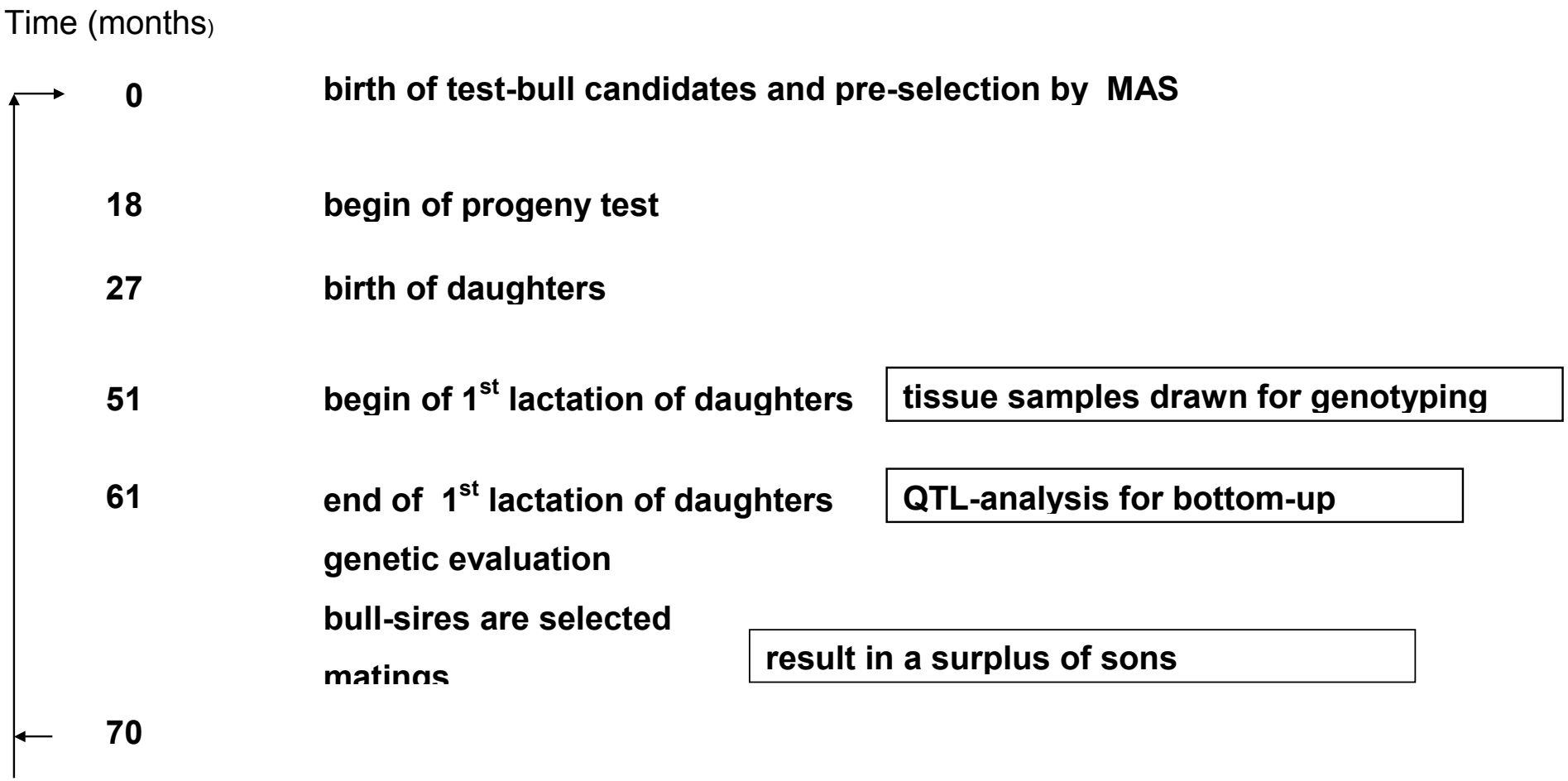
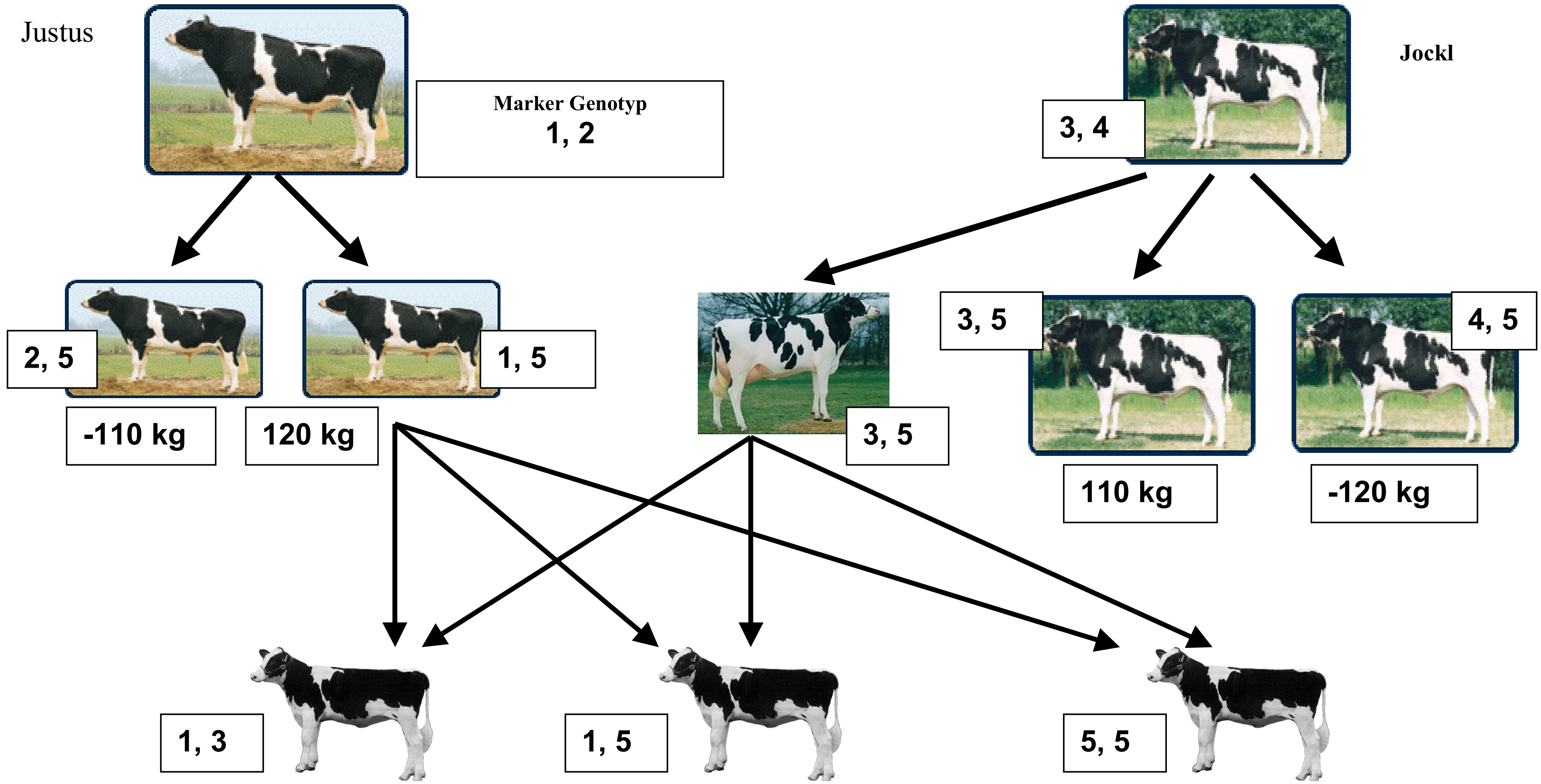


Figure 5: Bottom-up selection is used to pre-select the sons of bull-sires (test-bull candidates) early after birth by using marker information. Generation interval is the same as without marker-assisted preselection.

Pre-selection with MA-BLUP



polygenes:	0.0	polygenes:	0.0	polygenes:	0.0
1st QTL-gamete:	1.80	1st QTL-gamete:	1.80	1st QTL-gamete:	0.20
2nd QTL-gamete:	1.58	2nd QTL-gamete:	0.18	2nd QTL-gamete:	0.18
sum:	3.38	sum:	1.98	sum:	0.38

SHORT COMMUNICATIONS

THE RELATIONSHIP BETWEEN THE DIELECTROPHORETIC BEHAVIOUR OF BOVINE OOCYTES AND THEIR IN VITRO MATURATION COMPETENCE

ALJEDI Y.A.[1], TESFAYE D.[1], RINGS F.[1], GILLES M.[1], HÖLKER M.[1], JENNEN D.[1], THOLEN E.[1], SOUKHOROUKOV V.[2], HAVLICEK V.[3], BESENFELDER U.[3], SCHELLANDER K.[1]

[1] *Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Endenicher allee 15, 53115 Bonn, Germany.*
[2] *Biotechnologie Biozentrum, University of Würzburg, Am Hubland 97074 Würzburg, Germany*
[3] *Institute of Animal Breeding & Genetics, University of Veterinary Science, Veterinärplatz 1, A-1210 Vienna, Austria*

The developmental potential of an embryo is dependent on the developmental competence of the oocyte from which it originates. This study was carried out to examine the suitability of dielectrophoresis as a noninvasive approach to investigate bovine oocyte quality with respect to their maturation competence and to test if the variations in the dielectrophoretic behaviour of oocytes are driven by differences in their cytoplasmic constitution or the zona pellucida (ZP). For this purpose, the dielectrophoretic experimental conditions were determined as: 5 V, 2000 kHz AC electric field parameters, 300 µm electrodes distance, and the dielectrophoretic immersion medium with 6 mg PVA/ml 0.3 M sorbitol solution (300 mOsm), a conductivity of 80 µS/cm, and pH of 7.0. Oocytes obtained from abattoir-derived ovaries, were morphologically classified into four groups, namely: (A) good cumulus and good cytoplasm (n=355 oocytes), (B) good cumulus but poor cytoplasm (n=176), (C) poor cumulus and good cytoplasm (n=159), and (D) poor cumulus and cytoplasm (n=230). The cumulus free oocytes from each group were further dielectrophoretically categorized after exposure to the aforementioned experimental conditions into "fast" and "slow" moving oocytes. Moreover, the dielectrophoretic behaviour of zona intact oocytes, zona free oocytes and the zona pellucida alone were compared. The maturational status of the oocytes was determined after 24 h IVM in serum supplemented modified parker medium (MPM) based on the extrusion of the first polar body and the establishment of the second metaphase spindle. A general linear model was used to analyse maturation rate using SAS version 8.0 software package. Within morphologically good quality oocytes (group A), the fast moving oocytes showed a higher maturation rate (75 %) ($p \leq 0.05$) compared to the slow moving ones (60 %) and the control group (the oocytes that were not exposed to the electric field) (62%). Moreover, a similar trend was also observed within the other three morphological groups but differences were not significant. The dielectrophoretic behaviour of zona intact oocytes was consistent with that of zona free oocytes, while remarkable differences were observed between zona intact oocytes and zona pellucida alone, suggesting differences in dielectrophoretic behaviour of bovine oocytes is due to differences in their cytoplasmic constitution. Further investigation of differences in terms of the abundance of specific proteins, and to examine if this difference is expressed at the molecular level, will be a challenge for future research.

Notes

DEVELOPMENTAL COMPETENCE AND P34^{cdc2} EXPRESSION IN DIFFERENT SIZES OF PREPUBERTAL GOAT OOCYTES

ANGUITA B., JIMÉNEZ-MACEDO A.R., IZQUIERDO D., PARAMIO M.T.

Dpt. Ciencia Animal i dels Aliments. Facultat de Veterinaria, UAB. 08193 Bellaterra, Spain

Oocytes recovered from slaughtered females are highly heterogeneous and as a consequence, accurate selection of competent oocytes is necessary. So far, in vitro blastocyst production from prepubertal goat oocytes has been low and variable, possibly due to inefficient oocyte selection. The search of new markers of oocyte competence is needed. As a possible marker, we have analysed p34^{cdc2} protein expression, the catalytic subunit of Maturation Promoting Factor (MPF). This complex is the main meiotic regulator, and it is also thought to be a possible cytoplasmic maturation regulator (Naito et al, 1992).

Prepubertal goat oocytes were obtained from slaughterhouse ovaries and a sample of these oocytes were denuded and classified into four groups depending on their diameter (<110 µm, 110-125 µm, 125-135 µm, >135 µm); they were then frozen in N_2 liquid and stored at –80ºC until use in western blot. The remaining oocytes were matured in vitro in medium TC199 supplemented with hormones, 10% DBS and 400 µm cysteamine, for 27 h in 5%CO_2 and 38.5ºC. After IVM, a sample of oocytes were also denuded, classified by diameter and frozen as described above. The remaining oocytes were used for IVF in mDM with spermatozoa capacitated with heparin and ionomicin. After 24 h, presumptive zygotes were cultured for 7 days in SOF medium in 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC. At 48 h post-insemination, we added 0.1 µl FBS per embryo. Embryonic development was evaluated with Hoescht staining after IVC.

Frozen oocytes were used to perform protein extraction using TriReagent® (T9424, Sigma). The pelleted protein corresponding to 20 oocytes was used to perform electrophoresis in 10% acrylamide. Proteins were transferred to a nitrocellulose membrane. The membrane was incubated with the primary antibody (against p34^{cdc2}) for 1 hour. After washing, the incubation with the secondary antibody was performed for 30 minutes. Protein was detected by chemiluminiscence using the ECL-Plus Western Blotting Detection Kit (RPN2132, Amersham BioScences). Fisher's exact test was used to analyse IVC results, and ANOVA to analyse western blot results. We considered statistical differences when $P<0.05$.

Results are shown in Table 1. Embryonic cleavage and blastocyst rate increased with increasing oocyte diameter. The p34^{cdc2} protein detected was higher in the largest oocytes. As a consequence, we could conclude that oocytes with a higher p34^{cdc2} expression are more capable of maintaining embryonic development to the blastocyst stage. These results may indicate the important role that p34^{cdc2} and therefore, MPF, plays in cytoplasmic maturation.

Table 1. Cleavage, blastocyst rate and p34^{cdc2} expression in different sizes of prepubertal goat oocytes

Ø (µm)	CLEAVAGE		BLASTOCYST RATE		P34^{cdc2} PROTEIN		
	n	%	n	%	n	0 h	27 h
<110	30	0 a	30	0 a	3	0	1.15 ± 0.24 a
110-125	74	16.21 b	74	0 a	4	0.47 ± 0.54	2.02 ± 0.94 ab
125-135	152	40.13 c	152	1.97 a	4	0.69 ± 0.33	2.81 ± 1.21 ab
>135	72	61.11 d	72	12.5 b	5	1.35 ± 0.90	3.43 ± 0.18 b

Notes

EFFECTS OF PROGESTERONE TREATMENT ON QUANTITATIVE GENE EXPRESSION IN THE EARLY EQUINE EMBRYO

AURICH C.[1], KLEIN D.[2], PITTNER F.,[3] BUDIK S.[1]

[1]Centre for Artificial Insemination and Embryo Transfer, [2]Institute for Virology, University of Veterinary Sciences A-1210 Vienna, Austria, and [3]Institute for Biochemistry, Vienna Biocenter A-1030 Vienna, Austria

To decrease the incidence of embryonic death, mares are often treated with exogenous progesterone during early pregnancy. In cattle, higher concentrations of progesterone after ovulation have been shown to enhance development of the early embryo and its antiluteolytic mechanism. However, nothing about the effects of different concentrations of progesterone on early embryonic development is known in the horse. It was therefore the aim of the present study to investigate the effects of progesterone treatment during early pregnancy on the equine embryo and the expression of genes specifically involved in the regulation of early embryonic development.

In the equine embryo, α1/ β1 Na-K-ATPase and aquaporin 5 are suggested to be involved in formation of the blastocoele and pronounced growth of the conceptus by fluid transfer into the blastocoele. A precondition for maternal recognition of pregnancy is a constant movement of the horse conceptus through the uterine lumen which depends on the synthesis and secretion of prostaglandin E and F by the embryo itself. The antiluteolytic substance produced by the horse embryo is still unknown, but it synthesizes oestrogens in relatively large amounts, and therefore enzymes of the cytochrome P450 complex have to be expressed.

In our study, oestrus mares were inseminated with native or frozen semen until ovulation. Mares were treated with a progesterone-releasing intravaginal device (PRID, 1.55 mg progesterone) from one day after ovulation until Day 10 of the cycle or were left untreated. Embryos were flushed by routine techniques on Day 10 after ovulation. Embryo size and quality were determined and only embryos of very good quality were used for further analysis. Relative expression of α1-Na-K-ATPase (aATP) and β1-Na-K-ATPase (bATP) isoenzyme, aquaporin 5 (AQP5), cyclooxygenase 1 and 2 (Cox 1 and 2), prostaglandin E synthase (PGES) and cytochrome P450 (cP450) were determined by quantitative real time PCR in comparison to β-actin.

Seventeen embryos from untreated and 9 embryos from progesterone-treated mares were collected. Mean diameter of the embryos was 4.0 ± 0.3 mm in untreated and 4.0 ± 0.5 mm in progesterone-treated mares. Relative expression of the different genes is shown in Table 1. Treatment with progesterone resulted in a significant higher expression of AQP5. Relative expression of PGES tended to be lower in embryos after PRID-treatment, but the difference did not reach statistical difference (P=0.072). A higher expression of AQP5 after PRID-treatment during early pregnancy supports the idea of a positive influence of progesterone on early embryonic development in the mare.

Table 1: Expression of selected genes in relation to ß-actin in Day-10-embryos from untreated (control; n=17) and progesterone-treated (PRID; n=9) mares (means ± SEM)

Treatment	aATP	bATP	AQP5	Cox 1	Cox 2	PGES	cP450
Control	1.3±0.1	1.3±0.1	1.0±0.1	0.9±0.2	1.1±0.2	1.3±0.2	1.5±0.2
PRID	1.6±0.1	1.1±0.2	1.7±0.3*	1.8±0.6	0.8±0.2	0.8±0.1	2.1±0.6

*P<0.05 vs. control

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Notes

TWIN REDUCTION IN THE MARE BY TRANSVAGINAL ULTRASOUND-GUIDED ASPIRATION

BECKER F.[1], GÖLLNITZ K.[2], KLEINPETER A.[3], NEUBAUER J.[4] KANITZ W.[1]

[1]Research Institute for the Biology of Farm Animals, Department of Reproductive Biology Dummerstorf, Germany
[2]Veterinary Practice Hohen Schönberg, [3]Equine Clinic Alt-Sammit; [4]Equine Practice Güstrow, Germany

Early diagnosis and management of twins has reduced the incidence of abortion in mares due to twins; however, twin pregnancies still account for a notable amount of pregnancy wastage in the mare. They cause economic losses as they terminate in high rates of abortion, stillbirth or the delivery of dead or weak and deformed foals. Early transrectal ultrasonographic detection of twins and manual crushing of one conceptus is the method of choice for managing equine twins before Day 24 of gestation. Unilateral twins that do not undergo natural reduction to a singleton are challenging to manage. Manual reduction of unilateral twins is difficult without damaging the remaining conceptus. Transvaginal ultrasound-guided twin reduction techniques are described for twin pregnancies that advance beyond Day 25 of gestation. Reported success rates for the ultrasound-guided procedures vary between 20% and 50%.

In the present study, over a period of 4 years, 22 twin pregnancies were ultrasound-guided managed by aspiration of allantoic fluid. Mares with twin pregnancies from Day 23 to Day 52 after breeding were directed to the ultrasound-guided procedure. In total the success rate was 50 %. Ten mares got a therapeutic dose of Flunexine (Finadyne® RP; i.v., Essex) before and after aspiration. The success rate was 60 % in the treated group and 41.6 % in the control group. A direct relation between success rate and day of gestation and aspiration was observed. The survival rate of the remaining twin decreased as the day of initiation manipulation increased (decreasing to 10% beyond Day 43 of gestation).

Regarding management of twin pregnancies, we conclude: 1. All mares should be bred regardless of the number of preovulatory follicles. Withholding mares with multiple preovulatory follicles from breeding decreases the pregnancy rate per unit time and is followed by a loss of breeding time. 2. Check all mares for twins, regardless of the number of detected ovulations. 3. Ultrasound guided reduction of twins should be done before Day 36 of gestation.

Notes

BOVINE EMBRYO DEVELOPMENT IN SOF CULTURE MEDIA PREPARED FRESH BEFORE USE OR REFRIGERATED

BELTRAN-BREÑA, P., RIZOS, D., DE LA FUENTE, J., PEREZ-GARNELO, S., PALASZ, A.T.

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

Routinely final embryo culture media disregard of system used are prepare fresh before use. Rationales for such protocol are unstable nature of sodium pyruvate that decarboxylate quickly, inactivation of previously used preparations of antibiotics and potential decomposition of biological origin proteins if used. The objective of this study was to compare the effect of SOF culture media prepare fresh (SOF-f) 2 hr before use with the same SOF medium refrigerated (SOF-r) for 2 weeks on the in-vitro development and morphology of bovine embryos. Stock solution of SOF (Holm et al, 1999, Theriogenology 52:683-700) was prepared every 30 days. Final culture media were supplemented with 7.27 mM sodium pyruvate, 50 μg ml $^{-1}$ gentamicin and 0.8% BSA or 5% FCS and use at day of preparation (SOF-f) or were refrigerated (SOF-r) for 2 weeks before use. A total of 1254 presumptive zygotes (4 replicates) were evenly divided and cultured in the following 4 groups: Group 1, SOF+FCS-f, Group 2, SOF+BSA-f, Group 3, SOF+FCS-r and Group 4, SOF+BSA-r. Embryos were cultured in 30 μl drops (25 zygotes per drop) under paraffin oil at 39 ^{0}C and 5% CO_2 in humidified air. Cleavage rates were recorded on Day 2, number of zygotes that developed to 8≤ cells at Day 4 and number of blastocysts on Days 7, 8 and 9. Total number of nuclei was determined after Day 7 of embryo culture. At least 10 blastocysts from each treatment were fixed in 4% of paraformaldehyde solution in PBS, for 1 h at room temperature. Embryos were washed 3 times in 100 μl drop PBS/ET surfactant (Bionich Inc, Belleville, ON, Canada) and transfer to micro drop of Hoechst 33342 dye for 10 min. The total number of cells was counted under fluorescent microscope (ג 320). Also at least 5 blastocysts from each replicate and from each treatment were frozen for further evaluation of gene expression patterns. Embryos development, blastocyst formation and total number of nuclei counts were analyzed by Chi square analysis. No differences in cleavage rates at Day 2 (insemination Day 0) and number of zygotes that developed to 8≤ cells at Day 4 were observed between groups. There was no difference between fresh and refrigerated BSA supplemented SOF in the percentages of embryos that developed to the blastocyst stage at Days 7 and 8 (25.0% and 26.2% respectively). However, significantly ($P<0.05$) more blastocysts developed in fresh than refrigerated FCS supplemented SOF (41.5% and 16.3% respectively) and in both BSA supplemented groups (SOF-f 26.9% and SOF-r 26.4%). There was no effect of media refrigeration on average nuclei number on Day 7 blastocysts in either group: 111±22 (BSA-f, n 11), 101±31 (BSA-r, n 12), 126±20 (FCS-f, n 12) and 114±29 (FCS-r, n 12).

Based on the results of the present study we concluded that within our culture system SOF medium supplemented with sodium pyruvate, gentamicin and BSA but not with FCS can be refrigerated up to 2 weeks without compromising embryo development and total cell numbers.

Notes

INTERCELLULAR CONTACTS: A LIMITATION FOR THE ZONA-FREE CLONED BOVINE EMBRYOS?

BHOJWANI S.[1], POEHLAND R.[1], JONAS L.[2], BECKER F.[1], TOMEK W.[1], KANITZ W.[1]

[1]*Research Unit Reproductive Biology, Research Institute for the Biology of Farm Animals, Wilhelm-Stahl-Allee 2, D-18196, Dummerstorf, Germany*
[2]*Electron Microscopy Centre, Dept. of Pathology, Faculty of Medicine, University of Rostock, Strempelstrasse 14, D-18055, Rostock, Germany*

The intercellular communication system, for example gap junctions, allows small ions and molecules (molecular weight <1kDa), including second messengers such as cAMP (cyclic adenosine monophosphate), IP_3 (inositol 1,4,5-triphosphate), calcium and metabolites, to pass through and is also thought to be responsible for the passage of electrical signals from one cell to another in developing and differentiated tissues (Kumar & Gilula, 1996). Such cell-to-cell communication has been implicated in both directing developmental cell fate decisions and in tissue homeostasis / metabolite exchange.

Many features have been shown to differ between in vitro and in vivo bovine embryos (Massip et al. 1995). Darker cytoplasm, lower density (Pollard & Leibo, 1993), swollen blastomeres (Van Soom & DeKruif, 1992), slower growth rate, and higher thermal sensitivity (Leibo & Loskutoff, 1993) make in vitro-produced embryos weaker and more sensitive to cryotechnologies. We hypothesized that the process of removal of thezona pellucida from the oocytes destined for use in the production of zona-free cloned embryos might lead to a disturbance of cell-to-cell contacts between the blastomeres during early embryogenesis. Thus, in addition to the stress caused by the micromanipulations, zona pellucida removal might, thereby, cause an extra disadvantage to cloned embryos in terms of developmental competence. Hence, we decided to carry out ultrastructural analysis for the presence of cell-to-cell contacts in cloned, in vivo and in vitro produced (IVP) bovine embryos at the 8-cell stage, which is important since it coincides with the maternal-embryonic transition period.

Cloned bovine embryos were produced by HMC^{TM} (Hand-made-cloning) method (Vajta et al. 2003); in vivo embryos were collected after superovulation and slaughter on Day 3; in vitro embryos were obtained by normal IVP procedure; Transmission-Electron-Microscopy (TEM) facilitated ultrastructural analysis of intercellular contacts between blastomeres; western blot was carried out for qualitative and quantitative analysis of tubulin, actin and connexin 43.

Western Blot revealed the presence of tubulin, actin and connexin 43 from 4-cell stage up to the morula stage in IVP embryos with no quantitative differences between the various embryonic stages. These proteins were also detected in 8-cell stage cloned, in vitro and in vivo bovine embryos. Ultrastructure analysis revealed that tightly apposed plasma membranes of the blastomeres of the 8-cell bovine embryos were organized into microvilli. The points of apposition/contact points (CPs) between the blastomeres resembled tight junctions and were of the shortest order in cloned embryos and of highest order in the in vivo embryos clearly indicating a reduced intercellular contact in the zona-free cloned bovine embryos.

Notes

EXPRESSION OF SODIUM POTASSIUM ATPASES AND AQUAPORINS IN EARLY EQUINE EMBRYOS

BUDIK S.[1], WALTER I.[2], PITTNER F.[3] AURICH C.[1]

[1]Centre for Artificial Insemination and Embryo Transfer and, [2]Institute for Histology and Embryology, University of Veterinary Sciences and [3]Biocenter Vienna, A-Vienna, Austria

Mammalian blastocyst formation requires the establishment of a trans-trophectodermal ion gradient by the action of sodium potassium ATPases. Recently, it was shown that aquaporins (AQPs) are involved in murine blastocyst formation by enhancing fluid movement across the cell membranes (Anim Reprod Sci. 2004; 82-83:583-92). Aquaporins are members of the MIP (major intrinsic protein) family and transport small molecules like water, glycerol or urea. So far, 11 different aquaporins (0-10) have been detected that differ in selectivity for transportation and hormonal regulation. In the horse, nothing is known about the expression or AQPs during during early embryonic development; however, these molecules– together with sodium-potassium ATPase (Biol Reprod. 1997; 57:630-40) - could be involved in the formation of the blastocoele and the rapid expansion and growth of the equine conceptus.

In the present study, equine cumulus oocyte complexes (n=5, pooled) and preimplantation equine embryos between Days 8 and 14 of development (n=4; Day 8, 10, 12, 14) were analyzed for the abundance of sodium potassium and aquaporin transcipts by means of RT-PCR. After positive detection, immunohistochemistry was performed on embryonic sections using polyclonal antibodies. The same antibodies were tested in western blotting using protein preparations of the corresponding embryos.

Transcripts for $\alpha 1/\beta 1$ subunits of sodium potassium ATPase were present in all embryonic stages; simultaneously immunoreactive protein could be detected by immunohistochemistry and by western blotting, respectively. Equine AQP3 was expressed in cumulus oocyte complexes and all embryonic staged investigated. In contrast, expression of AQP5 did not occur before Day 8 of development. Immunohistochemistry of AQP3 showed a diffuse cytoplasmatic abundance in sections of Day 8 embryos, developing into a basolateral pattern on Day 12. AQP5 was detected on the apical side of the trophectoderm, only. A positive correlation of AQP5 expression with embryonic size and abundance of the $\alpha 1$ subunit of sodium potassium ATPase could be demonstrated by real time PCR.

Our results for the first time demonstrate the existence of aquaporin 3 and 5 gene products in early equine embryos. A possible involvement of AQPs in the rapid growth of the equine conceptus between Day 7 and 14 is supported.

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Notes

OPTIMIZATION OF THE EFFICIENCY OF DEMECOLCINE, NOCODAZOLE AND VINBLASTINE FOR CHEMICALLY ASSISTED ENUCLEATION OF MOUSE AND GOAT OOCYTES

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

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

With the aim of simplifying and improving the classical method of oocyte enucleation by micromanipulation, metaphase II (MII) oocytes have been treated with antimitotic drugs. Depolymerization of spindle microtubules results in the formation of a cortical protrusion in the oocyte that contains all the chromosomes and that allows their rapid localization during enucleation. The present study was designed to optimize the efficiency of this chemically assisted oocyte enucleation, by testing the ability of several antimitotic drugs to induce cortical protrusions in both mouse and goat oocytes.

Mouse MII oocytes were collected from the oviducts of CD1 and B6CBAF1 females at 14 h post-hCG. Cumulus cells were removed and the oocytes were immediately treated with the antimitotic drugs demecolcine (DEM, 0.4 µg/ml), nocodazole (NOC, 0.3 and 1µg/ml) or vinblastine (VIN, 0.1µg/ml) for 15, 30, 60 or 120 min. Immature goat oocytes were obtained from slaughterhouse derived ovaries from prepubertal goats and were matured *in vitro* in a conventional medium (TCM199 with serum, hormones and cysteamine). At 27 h post-maturation, oocytes were denuded and immediately treated with DEM (0.4 and 0.6 µg/ml) or NOC (0.3 and 1µg/ml) in the presence of 0.05M sucrose for 60 or 120 min. After treatments, both mouse and goat oocytes were fixed in a Microtubule Stabilization Buffer-Extraction Fixative for 30 min at 37ºC. A triple labelling protocol was then applied to stain microtubules, microfilaments and chromatin. Approximately 50 oocytes were analyzed for each treatment. Experiments were repeated at least three times and data were analysed by Chi-square or Fisher exact test.

Oocyte cortical protrusions were classified as type A or type B depending on their size. Type B protrusions, smaller in size than type A, are the most interesting for chemically assisted enucleation procedures. In mouse oocytes, cortical protrusion formation was affected by the length of the treatment but not by the type of drug used. In CD1 oocytes, the highest numbers of oocytes with protrusions were obtained in treatments with DEM (84%) and NOC 0.3µg/ml (81%) for 30 min and with VIN (84%) and NOC 1µg/ml (80%) for 60 min, and in 40-67% of these oocytes the protrusions were of type B. Similar results were obtained when these treatments were applied to B6CBAF1 oocytes. Interestingly, in goat oocytes the percentage of oocytes with cortical protrusions was not significantly affected by the length of the treatment, but was dependent on the type and concentration of the drug applied. The higher rates of protrusion formation were obtained when oocytes were treated with NOC 1µg/ml for 60 min (92.5%) or with DEM 0.6µg/ml for 120 min (91.1%). In these treatments, 78.4% and 81.1%, respectively, of the oocytes had a type B protrusion. Although it remains to be demonstrated that cytoplasts prepared from these treatments are competent to support embryo development after nuclear transfer, the results presented here may contribute to increase the efficiency of chemically assisted oocyte enucleation procedures.

Notes

EFFECT OF GROWTH FACTORS, IGF-I AND EGF, ON IN VITRO MATURATION OF SHEEP OOCYTES MATURED IN SEMI-DEFINED MEDIA: PRELIMINARY RESULTS

DATTENA M.[1], MARA L.[1], BIN A..[1,2], CAPPAI P.[1]

Istituto Zootecnico e Caseario per la Sardegna, 07040 Olmedo, Italy
Department of Technology and Biotechnology in Animal Production, Perugia University, Italy

It is well known that follicular fluid of several species contains growth factors including IGF and EGF. These factors seem to be related to the physiological status of the follicle, and to an improved nuclear and cytoplasmatic maturation in turn improving the developmental competence of oocytes. The objective of this work was to determine the effect of exogenous addition of insulin-like growth factor-I (IGF-I, 100 ng/ml, Sigma) and epidermal growth factor (EGF, 50 ng/ml, Sigma) to a semi-defined maturation medium on the in vitro development of sheep oocytes.

Sheep oocytes were collected from slaughterhouse-derived ovaries and divided into four different groups. All oocytes were matured in TCM199 supplemented with 0.4% BSA, 100 μM cysteamine, 0.1 UI/mL r-FSH (Gonal-F 75 UI, Serono, Italy) 5 μg/mL LH (Sigma), 1 μg/mL estradiol-17β (Sigma) plus the addition of IGF-I (IGF), EGF (EGF) or IGF and EGF together (IGF+EGF). The control group was cultured without the addition of growth factors. After maturation, oocytes were fertilized and cultured according to our laboratory protocol. Serum was present only in the fertilization medium, and 4 mg/ml of BSA fatty acid free (Accardo et al., 2004) was added to the culture medium. The embryos that reached expanded blastocyst stage (Day 6 to 7) of each group were vitrified (Dattena et al., 2003).

There were no significant differences among groups in rate of development to the blastocyst stage except when IGF+EGF was compared with the IGF group ($P<0.05$). In conclusion, groups with the addition of growth factors attained the blastocyst stage at rates comparable to those of the control group. Subsequent embryo transfer followed by pregnancy and lambing will clarify if the growth factors used at these doses improve the quality of embryos produced.

Table 1. Cleavage and blastocyst development rates after maturation of sheep oocytes in semi-defined media and growth factors

Maturation conditions	Ova n.	Cleavage %	Blastocyst %
IGF	80	72/80 (90.0)	36/80 (45.0)
EGF	84	78/84 (92.8)	42/84 (50)
IGF+EGF	97	88/97 (90.7)	59/97 (60.8)
Control	68	63/68 (92.6)	34/68 (50.0)

Notes

FACTORS AFFECTING PREGNANCY RATE FOLLOWING FROZEN EMBRYO TRANSFER IN SHEEP

EMSEN E., GIMENEZ DIAZ C.A.

Ataturk University, Department of Animal Science
25240, Erzurum, Turkey

The effect of the stage of embryo development and role of progesterone on subsequent embryo survival has been investigated in both sheep and cattle. While embryo survival has been reported to increase with an increase in plasma progesterone concentrations in cattle, little information of this type exists for sheep. Also, higher pregnancy rates have been reported after the transfer of two rather than a single embryo. We aimed to determine the effects of stage of embryo development, number of corpora lutea and twin or single transfer on pregnancy rate of Awassi recipients.

Multiparious Awassi ewes (n=17) were used as recipients for the transfer of frozen Romanov embryos in the late breeding season (January 10th). 33 Romanov embryos frozen with ethylene glycol were imported from Canada. The embryos were transferred singly or in pairs after holding them in holding media for 5 min following thawing. Full details of synchronization of recipients and transfer procedure have been described elsewhere (Bari et al., Theriogenology 2000;53: 727-742). Thawed embryos collected at Day 6 were transferred on the same day into recipients. Data were analyzed using Chi-squared analysis.

While some of studies indicated higher survival rates for blastocysts compared to morula (Armstrong and Evans, Theriogenology 1983; 19:31-42) in the current study, transferring embryos at the morula stage resulted in a significant ($P<0.05$) increase in pregnancy rate compared to transfer at the early blastocyst or blastocyst stage (Table 1). The number of corpora lutea did not improve the pregnancy rate contrary to reports in cattle. Pregnancy rate after twin transfer of frozen-thawed embryos was significantly higher ($P<0.05$) than single transfer inagreement with Brown and Radziewick (Theriogenology 1998; 49;1525-1536).

Table 1. Pregnancy rate of Awassi recipients received Romanov frozen embryos

Categories	Stage of development			Number corpora lutea		Transfer number	
	Morula	Early Blastocyst	Blastocyst	1	2	single	twin
Embryos transferred (n)	10	7	14	14	17	3	28
Pregnancy rate (%)	60^{a}	0^{b}	25^{c}	29	30	0^{a}	36^{b}

Values in the same row in each category with different superscript are significantly different ($P<0.05$)

Notes

FULL TERM DEVELOPMENT IN A COW CARRYING A CLONED EMBRYO DERIVED FROM FIBROBLASTS AND OOCYTES OF ITS OWN GENOTYPE

HEYMAN Y.[1], LE BOURHIS D.[1,2] , CHAVATTE-PALMER P.[1], PERON S.[1], AUDOUARD C.[1], RICHARD C.[3], RENARD J.P.[1], VIGNON X.[1]

[1]*INRA/ENVA - Biologie du Développement et Reproduction, 78352 Jouy en Josas, France*
[2]*UNCEIA, Dept R&D, 13, rue Jouet, 94703 Maisons-Alfort, France*
[3]*INRA-UCEA Bressonvilliers, 91630 Leudeville, France*

Cloned animals are a unique model for studying the role of maternal immune response in failure of pregnancies that have often been reported in bovine somatic cloning. Major Histocompatibity Complex Class 1 (MHC1) has been shown to be downregulated in placentomes during pregnancy and abnormal MHC1 expression by the fetal membranes has been suggested to be the cause of pregnancy failure in bovine clones. We designed this study to investigate if pregnancy losses would be decreased in cloned cows into which a genetically identical conceptus was transferred.

Bovine fibroblasts were established from a skin biopsy originating from the ear of an adult Holstein cow from which a clone of 12 females (clone C) had previously been derived. This set of cloned heifers was divided in one group for providing oocytes through OPU after stimulation (5 animals), and one group to be used as recipient heifers (7 animals). Fibroblasts from this genotype were thawed, grown to confluency and then used as the source of donor cells for NT. Recipient oocytes were matured in vitro before enucleation at 20-22 h post maturation (hpm). Embryos were reconstructed by fusion with donor cells at 23-24 hpm and activated in 10 µg/ml cycloheximide and 5µg/ml cytochalasin B for 5h after fusion, then cocultured on Vero cells for 7 days in microdrops of B2 medium. A control group of NT embryos was produced simultaneously with the same cell line and unrelated oocytes from slaughterhouse ovaries. Embryos were transferred into synchronous recipients (1 blastocyst/recipient). The pregnancies were followed by ultrasonography.

The reconstructed embryos fused at the same rate in both groups of oocytes, but the cleavage rate was significantly lower in the group of the same genotype (Table 1). The reason for this low rate is unclear. However, pregnancy rates after transfer of NT blastocysts were similar up to term between the related and non-related groups of transplanted embryos (Table 2). These results suggest that the maintenance of pregnancy in bovine is not linked to genetic disparity between fetus and dam. More investigations are required to elucidate why the in vitro development was so low, and if pregnancies established in cows with their genetically identical conceptus are less prone to abortion or physiopathological developments as is frequently seen in cloning procedures using unrelated oocytes, donor cells and recipients.

Table 1. In vitro development of embryos obtained with related or unrelated cell/oocyte-genotype

Recipient oocytes	Reconstructed embryos	Fused (%)	Cleaved (%) of fused	Morula (%) of fused	Blastocysts (%) of fused
Same genotype	113	97 (85.8)	10 (10.3)[a]	7 (7.2)[c]	7 (7.2)[e]
Unrelated	118	93 (78.8)	78 (83.9)[b]	48 (51.6)[d]	46 (49.5)[f]

[a,b,c,d,e,f] values in columns with different superscript are significantly different P< 0.01

Table 2. Pregnancies after transfer of related or unrelated clone/recipient-genotype

Recipient	Embryos Transferred	Recipients	Pg test D21	Confirmed Pregnancy D 35	Confirmed Pregnancy D90	Calving
Same genotype	7	7	4/7	1/7	1/7	1/7
Unrelated	10	10	6/10	3/10	3/10	1/10

Notes

REFINING PROPYLENE-GLYCOL DOSAGE PRIOR TO EMBRYO TRANSFER. A FIELD TRIAL

HIDALGO CO., GÓMEZ E., TAMARGO C., FACAL N., DÍEZ C.

SERIDA-CENSYRA. Camino de los Claveles 604. 33203 GIJON (SPAIN)

Nutritional status of the recipient is an important factor related with pregnancy rates after embryo transfer (ET). Oral administration of propylene glycol (PPG) 20 days before the predicted day of the ET increases corpus luteum quality and serum progesterone levels. Moreover, upon PPG administration, a higher proportion of recipients can be selected for transfer, and pregnancy and calving rates increase (Hidalgo et al., Theriogenology 2004;62:664-676). Long-term drenching is an expensive and intensive labour, so we planned a trial to reduce the treatment period up to 10 days.

Cyclic Holstein heifers (n=190) from 36 dairy farms, were used as potential recipients for embryos. For experimental design, heifers were oestrus synchronized with 2 doses of PGF2α given 11 days apart, and distributed as follows: 1) heifers fed daily with 250 ml PPG for 20 days, starting at day of first PGF2α injection, and 2) heifers fed with 250 mL PPG for 10 days. Provided that farmers were knowledgeable about the beneficial effect of PPG (see Hidalgo et al, 2004), they declined to set a untreated control group. Estrus detection (Day 0) was based on behavioral signs and serum P4 levels by ELISA (Ovucheck Plasma®, Vetoquinol Diagnostics, Spain). On Day 7, serum P4 was again analyzed and a CL quality score from 1 (excellent and good) to 3 (bad CL) was applied to each recipient by an experienced ET technician. In vivo frozen-thawed embryos (n=110) were non-surgically transferred into the uterine horn ipsilateral to the CL, irrespective of the recipient allocation to an experimental group. Data were analysed by one-way ANOVA and results are shown in table 1. Data of P4 and CL are mean values ± SEM. Superscripts express significant differences.

Table 1. Day 7 serum progesterone concentrations (P4; ng/ml), corpus luteum (CL) quality values, percentage of heifers selected for ET and Day-60 pregnancy rates (PR) within heifers transferred with a frozen embryo.

PPG Treatment	N	P4	CL	% Accepted for ET (N)	PR (%)
10 days	100	8.70±0.8	1.24±0.09	61.84±0.05 (54)	62.06±0.07
20 days	90	8.66±0.8	1.27±0.09	65.40±0.05 (56)	57.55±0.07

Comparable results were obtained between both groups of heifers, so we conclude that beneficial effects of PPG did not decrease by shortening the drenching period to 10 days. The use of PPG can improve economical benefit in the field of ET industry.

Notes

THE EFFECT OF DONOR MARE AGE ON EFFICIENCY OF A LARGE SCALE EMBRYO TRANSFER PROGRAMME

HUNT C.B.[1], AGUILAR J.J.[1], SPORLEDER C.[2], LOSINNO L.[3]

[1] *Department of Veterinary Clinical Studies, University of Edinburgh, EH25 9RG UK*
[2] *La Irenita, Equine Embryo Transfer Centre, Buenos Aires, Argentina*
[3] *Produccion Equina, Facultad de Agronomia y Veterinaria, Universidad Nacional de Rio Cuarto 5800, Argentina*

It is well known that domestic animals experience an age-associated decline in fertility. Embryo transfer (ET) is one reproductive technology that is proving to be of great benefit when breeding from aged, subfertile mares. The aim of this study was to assess how age of the donor mare affects the efficiency of an ET programme. Over 4 breeding seasons a total of 202 Polo Pony mares aged from 2 to 29 years were presented at an ET centre in Argentina. The mares were scanned daily to monitor follicle development. Once a preovulatory follicle was detected, insemination with fresh semen was performed. Uterine lavage was carried out 7/8 days after ovulation to recover the embryos. The embryos were transferred into synchronised mares. Pregnancy diagnosis in the recipient mare was carried out 15 and 30 days post ovulation. For analysis, the mares were categorised into 4 groups according to age: Young: ≤ 7 (n=30), Middle: 8-14 yrs (n=113), Old: ≥15 (n=49) and ≥20 (n=10). For each age category, the total number of flushings, positive flushings (at least one embryo was recovered per flush), embryos recovered and pregnancies achieved were noted from the records created. From these values embryo recovery rate (total number of embryos recovered/ total number of flushings), pregnancy rate (total number of pregnancies achieved/ total number of embryos transferred) and efficiency rate (embryo recovery rate x pregnancy rate) were calculated. The parameters were tested for statistical significance using chi square analysis.

Table 1: Number of flushings, positive flushings, embryo recovery rate, pregnancy rate and efficiency rate according to age category.

	Nº. Flushings	Positive Flushings (%)	Embryo Recovery Rate (%)	Pregnancy Rate (%)	Efficiency Rate (%)
Young (≤7)	103	70/103 (68)[a]	84/103 (81.5)[a]	54/84 (64.3)[a]	52.4[a]
Middle (8-14)	449	349/449 (77.7)[b]	456/449 (101.6)[b]	298/456 (65.4)[a]	66.4[b]
Old (15-20)	205	130/205 (63.4)[a]	157/205 (76.6)[a]	94/157 (59.9)[a]	45.8[a]
≥20 yrs	52	23/52 (44.2)[c]	24/52 (46.2)[c]	13/24 (54.2)[a]	25.0[c]

a, b, c,: different letters denote statistical differences (P<0.001)

In addition to the results shown in Table 1, there was a significant reduction in embryo recovery rate in old and ≥20 mares compared with young and middle age mares combined (97.8%; P≥0.001). Efficiency rate was significantly higher in young and middle age mares combined (63.6%) compared with old and ≥20 mares (P≥0.001). The efficiency rates suggested that the likely number of cycles needed to achieve a viable pregnancy increased from 2 in young and middle age mares to 3 in old mares and 4 in mares ≥20 years of age.

In conclusion, age does significantly affect the efficiency of an embryo transfer programme with increasing age being associated with decreasing efficiency. The observed decrease in efficiency rate in old mares was even more apparent in mares ≥20 years of age. This age associated decline in efficiency will increase the labour time and cost needed to achieve a viable pregnancy in older mares.

Notes

DOUBLE OVULATIONS: FREQUENCY AND IMPACT ON EFFICIENCY IN A COMMERCIAL EQUINE EMBRYO TRANSFER PROGRAMME

HUNT C.B.[1], AGUILAR J.J.[1], SPORLEDER C.[2], LOSINNO L.[3]

[1]*Department of Veterinary Clinical Studies, University of Edinburgh, EH25 9RG, UK*
[2]*La Irenita, Equine Embryo Transfer Centre, Buenos Aires, Argentina*
[3]*Produccion Equina, Facultad de Agronomia y Veterinaria, Universidad Nacional de Rio Cuarto 5800, Argentina*

The mare is considered a mono-ovular species despite considerable evidence of the occurrence of spontaneous multiple ovulations. Mares that naturally multiple ovulate are an asset to embryo transfer centres as more embryos could produce more pregnancies in a single cycle. The aim of this study was to determine firstly if there was any difference in frequency between unilateral and bilateral double ovulations and secondly if there was any differences in efficiency between the two types of double ovulation. Over 4 breeding seasons a total of 202 Polo Pony mares aged 2 to 29 years were presented at an embryo transfer centre in Argentina. The mares were scanned daily to monitor follicle development. Once a preovulatory follicle was detected insemination with fresh semen was performed. Uterine lavage was carried out 7/8 days after ovulation to recover any embryos. The embryos were transferred into synchronised mares. Pregnancy diagnosis in the recipient mare was carried out 15 and 30 days post ovulation. For analysis the mares were categorised into 3 groups according to age: Young: ≤ 7 (n=30), Middle: 8-14 yrs (n=113) and Old: ≥15 (n=59). For each age category the number and type of double ovulations were noted from records created. The double ovulations were also categorised irrespective of age into unilateral or bilateral. The total number of flushings, positive flushings (at least one embryo was recovered per flush), embryos recovered and pregnancies achieved were calculated from the records. From these values embryo recovery rate (total number of embryos recovered/ total number of flushings), pregnancy rate (total number of pregnancies achieved/ total number of embryos transferred) and efficiency rate (embryo recovery rate x pregnancy rate) were calculated. The parameters were tested for statistical significance using chi square analysis.

From the 202 mares there was a total of 808 cycles, 34.8% (281/808) were double ovulations and 1.4% were triple ovulations. The results showed that the frequency of double ovulations was consistently similar across age categories (Young: 28%, Middle: 35.6%, Old: 35.8%) and so no statistical significance was detected ($P \geq 0.1$). The frequency of unilateral double ovulations (irrespective of age) was significantly higher (58.7%) compared with bilateral double ovulations (41.3%; $P \geq 0.001$). For unilateral and bilateral double ovulations, the embryo recovery rate (101.2% vs. 113%), pregnancy rate (68.3% vs. 64.9%) and efficiency rate (69.1% vs. 73.3%) did not show statistical differences ($P \geq 0.1$). Efficiency rates increased significantly from 47% in single ovulations to 71% in double ovulations and to 153% in triple ovulations ($P \geq 0.001$).

In conclusion unilateral double ovulations would appear to occur more frequently than bilateral double ovulations. The type of double ovulation did not however affect the embryo recovery rate, pregnancy rate or efficiency rate. The occurrence of double and triple ovulations did dramatically increase the efficiency rate compared with single ovulations. The equine embryo transfer industry would benefit enormously in terms of increased efficiency if multiple ovulations could be induced on a regular basis.

Notes

EFFICIENCY OF INTRACYTOPLASMIC SPERM INJECTION (ICSI) DEPENDING ON DIFFERENT SIZES OF PREPUBERTAL GOAT OOCYTES

JIMENEZ-MACEDO A.R., ANGUITA B., IZQUIERDO D., PARAMIO M.T.

Departament de Ciència Animal i dels Aliments. Universitat Autònoma de Barcelona 08193 Bellaterra. Spain

Oocytes from prepubertal goats have lower rates of fertilisation than those from adults. Martino et al. (Theriogenology 1994 41:969-980) observed a positive correlation between oocyte diameter and competence to complete nuclear maturation. Moreover, studies done in our laboratory showed the same positive correlation between oocyte diameter and embryo development in IVF-oocytes as was observed in oocytes from adult goats (Crozet et al., J Reprod Fertil. 1995 103:293-298). Due to the low rates of fertilisation and embryo development obtained with prepubertal goats in this work, we carried out a study, using prepubertal goat oocytes, investigating the effect of oocyte size on embryo development following fertilisation by ICSI. The ability to fertilise each MII-oocyte using the ICSI methodology could permit the accurate study of oocyte cytoplasmic maturation.

Prepubertal goat oocytes were recovered from a local slaughterhouse and matured in a conventional IVM medium (TCM199 with serum, hormones and cysteamine) for 27 h. After that, they were divided in four groups according to diameter criteria (group A: <110µm Ø; group B: 110-125µm Ø; group C: 125-135µm Ø and group D: >135µm Ø) and they were fertilized by ICSI. The control group was a sample of oocytes, conventionally selected (groups C and D), and fertilised by IVF. After 24 h of culture in IVF medium (TALP), embryos were cultured in SOF medium for 192 h. The percentage blastocysts obtained in groups C and D was 15.9 and 11.8 %, respectively and they were statistically higher than the rest of the groups.

Table 1. Embryo development after ICSI and IVF of different sizes of prepubertal goat oocytes.

	N	cleavage	<16 cells	morulae	blastocysts
Group A	3	1 (33.3)b	1 (100)a	-	-
Group B	53	32 (60.3)a	30 (93.7)a	2 (6.2)c	-
Group C	103	69 (66.9)a	37 (53.6)c	21 (30.4)a	11 (15.9)a
Group D	48	34 (70.8)a	24 (70.6)b	8 (23.5)ab	4 (11.8)ab
IVF	169	75 (44.4)b	61 (81.3)ab	10 (13.3)bc	3 (4.0)b

Values with different superscripts within each row differ significantly (P< 0.05; Fisher test)

According to these results, the higher diameter (C and D) oocytes were more competent for embryo development than the smallest ones. It has been also improved the results in terms of blastocysts obtained by ICSI compared to IVF. Thus, fertilisation by ICSI has improved embryo development compared to IVF. In conclusion, according to this work, there is a positive correlation between the diameter of the oocyte and its competence to develop up to blastocyst stage

Notes

EFFICIENCY OF DONOR-WISE IVP USING SLAUGHTERED TOP BREEDING OOCYTE DONOR COWS, A RETROSPECTIVE SUMMARY

KANANEN-ANTTILA K.[1], LINDEBERG H.[1], REINIKAINEN E.[1], KAIMIO I.[2], PEIPPO J.[3], HALMEKYTÖ M.[1]

[1]University of Kuopio, Institute of Applied Biotechnology, FIN-70211 Kuopio, Finland; [2]ProAgria Cooperative Breeding Service, FIN-76101 Pieksämäki, Finland; [3]MTT Agrifood Research Finland, Animal Production Research, FIN-31600 Jokioinen, Finland

Oocyte recovery after slaughter and donor-wise *in vitro* embryo production is a means of increasing the number of offspring for top breeding milking cows. Here we summarize the efficiency of such embryo production during three years (2001, 2003-2004).

Ovaries of 15 slaughtered donor cows were removed and transported in physiological saline at room temperature to the laboratory. Oocytes were collected and matured in standard IVM medium, TCM-199 with glutamax-I (Gibco, Paisley, UK) containing 0.25 mM Na-pyruvate, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 10 μg mL^{-1} LH, 2 μg mL^{-1} FSH, 1 μg mL^{-1} β-estradiol and 10% FBS (Gibco, New Zealand). Fifteen different top breeding bulls were used in IVF with 10^6 spermatozoa ml^{-1} in IVF-Talp and 10 μg mL^{-1} heparin. Putative zygotes were cultured either in BOEC co-culture (zygotes of 3 donor cows) or in modified SOFaaci + 4 g L^{-1} faf-BSA (Holm *et al*. 1999 Theriogenology 52, 683-700). On Day 7 (Day 0=IVF), grade I-III embryos were selected for embryo transfer (heat=IVF day). Embryos of 4 donor cows were biopsied for sex determination prior to transfer. The embryos exceeding the number of recipients available were frozen in 1.5 M ethylene glycol (IcpBio, Auckland, NZ).

The summary of the donor-wise IVP of the 15 slaughtered top breeding donor cows is shown in Table 1. Eleven of the 15 donor cows (73%) produced transferable embryos after IVP resulting in total number of 82 Day 7 embryos (12% of all oocytes). Thirty-eight embryos were transferred as fresh and 12 resulted in pregnancy (32%). None of the nine embryos transferred after freezing resulted in pregnancy. To date, eight calves have been born (5 females, 3 males). In conclusion, despite of the high individual variation in the efficiency of donor-wise IVP, it is possible to increase the number of offspring for top breeding donor cows even after slaughter.

Table 1. Summary of oocyte recovery, Day 7 embryo production and pregnancy rate after donor-wise IVP using slaughtered top breeding oocyte donor cows.

Total number of donors	Total number of good quality oocytes (mean ± sd) (min-max)	Total number of Day 7 embryos (mean ± sd) (min-max)	Pregnancy rate of freshly transferred embryos (%)
15	669 (45 ± 34) (6-119)	82 (5.5 ± 5.9) (0-20)	12/38 (32%)

Notes

ALTERATIONS IN THE ABUNDANCE OF GENE TRANSCRIPTS DURING BOVINE PRE-IMPLANTATION DEVELOPMENT

KAŇKA J., NĚMCOVÁ L., KEPKOVÁ K.

Institute of Animal Physiology and Genetics, Laboratory of Developmental Biology, Czech Academy of Sciences, 277 21 Liběchov, Czech Republic

The main goal of this study was to identify mRNA transcripts, the content of which increases during bovine pre-implantation development, and which therefore may play a role in the activation of the embryonic genome. Bovine embryos were obtained after in vitro maturation of oocytes and their subsequent fertilization and culture in vitro (Pavlok et. al., 1992, Mol. Reprod. Dev. 31:63-67). We compared the gene expression profile between 4-cell stage embryos and bovine MII oocytes using the technique of supression subtractive hybridisation (Clontech PCR-Select cDNA Subtraction kit, BD Biosciences Clontech). Total RNA from 30 oocytes at metaphase II or late 4-cell stage embryos (45 hpf) was used for reverse transcription, cDNA amplification and subsequent subtractive hybridization. Differentially expressed amplicons were subcloned (TA cloning kit, Invitrogen) and 60 of them sequenced (ABI Prizm 310, PerkinElmer). Analysis of these sequences with the GenBank database resulted in identification of 41 known cDNAs and 19 novel sequences. From known cDNAs, 3 exist more then one time in our library. The expression of five differently expressed genes with apparent functions in cell cycle progression, chromatin remodelling, and splicing or translation initiation was further characterized during pre-implantation bovine development (MII oocyte, 2, 4, early 8, late 8-cell stage, morula, blastocyst, hatched blastocyst) by real-time RT-PCR (RotorGene 3000, Corbett Research). The data were obtained from three independent real-time RT-PCRs from three different batches of embryos. The significance of differences between stages was evaluated using a t-test (SigmaStat).

We observed an increase in the relative abundance of mitosin, nonhistone protein HMG17 and splicing factor SRp20 both in the 2-4-cell stage and late 8-cell stage. The transcription of splicing factor SRp20 is α-amanitin sensitive in 2-4-cell stage and late 8-cell stage embryos. This is the first time that expression of SRp20 has been described in preimplantation embryos; it is expressed during the period of minor gene activation. The transcription of mitosin and nonhistone protein HMG17 is α-amanitin sensitive only from late 8-cell stage. The mRNAs for translation initiation factors (eIF4A, eIF4E) show the highest abundance in MII oocytes; their content decreased gradually thereafter until the early 8-cell stage. The increase of expression to the late 8-cell stage can be inhibited by α-amanitin. All described genes may play an important role in the embryonic genome activation and during pre-implantation development of bovine embryos.

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Notes

INFLUENCE OF CYSTEAMINE SUPPLEMENTATION DURING IN VITRO CULTURE OF BOVINE EMBRYOS

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

[1]*Clinic for Reproduction and Obstetrics, Faculty of Veterinary Medicine, University of Zagreb, Croatia*
[2]*Center for reproduction and Animals Breeding of Croatia, Zagreb, Croatia*

The objective of this study was to evaluate the effect of cysteamine addition during *in vitro* culture on bovine embryo quality. Bovine oocytes collected from ovaries of slaughtered donors were matured *in vitro* in medium 199 supplemented with 10% FCS, FSH/LH (Pergonal, 75/75 IU, Serono), 1 µg/ml estradiol-17ß and 100 µM cysteamine. Matured oocytes were fertilized with modified Tyrode's solution supplemented with 10 µg/ml heparin, 0.5 µg/ml hypotaurine, 0.5 µg/ml epinephrine and 6 mg/ml BSA. Fertilization *in vitro* was performed with frozen/thawed bull semen prepared using BoviPure® separation protocol. Following fertilization, embryos were cultured in SOFaaBSA without glucose. After 48 h embryos were transfered to SOFaaBSA with 1.5 mM glucose (SOF g) with or without cysteamine (Cys). Embryos that had developed to the ≥4 cell stage after 48 h of culture were allocated to one of four different groups: 1) 0 Cys; 2) 50 µM Cys; 3) 100 µM Cys and 4) 200 µM. Cys. After 48 h of IVC in those media the embryos were transfered to SOF g without cysteamine and cultured until Day 9. A total of 958 oocytes were used in seven replicates. The number of cleaved embryos on Day 2, the total number of morulae (M) and blastocysts (Bl) on Day 7 and the numbers of hatched blastocysts (hBl) on Day 9 were recorded. Differential staining of the inner cell mass (ICM) and trophectoderm (TE) cells were performed on Day 9 blastocysts. The average values of the IVF results were: 83% cleaved on Day 2, 26.2% of M+Bl on Day 7 and 11.3% of hBl on Day 9 of development, without significant differences between treatments (P>0.05), although the difference in the percentage of morulae and blastocysts on Day 7 between 0 Cys group and 100 µM Cys group was over 10% (21.9% vs 33.3%).

Table 1. Number of total and inner cell mass cells (mean±SEM) in Day 9 Bl

Group	N	total cell no.	ICM cell no.	Proportion (%)
0 Cys	11	125.9±11.5 ^{a}	33.4±4.1 ^{b}	26.6±1.6 ^{b}
50µM Cys	12	129.9±10.9 ^{a}	37.9±3.9 ^{ab}	29.6±1.5 ^{ab}
100µM Cys	15	141.6±9.8 ^{a}	48.7±3.6 ^{a}	33.7±1.3 ^{a}
200µM Cys	7	85.0±14.4 ^{b}	22.6±5.2 ^{b}	26.5±1.9 ^{b}

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

The embryos cultured in the presence of 100 µM cysteamine had a significantly higher number and proportion of ICM cells which demonstrated that cysteamine supplementation during *in vitro* culture improves embryo quality. The addition of 200 µM cysteamine had a deleterious effect on embryo quality.

Notes

EFFECT OF FOLLICULAR SIZE IN THE DROMEDARY (CAMELUS DROMEDARIUS) ON THE IN VITRO DEVELOPMENTAL COMPETENCE OF OOCYTES AND THE VIABILITY OF EMBRYOS AFTER TRANSFER

KHATIR H.[1], ANOUASSI A.[1, 2], TIBARY A.[3]

[1] *Veterinary Research Centre, Abu Dhabi, United Arab Emirates*
[2] *Institut Agronomique et Vétérinaire Hassan II, Rabat, Morocco*
[3] *Department of Veterinary Clinical Science and Center for Reproductive Biology Washington State University, Pullman, WA, USA*
Corresponding author: Khatir Hadj e-mail: khatir67@emirates.net.ae

Several studies have shown that developmental competence of oocytes is acquired progressively during follicular growth; oocytes originating from large follicles resulted in greater in vitro embryo development than oocytes from small follicles (cattle: Lequarre et al., Theriogenology 2005, 63: 841-859).The aim of this study was to determine the effect of follicle size in camels on the quality of oocytes and their competence for development in vitro and in vivo.

Ovaries from a slaughterhouse were dissected to obtain two classes of follicle size: small (3-6 mm) and large (> 6) follicles.Quality of the oocytes was assessed after IVM, IVF and IVC of cumulus oocytes complexes (COCs). All cultures were done in 4 replicates at 38.5°C, under 5% CO_2 and high humidity (>95%). Only COCs with cumulus and homogenous (dark) cytoplasm were used. Maturation was completed by incubation in TCM-199 medium supplemented with 10% FCS, 10 ng/ml EGF and 250 µM cysteamine for 28 h. Nuclear maturation rate for each class of follicle size was determined by epifluorescence microscopy in a sample of oocytes (n=30) stained with Hoechst 33342.

In vitro fertilization was performed using fresh semen (0.5 x 10^6 spermatozoa/ml in modified TALP-solution). Fertilized oocytes were cultured in mKSOMaa, under 5% O_2 and 90% N_2. The viability of some good in vitro-produced hatched blastocysts from the two groups (3-6 mm: 15, >6 mm: 22) was assessed by transfer to synchronized recipients. Data were analysed by Chi-square and Fisher's exact test with $P<0.05$ considered significant.

The percentage of COCs reaching metaphase II (MII) after 28 h of maturation was 86% (26/30) and 67% (20/30) for oocytes originating from large and small follicles, respectively ($P>0.1$). The rate of total cleavage (2 cells to blastocyst stage) was greater ($P<0.05$) for oocytes originating from large follicles (72%; 116/162) than those derived from small follicles (59%; 140/237). The percentage of fertilized oocytes reaching the blastocyst stage was 35% (57/162) and 20% (48/237) for oocytes collected from large and small follicles, respectively ($P<0.05$). At the end of in vitro culture (IVC), more embryos (52%; 30/57) obtained from large follicles hatched than those form small follicles (32%; 17/48) but this difference was not significant ($P>0.1$). Regarding the viability of embryos after transfer, none of the hatched blastocysts from small follicles resulted in a detectable embryonic vesicle. However, 68% (15/22) of the transferred hatched embryos from large follicles developed until 60 days of pregnancy. Between the third and the fourth months, two females aborted and to date 45% (10/22) from this group (large follicles) remain pregnant.

This study shows that in dromedaries, oocyte developmental competence is acquired late during the final phase of follicular development and this developmental ability affects not only the in vitro quality of embryos but also their viability after embryo transfer.

Notes

EFFECT OF SOMATOTROPIN ON THE CYTOPLASMIC MATURATION OF BOVINE OOCYTES CULTURED IN VITRO

KUZMINA T.[1], TORNER H.[2] , ALM H.[2], DENISENKO V.[1], SHOKIN O.[1]

[1]Department of Genetics & Biotechnology, All-Russian Research Institute for Farm Animal Genetics & Breeding, 189620 St.Petersburg-Pushkin, Russia
[2]Department of Reproductive Biology, Research Institute for Biology of Farm Animals, 18196 Dummerstorf, Germany

In order to increase the quality of embryos, the basic fundamentals of in vitro cytoplasmic maturation of a donor's oocytes need to be completely understood. In previous studies we demonstrated that recombinant bovine somatotropin (rbGH) supplementation remarkably enhanced the proportion of the late morulae and blastocysts obtained from the oocytes in vitro. We had analysis mitochondrial activity, concentration of Ca^{2+} in intracellular stores of matured oocytes and morphology of 2, 8-16 cell's embryos (including chromatin stage (Tarkowsky, 1966). A total of 402 oocytes and embryos were evaluated. Oocytes were cultured for 24 h at 38.5° C under 5% CO_2 in TCM 199 with 10% FCS, $1x10^6$ granulosa cells (control - C). Culture medium of experimental group (E) was modified by addition of 10 ng/ml rbGH (Monsanto). After IVM, oocytes were fertilized using frozen bovine sperm and zygotes were cultured in TCM 199 with 20% FCS. Concentration of Ca^{2+} in intracellular stores of oocytes (fluorescence of complex membrane-bound calcium-chlortetracycline) was fixed with a fluorescent microscope (Emission: 530nm, using 40 μM chlortetracycline, Sigma). The results were expressed in arbitrary units of fluorescence (A.U.). We evaluated oxidative activity of mitochondria in matured oocytes using the fluorescence probe Mito Tracker Orange CMTM Ros (200 nm in PBS with 3% BSA, 30 min, Molecular Probes, Oregon, USA). The fluorescence intensity (μA) was measured by the Nikon Photometry System P 100 (red emission, 570 nm). Student's and Chi-square test was used for statistic analysis. Concentration of Ca^{2+} in the intracellular stores of oocytes matured in medium with rbGH was lower than in the intracellular stores of oocytes cultured in medium without rbGH (0.85±0.03 A.U.,n=39 v.s 0.96±0.03 A.U., n=48, $P < 0.05$). Intensity of Mito Tracker Orange CMTM Ros fluorescence in oocytes after 24 h of culture in the presence of rbGH was higher than in control group (309.21 μA n=34, v.s. 119.97 μA, n=39, $P<0.01$). There were no differences in the percentage of normal and degenerate 2-cell embryos in the control and experimental groups (C - 71.8%, E – 86.5%, n=123). 41.2% (21/51) of 8-16 cell embryos in the control group had different morphology and chromatin abnormalities (lysis of blastomeries, disruption of membranes, blastomeries without nucleus, pycnotic chromatin, chromatin degeneration, etc.). The proportion of degenerate 8-16 cell embryos obtained from oocytes matured in the medium with rbGH was significantly lower (20.6%, 14/68, $P<0.05$) than in control group. We have shown that under the influence of rbST oxidative activity of mitochondria is increased in matured bovine oocytes and content of Ca^{2+} in the intracellular stores of oocytes is decreased. Early bovine embryos obtained from the oocytes matured in the presence of rbGH have less percentage of morphology and chromatin abnormalities. These data demonstrate that the metabolic activity of in vitro matured oocytes is correlated with the quality of embryos.

Notes

PORCINE NUCLEAR TRANSFER WITH MESENCHYMAL STEM CELLS AND THEIR DIFFERENTIATED DERIVATIVES

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

[1]*Laboratorio di Tecnologie della Riproduzione, CIZ srl, Istituto Sperimentale Italiano Lazzaro Spallanzani, Italy.*
[2]*Dipartimento Clinico Veterinario, Università di Bologna, Italy*

Mesenchymal stem cells are a well known in vitro model of proliferating stem cells that can be efficiently differentiated along the osteogenic lineage. In this study we used porcine mesenchymal stem cells and their differentiated derivatives as nuclear donors in nuclear transfer experiments to investigate the effect of stemness on porcine preimplantation development.

For this purpose bone marrow cells were obtained by flushing the femurs and tibias of three one-week old piglets immediately after slaughter and cultures of mesenchymal stem cells were established in medium 199/DMEM (1:1) with 10% FCS. The undifferentiated cultures were exposed to osteogenic medium, containing dexamethasone 100 nM, ascorbic acid 0.25 mM and βglycerolphosphate 10 mM, for 8 or 21 days obtaining early and late osteocytes characterised by alkaline phosphatase and Von Kossa stainings. Adult skin fibroblast were used as control.

Pig oocytes collected from abattoir ovaries were matured for 44 h in DMEM/F12 supplemented with 10% FCS, ITS (Sigma, Insulin Transferrin sodium selenite, 1 µl/ml), 1 mM sodium pyruvate, 0.5 mM L-cystein, 10 mM glycine, 100 µM ß-mercaptoethanol, gonadotropins (FSH and LH 0.05 IU/ml each; Pergovet 75, Serono). Following zona-free enucleation and nuclear transfer, NT-embryos were activated 50h after the onset of maturation (2 to 3 h after fusion). For activation reconstructed embryos were washed in 0.3 M mannitol (1 mM Ca and100 µM Mg) solution and activated by double DC-pulse of 1.2 Kv/cm applied for 30 µsec followed by 4 h culture in m-SOFaa with cytochalasin B (5 µg/ml).

Table 1. Development of pig nuclear transfer (NT) embryos derived from adult fibroblasts, mesenchimal stem cells (MSC) and osteocytes, differentiated from MSC.

Cell type	N of NT embryos	Cleavage N(%)	CM D5 N(%)	BL D6 N(%)	BL D7 N(%)
Adult fibroblasts	86	74 (86)	41 (47.7)	30 (34.9)	32 (37.2)
MSC	85	77 (90.6)	39 (45.9)	34 (40)	38 (44.7)
Early osteocytes	82	71 (86.6)	37 (45.1)	26 (31.7)	27 (32.9)
Osteocytes	86	74 (86)	42 (48.8)	34 (39.5)	33 (38.4)

CM D5: compacted morulae on day 5; BL D6: blastocyst on day 6; BL D7: blastocyst on day 7

The data shown in Table 1 indicate that both undifferentiated and differentiated porcine mesenchymal stem cells give rise to similar rate of cleavage and high rate of preimplantation development to the blastocyst stage (ranging from 32.9 to 44.7%). When compared with adult fibroblasts used as control no difference was observed in developmental rates at all stages examined. In conclusion no effect of the stem cell nature of the nucleus was observed.

Notes

REPEATABLE USE OF HOLSTEIN-FRIESIAN HEIFERS AS EMBRYO DONORS IN A COMMERCIAL EMBRYO RECOVERY PROGRAM

LINDEBERG H.[1], KANANEN-ANTTILA K.[1], VARTIA, K.[1], AALTO, J.[1], HALMEKYTÖ M.[1], SALAHEDDINE M.[2], DE LOOS F.[2]

[1]Institute of Applied Biotechnology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland, [2]Pharming Technologies B.V., 2333 CN Leiden, The Netherlands

A total of 20 heifers were assigned to an embryo recovery program. Twelve were flushed 4 times (once a month) between November 2004 and March 2005. At the beginning of the program, oestrus was induced by 1.8 mg PG (Genestran vet 75 µg/ml, Vetcare Oy, Finland). Ten days after induced oestrus, the donors were treated with FSH (Folltropin-V, Bioniche, Canada) twice daily over 4 days on a decreasing dose regimen (50 mg + 50 mg, 50 mg + 50 mg, 30 mg + 30 mg, 20 mg + 20 mg). In December, the FSH dose was decreased (40 mg + 40 mg, 30 mg + 30 mg, 30 mg + 30 mg, 20 mg + 20mg) because of overstimulation of the ovaries. Luteolysis was induced by PG at 84 h (1.8 mg) and 96 h (1.8 mg) after commencing superovulation treatment. The donors were inseminated at 36 h, 40 h and 60 h after the second PG treatment with frozen-thawed semen of two different transgenic bulls (bull 1: 42 recoveries; bull 2: 6 recoveries). At 36 h and 40 h insemination, two straws were used and at 60 h insemination only one straw was used. Embryos were recovered on Day 7 (Day 0 = day of first insemination). After recovery, the heifers were treated with PG (1.8 mg) and a PRID device (CEVA, Santé, France) was inserted into the vagina for 10 days. One day before removal of the devices, the heifers were treated with PG (1.8 mg) to induce luteolysis. A new FSH treatment started 9-11 days after the induced oestrus. Recovered embryos were evaluated, grade I and II embryos were trypsin treated and frozen in 1.5 M ethylene glycol and stored in LN_2.

A total of 527 embryos were recovered from the 12 heifers (Table 1). In five recoveries of 48, neither embryos nor unfertilized oocytes were recovered. The majority of the transferable embryos were blastocysts (54.7%, 179/327) and grade I embryos (75.8%, 248/327).

Table 1. Embryo recovery results of 12 heifers that were synchronized for four consecutive flushings between November 2004 and March 2005.

	Total number of grade I-II embryos	Grade I-II embryos per donor	Total number of degenerated embryos	Total number of unfertilized oocytes	Total number of embryos and oocytes	Total number of corpora lutea	Recovery rate (%)
1. recovery	99	8.3	40	34	173	199	87
2. recovery	63	5.3	47	25	135	159	85
3. recovery	92	7.7	30	8	130	151	86
4. recovery	73	6.1	13	3	89	124	72
Total	327 (62%)	6.8	130 (25%)	70 (13%)	527	633	83

Total number of grade I-II embryos did not differ significantly between recoveries. Total number of degenerated embryos and UFOs decreased significantly after two recoveries ($P<0.007$, $P<0.001$, Nonparametric Friedman-test). Total number of corpora lutea decreased significantly from 1st to 4th recovery ($P<0.035$, Nonparametric Friedman-test). In conclusion, four consecutive recoveries in four months were easily performed with this program. The heifers steadily produced high number of grade I-II embryos and decreased the number of degenerated embryos and UFOs during the course of the program.

Notes

FIRST PREGNANCIES AFTER TRANSFER OF BOVINE IN-VIVO EMBRYOS CHARACTERIZED BY NANORESPIROMETRY

LOPES A.S.[1,2*], MADSEN S.E.[3], RAMSING N.B.[4] , LARSEN L.H.[4], GREVE T.[2], CALLESEN H.[1]

[1]Danish Institute of Agricultural Sciences, 8830 Tjele, Denmark; [2]Royal Veterinary & Agricultural University,1870 Frederiksberg C, Denmark; [3]Trans - Embryo Genetics, 8740 Brædstrup, Denmark; [4]FertiliTech ApS, 8000 Aarhus C, Denmark

Quantification of oxygen consumption by single embryos combined with a morphological assessment have potential to improve the selection of embryos by better prediction of their developmental competence. Earlier studies provided evidence that respiration rates of individual embryos were directly correlated with quality (Abe *et al.* 2003 J. Reprod. Dev. 49:193-202) and survival following embryo transfer (Overström *et al.* 1992 Theriogenology 37:269). This study further extended these observations by investigating if respiration rates of individual embryos, measured with nanorespirometer technology (Lopes *et al.* 2005 Reprod. Fertil. Develop. 17:151) are useful indicators of subsequent embryo viability.

Bovine in-vivo embryos (n=28) were recovered on Day 7 by non-surgical collection from 7 superovulated and inseminated cows. The embryos were morphologically classified as quality A, B and C by an experienced practitioner and according to the definitions specified in the IETS Manual (1998). Respiration rates were measured on each embryo as previously described (Lopes *et al*. 2005 Reprod. Fertil. Develop. 17:151) and 19 embryos were subsequently transferred individually to synchronized healthy recipients. A pregnancy diagnosis was performed on Day 35 and 60. Data were analyzed using Proc Mixed and values are given as mean ± SD.

The average respiration rate for in-vivo Day 7 embryos was 0.84 ± 0.34 nL/h. The average respiration rates for the different morphological qualities were: 1.17 ± 0.11^{a} (n=6), 0.84 ± 0.09^{b} (n=9) and 0.63 ± 0.09^{b} nL/h (n=9), for quality A, B and C embryos, respectively (^{a,b}: $P<0.05$; values with different superscripts vary significantly). After transfer of the measured embryos, 13 (68 %) of the cows were diagnosed pregnant on day 35 and 12 (63 %) on day 60. The average respiration rates did not differ significantly between embryos developing and not-developing into viable foetuses (0.72 ± 0.16 vs 0.58 ± 0.25 nL/h; $P>0.05$). Interestingly, neither respiration rate nor morphology of the embryos seemed to affect the subsequent pregnancy status ($P> 0.05$).

We concluded that measuring respiration rates of individual bovine embryos using the nanorespirometer technology does not influence their subsequent viability, as embryos developed into viable foetuses at rates considered normal under farm conditions. Respiration rates of in-vivo embryos were significantly lower than those of in vitro produced day 7 embryos (Lopes *et al.* 2005 Reprod. Fertil. Develop. 17:151), measured with the same technology ($P<0.05$). Furthermore, results were considerably lower than those documented by Overström (Theriogenology 1996 45:3-16) but higher than those reported by Thompson (Theriogenology 1995 43:337). Finally, we did not observe a significant correlation between respiration rates and the subsequent pregnancy status. However, the limited number of embryos transferred so far may be inadequate to see such effect. Further investigations involving a higher number of single transfers are therefore planned.

Notes

THE EFFICIENCY OF IN VITRO EMBRYO PRODUCTION FROM HIGH PERFORMANCE PARENTS IN CATTLE

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

Dept Gen Reprod, Veterinary Research Institute, 621 32 Brno, Czech Republic

An advanced method allowing better utilization of the female genetic material is in vitro production of embryos. In some donors, however, this method is not effective enough, because many factors, including donor follicular status, influence the outcome. In our preliminary study we have documented that, in experimental donors, the growth phase was more effective for embryo production than the dominant phase (Machatkova et al., 2004, Theriogenology 61:329). The aim of the present study was to assess data on embryo production from high performance donors and bulls. A total of 50 lactating Holstein and Siemmental donors, aged about 6-yr, with some reproductive disorder and five AI bulls (A, B, C, D and E) with good outcomes in non return rate were used. The oestrous cycles of donors were synchronized before being slaughtered on Day 3 after oestrus, in the growth phase of follicular development. Oocytes were matured, fertilized, and cultured by the standard protocol. The embryo production was expressed in terms of oocyte and embryo numbers collected per donor and embryo development rates for both the donors and the bulls. Data were analyzed by Student´s t-test, its variability was demostrated by box plots. A total of 1830 good quality oocytes were collected and 321 transferable embryos were prepared (36.6 and 6.4 per donor, respectively). According to the oocyte yield, the donors fell into Group 1 (n=22), Group 2 (n=15) or Group 3 (n=14) with low (≤ 25), moderate (26 to 50) or high (≥50) numbers of oocytes, respectively. There were significant differences ($p \leq 0.01$) in embryo numbers per donor amongst Groups 1, 2 and 3 (3.2±1.4, 6.5 ±3.1 and 12.2±3.0, respectively). High variability in embryo number per donor within each group is shown in Figure 1. No significant differences in embryo development rates were found amongst Groups 1, 2 and 3 (21.0 %, 17.3 % and 18.1% respectively), but high variability in embryo development rate within each group is evident from Figure 2. The embryo development rate did not differ significantly amongst bulls A, B, C and D (18.1%, 19.3%, 21.0 % and 26.4 %, respectively), it was significantly ($p \leq 0.01$) lower only in bull E (6.2%). But high variability in embryo development rates for bulls B and D contrasted with low variability in bulls A, C and E (Figure 3). One hundred and six embryos were transferred to 106 synchronized recipients, of which 51 (48.1%) became pregnant.

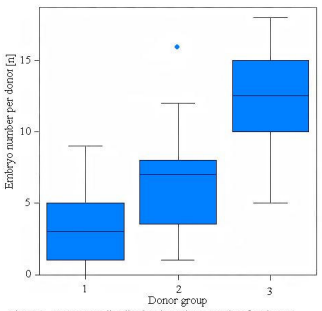

Figure 1. Frequency distribution in embryo number for donors

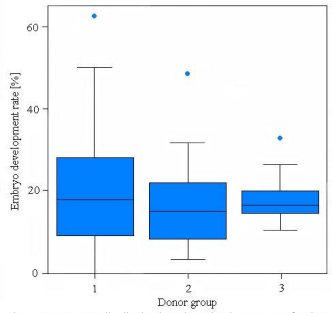

Figure 2. Frequency distribution in embryo development rate for donors

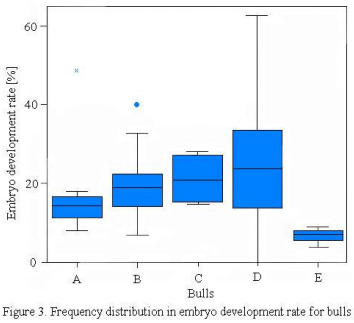

Figure 3. Frequency distribution in embryo development rate for bulls

In conclusion: a) ability to produce oocytes differs in donors of the same follicullar development status; b) yield of embryos from an individual donor is primarily influenced by the number of collected oocytes, but also by selection of an appropriate bull; c) some bulls are compatible with any donor, but others prefer one donor to other ones.

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Notes

UNIFORMITY IN IVP EMBRYO GRADING AMONG TECHNICIANS; DEVELOPMENT OF A MONITORING SYSTEM

MERTON J.S.[1], KOENEN E.P.C.[1], POULS A.[2], HAZELEGER W.[2]

[1]HG, R&D, PO Box 5073, 6802 EB Arnhem, The Netherlands
[2]Wageningen University, PO Box 338, 6700 AH Wageningen, the Netherlands

Evaluation of embryos is a qualitative method, which makes it susceptible to subjective interpretation. By means of a stereo microscope, different aspects of the morphological appearance are assessed to give the embryo its final grade: presence of cellular fragments, degree of compaction, color and texture of blastomeres and uneven cleavage divisions, as described in the international standardized coding system for stage of development and quality of the embryo (IETS Manual, 3rd Edition). Despite this code, uniformity in embryo grading among technicians needs continuous attention. This study aimed to develop a monitoring system, based on field data, in order to monitor and maintain uniformity in embryo grading among IVP-technicians within our company (HG).

Firstly, distribution of the embryos among the different qualities (IETS, grade 1,2, 3 and 4) was analyzed (PROC MIXED; SAS®, 1997). Data included 77,075 IVP embryo evaluations, performed between September 1993 and March 2003. Embryo evaluation was performed on Day 7 and was based on OPU batches from which the non-divided oocytes were removed at Day 4. Secondly, individual embryo evaluation results were compared with adjusted calving rates. Calving results were available on 8889 fresh and 4579 frozen/thawed (grade 1 and 2) IVP embryos, transferred between January 1995 and April 2002.

The average proportion of grade 1, 2, 3 and 4 embryos was 17.5%, 19.6%, 10.9% and 52.1%, respectively. Table 1 shows for each technician the deviation from the mean in combination with the difference in calving rate between grade 1 and grade 2 embryos. For most of the technicians, the deviations from the average proportion are inverse when comparing grade 1 and grade 2 embryos. The difference between the estimated calving rates for grade 1 and grade 2 embryos varied from – 0.1% (technician E) to 10.1% (technician J). It is generally accepted (and also found by us) that this gap in calving rate between grade 1 and grade 2 embryos should be at least 5%.

Table 1. Estimated deviation from the average proportion (percent points) and difference in calving rate for grade 1 and grade 2 embryos per technician.

		Embryo score; Deviation from the average		Calving rate of fresh IVP embryos; Difference between grade 1 and 2	
Technician	n	Grade 1	Grade 2	n	%
A	741	- 3.5[a]	1.4	78	1.5
B	968	0.2	0.6	108	5,3
C	2201	- 0.4	1.7	162	4.6
D	3184	0.6	- 0.3	168	3.8
E	3664	0.0	1.1	268	- 0.1
F	6079	2.7[a]	- 4.8[a]	1125	5.5
G	6875	- 1.6[a]	1.3	508	7.5
H	8015	3.3[a]	- 3.4[a]	1966	5.7
I	8495	- 0.3	1.2	586	5.9
J	9624	- 1.1	1.2	513	10.1
K	27229	0.1	0.1	3407	9.8

[a] significantly deviating from the mean ($P < 0.05$).

In conclusion, technicians grade embryos differently. Interestingly, technicians that apparently score in the same way (i.e. have the same deviation from the average), can have a different gap in calving rate between grade 1 and grade 2 embryos (e.g. technician E and K, with a gap of –0.1% and 9.8%, respectively). Since calving rate was considered as the best indicator of embryo quality, it was decided that a future monitoring system should be based on the differences between grade 1 and grade 2 embryos in either non return, pregnancy or calving rate.

Notes

EFFECT OF TRANSGENE CONCENTRATION ON THE EFFICIENCY OF ICSI-MEDIATED TRANSGENIC MOUSE PRODUCTION

MOREIRA P.N.[1], POZUETA J.[2,3], VALDIVIESO F.[3], MONTOLIU L.[2], GUTIÉRREZ-ADÁN A.[1]

[1]Departament of Animal Reproduction, INIA, Ctra. de la Coruña Km. 5.9 Madrid 28040, Spain; [2]Department of Molecular and Cellular Biology, CNB-CSIC, Campus de Cantoblanco, Madrid; [3]Departamento de Biologia Molecular, Centro de Biologia Molecular Severo Ochoa, CSIC-UAM, Madrid.

ICSI-mediated transgenesis production is a complex process and its overall efficiency can be influenced by many different factors including sperm and oocyte donor strain, transgene concentration, size and copy number, DNA vector, and others, whose influence remains poorly characterized. In this study, we have assessed the impact of transgene DNA concentration and size on the efficiency of mouse transgenesis production by ICSI. Presented data include assays with two DNA constructs; a plasmid EGFP of 5.4 kb (pEGFP, Clontech Laboratories, Inc., Palo Alto, CA), and a yeast artificial chromosome (YAC) of 520 kb. ICSI-mediated transgenesis was performed in the B6D2 hybrid mouse strain as previously described (Moreira et al, 2004, Biol Reprod 71:1943) using two concentrations (6 and 15 ng/µl) of the pEGFP transgene and three concentrations (2, 3.6 and 5 ng/µl) of the YAC construct. Fertilized embryos reconstituted after ICSI were cultured *in vitro* until 2-cell stage, transferred into pseudopregnant females, and at Day-14 or at term, fetuses were analyzed by PCR (Moreira et al., 2004) for the detection of the genomic presence of the integrated transgene.

Results are shown in Table 1. A Z-test analysis of the data generated with the 520kb YAC construct indicated that, although an increase in the transgene concentration did not significantly affected the proportion of transgenics among live offspring (integration rate), the number of liveborn transgenics, when expressed as a proportion of injected ova (efficiency rate), increased as the DNA concentration increased up to 3.6 ng/µl and then decreased at 5 ng/µl suggesting that this highest DNA concentration (and/or co-purifying contaminants) might have influenced embryo survival. Such transgene concentration threshold was not reached for the pEGFP construct with the maximum concentration tested (15 ng/µl), since the efficiency rate of the procedure was higher than the one observed with pEGFP concentrations of 6 ng/µl. The fact that much higher concentrations can be used for small constructs than for large ones, also indicate that the first are less toxic. Based on these results, the concentration to be used on ICSI-mediated transgenesis should be optimized for each construct in order to maximize the transgene integration rate among the liveborn offspring and the overall efficiency of the procedure.

Table 1: ICSI-mediated transgenesis obtained for a 520 kb (YAC) construct and a 5.4 plasmid EGFP used at different concentrations

Construct (ng/µl)	Injected Oocytes (No. Sessions)	2-Cells (%)	Embryos Transferred (No. Recipients)	Live Offspring or Fetuses (%)	Transgenic Offspring or Embryos (%)
YAC (2)	346 (7)	176 (51)	215 (10)	20 (9)	2 (10)[a]
YAC (3.6)	350 (7)	218 (62)	228 (12)	52 (23)	11 (21)[b]
YAC (5)	224 (5)	146 (65)	175 (8)	30 (19)	4 (13)[a]
pEGFP (6)	219 (6)	167 (76)	163 (8)	22 (13)	10 (45)[*]
pEGFP (15)	381 (8)	271 (71)	270 (14)	35 (13)	24 (69)[†]

[a, b] and [*, †] Values with different superscripts are significantly different ($P<0.05$)

Notes

ABUNDANCE OF GENE TRANSCRIPTS IN BOVINE BLASTOCYSTS DERIVED FROM OOCYTES WITH DIFFERENT MEIOTIC COMPETENCE COLLECTED AT A DEFINED FOLLICULAR DEVELOPMENT STAGE

NEMCOVA L.[1], MACHATKOVA M.[2], HANZALOVA K.[2], HORAKOVA J.[2], KANKA J.[1]*

[1] *Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburská 89, 277 21 Liběchov, Czech Republic*
[2] *Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic*

The aim of the present study was to compare the expression of selected genes in bovine embryos developed from oocytes with different meiotic competence collected from different-sized follicles at two stages of follicular development. Oocyte subpopulations were recovered separately from small (2-5mm) or medium (6-10mm) follicles either in the growth/stagnation (G/S) or in the dominance/regression (D/R) stages of follicular development. They were collected from slaughtered cows after the characterization of the ovaries of individual donors in terms of morphology. Evaluation criteria for the growth/stagnation and the dominance/regression stages included the presence of hemorrhagic corpus luteum with signs of ovulation and no follicle larger than 11 mm in diameter and the presence of an advanced corpus luteum and two large follicles, 14-15mm and 8-11mm in diameter, respectively (Machatkova et al., Theriogenology 2004, 61:329-335). Oocytes were matured, fertilized and cultured by a standard protocol. Oocytes were matured in TCM 199, fertilization was carried out in Tyrode's medium, zygotes were transferred on the Buffalo rat liver cell line monolayer in B2 Menezo medium at 39ºC and 5% CO_2 (Machatkova et. al., 2004). Oocytes developmental competence was expressed as the percentage of oocytes cleaved on Day 2 (D2) and developed to blastocysts on Days 7 (D7) and 8 (D8) from fertilized oocytes. The data was analyzed by the Chi-square and Student's tests, using the SPSS Version 11.5. for Windows software. The D2 cleavage rates were significantly higher ($p\leq0.05$ and $p\leq0.01$) in oocytes from either medium or small follicles in the growth/stagnation than in the dominance/regression. There were no significant differences in the D7 blastocyst rates between oocytes collected from both medium and small follicles in the G/S or D/R stage. But the D8 blastocyst rate was significantly higher ($p\leq0.01$) in oocytes collected from small follicles in the G/S stage (32.3%) compared with those collected from small follicles in the D/S stage (25.5%). The transcripts of apoptosis regulator box-α (Bax), and gap junction proteins, connexin 43 (Cx 43) and connexin 31 (Cx 31) were studied using real-time RT-PCR. The relative abundance of Bax and Cx 31 exhibited no significant differences in both D7 and D8 blastocysts developed from oocytes collected either from medium or small follicles in the G/S or D/R stage. But the relative abundance of the Cx 43 transcript was significantly higher ($p\leq 0.05$) in D8 blastocysts developed from oocytes collected from both medium and small follicles in the G/S stage compared with those collected in the D/R stage. We conclude that, a) the relative abundance of Cx 43 can be used as a marker of differentiation in blastocyts originated from oocytes with different meiotic competence, b) the level of Cx 43 transcript was greater in embryos derived from oocytes with greater meiotic and developmental competence compared with those derived from oocytes with lesser meiotic and developmental competence.

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Notes

SUPPRESSION OF E-CADHERIN GENE EXPRESSION IN BOVINE PREIMPLANTATION EMBRYOS BY RNA INTERFERENCE TECHNOLOGY USING DOUBLE-STRANDED RNA

NGANVONGPANIT N.[1], MÜLLER H.[1], RINGS F.[1], GILLES M.[1], JENNEN D.[1], HÖLKER M.[1], THOLEN E.[1], HAVLICEK V.[2], BESENFELDER U.[2], SCHELLANDER K.[1], TESFAYE D.[1]

[1] *Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany*
[2] *Institute of Animal Breeding & Genetics, University of Veterinary Science, Veterinärplatz 1, A-1210 Vienna, Austria*

RNA interference (RNAi) has become acknowledged as an effective and useful tool to study gene function in diverse group of cells. We aimed to suppress the expression level of the E-cadherin gene during in vitro development of bovine preimplantation embryos using RNAi approach. In this experiment the effect of microinjection of bovine E-cadherin and Oct-4 double- stranded (ds) RNA on the mRNA and protein expression level of the E-cadherin gene was investigated. For this, a 496 bp long bovine E-cadherin (AY508164) and 341 bp long Oct-4 (AY490804) were prepared using Promega RiboMax^{TM} T7 system (Promega, Madison, USA), where sense and antisense strands were transcribed from the target DNA template. In vitro produced bovine zygotes were categorized into four treatment groups (at least 300 zygotes in each group) including those injected with E-cadherin dsRNA, Oct-4 dsRNA, RNase-free water (which was used for resuspension of dsRNA) and non injected control. Statistical analysis was performed using SPSS versions 13. While the injection of E-cadherin dsRNA resulted in the reduction of E-cadherin mRNA and protein levels at the morula and blastocysts stage, the same transcript and protein products remained unaffected in the Oct-4 dsRNA, water injected and non treated control groups. The relative abundance of E-cadherin mRNA in the E-cadherin dsRNA injected morula stage embryos was 73% reduced compared to the control group (P<0.05). The western blot analysis also showed a significant decrease in the E-cadherin protein (119 kDa) in E-cadherin dsRNA injected embryos compared to the other three groups. Assessment of the effect of the E-cadherin dsRNA on the developmental potential of the embryos showed that both morula and blastocyst rate were lower in E-cadherin dsRNA treated group compared to the other groups. While 22 ± 5.34% of the E-cadherin injected zygotes developed to morula stage, 23 ± 7.73%, 27 ± 7.31% and 38 ± 5.96% of zygotes developed to morula stage in Oct4 dsRNA (n=308), water injected (n=326) and non treated control (n=322), respectively. Moreover, 19 ± 8.11% of the E-cadherin injected zygotes developed to blastocyst stage forming blastocoel cavity compared to 17 ± 9.13%, 27 ± 7.40% and 37 ± 5.73% in Oct4 dsRNA, water injected and non treated control, respectively. In conclusion, our results indicated the RNAi technology is a promising approach to suppress the mRNA and protein products of target genes in bovine embryos in order to study their function in early embryogenesis.

Notes

PARALLEL INVESTIGATION INTO MITOCHONDRIAL ACTIVITY AND CHROMATIN CONFIGURATION IN BOVINE OOCYTES DURING MATURATION IN VITRO

OTZDORFF C.[1], FRIEDRICH M.[2], ALM H.[2], NOHNER H-P.[1], LEIDING C.[1], KANITZ W.[2], TORNER H.[2]

[1] *Besamungsverein Neustadt/Aisch, 91413 Neustadt/Aisch, Germany*
[2] *Research Institute for the Biology of Farm Animals, 18196 Dummerstorf, Germany*

Mitochondria play a vital role in the oocyte to provide ATP for fertilization and pre-implantation embryo development. Data in human and bovine oocytes suggest that the efficiency of mitochondrial respiration in oocytes is closely correlated with the rate of embryo development after fertilisation (Stojkovic et al. 2001; Wilding et al. 2001).

Electron microscopy studies of oocytes have revealed dynamic morphological changes of mitochondria during the pre-ovulatory period. At this time, they are the most prominent organelles in the ooplasm (Motta et al. 2000). They form voluminous aggregates with the smooth endoplasmic reticulum (SER), tubules and vesicles. These mitochondrial-SER aggregates (m-SER) could be involved in the production of a reservoir of energy prior to fertilization. However, the role of mitochondria during maturation, fertilization, and embryonic development is not fully understood (Bavister, Squirrell 2000). There appears to be large differences in the timing of changes in activity and in distribution of mitochondria during oocyte maturation among species (Wilding et al. 2001; Stojkovic et al. 2001; Smaili et al. 2000; Torner et al. 2004).

In this study, we evaluated the chromatin configuration of the nucleus and the distribution and oxidative activity of mitochondria in in vitro matured bovine oocytes using the fluorescence probe MitoTracker Orange CMTM Ros. Cumulus-oocyte complexes (COCs) were classified according to cumulus morphology (compact, dispersed, expanded, without of cumulus) and cultured up to 24 h. At frequent intervals (4 h) during IVM COCs from these 4 categories of cumulus morphology were randomly selected and parallel analyzed for nucleus and mitochondria.

The type of mitochondrial distribution in the oocytes (n = 1473) changed from fine homogeneous to crystalline homogeneous as the interval after start of IVM increased ($P<0.01$) and was associated with the cumulus morphology.

The oxidative activity of mitochondria measured by fluorescence intensity (Em: 570 nm) per oocyte after MitoTracker Orange CMTM Ros labeling increased in the oocyte during first phase of IVM (0 to 12 h), and became lower up to the end ($P<0,05$). The mitochondrial activity in the oocyte depended on the type of surrounding cumulus cells at the beginning of IVM. Oocytes with initial compact cumuli achieved highest level of respiratory activity during IVM.

These results indicate there is a relationship between meiotic progression, cumulus quality and mitochondrial redistribution and their oxidative activity during in vitro maturation in bovine oocytes.

Notes

INFLUENCE OF THE DIFFERENT TIME COMPONENTS BETWEEN FLUSHING AND TRANSFER ON PREGNANCY RATES OF FRESH CATTLE EMBRYOS

PONSART C.[1], QUINTON H.[2], ROHOU A.[2], KEHLEMBO J. [1], BOURGOIN G.[1, 2], HUMBLOT P. [1]

[1] UNCEIA, Département R & D, 13 rue Jouët, BP 65, 94703 Maisons-Alfort cedex
[2] EMBRYO TOP, URCEO, 69 rue de la Motte Brûlon,BP 1978, 35019 Rennes cedex, France

Previous studies have shown that the time interval between flushing and transfer of fresh embryos can influence pregnancy rates following embryo transfer (ET). The aim of this study was to determine which time components can influence embryo survival following ET. The different time components between flushing of a superovulated donor cow and transfer of the collected embryos were investigated under field conditions. For each embryo, the total time spent in vitro (TIV) was recorded. During January 2003, ET technicians (Embryo Top) recorded systematically times corresponding to each step composing the TIV from 231 collections : end of uterus flushing, beginning and end of search /evaluation of embryos, straw loading, first and last transfer of fresh embryos (if different groups of recipients were transferred, times were recorded for each group). Numbers of donor cows and ET technicians were noted for each recovery session (RS). Embryo (stage and quality) and recipient (breed, parity) characteristics were also recorded. A total of 806 fresh embryos (181 RS) were transferred and pregnancy was assessed. Variability of each time components was investigated (Bourgoin et al., AETE 2004). Student t-tests and Chi2 analysis were performed to study the influence of each time component and other variation factors on pregnancy rates. The TIV averaged 190±90 minutes and did not influence pregnancy rates (≤ 4 h = 50.7% (n=621) vs > 4 h = 51.9% (n=185) ; p> 0.05), as well as interval between end of flushing and search (31 ± 26 min) and interval between evaluation and first ET (64 ± 59 min). No effect of embryo stage and breed of recipient was observed. Only significant factors were kept for further analysis. The effects of search duration, intervals between search and straw loading and between first and last ET were tested together with operator, embryo quality, parity and synchronization of recipients in a multivariate logistic model. Pregnancy rates varied with embryo quality (1 = 60,4%, 2=46%, 3=36% ; P=0.001) and were higher in heifers than in cows (53,8% vs 34,8% ; P=0.03). When the time between first and last transfers was higher than 30 minutes, pregnancy rates were strongly decreased (P=0.004 ; Table 1). This interval increased mainly with number of transfers per group, number of herds in which embryos had to be transferred and number of donor cows per operator.

Table 1 : Influence of time between first and last transfers on pregnancy rates following transfer of fresh embryos (P=0.004).

Time (min) between first and last ET	0-10	11-19	20-29	≥ 30
Frequency	215	152	186	253
Observed pregnancy rates (%)	54.0	59.9	54.8	40.3
Odds ratio (95% confidence limits)	1	1.12 (0.75 - 1.9)	0.96 (0.6 - 1.5)	0.56 (0.37 - 0.85)

These results show that good pregnancy results may be achieved with a delay of several hours between flushing and ET, when embryo quality and recipient parity are optimal. However, the total time during which the embryo stays in straw (time between first and last ET) seems to be critical and should not be longer than 30 minutes. This could be related to the small volume of straws, in which characteristics of medium may vary rapidly with changing environmental conditions such as temperature.

Notes

EFFECT OF DIFFERENT PROSTAGLANDIN TREATMENT PROTOCOLS ON THE AREA OF CORPUS LUTEUM, THE LARGEST FOLLICLE AND PROGESTERONE CONCENTRATION IN DAIRY COWS

RÉPÁSI A.[1], BECKERS J.F.[2], SULON J.[2], REICZIGEL J.[3], SZENCI O.[4]

[1]Kenézlő Dózsa Agricultural Ltd, Kenézlő, Hungary; [2]Department of Physiology of Reproduction, Faculty of Veterinary Medicine, University of Liege, Liege, Belgium; [3]Department of Biomathematics and Informatics, Faculty of Veterinary Science, Szent István University, Budapest, Hungary and [4]Clinic for Large Animals, Faculty of Veterinary Science, Szent István University, Üllő – Dóra major, Hungary

Lactating dairy cows with a mature corpus luteum with a diameter of ≥ 17 mm determined by ultrasonography and having a follicle with a diameter of ≥ 10 mm were randomly assigned to different groups. In the first trial, cows (n=49) were treated with a single dose of exogenous prostaglandin (25 mg /Group 1/ vs. 35 mg /Group 2/), and the third group served as control. In the second trial, cows (n=72) were treated with cloprostenol (single vs. double dose at 8-h apart) or dinoprost (single or double dose at 8-h apart). The ovaries of each cow were scanned daily by transrectal ultrasonography to measure the changes in the areas of corpus luteum (CL) and the largest follicle (LF) during the 5-day experiment. Oestrus was detected twice daily. In addition, blood samples were withdrawn from each cow daily for measuring the plasma progesterone (P4) concentrations by a direct solid-phase ^{125}I RIA method.

The area changes of the CL and the LF, and the changes in P4 concentrations were expressed as percentage changes relative to initial value on Day 0. The reason for this was that absolute changes showed strong dependence on the initial area, while relative changes did not. As changes were not normally distributed, the sign test was used. To correct for multiple testing, Bonferroni correction was applied. Besides these Mann-Whitney U-test, Kruskal-Wallis test, Fisher's exact test, chi-square analysis and analysis of variance were used (S-PLUS 2000).

In the first trial, the tendency for the percentage changes relative to the CL area decreased, and for the percentage changes relative to the LF area increased faster, and even the oestrus started sooner in cows treated with 35 mg vs. 25 mg dinoprost. However, these differences between groups were not statistically significant. At the same time, the decrease in the percentage changes relative to the area of CL, and the concentrations of P4 were significant in both groups.

In the second trial, treatment of dairy cows with double injections of prostaglandins (cloprostenol or dinoprost) at an 8-h interval resulted in more cows being observed in oestrus within 5 d after treatment and having significantly higher pregnancy rate ($P < 0.0309$) than those treated with a single prostaglandin injection. Further studies in progress should confirm the benefit of the higher doses of prostaglandin treatments in a larger scale.

Notes

EMBRYO TRANSFER OF BEEF CATTLE BREEDS IN THE CZECH REPUBLIC

ŘIHA J.[1,2], HEGEDÜSOVA Z.[1], PUDILOVA K.[1], LAKOMA Z. [2], SLEZAKOVA M.[2]

[1]*Research Institute for Cattle Breeding Rapotín Ltd., Vikyrovice, 788 13, Czech Republic*
[2]*Agrovýzkum Rapotín, Ltd., Vikyrovice, 788 13, Czech Republic*

Corresponding author: Pudilova Katerina e-mail: katerina.pudilova@vuchs.cz

Embryo transfer as modern biotechnology method can significantly contribute to development and rationalization of beef cattle rearing. The aim of this study was to evaluate the development of embryo transfer in the Czech Republic during the period 1991 – 2004, by examining the recovery rate and the quality of embryos after superovulation treatment and viability after transfer of fresh and frozen embryos. Furthermore, the influence of the morphological quality of transfered embryos and of the interval from freezing to transfer on viability after embryo transfer were evaluated.

The highest average number of available embryos was obtained in 2001 (11.32 ± 7.41 embryos) and the lowest average number was obtained in 1991 (3.82 ± 3.01 embryos). The recovery rate of embryos in the period 1999 – 2003 was similar. The portion of transferable embryos as a proportion of the total number of recovered embryos was highest in 1992 (73.03 %), compared with the lowest portion of transferable embryos which was obtained in 1993 (32.88 %); in this year a high portion of degenerate embryos was recorded.

The results of superovulation were very variable, which may have been related to many factors of varying importance (quality of preparation, animal sensitivity, optimal physiological condition, season, efficiency level, rearing, breeding competency etc).

Conception rate ranged between 47.0 % and 61.6 % in the period 1998 – 2004. The viability of embryos was higher after transfer of fresh embryos in particular years. The conception rate after the transfer of frozen embryos was higher then conception rate after the transfer of fresh embryos from 1999 – was resolved the problems of the long-term preservation of embryos by freezing. From the aspect of development stage and morphological quality of the transfered embryo was achieved the highest conception rate (47.19 %) by morula and from the aspect of freezing interval of embryos was achieved the highest conception rate after transfer of 2401 days (6.5 years) frozen embryos.

In conclusion, the long-term preservation of frozen embryos does not impact on the viability of transfered embryo.

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Notes

DIFFERENTIAL AUTOSOMAL GENE TRANSCRIPTION BETWEEN MALE AND FEMALE IN VITRO PRODUCED BOVINE BLASTOCYSTS

RIZOS. D.[1], DE LA FUENTE J.[1], LONERGAN P.[2], GUTIÉRREZ-ADÁN A.[1]

[1]Dpto. de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, Madrid 28040, Spain. [2]Department of Animal Science, University College Dublin, Lyons Research Farm, Newcastle, County Dublin, Ireland.

In preimplantation bovine embryos, total glucose metabolism is twice as high in male embryos as female embryos, and the activity of the pentose phosphate pathway is four times greater in female than in male blastocysts. We have shown that the mRNA abundance of two X-linked genes (glucose 6-phosphate dehydrogenase -G6PD- and hypoxanthine phosphoribosy transferase - HPRT) are expressed at higher levels in female bovine embryos than in male embryos at the early blastocyst stage (Gutierrez-Adan et al., 2000, Mol Reprod Dev 55:146-151). The objective of this study was to quantify the relative transcript abundance of some important autosomal linked genes between male and female embryos produced in vitro. The quantification of all gene transcripts was carried out by real time quantitative RT-PCR. Embryos (114: 54 females and 60 males) were produced following in vitro oocyte maturation, fertilization with frozen-thawed semen and culture of presumptive zygotes up to Day 7 in synthetic oviduct fluid. Embryos were sexed using specific primers for ZFX-ZFY (Gutierrez-Adan et al., 1996, Theriogenology 46:515-525). The relative abundance of sex-specific mRNA was investigated for genes implicated in resistance to apoptosis (survivin and BAX), response to stress (sarcosine oxidase -Sox, interferon tau -IFNτ, and the heat shock protein 70 -Hsp70), metabolism (GADPH and β-actin), compaction (Na/K ATPase -Na/K- and desmocollin II -Dc II), methylation (transcription factor activator CpG binding protein -CGBP- that binds specifically to DNA elements containing unmethylated CpG motifs), and related to pluripotency (Oct-4). In female bovine embryos a significantly higher expression of IFNτ, Na/K and Dc II was observed. On the other hand, Sox and Oct-4 were up-regulated genes in male embryos (Figure 1). The differences in mRNA transcription of these genes between genders may implicate a new epigenetic process occurring in early embryos; alternatively, the differences in transcription of the autosomal genes may be a consequence of the differential transcription level observed to some X chromosome linked genes.

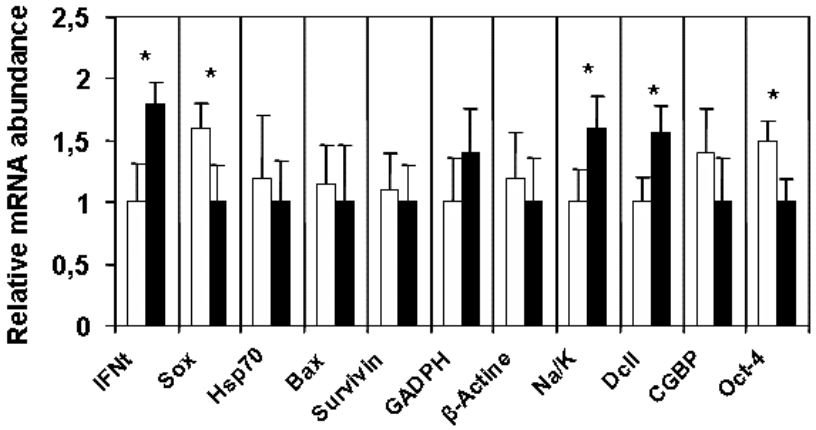

Fig.1: Relative transcription quantity by Real Time PCR of bovine blastocysts produce in vitro. An asterisk (*) indicates a significant difference in relative transcript abundance between male (white bars) and female (black bars) embryos (ANOVA analysis; $P<0.01$)

Notes

GENERATION OF PORCINE TRANSGENIC NUCLEAR-TRANSFERRED EMBRYOS USING ADULT FIBROBLAST CELLS TRANSFECTED BY NOVEL TECHNIQUE OF NUCLEOFECTION

SKRZYSZOWSKA M., SAMIEC M., SŁOMSKI R., LIPIŃSKI D.

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

The aim of our study was to determine the *in vitro* developmental competences of porcine nuclear-transferred (NT) embryos reconstructed with sow ear skin-derived fibroblast cells, which had been subjected to the novel technique of transgenesis by nucleofection with *pWAPhGH-GFPBsd* gene construct. This method of transfection as a combination of lipofection and electroporation enables targeted transduction of the gene construct directly into the cell nucleus of somatic cell, which is mediated through liposome carriers. Moreover, compared to the standard transfection methods of somatic cells such as lipofection or electroporation, nucleofection technique allows considerable shortening of the verification timing for transgenesis efficiency, which is performed through live-eGFP reporter gene expression control, from 24 to 48 h up to even 4 to 6 h after the transfection procedure. The nucleofection samples consisting of 4-5 □ 10^5 cells per 100 □L Dermal Fibroblast Nucleofector™ Solution (Amaxa Biosystems) with the 3.5 □g highly purified linear DNA in the 2 □L Tris/HCl-EDTA buffer were transferred into the amaxa certified cuvettes. The cuvettes were inserted into the Nucleofector™ apparatus, in which AC pulses were generated using U-23 program for high transfection efficiency. Positively-selected transgenic adult fibroblast cells, which had been evaluated by live-eGFP fluorescence excitation, were *in vitro* cultured up to a total confluence state and then used for the somatic cell cloning. The source of recipient cells were *in vitro* matured oocytes. Maternal chromosomes were removed by chemically assisted microsurgical technique. Then, single nuclear donor cells were injected into a perivitelline space of previously enucleated oocytes. Fibroblast cell-ooplast couplets were simultaneously fused and activated with two consecutive DC pulses of 1.2 kV/cm for 60 μsec. Reconstructed embryos were *in vitro* cultured at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air in 50-μL drops of NCSU-23 supplemented with 0.4% BSA-V for 4 days, followed by NCSU-23 with addition of 10% FBS for 2 to 3 days. The rates of cleavage and development to morula/blastocyst stages were examined on Days 2 and 6/7, respectively. A total of 79 enucleated oocytes were successfully fused with transgenic nuclear donor cells and intended to be *in vitro* cultured. Out of 79 reconstructed oocytes, 52 (65.8%) NT embryos were cleaved. The frequencies of cloned embryos, that reached the morula and blastocyst stages, were 29/79 (36.7%) and 14/79 (17.7%), respectively. Nucleofection efficiency of *in vitro* cultured porcine dermal fibroblast cells as estimated by nuclear donor live-fluorescent evaluation based on expression index of eGFP reporter transgene was nearly 100%. It was also found that porcine nuclear-transferred morulae and blastocysts exhibited approximately 100% index of xenogeneic eGFP gene transcriptional activity, which revealed the live diagnostics of emission intensity for green fluorescent protein-derived biochemiluminescence.

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Notes

THE PROTEIN KINASE AKT (PKB) IS INVOLVED IN THE REGULATION OF MEIOTIC MATURATION OF BOVINE OOCYTES IN A MPF AND MAPK INDEPENDENT MANNER

TOMEK W., SMILJAKOVIC T., STERZA F., NOHNER H.P., LEIDIG C., KANITZ W.

Dep. of Reproductive Biology, FBN-Dummerstorf, 18196 Dummerstorf, Germany; BVN Neustadt / Aisch, 91413 Neustadt, Germany

In general, cellular maturation and differentiation processes are accompanied by the expression of specific proteins. Furthermore, the activity of proteins is modulated by the action of specific kinases and phosphatases, which contribute in the control of basic cellular processes, like cellular growth and differentiation, cell cycle regulation and meiosis. In bovine oocytes two distinct protein kinase pathways, namely mitogene-activated protein kinase (MAPK) and maturation promoting factor (MPF, cdc2K) become activated during meiotic maturation (transition from prophase I to metaphase II) and inhibition of these kinases arrest oocytes in the GV-stage. Our previous observations have shown that another kinase, namely the Ser/Thr kinase Akt (PKB) is expressed in bovine oocytes without significant changes in the abundance during IVM. Now we have analysed the activation profile and functional properties of Akt. Performing western blot analysis with Akt and phospho-Akt specific antibodies, our results show that this kinase is highly phosphorylated at Thr 308 and Ser 473 in M I stage of maturation. In contrast, in GV and M II stage, only low, basal levels of phosphorylation could be observed. The activity of this kinase was determined by an in vitro kinase assay with GSK as an external substrate. These results show that Akt is activated in M I stage and therefore the activity is tightly correlated with the phosphorylation of the kinase. These investigations revealed that Akt exhibits a fundamentally different phosphorylation and activation pattern compared to those of MAPK and MPF. Functional properties of Akt were investigated by the specific inhibition of the kinase during IVM by the phosphatidylinositol analogue SH6 and will be discussed.

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Notes

EFFECT OF OPEN PULLED STRAW (OPS) VITRIFICATION ON FERTILIZATION ABILITY AND DEVELOPMENTAL COMPETENCE OF IN VITRO MATURED AND IMMATURE DENUDED OR CUMULUS COVERED PORCINE OOCYTES

VARGA E.[1], GARDÓN J.C. [2], BALI PAPP Á.[1]

[1]*Institute of Animal Breeding, University of West Hungary, Mosonmagyaróvár, Hungary*
[2]*Faculty of Agrarian Sciences, Universidad Nacional de Lomas Zamora, Buenos Aires, Argentina*

The aim of our present work was the comparison of immature and in vitro matured porcine oocytes regarding their morphology and ability to be fertilized after vitrification with the OPS method. Cumulus-intact porcine oocytes were collected from slaughterhouse ovaries of Hungarian Large White gilts. Oocytes were matured in vitro for 42 h in maturation medium at 39˚C, 5 % CO_2 in air. Vitrification of oocytes was performed as described by Vajta et al. (Mol Reprod Dev. 1998, 51:53-8). Matured oocytes were fertilized in vitro with a final concentration of was 1×10^5 spermatozoa/ml. After IVF, embryos were cultured for 24 h. The rate of normal morphology and the rate of fertilization were examined after orcein staining. In the present study the following treatment groups of oocytes were compared in four treatments:

1. cVM group: cumulus enclosed immature oocytes were first vitrified, and then in vitro matured.
2. VdM group: denuded immature oocytes were vitrified and then cultured for 48 h.
3. McV group: cumulus covered oocytes were first in vitro matured, and then vitrified.
4. MdV group: oocytes were in vitro matured first, then denuded and vitrified.

After vitrification there was no significant difference between morphology and fertilization ability of immature and in vitro matured cumulus enclosed oocytes. It appears that the order of maturation and vitrification did not affect the normal morphology rate and on the fertilization ability of COCs. The normal morphology rate for MdV was significantly higher ($P<0.05$) than for VdM (22.35±1.75 % and 13±2.05 % respectively). The rate of normal morphology was significantly higher in cVM group than that of VdM group (21.15±2.18 % and 13±2.05 % respectively). The germinal vesicle injuries were higher in denuded oocytes (VdM) than cumulus covered (cVM) oocytes. These data suggest that vitrification of cumulus enclosed matured (M II stage) oocytes is preferable to vitrification than the denuded M II stage oocytes. The fertilization rate was higher in cumulus-enclosed oocytes than the denuded ones. After vitrification there was no significant difference between M II stage (McV group) and GV stage (MdV group) cumulus covered oocytes. It seems that the beneficial effect of cumulus cells to prevent oocyte damage caused by the vitrification procedure is expressed only on immature (GV) but not on in vitro matured oocytes. After vitrification immature oocytes were matured for 42 hours and it is possible that cumulus cells prevent the oocytes during vitrification.

Our results demonstrate that pig oocytes at the germinal vesicle stage have less survival ability than matured (M II stage) oocytes. Our results suggest that the vitrification/warming procedure is the most effectiveness in cumulus-enclosed oocytes. But there is no difference between the order of maturation and vitrification in cumulus enclosed oocytes. Less immature, denuded oocytes had normal morphology than in vitro matured and denuded ones.

This work was supported by T 031758, 00796/2003 and ARG-13-02.

Notes

FOLLICULAR DEVELOPMENT IN RESPONSE TO A SUPEROVULATORY TREATMENT IN SHEEP IS DELAYED BY THE PRESENCE OF A DOMINANT FOLLICLE

VEIGA-LOPEZ A.[A], COCERO M.J.[A], DOMINGUEZ V.[A], GARCIA-GARCIA R.M.[B], SOUZA C.J.H.[C], MCNEILLY A.S.[D], GONZALEZ-BULNES A.[A]

[A]Dpto Reproducción Animal, INIA. Avda P Hierro s/n, Madrid, Spain. [B]Dpto Fisiologia Animal, Facultad de Veterinaria, UCM, Avda. Puerta de Hierro s/n, Madrid, Spain. [C]Embrapa Pecuaria Sul, C.P 242, CEP 96401-970, Bage, Brazil. [D]MRC, Human Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh, Edinburgh, EH16 4SB, UK.

The presence of a dominant follicle at the start of a superovulatory treatment in sheep affects final embryo outputs, although ovulation rates remained unaffected (Gonzalez-Bulnes et al. Theriogenology, 2002 57:1263-72). The aim of this study was to evaluate whether these effects may be related to alterations in the follicular development during the FSH treatment. Fourteen Manchega ewes were treated with an intravaginal progestagen for 14 days and superovulated with a step-down superovulatory protocol, consisting of 8 doses of oFSH (OVAGEN^{TM}, ICP, Auckland, New Zealand) twice daily, starting on Day 12 after sponge insertion. The follicle population (≥2mm) was assessed by ultrasonography every 12 h from first FSH injection (0 h) to progestagen withdrawal (60 h). Newly recruited follicles were considered as those not previously detected and growing follicles were considered as those increasing in diameter in comparison with the previous observation. In addition, the number of ≥4mm follicles (those able to ovulate in response to the FSH treatment) and the number of ≥6mm follicles (those fully mature and able to ovulate a viable oocyte) were assessed at oestrus detection by direct observations of the ovaries. Data were analysed by one-way ANOVA. All results were expressed as mean ± SEM and the statistical significance was accepted from $P<0.05$. Four ewes were identified as bearing a large presumptive dominant follicle (≥6mm) at the beginning of the FSH treatment (group DF) while the remaining sheep showed follicles ≤ 5mm (group NF; n = 10). A higher number of newly recruited follicles was observed from 12 to 36 h in the NF group, reaching significant differences with the DF group at 12 h (10.7 ± 1.4 vs 7.0 ± 0.5, $P < 0.01$). Follicular recruitment started to decrease at 36 h ($P=0.07$) in the NF group, and 12 h later in the DF group ($P<0.0005$). Overall, higher follicular recruitment was observed in the NF group throughout the observation period (7.7 ± 1.0 vs 6.0 ± 0.4, $P=0.07$). The number of growing follicles increased in the NF group from 12 to 36 h (3.7 ± 1.3 to 10.7 ± 2.5, $P<0.05$), remaining stable thereafter until 60 h (9.7 ± 2.3). However, in the DF group follicles started to grow 12 h later, reaching similar values than NF at 60 h (9.2 ± 0.8). Likewise, a higher number of growing follicles was also found in the NF group throughout the FSH treatment (8.2 ± 1.1 vs 6.5 ± 0.4, $P=0.08$). Finally, the number of ≥4 mm follicles at 60 h was higher in the NF group (21.5 ± 3.3 vs 17.5 ± 0.5, $P=0.08$). The number of ≥4mm follicles (24.2 ± 1.8 vs 16.5 ± 0.6, n.s.) and ≥6mm follicles (14.2 ± 1.4 vs 9.1 ± 0.6, n.s.) at oestrus detection were also higher in NF group, although the differences were not statistically significant. In conclusion, a delay in the follicular recruitment and growth has been found in ewes bearing a dominant follicle at start of the superovulatory treatment. However, no significant differences in the number of follicles reaching preovulatory sizes were found, which would explain the lack of differences in the ovulation rate previously reported. Thus, because of the known differences on subsequent embryo yields between DF and NF groups, further studies should be focused on the functionality of both these preovulatory follicles and their oocytes.

Notes

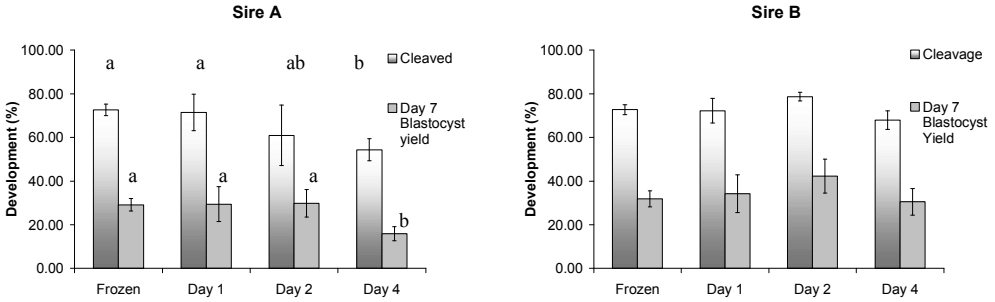
EMBRYO DEVELOPMENT FOLLOWING IVF USING FROZEN OR FRESH SEMEN STORED FOR 1 TO 4 DAYS POST COLLECTION

WARD F.[1], MONAGHAN R.[2], EIVERS B.[2], LONERGAN P.[1]

[1]Department of Animal Science, University College Dublin, Lyons Research Farm, Newcastle, County Dublin, Ireland.
[2]National Cattle Breeding Centre, Enfield, County Meath, Ireland

Although the use of fresh semen in the Irish dairy AI industry only accounts for approximately 5% of total AI usage throughout the year, this may peak to over 25% during the spring breeding season due to the increased demand for proven sires of high genetic merit. The aim of this study was to evaluate the ability of sperm to fertilise oocytes in vitro following freezing and thawing or storage at ambient temperature (15-20 °C) for up to 4 days.

Fresh and frozen semen from the same ejaculate was obtained from 2 sires and diluted in a Caprogen-based (fresh) or Biociphos (frozen) extender. Day 0 was defined as the day of collection. Immature oocytes collected from the ovaries of slaughtered heifers were matured in TCM 199 supplemented with 10% fetal calf serum and 10 μg epidermal growth factor/mL for 24 h at 38.8 °C in a gas phase of 5% CO_2 in air at maximum humidity. Matured oocytes were inseminated in TALP medium, with Percoll-separated frozen-thawed or fresh semen stored at ambient temperature for 1, 2 or 4 days. After 24 h of co-incubation presumptive zygotes were stripped of cumulus cells and cultured for 7 d in modified synthetic oviduct fluid medium. Cleavage rate and blastocyst formation were determined on Days 2 and 7 post insemination, respectively. Four replicates were carried out with a minimum of 40 oocytes per semen type per bull per day. Data were analysed using Chi-squared analysis.



Different superscripts differ significantly ($P<0.05$).

Irrespective of sire, the cleavage rate and blastocyst development were not different between frozen-thawed semen and fresh semen used on Day 1 or Day 2. For Sire A, both cleavage and blastocyst development rates were significantly lower when fresh semen that had been stored for 4 days was used. In contrast, duration of storage (up to 4 days) did not affect the developmental outcome in the case of Sire B. These results would suggest that semen from individual sires may vary in its suitability for use on a fresh semen programme based on its ability to fertilise oocytes in vitro.

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Notes

EFFECT OF EMBRYO SOURCE AND RECIPIENT PROGESTERONE ENVIRONMENT ON EMBRYO DEVELOPMENT IN CATTLE

WOODS A., WARD F., QUINN K., EVANS A.C.O., LONERGAN P.

Department of Animal Science, University College Dublin, Lyons Research Farm, Newcastle, County Dublin, Ireland.

There is increasing evidence of an association between the concentration of progesterone in the first few days after fertilization and embryo survival in cattle. The aim of the present study was to examine the effect of embryo source (in vivo vs in vitro) and the progesterone environment into which it was transferred on Day 7 on embryo size on Day 13. Forty nine continental cross heifers (18-24 months of age) were used. Day 7 blastocysts were produced either in vivo using superovulation, artificial insemination and non-surgical embryo recovery or in vitro using in vitro maturation of immature oocytes recovered from the ovaries of slaughtered heifers, in vitro fertilization and in vitro culture.

In order to produce animals with divergent progesterone concentrations, following synchronization, recipients were either superovulated (n = 10), or not (n = 10). Ten blastocysts, produced either in vivo or in vitro, were transferred to each recipient on Day 7. Both groups were slaughtered on Day 13. A daily blood sample was taken from all animals by jugular venipuncuture from Day 0 to Day 13 to profile progesterone concentrations (by time-resolved fluoroimmunoassay using an AutoDELFIA™ Progesterone kit).

Following slaughter, the reproductive tract was removed, sealed in a plastic bag and placed in a sealed polystyrene box for transportation to the laboratory. The number of CL and total weight of CL tissue per animal was recorded. The oviducts were removed and the uterine horns were trimmed free of excess tissue before being flushed with 100 ml PBS. Embryos were then located under a stereomicroscope and individually measured. Data were analysed using the GLM procedure of SAS (SAS version 8.2, Cary, NC, USA).

The mean superovulatory response, mean recovery rate (embryos recovered as a proportion of the number of CL) and number of transferable embryos per recipient was 12.21 ± 1.28, 7.04 ± 0.75 and 4.52 ± 0.53, respectively. The overall recovery rate was 57.6% (162 oocytes/embryos from 281 CL) and the proportion of transferable embryos was 64.2% (104/162). The mean P4 concentration from Day 0 to Day 6 in the donors was 10.06 ± 3.50 ng/ml. The P4 concentrations between the superovulated and non-superovulated recipients diverged significantly from Day 3 onwards. The mean P4 concentration from Day 7 to Day 13 (the period when the embryos were in the uterus) in the High and Low P4 recipients was 32.70 ± 4.77 and 9.77 ± 2.13 ng/ml, respectively.

The mean embryo length from the High P4 group was 3.56 ± 1.66 mm (n = 29) compared with 1.36 ± 0.24 (n = 24, $P<0.05$) for the Low P4 group. Progesterone concentration was positively correlated with CL weight and CL number. No significant differences were observed due to embryo source. In conclusion, embryos exposed to high progesterone concentrations were significantly larger than those transferred to a normal progesterone environment.

Supported by Science Foundation Ireland

Notes

AUTHOR INDEX

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