20ème COLLOQUE SCIENTIFIQUE

20th SCIENTIFIC MEETING

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Professor Torben Greve

Special Celebration

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Lyon 10th and 11th September 2004

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Professor Torben Greve

A.E.T.E. Medalist 2004

Torben Greve
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Torben was born in 1945 on one of the many Danish islands. He went to the Royal Veterinary and Agricultural University (KVL) in Copenhagen, Denmark, from where he graduated as a veterinarian in 1970. His career started in USA where he spent three years first completing a MSc in dog endocrinology at Kansas State University, then being resident veterinarian at University of California, Davis. After returning to Denmark in 1973, he continued in private veterinary practice in the western part of the country, until 1975 when he was appointed as Assistant Professor at KVL, urged by the late Professor Rasbech. One of the reasons for this change was that Torben had been involved in some of the first practical attempts with the new technology of embryo collection and recovery in cattle; one milestone achieved in 1976 was one pregnancy following two transfers - a 50% rate that would be acceptable even today. At KVL, Torben was appointed Associate Professor in 1979, before becoming Professor in Animal Reproduction in 1987.

Superovulation and embryo handling in cattle became a focus area of Torben's research at KVL. In a strong collaboration with Henrik Lehn-Jensen and Inger Heinze, embryo transfer including cryopreservation was established for practical application in Denmark, and for Torben it resulted in a DrVetSci thesis that he defended successfully at KVL in 1981. Many of the practical problems and considerations were discussed in this thesis, based on, for that time, a large experiment performed in close collaboration with practice. Through this work Torben created a basis for the introduction and further development of embryo technologies in cattle and other farm animals over the next 20 years. He initiated and participated in several new research programmes, and among the many results obtained, the first European calf born at KVL in 1987 as a result of in-vitro maturation and fertilization was a significant milestone. Other technologies have had Torben as the initiator, e.g. use of ultrasound in farm animals for Ovum-Pick-Up and fetal investigations, studies of follicular temperatures in vivo, in vivo development of embryos and fetuses, cloning of embryos, and in more recent years Torben has also been involved in the molecular area such as FISH technology. In such a broad field, covering several technologies and most farm animal species, some areas or disciplines will be favourites, and for Torben it has always been the clinical and the surgical part, but also to have hands and eyes on the embryos. As one side of that, he learned micromanipulation during a study visit in 1991 to Guelph, Canada.

The importance of Torben's research achievements have been very many. Through his own work and subsequent activities, he has contributed to more than 100 articles and book chapters in

the scientific literature. His work has been a basis for the development and practical implementation of embryo technologies in farm animals in Denmark. Another important aspect of Torben's work has been through his involvement in the work of many research students, PhDs, post-docs or other colleagues that have spent time at KVL for varying periods under Torben's supervision and guidance. All these people have learned KVL and Torben's lab as a pleasant place to be, filled with good research and a good atmosphere. The most serious research work can very well be combined with a good laugh.

Over the years, Torben has received many recognitions for his scientific achievements. He has been awarded several academic honours, including an honorary degree from the Swedish University of Agricultural Sciences, as well as research prices from e.g. the Carlsberg Foundation in Denmark. He has been invited as main speaker and as session chairman at many scientific meetings and conferences, and he has been a Board member of the AETE, the ICAR and the IETS, where he served as President in 1984-1985.

An equally important part of Torben's work has been as a teacher in a number of ways: Courses at KVL for the veterinary students; courses in embryo transfer for his veterinary colleagues in Denmark; reviews of numerous scientific papers for various journals; active participation in the public debate on subjects related to his work. In all contexts, Torben is respected for his scientific knowledge, but also for his positive attitude and willingness to discuss also difficult issues, with ethical aspects of the controversy in handling mammalian embryos as one such example.

All the years, Torben has taken up a lot of administrative work within the research and teaching community in research councils, advisory boards, editorial boards etc., both at KVL but also in other institutions and organizations in Denmark, in the Nordic countries, in EU and also more internationally. In all these situations, Torben has struggled to combine the administrative tasks with his strong dedication to research.

Torben Greve is a true scientist that has made very many contributions over the last 20-25 years to areas that are fundamental for our society, and that are recognized worldwide. He is also a person who is good colleague and friend, and who has a humoristic and serious attitude to life and science. As such, Torben Greve is a very worthy 2004 A.E.T.E. Medalist.

Henrik CALLESEN

FROM EMBRYO-TECHNOLOGY TO FERTILITY IN CATTLE

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Introduction

The first 6-7 days old embryos were recovered from superovulated cattle by non-surgical means in the middle of 1970ies. This provided not only a new tool for cattle breeding but it also opened an entirely new and exiting research era in reproductive biotechnology in cattle. The traditional superovulation and non-surgical embryo recovery and transfer were rapidly succeeded by more advanced methods such as cryopreservation, bisection, sexing, in-vitro production and cloning of embryos, as well as transgenic animal production and finally cloning by somatic cell nuclear transfer. Perfection of each of these technologies has required intensive research and yielded information of importance not only to that specific technique but also information which may be used in other contexts. The techniques have focused mainly on the embryos being produced or handled in various ways, but the subsequent steps leading to birth of animals based on these embryos are equally important. Thus, the extent of conceptus loss and its causes is far better understood today, and this is by and large due to deeper insight into the structure and function of the follicle, the oocyte, the early developing embryo and the progress of pregnancy and even parturition. The embryo-technologies have visualized this continuum, therefore also learning us more about the phenomenon of fertility. In this very brief paper we will try to address the question about what embryo-technologies and related disciplines have learned us in terms of normal and deviating fertility in cattle.

The follicle and its growth

At the time of birth a very high number (> 100.000) of resting primordial follicles is present in the ovary of the bovine female. These follicles are formed during fetal life and will through the animal's life continuously be recruited to either ovulation or to atresia; the latter is the fate of most for them. The duration of growth from the early stages and until ovulation is around three months (Fair 2003). By using daily rectal ultrasonography it has been well established that more than 95% of all estrous cycles consists of 2 or 3 follicular waves (Adams 1999; Evans 2003), that the pattern seems to be fairly repeatable within a given animal (Ireland and Burns 2003) but that it apparently is independent of age and species (Evans 2003).

The pattern of growth and selection of the dominant follicles of each follicular wave has been described in details in several reviews and shall not be addressed here (Adams 1999; Ginther 2000; Evans 2003). The hormonal and molecular mechanisms regulating growth, deviation and final selection of the dominant follicle are very complex and still not fully elucidated. It is clear that circulating FSH and LH concentrations in concert with inhibin, activin, follistatin and several growth factors play major roles (Ginther 2000; Mihm and Bleach 2003; Webb *et al*., 2003). The ever increasing knowledge about follicular growth, deviation and atresia has given us a better understanding of the variation in length of cattle estrous cycles (3 wave pattern being 1-2 days longer than two wave pattern) and has helped us to explain why a high proportion of heifers and cows are inseminated wrongly around day 10-12. These days coincide with the existence of the estrogen producing dominant follicle of the first follicular wave giving rise to estrous like symptoms. Although the oocyte contained in this follicle is competent and may be fertilized and undergo normal embryonic development, ovulation of this follicle is extremely rare in cattle because of the high progesterone level and lack of an LH-peak. Reports on birth of twins approximately 10-14 days apart may well be a consequence of ovulation and fertilization of both the oocyte of the first follicular wave and the oocyte of the second follicular wave. It can be added

that the knowledge about the very fine tuned regulation of follicular deviation and ovulation may explain why even subtle changes in feeding, stress etc. may disrupt these processes and thus result in for example cystic ovaries (Wiltbank *et al*. 2002).

The knowledge about the follicular wave pattern has been useful also in an embryo-technology context. Firstly, it has enabled us to achieve better superovulation protocols and even manipulate the follicular waves (Bo *et al.* 2002), and secondly it explains why it is better to recover oocytes by OPU twice weekly rather than once weekly (Petyim 2002), namely because the twice weekly OPU sessions suppresses normal follicular wave pattern and thus gives a more consistent amount and even a more uniform population of oocytes.

The oocyte

The oocytes present in the ovaries of the newborn heifer calf are maintained in the prophase of the first meiotic division and they remain in this state until the final nuclear maturation starts approximately 24 h prior to ovulation. However, to be developmentally competent and ready for ovulation the oocyte must undergo a series of specific changes, encompassing oocyte growth, capacitation and final preovulatory maturation.

The *growth phase* is by far the longest period and is estimated to be around 6 months. During this period the oocyte diameter increases from around 30 μm in the primordial follicle to more than 130 μm in the tertiary follicle. Very important structural and molecular changes occur within the ooplasm during this growth period and these really aim at giving the oocyte the required developmental competences for subsequent normal embryonic and fetal development (Hyttel *et al.* 1997; Mermillod *et al*. 1999; Fair 2003; Sirard *et al*. 2003). In the primary and secondary follicles the oocyte builds up a reserve of RNA, proteins, lipids and carbohydrates for later use until the embryo itself become transcriptionally active. Concomitant and obviously dependent upon these, certain organelles are appearing, namely the Golgi complex, the endoplasmatic reticulum, mitochondriae, vesicles and lipid droplets. In the secondary follicle, the zona pellucida is formed and the oocyte´s communication with the surrounding follicular vicinity is assured through the cumulus cell projections through the zona. At this stage the cortical granules are formed as well.

Most of the processes that take place in this growth phase are aimed at improving and fine-tuning the ooplasmic competence whereas the nuclear changes take place later. The duration is as mentioned fairly long, namely approximately 6 months, and during this period the oocytes may be exposed to a number of disrupting compounds (hormones for example), adverse feeding regimen, heat stress, infections etc. All of these factors may affect not only the oocyte quality and later embryonic development (Butler 2003) and thus have a significant effect on the post partum fertility, but the embryo-technology era has also learned us that there may be significant effects on pregnancy, parturition and postnatal development when the normal oocyte development is even slightly disrupted.

The changes occurring within the oocyte, from the time where its follicle becomes selected for dominance and until the preovulatory LH-surge, is denominated *oocyte capacitation* (Hyttel *et al.* 1997). The processes aim more specifically at preparing the oocyte for the imminent ovulation and fertilization and include peripheral localization of organelles with fewer Golgi complexes, more lipid droplets as well as peripheral migration of the cortical granules to under the plasma membrane. In addition the nuclear membrane shows signs of initial breakdown and the nucleolus undergoes specific changes.

The final phase, *the preovulatory period*, is initiated by the preovulatory LH-surge and ends with ovulation approximately 24 h later. The ultrastructural changes which occur during this period have been described in detail by Hyttel *at al.* (1997) and they encompass for example loss of contact between the cumulus projections and the oocyte, peripheral distribution of the cortical granules, increase in lipid and protein stores and finally resumption of meiosis. Just prior to ovulation the oocyte is at metaphase II in the second meiotic division.

The follicular-oocyte interaction

It has become increasingly evident through the era of superovulation and embryo transfer (Greve *et al*. 1984, 1995) and the experience from both human and animal in vitro fertilization studies (Greve *et al*. 1989) that the follicular microenvironment may affect oocyte quality, fertilizability and subsequent embryonic development. This is on the other hand profoundly affected by the donor's endocrine balance and hormonal profiles (in vivo; Callesen *et al.* 1986, Greve *et al.* 1995) and the culture in which the oocytes are matured (Greve *et al.* 1989). The subtle and fine tuned structural, molecular and endocrine changes are well synchronized and in homeostatic balance in the "normal" heifer or cow, and they can obviously easily be disrupted by factors interfering with the normal endocrine regulation, the follicular microenvironment and hence the oocyte's final maturation and ovulation (delayed). The consequences are as mentioned not only immediate in terms of lack of fertilization and early embryo development to the blastocyst stage. They are long lasting as clearly seen in terms of e.g. the large offspring syndrome (LOS).

In addition to the clear differences between oocytes from the same source (vivo or vitro), newer experiments from Utrecht have very strongly reiterated the differences between the quality of in vivo versus in vitro matured oocytes (Dieleman *et al*. 2002). These changes are also reflected in differences in the transcriptional pattern of the resulting embryos produced in vivo versus in vitro (Niemann *et al*. 2002; Wrenzycki *et al.* 2002).

It may be added that it is still to be elucidated whether the follicle or the oocyte is the determining element in achieving the final competence but newer research seems to indicate that the oocyte is a very important player in this game (Fair 2003).

Ovulation and the oviduct

Recent experiments have clearly substantiated that as the follicle approaches ovulation, its temperature decreases and is about 1-1.5 ^{0}C cooler than the surrounding stroma (Greve *et al*. 1996; Hunter *et al*. 2000). The precise mechanisms still await to be elucidated but according to Hunter (2003) it is probably due to chemical reactions in the follicle combined with some kind of a counter-current system in both the follicle and the ovary. Since the temperature is lower in the follicles, one might argue that it would be advantageous to perform the final in vitro oocyte maturation at a lower temperature than the normally used 39 ^{0}C. This was tested some years ago without giving specific conclusions as to the advantage of lowering the maturation temperature (Shi *et al*. 1998).

Hunter (2003) has recently described the normal ovulation process in great detail. Among the changes taking place are reduced permeability of the vessels of the theca layer which migrates through the basal membrane of the granulosa cell layer, increased viscosity of the follicular fluid, increased volume and increased intrafollicular pressure. The ovulation time per se lasts for about 1-3 minutes and occurs as oozing where the oocyte with its surrounding cumulus cells is caught by the fimbriae of the oviduct (Hunter 2003).

From studies in superovulated cattle it is very clear that the oocyte must leave that follicle at a very specific and predetermined time interval following the LH-surge. Delayed or entirely disruption of ovulation will inevitably lead to oocytes of inferior quality (Greve *et al*. 1984), observations which have been confirmed by studies in which ovulation has been delayed by means of abolition or delay of the LH-surge (Goff *et al*. 1986). In unstimulated animals a similar situation may well arise in conjunction with stress (high yield, improper feeding) which is known to negatively interfere with the hormonal regulation of ovulation and thus produce "overmatured" oocytes which are unable to be fertilized and thus give rise to pregnancy.

The oviductal function around ovulation and through the early embryonic development has been very well described by Hunter (1988, 2003) and is tightly regulated by the relative concentrations of progesterone and estradiol-17_ which reaches the Fallopian tube through the counter-current system of the oviductal vessels. The hormonal profiles of superovulated animals prior to, during and after ovulation may be disrupted (Callesen *et al.* 1986), leading to an adverse environment of the oviduct ultimately resulting in improper sperm transport and reduced or abolished fertilization evidenced by a reduced number of supernummary spermatozoa in the zona pellucida (Saacke *et al*. 1994). Oedema at the site of the utero-tubal junction and enhanced or reduced oviductal motility are other factors leading to lack of fertilization or improper embryonic development. Again, it may stressed that similar conditions may occur in animals not submitted to superovulation, but we can now from the superovulatory data deduct to unstimulated animals.

From the embryo in vivo and in vitro to fertility: what have we really learned?

The processes described above, namely follicular development, oocyte growth, capacitation and maturation, ovulation, fertilization, conceptus development and parturition are under increasing pressure leading to a reduced fertility in our dairy herds (Lucy 2003). The overall calving rate may be as low as 33% on day 28 following one AI (Lopes *et al*. 2004). Many factors such as nutrition (Boland *et al*. 2001), genetics (Royal *et al*. 2002) and yield (Butler 2003) will contribute to this trend. In addition the calf mortality is experiencing an increasing incidence that leads to an even more drastic trend for dairy cattle production: there are quite simply not enough replacement heifers.

Superovulation studies including measurements of peripheral as well as follicular endocrine parameters clearly indicated that oocyte quality may be reduced in superstimulated animals. However, when the embryo had reached the blastocyst stage on day 7 their quality were apparently similar to non-recovered embryos since the pregnancy rate and conceptus loss rate following transfer to recipients were similar to AI data (Callesen *et al.* 1996). Deviant endocrine patterns following superstimulation might also contribute to adverse oviduct environment and thus reduced fertility. Again, the periods in question are around fertilization and during the early embryo development. The superovulatory era also taught us to categorize embryo quality and there are now well-defined standards set by the IETS. This in itself was an achievement.

The in vitro era has given important new information the fertilization process, early embryo development and embryo quality although in vitro embryos are fundamentally different from the in vivo counterparts in terms of for example morphology (Crosier *et al.* 2001; Maddox-Hyttel *et al*. 2003), gene expression patterns (Niemann *et al.* 2002; Wrenzycki *et al*. 2003; Lonergan *et al*. 2003) and chromosomal abnormalities (Viuff *et al.* 2001, 2002).

The in vitro era has also made it possible to get a much better handle on oocyte quality per se and factors which may affect the quality (Merton *et al*. 2003). Through detailed morphological and molecular studies it has become possible to predict the quality of a given oocyte and thus be able to estimate whether it may give rise to normal embryo development. What is even more important is the possibility of quantifying factors that might lead to a poorer oocyte quality such as adverse endocrine environment, heat stress etc. because this may explain why the in vivo processes often deteriorates.

Probably the most important information we have gained from the in vitro production era including production of embryos by cloning is the so-called large offspring syndrome (LOS) which was in fact first reported by Willadsen *et al*. (1991) and then later addressed in a larger review by Kruip and den Daas (1997). It is now well accepted that transfer of in vitro produced embryos and cloned embryos frequently results in a high incidence of conceptus loss, abnormal fetal and placental development (hydrallantois), larger and weaker calves and a weak labour (Hasler 1998; Wagtendonk-de Leeuw *et al*. 1998, 2000; Bertolini *et al.* 2002; Heyman *et al*. 2002; Wells *et al.* 2003). It is also well established that these deleterious effects can be exerted up till approximately 9 months prior their appearance and we know now that disruption of the expression pattern of

certain developmentally important genes including imprinted genes is involved in this syndrome (Young *et al.* 2000).

In addition to the aforementioned factors that drive reproductive capacity in a wrong direction there is a new player, namely epigenetics. There is no doubt that not just under artificial but also under normal conditions is epigenetics important for embryo and fetal losses in cattle. One may say that in vitro embryo production and in particular cloning by somatic cell nuclear transfer are overexpressing the problems and thus leading to conceivable causes for reduction in embryo, conceptus and neonatal survival.

Concluding remarks

Over the past 25 years superovulation and embryo-technology have become an integrated part of cattle breeding in most parts of the world showing that the technology per se is justified. In addition the underlying research has elucidated features of oocyte quality which may be used to explain reduced fertility in cattle.

The science and practice of artificial embryo production (in vitro produced and cloned) has given us even more insight into the importance of the early embryo period for the later fetal and neonatal development. How one may affect the epigenetics and how disruption in the normal imprinting pattern may occur and to which extent this contributes to abnormal development remains to be clarified. By studying in greater detail the aberrant features of artificially produced embryos, one may find that these very same mechanisms lie behind the so-called "normal" fetal and neonatal loss. In this way we may be able to alleviate the situation.

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National Statistical Data of Bovine Embryo Transfer Activity in Europe (2003).

TABLE : 1 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: AUSTRIA

A.E.T.E 2003

Data collected by
Drs. Karl Bauer/Michaela Gruber

Total number of approved E.T. teams in the country	15
Number of teams providing data	6

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	80	B / A=	16.3
	Embryos collected	**B**	1307	C / A=	10.1
	Embryos transferable	**C**	805	C / B=	61.6%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total *in vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	805	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	157		
In vivo	Frozen	**I**	393		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	550	H+I+J+K=	
Number of frozen stored embryos		**M**	515		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	71.4%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 2 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: **BELGIUM**

A.E.T.E 2003

Data collected by
Dr. Jean-François Beckers

Total number of approved E.T. teams in the country	30
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1146	B / A=	6.79
	Embryos collected	**B**	7783	C / A=	4.49
	Embryos transferable	**C**	5143	C / B=	66.1%
In vitro *(OPU)*	Nb of oocyte donors		105		
	Nb of OPU sessions		189		
	Nb of transferable embryos	**D**	479		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	84		
Total i*n vitro* embryos		**F**	563	=(D+E)	
Total number of transferable embryos		**G**	5706	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1322		
In vivo	Frozen	**I**	3318		
In vitro	Fresh	**J**	397		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	5037	H+I+J+K=	
Number of frozen stored embryos		**M**	1258		
% of *in vitro* embryos transferred		**N**	7.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	65.9%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 3 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: **CROATIA**

A.E.T.E 2003

Data collected by
Drs. Jak_a Petri_/Iva Getz

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	34	B / A=	6.97
	Embryos collected	**B**	237	C / A=	4.0
	Embryos transferable	**C**	136	C / B=	57.4%
In vitro	Nb of oocyte donors		10		
(OPU)	Nb of OPU sessions		38		
	Nb of transferable embryos	**D**	125		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	433		
Total i*n vitro* embryos		**F**	558	=(D+E)	
Total number of transferable embryos		**G**	694	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	7		
In vivo	Frozen	**I**	5		
In vitro	Fresh	**J**	6		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	18	H+I+J+K=	
Number of frozen stored embryos		**M**	124		
% of *in vitro* embryos transferred		**N**	33.3%	(J+K) / L=	
% of frozen embryos transferred		**O**	28.0%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 4 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: CZECH REPUBLIC *A.E.T.E 2003*

Data collected by
Dr. Jirina Petelikova

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1073	B / A=	9.78
	Embryos collected	**B**	10495	C / A=	5.26
	Embryos transferable	**C**	5648	C / B=	53.8%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	80		
Total *in vitro* embryos		**F**	80	=(D+E)	
Total number of transferable embryos		**G**	5728	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3082		
In vivo	Frozen	**I**	2726		
In vitro	Fresh	**J**	84		
In vitro	Frozen	**K**	28		
Total embryos transferred		**L**	5920	H+I+J+K=	
Number of frozen stored embryos		**M**	2478		
% of *in vitro* embryos transferred		**N**	1.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	46.5%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 5 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: DENMARK

A.E.T.E 2003

Data collected by
Dr. Henrik Callesen

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	605	B / A=	10.0
	Embryos collected	**B**	6055	C / A=	6.94
	Embryos transferable	**C**	4199	C / B=	69.3%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total *in vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	4199	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2256		
In vivo	Frozen	**I**	2327		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	4583	H+I+J+K=	
Number of frozen stored embryos		**M**	2324		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	50.8	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	1846
Number of calves born from *in vitro* embryos	0
Total	1846

* data not available

TABLE : 6 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: ESTONIA *A.E.T.E 2003*

Data collected by
Dr. Y. Jaakma

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	4	B / A=	11.5
	Embryos collected	**B**	46	C / A=	7.75
	Embryos transferable	**C**	31	C / B=	67.4%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro	Nb of transferable embryos	**E**	0		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	0	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	8		
In vivo	Frozen	**I**	14		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	22	H+I+J+K=	
Number of frozen stored embryos		**M**	23		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	63.6%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	35
Number of calves born from *in vitro* embryos	-
Total	35

* data not available

TABLE : 7 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: FINLAND

A.E.T.E 2003

Data collected by
Dr. Marja Mikkola

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	500	B / A=	8.84
	Embryos collected	**B**	4421	C / A=	5.47
	Embryos transferable	**C**	2733	C / B=	61.8%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	35		
Total i*n vitro* embryos		**F**	35	=(D+E)	
Total number of transferable embryos		**G**	2766	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1100		
In vivo	Frozen	**I**	1201		
In vitro	Fresh	**J**	10		
In vitro	Frozen	**K**	1		
Total embryos transferred		**L**	2311	H+I+J+K=	
Number of frozen stored embryos		**M**	1339		
% of *in vitro* embryos transferred		**N**	0.5%	(J+K) / L=	
% of frozen embryos transferred		**O**	52.0%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 8 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: FRANCE

A.E.T.E 2003

Data collected by
Dr. Bernard Guérin

Total number of approved E.T. teams in the country	27
Number of teams providing data	4

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5665	B / A=	11.5
	Embryos collected	**B**	64925	C / A=	6.60
	Embryos transferable	**C**	37433	C / B=	57.6%
In vitro	Nb of oocyte donors		77		
(OPU)	Nb of OPU sessions		77		
	Nb of transferable embryos	**D**	261		
In Vitro	Nb of transferable embryos	**E**	22		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	281	=(D+E)	
Total number of transferable embryos		**G**	37714	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	18415		
In vivo	Frozen	**I**	15076		
In vitro	Fresh	**J**	231		
In vitro	Frozen	**K**	7		
Total embryos transferred		**L**	33729	H+I+J+K=	
Number of frozen stored embryos		**M**	14655		
% of *in vitro* embryos transferred		**N**	0.7%	(J+K) / L=	
% of frozen embryos transferred		**O**	44.7%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	5122
Number of calves born from *in vitro* embryos	113
Total	5235

* data not available

TABLE : 9 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: GERMANY

A.E.T.E 2003

Data collected by
Dr Hubert Cramer

Total number of approved E.T. teams in the country	42
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2687	B / A=	9.80
	Embryos collected	**B**	26350	C / A=	5.54
	Embryos transferable	**C**	14889	C / B=	56.5%
In vitro *(OPU)*	Nb of oocyte donors		*		
	Nb of OPU sessions		*		
	Nb of transferable embryos	**D**	*		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	3120		
Total *in vitro* embryos		**F**	3120	=(D+E)	
Total number of transferable embryos		**G**	18009	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5849		
In vivo	Frozen	**I**	4106		
In vitro	Fresh	**J**	1878		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	11833	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**	15.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	34.7%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 10 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: GREECE

A.E.T.E 2003

Data collected by
Dr. Foteini Samartzi

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	27	B / A=	17.2
	Embryos collected	**B**	465	C / A=	3.78
	Embryos transferable	**C**	102	C / B=	21.9%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions				
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	102	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	48		
In vivo	Frozen	**I**	0		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	48	H+I+J+K=	
Number of frozen stored embryos		**M**	51		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	0	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	29
Number of calves born from *in vitro* embryos	0
Total	29

* data not available

TABLE : 11 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: HUNGARY

A.E.T.E 2003

Data collected by
Dr. Laszlo Solti

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	224	B / A=	9.92
	Embryos collected	**B**	2222	C / A=	5.45
	Embryos transferable	**C**	1220	C / B=	54.9%
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**	607		
Total *in vitro* embryos		**F**	607	=(D+E)	
Total number of transferable embryos		**G**	1827	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	275		
In vivo	Frozen	**I**	347		
In vitro	Fresh	**J**	*		
In vitro	Frozen	**K**	*		
Total embryos transferred		**L**	622	H+I+J+K=	
Number of frozen stored embryos		**M**	894		
% of *in vitro* embryos transferred		**N**	*	(J+K) / L=	
% of frozen embryos transferred		**O**	55.8%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	303
Number of calves born from *in vitro* embryos	*
Total	303

* data not available

TABLE : 12 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: IRELAND

A.E.T.E 2003

Data collected by
Dr. Pat Lonergan

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	272	B / A=	8.82
	Embryos collected	**B**	2398	C / A=	5.21
	Embryos transferable	**C**	1418	C / B=	59.1%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	1418	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	550		
In vivo	Frozen	**I**	714		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	1264	H+I+J+K=	
Number of frozen stored embryos		**M**	868		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	56.5%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 13 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: ITALY

A.E.T.E 2002

Data collected by
Dr. Francesco Brun

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1002	B / A=	14.32
	Embryos collected	**B**	14350	C / A=	7.06
	Embryos transferable	**C**	7076	C / B=	49.3%
In vitro *(OPU)*	Nb of oocyte donors		147		
	Nb of OPU sessions		305		
	Nb of transferable embryos	**D**	632		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	3983		
Total i*n vitro* embryos		**F**	4615	=(D+E)	
Total number of transferable embryos		**G**	11691	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2787		
In vivo	Frozen	**I**	3255		
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	3457		
Total embryos transferred		**L**	9599	H+I+J+K=	
Number of frozen stored embryos		**M**	6646		
% of *in vitro* embryos transferred		**N**	37.0%	(J+K) / L=	
% of frozen embryos transferred		**O**	69.9%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 14 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: (The) NETHERLANDS ***A.E.T.E 2003***

Data collected by
Dr. Bas Landman

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	3119	B / A=
	Embryos collected	**B**	*	C / A= 5.74
	Embryos transferable	**C**	17915	C / B=
In vitro *(OPU)*	Nb of oocyte donors		222	
	Nb of OPU sessions		2492	
	Nb of transferable embryos	**D**	2084	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	77	
Total i*n vitro* embryos		**F**	2161	=(D+E)
Total number of transferable embryos		**G**	20076	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	3364	
In vivo	Frozen	**I**	11988	
In vitro	Fresh	**J**	901	
In vitro	Frozen	**K**	1224	
Total embryos transferred		**L**	17477	H+I+J+K=
Number of frozen stored embryos		**M**	*	
% of *in vitro* embryos transferred		**N**	12.1%	(J+K) / L=
% of frozen embryos transferred		**O**	75.6%	(I+K) / L= %

Number of E.T. calves born (2003)

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

* data not available

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: NORWAY

A.E.T.E 2003

Data collected by
Dr. Elisabeth Kommisrüd

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	24	B / A=	8.17
	Embryos collected	**B**	196	C / A=	4.95
	Embryos transferable	**C**	119	C / B=	60.7
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	0	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	20		
In vivo	Frozen	**I**	98		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	118	H+I+J+K=	
Number of frozen stored embryos		**M**	106		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	83.0%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 16 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: POLAND

A.E.T.E 2003

Data collected by
Dr. Jedrzej Jaskowski

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

3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	67	B / A=	8.12
	Embryos collected	**B**	544	C / A=	5.74
	Embryos transferable	**C**	385	C / B=	70.8%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro	Nb of transferable embryos	**E**	19		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	19	=(D+E)	
Total number of transferable embryos		**G**	404	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	273		
In vivo	Frozen	**I**	87		
In vitro	Fresh	**J**	13		
In vitro	Frozen	**K**	6		
Total embryos transferred		**L**	379	H+I+J+K=	
Number of frozen stored embryos		**M**	31		
% of *in vitro* embryos transferred		**N**	5.0%	(J+K) / L=	
% of frozen embryos transferred		**O**	24.5%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 17 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: PORTUGAL ***A.E.T.E 2003***

Data collected by
Dr. Joao Nestor das Chagas e Silva

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	61	B / A=	5.67
	Embryos collected	**B**	346	C / A=	5.15
	Embryos transferable	**C**	314	C / B=	90.7%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	551		
Total *in vitro* embryos		**F**	551	=(D+E)	
Total number of transferable embryos		**G**	865	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	219		
In vivo	Frozen	**I**	135		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	354	H+I+J+K=	
Number of frozen stored embryos		**M**	245		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	38.1%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	35*
Number of calves born from *in vitro* embryos	0
Total	35

* data from 1 team

TABLE : 18 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: ROMANIA

A.E.T.E 2003

Data collected by Drs. Stela Zamfirescu/George Toba

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	22	B / A=	8.09
	Embryos collected	**B**	178	C / A=	5.18
	Embryos transferable	**C**	114	C / B=	64.0%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	114	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	36		
In vivo	Frozen	**I**	47		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	83	H+I+J+K=	
Number of frozen stored embryos		**M**	69		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	56.6%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	6
Number of calves born from *in vitro* embryos	0
Total	6

* data not available

TABLE : 19 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: SLOVAKIA *A.E.T.E 2003*

Data collected by
Dr. Peter Cesnak

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	75	B / A=	9.49
	Embryos collected	**B**	712	C / A=	4.87
	Embryos transferable	**C**	365	C / B=	51.3%
In vitro *(OPU)*	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	365	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	248		
In vivo	Frozen	**I**	271		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	519	H+I+J+K=	
Number of frozen stored embryos		**M**	116		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	52.2%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	287
Number of calves born from *in vitro* embryos	0
Total	287

* data not available

TABLE : 20 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: SPAIN

A.E.T.E 2003
Data collected by
Dr. Julio de la Fuente

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	303	B / A=	10.6
	Embryos collected	**B**	3207	C / A=	4.73
	Embryos transferable	**C**	1436	C / B=	44.8%
In vitro	Nb of oocyte donors		2		
(OPU)	Nb of OPU sessions		7		
	Nb of transferable embryos	**D**	22		
In Vitro	Nb of transferable embryos	**E**	0		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	22	=(D+E)	
Total number of transferable embryos		**G**	1458	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	314		
In vivo	Frozen	**I**	1041		
In vitro	Fresh	**J**	15		
In vitro	Frozen	**K**	6		
Total embryos transferred		**L**	1376	H+I+J+K=	
Number of frozen stored embryos		**M**	773		
% of *in vitro* embryos transferred		**N**	1.5%	(J+K) / L=	
% of frozen embryos transferred		**O**	76.1%	(I+K) / L=	%

Number of E.T. calves born (2003)

Number of calves born from superovulated embryos	426
Number of calves born from *in vitro* embryos	2
Total	428

* data not available

TABLE : 21 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: SWEDEN

A.E.T.E 2003

Data collected by
Dr. Hans Gustafsson

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	270	B / A=	7.22
	Embryos collected	**B**	1950	C / A=	4.90
	Embryos transferable	**C**	1322	C / B=	67.8%
In vitro	Nb of oocyte donors		0		
(OPU)	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0		
Total *in vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	1322	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	417		
In vivo	Frozen	**I**	905		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	1322	H+I+J+K=	
Number of frozen stored embryos		**M**	0		
% of *in vitro* embryos transferred		**N**	0	(J+K) / L=	
% of frozen embryos transferred		**O**	68.4%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 22 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: SWITZERLAND *A.E.T.E 2003*

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**	243	B / A=	11.0
	Embryos collected	**B**	2686	C / A=	7.91
	Embryos transferable	**C**	1923	C / B=	71.6%
In vitro (*OPU*)	Nb of oocyte donors		0		
	Nb of OPU sessions		0		
	Nb of transferable embryos	**D**	0		
In Vitro (*Slaughtered donors*)	Nb of transferable embryos	**E**	0		
Total *in vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	1923	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	542		
In vivo	Frozen	**I**	1165		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	46		
Total embryos transferred		**L**	1753	H+I+J+K=	
Number of frozen stored embryos		**M**	1381		
% of *in vitro* embryos transferred		**N**	2.6%	(J+K) / L=	
% of frozen embryos transferred		**O**	69.1%	(I+K) / L=	%

Number of E.T. calves born (2003)

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

* data not available

TABLE : 23 EMBRYO TRANSFER ACTIVITY IN 2003

COUNTRY: UNITED KINGDOM ***A.E.T.E 2003***

Data collected by
Dr. Alison Liddle

Total number of approved E.T. teams in the country	17
Number of teams providing data	10

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	*	B / A=
	Embryos collected	**B**	*	C / A=
	Embryos transferable	**C**	*	C / B=
In vitro *(OPU)*	Nb of oocyte donors		*	
	Nb of OPU sessions		*	
	Nb of transferable embryos	**D**	*	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	*	
Total i*n vitro* embryos		**F**	*	=(D+E)
Total number of transferable embryos		**G**	*	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	*	
In vivo	Frozen	**I**	*	
			*	
In vitro	Fresh	**J**	*	
In vitro	Frozen	**K**	*	
Total embryos transferred		**L**	4109	H+I+J+K=
Number of frozen stored embryos		**M**	3463	
% of *in vitro* embryos transferred		**N**	*	(J+K) / L=
% of frozen embryos transferred		**O**	*	(I+K) / L= %

Number of E.T. calves born (2003)

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

* data not available

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY IN EUROPE IN 2003

I. EMBRYO PRODUCTION

(Data collected from 23 countries)

***In vivo* produced embryos (superovulation)**	
- number of flushed donors	**17, 503**
- number of transferable embryos	**104, 726**
- mean number per flushed donor	**5.98**
***In vitro* produced embryos:**	
From OPU	
- number of oocyte donors	**563**
- number of OPU sessions	**3, 108**
- number of transferable embryos	**3, 683**
From slaughterhouse collected ovaries	
- number of transferable embryos	**8, 498**
Total *in vitro*	**12, 181**
Total number of transferable embryos	**116, 907**

(P. Lonergan, AETE Lyon, 2004)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2003

II. EMBRYO TRANSFERS

(Data collected from 23 countries)

***In* vivo produced embryos**	**Number of embryos transferred** **90, 508*** **(41, 289 fresh/49, 219 frozen)**
***In vitro* produced embryos**	**8, 410*** **(3, 635 fresh/4, 775 frozen)**
Total number of embryos transferred	**98, 918**
Proportion of IVF embryos transferred	**8.50 %**
Proportion of frozen embryos transferred	**54.6 %**

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

(P. Lonergan, AETE Lyon 2004)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES*
EUROPE year 2003

Species	Embryo Production	Embryo Transfers	Countries
Sheep	**326**	**167**	Greece Portugal Romania
Swine	**1221**	**816**	Czech Republic Hungary Poland Romania Switzerland
Goat	**61**	**52**	Romania
Horse	**443**	**344**	Austria Czech Republic Finland France Hungary Italy Poland Sweden

(P. Lonergan, AETE Lyon, 2004)

INVITED LECTURES

RESPECTIVE IMPACTS OF ET AND OPU/IVP ON GENETIC SELECTION EFFICIENCY IN THE MONTBELIARDE BREED

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Introduction

In dairy cattle, breeding programmes aim to optimise the genetic progress for different characters. Three groups of selection criteria are usually distinguished. Milk production characters are predominant for dairy breeds. Morphological criteria are necessary to complete the milk production evaluation in terms of body and udder conformation. However, the muscular mass is also used as a selection criteria for mixed breeds in which equilibrated characteristics are valuable. Functional criteria, such as resistance to mastitis, fertility, longevity, birth or calving abilities and behaviour are currently becoming more important, because of their economical benefit through control of animal health status and culling, together with improved working conditions for breeders (Aswhell et al., 2004 ; Pryce et al., 2004).

The multiplication of the characters of interest leads to more complex breeding programmes since there is a need to select animals combining the maximum number of characters of interest. Therefore, the challenge of these programmes is to obtain the best result derived from a combination of characters with different genetic determinism. This has lead to the estabnlishment of strategies to optimise genetic progress while restricting inbreeding (Quinton et al., 1995 ; Van Arendonk et al., 2003).

During the last decades, biotechnologies such as embryo transfer (ET) and, more recently, ovum pick up associated with in vitro embryo production (OPU-IVP ; Colleau et al., 1998; Marquant Leguienne et Humblot 1998, Marquant Leguienne et al., 2001, Galli et al., 2003, Merton et al., 2003), have been used in breeding programs. Those techniques are more or less used intensively depending on the country. In Europe, the intensive use of OPU and IVP has been reported in the Netherlands and Italy (Heyman, 2002, Galli et al., 2003 ; Merton et al., 2003), whereas ET remains the main biotechnology used in breeding schemes in France. With increased knowledge in genomics, the use of marker assisted selection (MAS) is currently emerging.

This paper aims to investigate and compare the results and realised value of ET and OPU-IVP in the breeding programme conducted by UMOTEST (Union of AI cooperatives) in the Montbeliarde breed, which is one of the main red coloured mixed breed in Europe.

The Montbeliarde breeding program

In the Montbeliarde breed, 25% of females are inseminated with beef bulls, which are used to produce crossbred animals. This illustrates the ease of calving and the morphological qualities of this breed (Table 1). However, the Montbeliarde breed is mainly a dairy breed with a large morphological format (sacral height : 1.48 m) and a high protein content used to produce cheese with local/regional labels (Comté, Bleu de Gex, Morbier, Cantal…).

Table 1 : The UMOTEST breeding program

Type of animals	Number
Total females	600 000

Inseminated females - pure breed - cross breed	560 000 410 000 150 000
Controlled females	330 000
Nucleus females	4 200
Bulls tested	130

The selection has always taken into account the morphological quality, aiming to produce male calves and culled cows of a high commercial value (Table 2).

Table 2. Production characteristics in the Montbeliarde breed

- Milk production (adult lactations > 200 days)

Days	Milk (kg)	% Protein content	% Fat content
317	7275	3.4	3.88

- Beef

	Weight (carcass)	Class (Europe)
Bulls (18-20 months	365 kg	R to R^+
Culled cows	345 kg	O^+ to R^-

Use of embryo transfer (ET) on the nucleus females

The realized value of embryo transfer (ET) for females of the selection scheme has been estimated from embryo collections performed from 1994 to 2003. The number of flushes was quite stable before 1994, ranging from 150 to 200 between 1988 and 1993, but has doubled within the last ten years (Table 3). This contributed to the multiplication of calves born from females of high genetic merit in order to increase the genetic progress.

Table 3. Number of embryo collections for the past 10 years

Year	Number of embryo collections
1994	263
1995	339
1996	327
1997	447
1998	508
1999	470
2000	443
2001	560
2002	608
2003	523

Embryo transfer may potentially impact positively on the components contributing to genetic progress ($\Delta G = i \times p \times \sigma_G / T$) by :

- Increasing the intensity of selection (i), which corresponds to increased numbers of male and female calves to enter the selection process,
- Increasing the precision of selection (p), with the use of the BLUP model, which allows to take into account more animals within a family of mothers and leads to higher coefficients of determination.

- Increasing the genetic variability (σ_G), obtained with higher numbers genetic combinations Father/grand-father (embryo collections with different bulls)
- Shortening the generation interval (T), with the transfer of embryos collected from heifers and birth of several calves within 9 months.

Embryo collections are programmed among ages and the genetic value of the nucleus females in which a majority of bull dams (BD) are submitted to ET. They represent 59% of total collections with 23% performed in young bull dams (YBD ; ET performed 13 to 15 months before first breeding) and 36% in adult bull dams (ABD ; ET performed during the first, second, 3rd lactation or more). The percentage of treated heifer Dams (HD) is 41 %. This shows that efforts are made to control the generation interval, because 2/3 of bull dams are young females. Heifer dams are also mainly collected as young females, in order to renew the nucleus.

The use of ET resulted in a decrease of the generation interval. Between 1994 and 1996, the age of the dam at birth of its offspring averaged 4.7 years, compared to 4.2 since 2001. This corresponds to a decrease of T of 11% within 7 years.

Approximately, half of the bulls and heifers dams selected in 2004 are originating from families with at least 2 offspring (table 4). The families represented by numerous offspring allows to increase the intensity and accuracy of selection. For instance, in those families, 7 different bulls and breeding combinations have been used. Generally, the number of different fathers per family corresponds to half of the number of calves.

Table 4. Repartition of the nucleus females according to the number of offsprings per origin

	Number of families	Number of offspring per family				
		1	2-3	4-5	6-9	≥ 10
Bull Dams	264	42 %	40 %	8 %	7 %	3 %
Heifers dams	179	42 %	37 %	10 %	6 %	4 %

The genetic value of the females used for ET increased regularly of 4 to 5 points (points of ISU; composite index summarising milk, morphological and functional characters). Consequently, the difference between the ISU index of the dams issued from AI and dams issued from ET did increase, ranging from 1 point in 1994 to 10 points in 2003. This is easily explained as females used for ET are more frequently originating from dams or at least from families already used for ET. For instance, about 3/4 of the nucleus females selected in 2004 are originating from families where ET was used, either as bull dams or as heifers dams. The use of ET gives the opportunity to get rapidly male calves and potential dams, which can be chosen within the female calves.

The realised value of ET on the annual genetic progress has been estimated from male calves, which entered the progeny testing procedure in station. The genetic progress has been compared to the reference 100, using the INEL index (mother-calf component), which corresponds to the genetic progress observed only with AI. The use of ET in this selection scheme led to obtain 105-106 points of INEL when 1 bull calf per origin entered the station and 108-109 points when 2 calves per origin were progeny tested.

The mean cost per embryo collection for the selection unit is 700 euros. Additional costs corresponding to organisation and application of the breeding strategy by geneticists and to the transfer of frozen embryos represents 350 euros per ET session. Therefore the additional cost of an ET session when compared to AI is around 1000 euros. However, the mean number of embryos per session calculated from those ten years of records was 5.5 allowing to obtain 1.5 males/ session. The supplement of cost when compared to AI can be estimated as 700 euros per male calf and is even reduced to 350 euros when considering the advantages of ET related to the renewal of the selection nucleus and to the conservation of families of interest.

Use of OPU and IVF in the nucleus herd

Since 1996, UMOTEST has been associated in setting up the OPU-IVF techniques developped by UNCEIA and UCEAR in the field station of Chateauvillain. Results initially obtained in high genetic merit females kept and collected in station have been progressively applied to farm conditions (Guyader-Joly et al., 1997, Marquant-Leguienne et al. 2001). In the Montbeliarde breed, from OPU sessions performed in station, a mean of 2 born calves per week was observed (with one oocyte collection per week and per female following superovulation. This allowed to produce one male calf per session.

From 137 on farm OPU sessions performed in very high genetic merit cows (85% of sessions performed in sire dams) since year 2000, results are slightly lower with 1.5 calf and 0.75 male calf per session respectively. For those sire dams, technical results were not significantly different between heifers (lactation 0 ; L0), primiparous (Lactation 1 ; L1) or which had 2 or more lactations before starting OPU (L2 or more).

Due to the supplement of cost when using OPU and IVF under farm conditions, ie 1500 euros to be compared to 1000 euros (when considering equivalent costs for ET), these techniques appears to be profitable if applied to young cows (L1) when compared to heifers and older cows. Effectively, the collected heifers are known for morphology and for the performances of their parents (grand parents) but only 50% of them are finally selected on milk characteristics, udder conformation and other functional traits when being primiparous cows. Due to this selection, the genetic progress obtained from superovulating (for ET or OPU) 100 heifers is equivalent to the progress obtained from 50 primiparous cows. The supplement of cost due to the use of OPU and IVF in large number of heifers (which will be eliminated at a later age and provides large number of offspring non suitable to enter subsequent progeny testing), prohibits the use of those technologies in this kind of genetic scheme.

Older cows (≥ L2) produce more viable embryos than heifers (+ 1 embryo per collected female; Manciaux et al., 2000) or primiparous cows when using conventional ET protocols. The cost of ET sessions is lower and due to a better knowledge of such donor cows, the advantage of using OPU and IVF to shorten the generation interval is very low.

On the contrary, due to significant advantages in terms of precision (p) and to a generation interval still acceptable for L1 cows, applying OPU-IVF in such females appears as the best compromise to optimise the genetic scheme efficiency. In addition to this, the advantages of collecting pregnant females, from which the birth of a calf is highly probable, without any increase in days open (as observed when using conventional ET) are of a major interest for the geneticist and the farmer.

Considering these observations, a technical and financial simulation was made to compare the respective costs and advantages of conventional ET and OPU-IVF when used in the Montbeliarde selection scheme. It was considered that the objective of the geneticist was to produce 30 young bulls that will enter the progeny testing process from an initial pool of 100 young females , candidates to be sire dams. Four scenarios (S1 to S4) were analysed depending on the use of AI, ET and OPU-IVF technologies when applied in heifers or in L1 females (Tables 5 and 6). It was hypothesised that half of these young heifers will not be confirmed as sire dams when being primiparous due to initial lack of precision of their genetic value.

In scenario 1, 100 heifers are systematically superovulated and collected once for conventional ET by 13-15 months of age, before their first breeding (Table 6), and finally AI is subsequently used to produce additional calves. The resulting number of male produced and session per female, depending on the observed numbers of embryos and calves per session are presented in table 5. After 1 ET session and subsequent AI, a total of 150 males are born. From those, 50% will be eliminated due to a lack of confirmation of their mother's genetic value (Table 6). Additional elimination will take place before they enter progeny testing after individual morphological evaluation and control of their reproductive performances (behavioural traits and sperm production and quality traits).

In scenario 2, AI is used in 100 heifers and the best 50 primiparous cows are chosen to be collected by ET. Around 25 calves are kept from AI made previously in those females. Half the number of calves and males are produced by ET when compared to the first scenario (Table 5). All males will be kept for milk production traits and elimination will be based only on individual performances (Table 6). The cost for producing the 30 males which will enter progeny testing is considerably reduced when compared to the first scenario.

Table 5. Numbers of embryos and calves (Total and per session) and number of session per female to produce the desired number of males necessary for progeny testing.

N°	Type of females	Number of females	Reproductive techniques	Emb /sess.	Calf /sess	Number of Sess/female	Number of calves	Number of males
S1	Heifers	100	ET AI Total	5	2.5 0.5	1	250 50 300	125 25 150
S2	L1 cows	50	AI ET Total	5	0.5 2.5	1	25 125 150	12.5 62.5 75
S3	L1 cows	50 (station)	AI OPU-IVF Total	4	0.5 2	1.25	25 125 150	12.5 62.5 75
S4	L1 cows	50 (farm)	AI OPU-IVF Total	3	0.5 1.5	1.66	25 125 150	12.5 62.5 75

The 3rd and 4th scenarios are similar to the second one but OPU and IVF are used instead of ET with collections taking place either in station (S3) or on farm (S4). The culling rate is the same as in S2. From the 50 primiparous cows, 1.25 and 1.66 sessions are needed with S3 and S4 respectively to obtain the planned 75 male calves (Table 5). The costs of these procedures represents approximately 140% and 150% when compared to S2 (100%) for S3 and S4 respectively.

Table 6. Respective costs (Kilo euros) of conventional ET and OPU-IVF to produce 30 males potentially selected for progeny testing.

N°	Reproductive techniques	Type of females	Number of collections	Number of males	Eliminated (1)	(2)	Remaining	Costs (K€)	%
S1	ET+AI	Heifers	100	150	- 75	- 45	30	142	195
S2	AI+ET	L1 cows	50	75	0	- 45	30	72	100
S3	AI+OPU-IVF (station)	L1 cows	63	75	0	- 45	30	103	140
S4	AI+OPU-IVF (farm)	L1 cows	83	75	0	- 45	30	106	150

(1) eliminated for non confirmation of the performances of their mother for milk production traits
(2) elimination for morphology and reproductive performance traits

S1 appears as the most expensive scenario. It's main advantage when compared to the other alternatives tested here is to shorten the generation interval by one year. However, this is accompanied by a lack of precision of the selection process especially for traits than can be estimated late (fertility, longevity, udder conformation). S2 looks to be the more economical scenario. However, due to the use of primiparous females, one year of generation interval is lost but the gain in precision is important since young bulls enter progeny testing and are evaluated after the second lactation of their mother. S3 and S4 are apparently more expensive than S2. The characteristics of the selection scheme are the same (long generation interval, good precision). Due to the higher number of sessions, the variability and precision can even be increased when compared to S2. When comparing S4 to S1 and S2, due to the high number of on farm OPU sessions, the combinations (Father / maternal Grand Father) can be multiplied by 2 and this contributes to increase variability. An additional advantage when compared to S2 is that milk production during 1st lactation and subsequent reproductive efficiency are less affected by the superovulation treatment when using OPU protocols instead of conventional ET procedures. Despite additional cost are not negligible, the last scenario represents probably the best compromise for the geneticist.

Conclusions

The use of embryo technologies allows to optimise the genetic progress for several traits of interest for the farmer and the consumer. Embryo transfer has been used extensively in the Montbeliarde breed since the end of the eighties and results improved very significantly to reach a production of 5 to 6 transferable embryos per session. However individual variability in response to superovulation treatment is very high and the percentage of sessions for which 0 transferable embryo is produced is still close to 20%. Lowering this percentage is of a major interest for the geneticist to conserve families which may disappear due to the low number of progeny of a high genetic value.

OPU combined to IVF represents an alternative to ET allowing potentially to obtain a lot of progeny from a given high genetic merit mother bred with different sires (theoretically up to 9 sessions with 9 different sires). Another advantage when compared to ET is the possibility of collecting pregnant females. In such a situation, one can expect to get embryos in addition to a calf produced by AI without any delay in the reproductive life of the cow. However the results are still highly variable due to unpredictable individual response. The cost of OPU and IVF should be reduced by 20 to 30% to make this techniques really attractive when compared to ET.

The low number (and associated variability) of transferable embryos produced by OPU and IVF and the difficulties encountered to apply the technique on farm (both for collection and transfer) represents major limitations when compared to ET. These limitations explains probably why the use of these technologies is highly variable from one breed to another or/and in different countries. However, due to the possibility of repeating OPU sessions without affecting the reproductive efficiency, OPU and IVF may help in a near future to really optimise multiple traits selection when associated to the use of the arising technologies based on genomics and marker assisted selection.

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PRESERVATION OF GENETIC DIVERSITY OF FARM ANIMALS : GENE-BANKING OF GERMPLASM

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Introduction

Currently, the world faces the global challenge of declining genetic diversity in most species of farm animals. There is a tendency of 'globalisation' in the use of breeds. A limited number of breeds are used more and more world-wide, while the use of other breeds is declining. The less popular breeds may be maintained only locally, and most often in only small populations. In small populations, genetic diversity is lost as a result of genetic drift. While the commercial breeds are represented in large numbers of animals, the genetic diversity of these breeds may also be quite small, because a small number of sires are selected to have a multitude of progeny, leading to high rates of inbreeding.

One reason for maintaining rare and local breeds is because these breeds may fulfil specific requirements with respect to local terrain or climate, or product quality needed for typical regional products. Also, local breeds are seen as cultural heritage. However, also with respect to the more widely used breeds, it is necessary to preserve genetic diversity. Generally, inbreeding is associated with increased frequency of heritable diseases, malformations or dysfunctions. Furthermore, we need genetic diversity as a toolbox for continued breeding. This is especially true in the situation that future breeding goals are different from those of today.

In many countries, steps are being taken to preserve genetic diversity of farm animals, e.g. by stimulating the use of indigenous rare breeds. In addition to this so-called *in situ* conservation, gene banks are set up to preserve germplasm of rare breeds as well as the more widely used commercial breeds (so-called *ex situ* conservation). Long time storage of material is used to preserve the genetic diversity as it is today for any future use. Furthermore, gene bank resources are actively used to support breeding schemes of rare breeds. This requires a regular update of material in the gene bank, and a ready availability of gene bank resources for use in the field. In the Netherlands today, the gene bank is largely based on storage of semen (Danchin and Hiemstra, 2003). Other possibilities are considered. Advances in cryobiology can be important for increasing the efficiency and safety of freezing, storage, and use of semen or other cells for gene banking. Some examples of developments will be discussed.

Purposes of ex situ genetic resources

Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Mazur 1985), but probably much longer. This means that we can preserve the present wealth of genetic diversity in long-time storage in a biological 'safe deposit vault'. A germplasm repository may serve a number of different purposes (Hiemstra, 2003). One purpose of a germplasm repository is to provide the possibility of recreating breeds or breeding lines in case they are lost as a consequence of a calamity. For example, during an outbreak of foot and mouth disease in the Netherlands, several years ago, culling measures that were taken to contain the epidemic placed a number of rare Dutch sheep breeds in danger of total extinction. If such an event would occur, recreation of that breed would be possible provided that germplasm from an adequate sample of animals is cryopreserved. Storage of germplasm for this purpose would typically be long time storage, without frequent use of the stored material and without the need of regular updating of the collection.

A second way to make use of gene bank resources is to support *ex situ* conservation. Frozen semen and embryos can be used to minimise inbreeding and genetic drift in small-managed populations, and the combination of live animals and cryopreserved germplasm can be a powerful tool in conservation of small populations (Meuwissen, 1999). Sonesson et al. (2002) proposed a scheme where semen is collected from the first two generations and used alternatively on dams, allowing a reduction of the rate of inbreeding.

Additionally, gene bank resources may be used as a back-up in case genetic problems would occur. Decrease of effective population size and the resulting high level of inbreeding can lead to an increased relative frequency of deleterious alleles that were not apparent in a larger population. This happens not only in 'rare' breeds but can also be found in large commercial breeds, e.g. when a very small number of sires is responsible for a very high number of offspring. In such cases, the effective gene pool size is still very small. Gene bank resources may be needed to remove deleterious genes from the population by introducing new genotypes (e.g. semen doses) from the original (larger) population.

A fourth important use of the cryopreserved genetic resources is to allow development of new lines or breeds, or to quickly modify or reorient the evolution /selection of the population. For instance, storage of original or extreme genotypes can be of use to quickly modify or reorient the evolution /selection of a selected population. It has been suggested (Verrier et al., 2003) to store original and extreme genotypes identified as having extreme breeding values for specific traits, or that carry rare alleles, or that represent specific founders or pedigree lines. A specific example can be the storage of material from dual-purpose cattle, reoriented to beef or milk production.

Types of genetic resource material

For the conservation of genetic diversity, storage of semen, embryos, oocytes, and somatic cells, among others, have been considered. There are large differences in present day feasibility and practicality of these possibilities.

Semen

Semen has the disadvantage that only a single complement of chromosomes is preserved. For recovery of a lost breed it will take at least 6 generations of backcrossing to restore the original genotype (Ollivier and Renard 1995). Moreover, mitochondrial genes are not preserved, whereas variation in mitochondrial genes between breeds and within breeds does exist (Loftus et al 1994; Troy et al. 2001). The advantage of semen is that it can be collected and used in most species. Moreover, for a number of species, notably in cattle and pigs, an existing AI infrastructure allows easy collection and future use of semen. In other species, however, semen collection can be a problem, as we have experienced in an effort to cryopreserve the genotype of endangered breeds of Dutch heath sheep. It appeared that in some of the breeds, training of the rams for semen collection was very difficult. In these breeds we have been able to cryopreserve epididymal sperm collected from the caudae epididymides of slaughtered rams. From epididdymides of a total of 34 rams we have collected an average of 20 billion epididymal spermatozoa per ram. This provided an average of 108 doses of 0.2 billion sperm/dose per ram. The post-thaw motility of the epididymal spermatozoa was at least as good as that seen in frozen-thawed ejaculated ram sperm. This year we have planned insemination experiments to test the fertility of the frozen-thawed epididymal sperm.

Semen plus oocytes

In the last 10 years, considerable progress has been made with cryopreservation of oocytes. Restoration of a lost breed or genotype by using cryopreserved oocytes plus semen would not require backcrossing. This advantage is also obtained with freezing embryos. However, in

comparison to blastocyst-stage embryos, cryopreservation of oocytes is not very successful. Viable oocytes have been recovered after freezing and thawing in a great number of species, but, i.e. cattle, pig, sheep, rabbit, mouse, monkey and human (as reviewed by Critser et al. 1997) and in goat (Le Gal and Le Gal, 1996; Agrawal, 1999), horse (Hochi et al., 1994), and buffalo (Das et al., 1999). Some successes have been reported as to oocyte maturation, fertilisation, and even embryo development to the morula and blastocyst stage using frozen-thawed oocytes. In the bovine, pregnancies and even a live born calf (Otoi et al., 1996) have been reported in just a few studies. Live born young have also been obtained in the mouse (Frydman et al., 1998). The present efficiency and reliability of using frozen thawed oocytes for generating embryos and young is extremely poor.

Embryos

In cattle, cryopreservation of embryos is highly successful. It is used as a routine procedure. Both slow-freezing and vitrification protocols are effective. The success of cryopreservation is dependent on the stage of the embryo; that is, especially good results are obtained with blastocysts. The possibilities for cryopreservation of sheep embryos are similar to that for cattle (e.g. Fogarty et al 1999). Cryopreservation of pig embryos has long been quite problematic, due to extreme chilling sensitivity and the high lipid content of the pig embryos. However, recent studies have focussed on overcoming particularly these problems and produced successful methods for cryopreservation of pig embryos (e.g. Vajta, 2000; Nagashima et al. 1999; Dobrinsky et al., 2000). Especially for these species for which collection and transfer techniques are available and operational, embryo banking is a very good possibility for preservation of genetic diversity, and the fastest way to restore an original genotype when needed.

Somatic cells

Somatic cells, e.g. skin fibroblasts, can be readily cryopreserved. Collection of suitable somatic cells is possible and easy. Cryopreservation protocols for somatic cells are relatively simple and do not require controlled-rate freezing equipment. This means that establishing the collection is easy and cheap. The opposite is true for using the material when needed. This involves culturing the cells after thawing, reprogramming of the nuclei, collection of oocytes by ovum pick-up or from slaughtered animals, culture and in vitro maturation of the oocytes, enucleation of the oocytes, transfer of the somatic nucleus to (or fusion of the somatic cell with) an enucleated oocyte, culture of the resulting embryos, and finally, embryo transfer in recipients of the same species. The use of nuclear transfer means that the original mitochondrial genotype is lost.

In animals, live offspring have been obtained from embryos generated by transfer of nuclei of somatic cells in a number of species, i.e. in sheep, cattle, mice, pigs, goats, rabbits and cats (Wilmut et al., 2002). Until now, cloning has failed in the case of the rat, rhesus monkey and dog. Amazingly, some success (at least to the extent of embryo development, not live offspring) was even obtained when bovine oocytes were used as recipient oocytes for somatic nuclei from other mammalian species (Dominko et al. 1999; Saikhun et al. 2002). However, it must be emphasised that current techniques are inadequate to be used safely and efficiently for procreation at this point in time. In all published research until now, only a small proportion of embryos produced using somatic cells developed to become live young, typically between 0 and 4%. The low overall success rate is the cumulative result of inefficiencies at each stage of the process. Many pregnancies are aborted, and full term pregnancies often result in malformed young. Therefore it seems that the present cloning techniques introduce errors that affect embryonic and foetal development. Even apparently healthy live born young could have anomalies that only become apparent later in live, or in the next generation of animals. On the other hand, development of our knowledge on the mechanisms of reprogramming the genome is likely to increase dramatically. On a very long time horizon of e.g. one hundred years it is very likely that cloning methodology will have become both reliable and efficient.

The type of genetic resource material to be preserved in the gene bank may depend on the purpose of the gene bank, i.e. whether the gene bank is intended to save and preserve present day biodiversity for ‘eternity’, or must (also) serve to support breeding schemes (Sonesson et al. 2002).

In the latter case, it seems sensible to store semen (and embryos), which can be updated regularly and can also be regularly taken from the gene bank and be readily used in the field. If gene banking is intended to save and preserve present day biodiversity for 'eternity' one would like to have a cheap and fast collection of as many species and breeds as possible. Wherever financial resources and existing expertise and facilities are available, embryos are probably the best choice, but in lack of that, collection and cryopreservation of somatic cells could be considered as a possibility.

Table 1. An overview of some characteristics of several ways to cryopreserve genetic diversity (from Woelders et al. 2003)

	semen	**semen plus oocytes**	**embryos**	**somatic cells**
Samples needed to restore a breed	10000	2 x 100	200	Depends on future efficiency of cloning
Backcrossing needed	Yes	No	No	No
Mitochondrial genes included?	No	Yes	Yes	No
collection possible	Mostly, not always	Yes, some species. Operational for bovine	Yes, some species. Operational for bovine	Always
Cost of collection	€€	€€€	€€	€
Cryopreservation possible?	Yes	No! Despite some (very poor) successes	Bovine, Horse and Sheep are OK. Pigs is promising. Poultry is impossible	Yes
How to use	Surgical or nonsurgical Insemination/backcross-ing ≥ 6 generations	IVM/IVF → surgical or nonsurgical ET	Surgical or nonsurgical ET	Transfer in enucleated oocytes, → surgical or nonsurgical ET
Possible?	Yes	Yes	Yes	Low efficiency and many risks. Future development is likely!

In the Netherlands, we have started with semen for practical reasons. In addition, we will include embryos, probably beginning with rare/endangered cattle breeds. We have just started considering the possibility of gene banking of somatic cells. The present semen collection comprises 8 breeds of cattle, 3 breeds of horse, 5 breeds of sheep, 16 breeding lines of pigs, and 6 breeds of poultry (Tables 2, 3). The Dutch gene bank collections are managed by the Centre for Genetic Resources, the Netherlands (CGN), and are partly owned by the Dutch Gene Bank Foundation (SGL) and the CGN.

Table 2. Present numbers of semen doses available in the Dutch gene bank for various livestock species (SGL and CGN collections)

	# of breeds/lines	**# males per breed**	**# doses per breed**
Cattle	8	2-2000	400-50000
Sheep	5	12-27	1100-3000
Pig	16	6-34	200-800

Poultry	6	10	70-600
Horse	3	4-8	60-100

Table 3. Breeds of various livestock species in the Dutch Gene bank

Cattle	**Horse**
Groningen White Headed	Gelderland Horse
Deep Red	Draught Horse
Friesian Red Pied	Groningen Horse
Line Back	
Dutch Belted	
Dutch Friesian Black and White	**Pig**
Meuse-Rhine-Yssel	16 breeding lines of Dutch breeding organisations
Holstein Friesian	
	Poultry
Sheep	Welsumer
Drente Heath Sheep	Barnevelder
Schoonebeeker	Drente Fowl
Mergellander	Dutch Bantam
Kempen Heath Sheep	Dutch Uilenbaard
Veluwe Heath Sheep	Twente Fowl

Developments in cryopreservation

Some of the developments in cryopreservation of oocytes and embryos have been mentioned above. Both in oocyte and embryo cryopreservation respectable advances have been obtained by improvement of vitrification techniques (e.g. Vajta, 2000). This technique is especially important for freezing cells that are sensitive to chilling, like oocytes, particularly pig oocytes. Vitrification allows extremely rapid cooling rates, so that the duration of exposure to the temperature range in which chilling injury can occur is extremely short. This proved to be vital for freezing pig oocytes (Vajta , 2000), but is also reported to be important for freezing cattle oocytes and embryos (Martino et al., 1996; Vajta, 2000). Nevertheless, very good results are also obtained using more conventional, slow-cooling methods (e.g. Otoi et al, 1996). In fact, the procedures for cryopreservation of embryos used in the cattle ET industry today are still based on slow-cooling protocols (Vajta, 2000).

Methods for cryopreservation of semen are available for a number of livestock species but further development of cryopreservation methods is wanted in many cases. Progress in this field can improve the efficiency, because less semen per insemination and fewer inseminations per pregnancy or per live born young may be needed when post thaw sperm quality is improved. Another important reason to strive for further improvement is that there is a considerable variation among males in 'freezability' of the semen. This means that semen of some (possibly genetically important) males cannot be frozen. This is particularly true for some species like pigs and horses.

Theoretical approach; fundamental cryobiology

Further progress could come from studying the mechanisms of cryoinjury. One of the hypotheses is that cell death can occur at too high cooling rates as a consequence of intracellular ice formation (IIF) (see e.g. Mazur 1985), while too slow cooling leads to cell damage as a result of too excessive dehydration of the cells. The likelihood of IIF can be estimated for a given cell type frozen in a given medium at a given cooling rate, by computer simulations of the osmotic behaviour of cells during freezing. For such simulations it is necessary to have reliable estimates of the osmometric and membrane permeability characteristics of the cells.

In previous simulation models it was only possible to simulate the osmotic response of cells during freezing at a constant cooling rate. There is ample theoretical and empirical evidence indicating that a non-linear freezing programme (not a constant cooling rate) is better for post-thaw cell survival than a linear programme. We have recently developed a model to simulate the osmotic behaviour of cells during freezing and thawing (Woelders and Chaveiro, 2003). The model is designed to generate freezing programmes in which at all times the cooling rate is maximized (to minimize slow cooling damage), while preventing conditions that could lead to fast cooling damage, like IIF (see figure 1). The most important difference with current cryopreservation protocols is that the simulations predict that the freezing rate should not be constant, but should vary as a function of subzero temperatures. With our present non-linear model we are able now for the first time to optimise the freezing protocol without being confined to linear cooling programmes.

Application of the theoretical approach to cryopreservation of embryos

Embryos are usually frozen with a very simple linear cooling programme, using a constant cooling rate between 0.2 and 1 °C/min, depending on the species. As example, we have applied our theoretical model to embryos, using values for the membrane permeability for water and cryoprotectants as published for rat embryos (Pfaff, et al., 2000), and using three different cryoprotectant concentrations. As is shown in figure 1, the optimal way to freeze the embryos would depend on the concentration of cryoprotectant (c_s) used. The figure also shows that a substantial reduction of the cryoprotectant concentration would become feasible. To make a proper theoretical simulation for e.g. human or animal embryos, we would need to have reliable estimates of the osmometric and membrane permeability parameters for these embryos, which in the case of embryos can in fact be obtained quite easily, due to their large size and their slow volume changes. The model would then give predicted optimal freezing programmes for a number of chosen media compositions (cryoprotectant concentrations). This can be repeated using values for the cell parameters around those measured experimentally. This will result in a number of different combinations of freezing programs and medium composition that can be tested empirically. The advantage of having the theoretical simulation model available is that we do not need empirical testing of all thinkable possibilities.

Other mechanisms of cryodamage

Considerable mechanical and temperature shock may result from supercooling. Samples may become supercooled to temperatures as low as _12 °C in straws, and even lower in other packages. As soon as ice formation sets in, the temperature jumps to the freezing point due to release of latent heat of fusion.

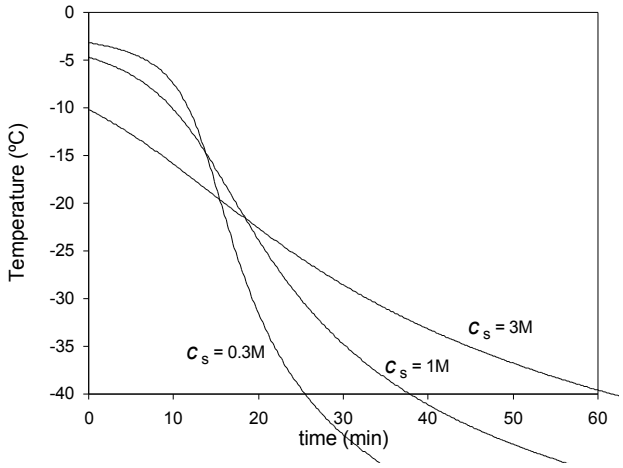

Figure 1. Theoretical predicted optimal freezing programmes for embryos using a cryoprotectant concentration (c_s) of 0.3, 1, and 3M, respectively. As example, membrane permeability data for water and cryoprotectant for rat embryos (Pfaff et al, 2004) were used in the model. The model used was that described by Woelders and Chaveiro (2003).

At the same time there is a very abrupt growth of ice masses and a very sudden increase in osmotic strength. It has been tried to prevent supercooling by inducing ice nucleation ('seeding') in protocols for freezing semen, However, seeding of straws did not result in improvement of post-thaw sperm survival (Fiser, 1988). This is probably due to the fact that the ice front propagates only slowly from the point of seeding, which allows large parts of the straw to supercool extensively (Woelders et al, 2004). We have designed a novel type of freezing apparatus for controlled rate freezing of straws, in which supercooling can be effectively prevented in the entire straw. In a split-sample comparison of semen frozen in straws we found significantly higher percent membrane-intact sperm for the new apparatus, compared to a conventional programmable controlled rate freezer (Table 4).

Table 4. Post-thaw percentage sperm with intact cell membrane (DAPI staining) after freezing in straws in a conventional programmable controlled-rate freezer and in our new apparatus (mean ± SE).

	conventional	new apparatus
Boar semen; 8 boars 9 ejaculates	54.8 ± 1.9	40.7 ±1.7
Bull semen; 6 boars 8 ejaculates	67.3 ± 3.0	59.3 ±2.9

Conclusion

Strategic choices for gene banking depend on the objectives of the gene bank. If the gene bank must have frequent exchange with an *in situ* population, semen is probably the most sensible choice. For the purpose of preserving particular breeds and to be able to restore a breed when necessary, embryo storage may be the best choice. For some species, e.g. the pig, more research developments are needed to improve safety and efficiency of using frozen-thawed embryos. Storage of somatic cells is a possibility to consider for use of very long-time storage, but future use depends on the (likely) assumption that scientific developments will render nuclear transfer techniques both safe and efficient. In the Netherlands, we will continue to use semen for cattle, pigs, sheep, poultry and horse. The semen will be used to support breeding schemes of e.g. breeder associations for rare breeds. Additionally, we will store embryos, beginning with rare cattle breeds.

Improvement of cryopreservation methods is necessary when adequate methods are not available or when large animal differences in freezability are seen with existing methods. Improvement of current methods will also improve efficiency of collection and storage. Further improvement of cryopreservation methods may be obtained by using theoretical modelling to

predict optimal freezing programmes, and by using freezing methods or freezing devices in which supercooling can be prevented.

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RELATIONS BETWEEN ENERGY BALANCE AND REPRODUCTION IN HIGH YIELDING DAIRY COWS

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The upward trend in milk production over the past 30 years has been accompanied by a steady decline in fertility. Over the same period there have been considerable changes to dairy management systems in terms of increased herd size, reduced manpower and altered diets. Nutritional status is undoubtedly one of the major influences on fertility, The most important factor appears to be the animal's energy balance (EB) status in the periparturient period. When the energy supply is inadequate, the cow mobilizes more of her body tissues for milk production and the extent of this mobilization is related to genetic merit. Higher yielding cows remain in a state of negative energy balance (NEB) for longer; in some cows this may last until 20 weeks after calving. This is associated with a longer interval from calving to first ovulation, a higher proportion of cows showing abnormal oestrous cycles and a decreased likelihood of conception.

Cows in NEB experience a number of metabolic and hormonal changes. NEFA and BHB concentrations reflect the amount of tissue mobilisation and are thus indirectly associated with fertility. Glucose, insulin and IGF-I all influence reproductive function directly via actions on the brain, ovary and reproductive tract. Of these, we have found that IGF-I links most closely with both return to cylicity and to conception. Concentrations of IGF-I fall precipitously around calving and then slowly recover. Multiparous cows which failed to conceive had lower IGF-I concentrations both before and immediately after calving associated with a higher yield and a greater body condition score (BCS) loss. Both high and low BCS before calving can be deleterious. When it is too low, the cows have little adipose tissue available for mobilization. When it is too high, appetite in the *post partum* period can be depressed, leading to a lower dry matter intake and a higher rate of BCS loss.

Another metabolic parameter often associated with fertility is urea. Rumen degradable protein (RDP) provides a source of metabolic fuel for rumen microbes. Urea is produced during this process and urea levels therefore rise as the amount of dietary protein increases, particularly if there is inadequate dietary energy supplied at the same time Low urea concentrations are indicative of an inadequate protein intake, whereas high levels are associated with elevated concentrations of ammonia, which may have direct toxic effects on the oocyte and/or early embryo.

Metabolic profiles differ significantly between first lactation cows (which are still growing) and older cows (which are producing more milk). Both rapid pre-pubertal growth and first conception at too early an age can reduce mammary development and result in poor fertility in the first lactation. The main factors associated with a delay in conception during the first lactation were all identifiable around calving and included high IGF-I and urea in late gestation and a large BCS loss early *post partum*. More work is required to determine the optimum growth rates, age at first conception and nutritional management during the first pregnancy for Holstein heifers in terms of both future production and fertility parameters.

EMBRYONIC LOSSES IN CATTLE

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Introduction

Genetic improvement of dairy cows over the past 50 years has led to a dramatic increase in milk yield, and an associated increase in dry matter intake (Van Arendonk et al., 1991). This increase in milk output has been associated with an overall decrease in reproductive performance (Pryce et al., 1999; Starbuck et al., 2004). Reduced fertility is particularly obvious in cows where milk yields are above 6000 L per lactation (Nebel and McGilliard, 1993; Macmillan et al., 1996), and in cows fed in excess during the previous dry period (Kruip et al., 1998). High genetic merit cows require up to four times their daily maintenance energy requirements during peak milk production. The decline in pregnancy rate to a single service has been reported to be approximately 0.5-1% per annum (Royal et al., 2000).

Following insemination, embryo mortality is recognised as the major cause of reproductive failure in cattle resulting in fewer calves born, slower genetic progress and significant financial loss to the cattle industry. The aim of this paper is to review recent data relating to the timing and possible causes of this mortality.

Timing of Embryonic Loss

Published data indicate a fertilisation rate of 90% and average calving rates of about 55% are normal for heifers and moderate yielding dairy cows, indicating an embryonic and foetal mortality rate of about 35%. Very few embryos are lost between fertilization and Day 8 of gestation (corresponding to the blastocyst stage); 70-80% of the total embryonic loss is sustained between days 8 and 16 after insemination (corresponding to the day of maternal recognition of pregnancy in cattle; a further 10% between days 16 and 42, by which time implantation is complete, and further 5-8% between day 42 and term (reviewed by Peters, 1996; Ball, 1997; Sreenan et al., 2001). Over the 20-year period from the 1975/82 to 1996/99, Lamming and Royal (2001) reported an increase in early embryonic loss from 24% to 40%, highlighting the importance of embryo mortality to the dairy industry.

A more detailed study by Dunne et al. (2000) reported that embryo survival rates on Days 14, 30 and at full-term were similar (68%, 76%, 72%, respectively), indicating that most embryo loss occurs before Day 14. Silke et al. (2002) reported embryonic loss of 6-7% between Day 28 and 84 of gestation. Starbuck et al. (2004) reported embryonic loss of about 11% between Day 30-60 and related this loss to concentration of progesterone at week 5 of gestation, twin ovulation, body condition, age and sire.

Maternal Recognition of Pregnancy – Role of Progesterone

An objective method to study fertility is to examine endocrine parameters associated with reproductive success (Lamming & Royal, 2001). The correct activity of the hypothalamus-pituitary-ovarian–uterine axis is necessary for the establishment and maintenance of pregnancy. The measurement of progesterone concentrations is one robust means of assessing whether normal oestrous cycles are occurring. In addition, normal progesterone profiles are required for the establishment and maintenance of pregnancy, particularly during early embryo development and during the critical period of maternal recognition of pregnancy when luteolysis occurs in non-pregnant cows. Lamming & Royal (2001) examined such patterns in two populations of cows in the UK; one group from 1975-82 and the second group from 1996-98. The disturbing factor from this study was an increase from 32% to 44% in the incidence of atypical progesterone values. This was manifested by a major increase in cows showing delayed luteolysis such that the average cycle length increased from 20.2 to 22.3 days. Animals with atypical progesterone concentrations had lower pregnancy rates. In addition, there has been a significant decrease in pregnancy rate (66-45%) in animals with atypical milk progesterone patterns, which may provide some insight into subfertility mechanisms. The data indicate that abnormal ovarian activity may be a major contributing factor to the decreased fertility observed in dairy cows. Whether this is a result of abnormal luteal activity or the result of abnormal follicular development associated with ovulation of

incompetent oocytes is unclear at this time. However, it is most likely a combination of factors resulting in fertilization of poor quality oocytes less capable of establishing and maintaining a pregnancy.

There is increasing evidence of an association between the concentration of progesterone and embryo survival but not all studies are in agreement on the timing of this association. A number of studies have revealed lower maternal progesterone concentrations in both milk and plasma from Day 10 in inseminated cows in which pregnancy fails compared to those in which pregnancy is successfully established (Henricks et al., 1971; Mann and Lamming, 2001; Lamming and Royal 2001) while supplementary progesterone from Days 2-5 has been reported to enhance development by Day 14 (Garrett et al., 1988). Antiluteolytic strategies aimed at improving fertility in cattle have been described (Binelli et al., 2001) including hormonal and nutritional manipulations to decrease plasma concentrations of oestradiol 17 beta while increasing those of progesterone and inhibiting the PGF2alpha-synthesizing enzymatic machinery in the endometrium. Progesterone supplementation results in a 5% enhancement in pregnancy rate but the time of treatment post insemination and the initial pregnancy rate of the herd have a major influence on the outcome; early supplementation of progesterone in cows with low initial pregnancy rates can be of significant benefit and knowledge of milk progesterone concentrations during the early post mating period can be used to select animals requiring supplementation at the appropriate time (Mann and Lamming, 1999). Stronge et al. (2004) examined the relationship between the progesterone concentration of milk on Days 4, 5, 6 and 7-post insemination in spring-calving dairy cows and early embryo survival and identified an optimum range of progesterone on Days 5, 6 and 7 within which embryo survival was maximized, with concentrations above and below this range being detrimental to survival.

Maternal Recognition of Pregnancy –Role of IFN-tau

The success of early pregnancy depends on the successful maternal recognition of pregnancy (Thatcher et al., 1995; Mann et al., 1999). To achieve this the embryo must prevent the regression of the corpus luteum by the production of a protein, IFN-tau, the embryonic signal which inhibits the development of the luteolytic mechanism. IFN-tau acts locally within the uterus to inhibit luteolytic PGF2a secretion by inhibiting the development of oxytocin receptors on the luminal epithelium (Robinson et al., 1999).

Mann and Lamming (2001) examined IFN-tau production by Day 16 bovine embryos and compared them with maternal endocrine profiles to identify hormonal patterns conducive to good embryonic development. They showed that cows with poorly developed embryos on Day 16 that were producing little or no IFN-tau exhibited a delayed increase in progesterone concentrations after ovulation and had a lower luteal phase plateau than did cows with well developed embryos.

Bovine embryos begin to express IFN-tau as the blastocyst forms (Farin et al., 1990), although there is considerable variability between individual embryos in the amount they produce (Hernandez-Ledezma et al., 1992) which may be related to the age at which blastocyst formation occurs (Kubisch et al., 1998; 2001), the group size in which culture takes place (Larson et al., 1999), the medium composition (Stojkovic et al., 1995; Kubisch et al., 2001) or to the sex of the embryo (Larson et al., 2001). It has been speculated that this may be related to their developmental potential (Kubisch et al., 1998, 2004) Several studies have shown that the relative abundance of IFN-tau transcripts are affected by the post fertilization culture conditions; a significantly higher level of expression of IFN-tau mRNA in blastocysts produced in the absence of serum which was correlated with significantly higher cryotolerance (Wrenzycki et al., 1999; Rizos et al., 2003).

Effect of Genetic Merit on Fertility

Genetic improvement of dairy cows has mainly focused on breeding strategies that increase milk production. However, selection for milk production alone can lead to deterioration in fertility (Pryce et al., 1999). In earlier studies, increased milk production was associated with delayed ovulation (Butler and Smith, 1989), an increased incidence of silent heats and ovarian cysts (Gröhn et al., 1994), a greater number of services per conception and a lower first service conception rate (Dhaliwahl et al., 1996). Whether the reduced reproductive performance recently reported in cows is due to the increased breeding value for milk production as suggested by Hoekstra et al. (1994), or to environmental causes as suggested by Butler and Smith (1989) is not clearly established.

Silke et al. (2002) examined late embryonic mortality in maiden heifers and cows. Animals diagnosed as pregnant at 28 days after breeding were scanned at 2-week intervals until day 84 of pregnancy. The results of this study showed that the overall incidence of late EM is similar between heifers and lactating cows (6.1% and 7.2%, respectively) and was not affected by calving to conception interval, parity or milk production.

To study this in more detail, Snijders et al. (1999) selected cows based on pedigree indices for milk production traits. High and medium genetic merit cows were studied in each of two years to determine the possible relationships between genetic merit, milk production, body condition, feed intake and reproductive performance.

There were no significant differences between the high genetic merit cows that did not conceive and those that conceived to first service in terms of milk production (3838 ± 79 kg vs 3843 ± 95 kg in 120 days), body condition score change between calving and first service (-0.47 ± 0.06 vs -0.50 ± 0.07) or feed intake (18.3 ± 0.59 vs 18.8 ± 0.66 kg/day), respectively. Medium genetic merit cows that did not conceive to first service lost more body condition between calving and first service than those that conceived to first service (-0.27 ± 0.09 vs -0.14 ± 0.07; $P < 0.05$).

Table 1. Milk production, body condition, dry matter feed intake (grass and concentrates) and pregnancy rates in high (n = 48 in year 1 and n = 46 in year 2) and medium genetic merit dairy cows (n = 48 in year 1 and n = 48 in year 2).

Genetic Merit	High	Medium
Milk production at 1st service (kg/day)	34.6 ± 0.64[a]	31.0 ± 0.64[b]
Milk production over 120 days (kg)	3819 ± 65.2[a]	3433 ± 64.5[b]
Body condition score (BCS) at calving	2.88 ± 0.05[b]	3.31 ± 0.05[a]
Change in BCS from calving to 1st service	-0.47 ± 0.05[b]	-0.23 ± 0.05[a]
Total feed intake at 1st service (kg DM/day)	18.6 ± 0.43[a]	16.7 ± 0.47[b]
Day of first ovulation postpartum	18.1 ± 2.28	19.7 ± 2.44
Conception rate % (No.)		
Overall (during 13 wk breeding season)	77.7 (73/94)[b]	93.8 (90/96)[a]
To 1st service	41.5 (39/94)[d]	52.1 (50/96)[c]
No. of services (in cows that conceived only)	1.75 ± 0.11	1.70 ± 0.11
No. of services (No services/no. pregnant.)	2.67 (195/73)[a]	1.91 (170/90)[b]

Values within rows with different superscripts [a, b] $P < .05$ [c, d] $P = .09$ (From Snijders et al., 1999).

Nutritional Effects on Fertility

Body condition, food intake, and stage of lactation or pregnancy can influence nutritionally-mediated effects on the efficiency of the reproductive system. Dietary factors can affect reproduction by influencing the animal at the level of the hypothalamus and pituitary gland, the ovary or the uterus. Ovulation occurs following a clearly defined period of follicle growth and oocyte maturation. While the initial stages of follicle growth are independent of gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) support is required for a follicle to proceed to the ovulatory stage and ultimately to allow resumption of meiosis and ovulation. Although the final manifestation of a detrimental effect of nutrition on fertility is the death of the embryo, it is also possible that nutrition affects embryo quality through changing the developmental capacity of the oocyte or through changes occurring during embryo development.

Effect of feed intake on fertility

The effect of high milk production on the incidence of reproductive disorders may be related to the degree of negative energy balance (NEB) in the early postpartum period (Gröhn et al., 1994). During early lactation, many high producing cows are unable to consume enough feed to meet their energy demands, resulting in a period of NEB. In terms of body tissue mobilization, early lactation is similar to severe undernutrition. It is typical for lactating cows to lose 30-40% of their initial lipid reserves at

parturition (Chillard et al., 2000). Many metabolic and endocrine changes are associated with NEB and are implicated in reduced fertility including diminished release of luteinizing hormone, reduced circulating concentrations of insulin, IGF-I, leptin and glucose and elevations in nonesterified fatty acids, beta-hydroxybutyrate and growth hormone (see Diskin et al., 2003, for review).

Although a positive link between early postpartum NEB on the calving to first ovulation interval was shown by Butler and Smith (1989), others have been unable to show this relationship (Fonseca et al., 1983; Harrison et al., 1990). Furthermore, Beam and Butler (1997) reported that the initiation of follicular development postpartum occurred regardless of metabolic status and EB. Changes in body condition score (BCS) during early lactation follow changes in EB (Villa-Godoy et al., 1988). Thus, cows losing more BCS early postpartum should experience more severe NEB, which could result in reduced reproductive performance. Indeed, Butler and Smith (1989) observed that severe body condition losses postpartum were associated with lower first service conception rates. NEB is also associated with reduced blood concentrations of glucose and insulin-like growth factor-1 (IGF-1) and elevated concentrations of non-esterified fatty acids (NEFA) (Spicer et al., 1990). Glucose is a major source of energy for the ovary (Villa-Godoy et al., 1988) and a deficiency in glucose may impair ovarian function, resulting in follicles and oocytes of poorer quality. Plym Forshell et al. (1991) noted that cows that did not conceive to first service had lower serum glucose concentrations than cows that conceived to first service. The relative importance of peripheral IGF-1 and locally produced IGF-1 to follicular development remains unclear, but studies suggest that higher concentrations of IGF-1 are related to an earlier resumption of oestrous cycles (Formigoni et al., 1996; Beam and Butler, 1998). However, it is not clearly established how concentrations of IGF-1 are associated with the reduced conception rates observed in cows with higher milk production. While several studies have indicated associations between energy, milk production, glucose, NEFA and IGF-1 and reproductive performance, there has been little evidence of differential effects between cows of different genetic merit.

Total dietary intake can affect fertility, both at the level of the oocyte and embryo. In dairy cows, oocytes aspirated transvaginally from early postpartum dairy cows fed ad libitum grass silage and 1 kg of concentrate per day had better morphological grading and cleavage rate and a trend towards a higher blastocyst formation rate than those aspirated from cows on ad libitum grass silage and 5 or 10 kg of concentrate per day (Lozano et al., 2000a; Table 3). In other studies ewes were used as experimental models to determine the effect of nutrition on embryo quality, and superovulation was used to increase the yield of embryos. A lower percentage of good quality embryos was recovered on day 4 from superovulated ewes on both a low diet (0.5 times the maintenance energy requeriments (M)) and an ad libitum diet, compared with animals on a control diet (1.5 M; Lozano et al., 2000b) However, while the embryos from the ad libitum group were delayed in their development, oocytes from the low dietary intake group had a lower fertilization rate. Overall, due to a poorer response to the superovulatory treatment in terms of the number of animals showing oestrus and lower ovulation rate, the embryo yield was lower in the ad libitum group when compared with the control and the low group (Lozano et al., 2000b). It is possible that similar effects of high dietary intake and metabolic load are affecting the quality of embryos produced in high yielding dairy cows. Preliminary evidence in dairy cows suggests that embryo quality as recorded by total cell number after recovery on day 7, is reduced in dairy cows offered large quantities of food in early lactation (Snijders, 2000). Thus, high intake diets exert a negative effect on the developmental capacity of embryos. Experimental diets offered below maintenance requirements resulted, in this study, in improved embryo development compared with animals on ad libitum diets. The effect of nutrition is exerted very early in embryo development, possibly before fertilization during the acquisition of developmental competence by the oocyte. This negative effect of high dietary intake or metabolic load on fertility is a fundamental challenge that needs to be addressed in high production cows, where fertility is compromised.

Table 2. Percentage of grade 1 oocytes (good oocytes) obtained by transvaginal aspiration and cleavage and blastocyst formation rates when cultured in-vitro in groups. Oocytes were aspirated from early postpartum dairy cows fed grass silage ad libitum and 10 kg (High), 5 kg (Control) or 1 kg (Low) of concentrate daily and oocytes obtained from bovine slaughterhouse ovaries were cultured as controls.

	Slaughterhouse	High	Control	Low

Good oocytes (%)		94/232 (40.7)[a]	66/192 (34.4)[a]	27/56 (48.2)[b]
Cleavage rate (%)	65	11/28 (39)[a]	22/47 (47)[a]	12/18 (67)[b]
Blastocyst rate (%)	30.5%	1/28 (3.5)	0/47 (0)	4/18 (22)

[a,b]$P<0.05$ [From Lozano et al. (2000a)].

Effects of dietary fat on fertility

The modern high genetic merit dairy cow prioritises nutrient supply towards milk production in early lactation. This demand appears to take precedence over providing optimal conditions for reproduction. Early in lactation, milk output rises faster than nutrients can be supplied from feed intake. Thus, in order to meet the nutritional demands of milk production, the cow mobilises energy reserves from her body and enters a phase of negative energy balance. Increasing the energy density of lactating rations can reduce the energy deficit experienced. Fats are incorporated into the diet of dairy cows in early lactation to minimize the difference between energy input and energy output.

A review examining the influence of supplemental fats on reproductive tissues and performance of lactating cows (Staples et al., 1998) showed that 11 of 20 articles reported improvement in either conception rate to 1st AI, overall pregnancy rate, overall conception rate, days open or AI per conception. Studies that report a negative influence of dietary fat supplementation on reproductive performance are often accompanied by large increases in milk production (Erickson et al., 1992; Sklan et al., 1994).

Several putative mechanisms have been suggested to explain the positive effects of dietary fat on reproduction. Dietary fat supplementation has been reported to increase the size of preovulatory follicles (Lucy et al., 1993; Beam and Butler, 1997), increase the number of follicles, (Wehrman et al., 1991) and increase the growth rate of the dominant follicle (Oldick et al., 1997). Supplemental fat may partially reduce energy deficits, however, early postpartum improvement in fertility occurred in some studies independent of the energy status of cattle (Lucy et al., 1993). Fat supplementation has been reported to increase the lifespan of corpora lutea (Williams, 1989). The concentration of plasma cholesterol, which is a precursor of progesterone, is increased consistently under regimes of supplemental fat (Spicer et al., 1993). Total progesterone production during an oestrous cycle has been increased in cows offered dietary fat (Hightshoe et al., 1991; Spicer et al., 1993). The increased progesterone was associated with increased lipid accumulation in luteal cells of the corpus luteum and a slower rate of disappearance of progesterone from serum. Increases in cholesterol and progesterone have also been reported in the follicular fluid of cattle on supplementary fat diets (Ryan et al., 1992).

Effect of crude protein intake on fertility

Much of the crude protein in the diet is hydrolysed in the rumen to ammonia, which is used as a substrate by rumen microbes for microbial protein synthesis. Excess ammonia is transported to the liver where it is metabolised to urea or used in the synthesis of amino acids.

Excess dietary crude protein or direct supplementation of the diet with urea as a source of nitrogen can result in increased plasma urea concentrations (Canfield et al., 1990; O'Callaghan et al., 1997). Urea is a relatively small molecule which has the ability to move freely through cell membranes. It is therefore logical that when urea concentrations are high, urea will move from the blood into other areas of the body, for example, the reproductive tract and mammary gland. The association between high dietary protein which induces high milk and serum urea, and reduced fertility has been reported previously (Jordan et al., 1983; Kaim et al., 1983; Canfield et al., 1990; Elrod and Butler, 1993; Butler et al., 1996). In most cases, as crude protein increases, services per conception and days open also increase.

Urea is offered in the diet of cattle as a source of nitrogen for rumen micro-organisms to synthesise microbial protein. Reduced fertility and embryonic loss can occur when dietary urea is in excess (McEvoy et al., 1997) or rumen degradable protein is increased (Blanchard et al., 1990). In an experiment of Fahey et al. (1998), the effect of high levels of urea on embryo quality and embryo survival was evaluated in a sheep model. Specifically, the effect of dietary urea on yield and quality of embryos in superovulated donor ewes and on embryo survival (to day 34-36) in recipient ewes following embryo transfer was evaluated. Despite high levels of dietary urea and blood urea concentrations, there was no effect of dietary urea on ovulation rate in donor or recipient ewes. However, embryo quality was

reduced as fewer embryos with more than 8 cells were recovered at day 4 from urea-treated ewes. The diet offered to recipient ewes had no effect on embryo survival.

It was shown that when heifers were supplemented with urea on a basic diet designed to provide approximately 50% of maintenance ME requirements or 200% of maintenance ME requirements, serum urea was higher in heifers on the low plane of nutrition. This occurred despite similar intakes of exogenous urea and reduced dietary intakes of dietary crude protein in the animals on the low dietary intake, and supports the suggestion that liver function is reducing metabolic clearance of urea in animals on a low plane of nutrition. When good quality in-vitro produced embryos were transferred into these heifers, no difference in pregnancy rate was observed. This suggests that uterine environment is not the main contributor to embryo loss when high urea diets are offered, even in a situation of relative energy deficit (which is common in the lactating dairy cow). Little is known about the effects of high concentrations of urea on oocyte quality and subsequent development capacity.

Nutrition and embryo quality

Short-term restrictions in dietary intake have been shown to increase the subsequent pregnancy rates in cattle (Dunne et al., 1997). Mantovani et al. (1993) reported that the yield of transferable embryos was significantly reduced in beef cattle following superovulation when heifers had ad-libitum access to concentrates compared with restricted concentrates. Yaakub et al. (1996) demonstrated that concentrate type and level can affect the subsequent yield of transferable embryos following superovulation in beef cattle. Heifers on a restricted diet, where the predominant concentrate supplement was in the form of citrus and beet pulp, produced more transferable embryos compared with those where barley was the predominant concentrate or with those on ad-libitum quantities of concentrates (Table 4). Thus, nutrient requirements for optimum follicle growth and embryo development may be quite different. This highlights the importance of diet around the time of mating, and in particular the significance of overfeeding, in regulating pregnancy rate.

Table 3. Concentrate intake (3 kg or ad-libitum) and type [barley or citrus/beet pulp] influenced mean±sem superovulatory response and embryo yield in heifers.

Concentrate intake	3 kg	*ad-libitum*	Barley	Citrus/beet pulp
Corpora lutea	15.5 ± 1.6^{a}	12.3 ± 1.4^{b}	13.4±1.5	14.4±1.5
Ova/embryos recovered	9.5 ± 0.9^{c}	6.5 ± 0.9^{d}	7.9±0.9	8.1±0.9
Grade 1 and 2 embryos	2.7 ± 0.5^{e}	1.0 ± 0.3^{f}	1.3 ± 0.3^{a}	2.4 ± 0.5^{b}
Grade 3 embryos	2.1±0.3	1.8±0.3	1.5±0.2	2.3±0.3

Within row and concentrate quantity type and quantity a, b ($P<0.06$); c, d ($P<0.05$); e, f ($P<0.001$).
From: Yaakub et al. (1996).

Heat Stress

While perhaps not of major significance in Europe, heat stress is a major contributing factor to the low fertility of dairy cows inseminated in the late summer months (reviewed by Hansen et al., 2001; De Rensis and Scaramuzzi, 2003). Apart from potential affects on both gametes, in terms of embryo development, the intrauterine environment is also compromised in heat-stressed cows including a decreased blood flow to the uterus and an increase in uterine temperature (Roman-Ponce et al., 1978; Gwazdauskas et al., 1975). These changes can potentially inhibit embryo development (Rivera and Hansen, 2001) increase embryonic loss and reduce the proportion of successful inseminations. The magnitude of the effect tends to decrease as embryo development proceeds with most embryo loss occurring before Day 42 in heat stressed cows (Vasconcelos et al., 1998).

In Vitro Produced/Manipulated Embryos

Pregnancy rates achieved following the transfer of both fresh and frozen IVP embryos are significantly lower than the rates observed with in vivo embryos (Hasler, 1998). Problems with pregnancies derived from in vitro produced embryos were described in a worldwide survey (Kruip and Den Daas, 1997) and by several commercial ET operations (Hasler, 1998; van Wagtendonk de Leeuw et al., 1998, 2000). Both an increased rate of embryonic, foetal and perinatal losses, and the occurrence of deviations in foetal and placental development are associated with bovine pregnancies obtained from in

vitro produced embryos (Kruip and Den Daas, 1997; Young et al., 1998; Taverne et al. 2002). Comparing the overall efficiency of AI, MOET and IVP, it has been estimated that for every 100 immature oocytes, an average of 55, 30 and 8 result in the birth of a live calf (van Wagtendonk-de Leeuw et al., 2000).

In their world-wide retrospective analysis on results from transfer of IVP embryos, Kruip and Den Daas (1997) reported that while 70% of the embryonic losses after AI or ET occurred within the first 21 days this was only 58% for IVF embryos. Van Wagtendonk-de Leeuw et al. (2000) compared pregnancy rates after single transfers of MOET, IVP-coculture or IVP-SOF embryos. Pregnancy rates were 45.6, 48.5 and 53.9%, respectively. Total return rates were not significantly different between the groups (54.4, 51.5 and 46.1, respectively) but return rate between 0 and 31 days was higher in recipients with a MOET of IVP-SOF embryo. However, the relative proportion of returns between Days 24 and 31 was significantly higher in the IVP-coculture group, while losses beyond Day 53 were also highest in this group(though not significantly different (6.3%, 11.8% and 8.5%, respectively). In another study, there were no differences in the relative proportions of early embryonic losses (before Day 24) and late embryonic/early foetal losses (between Days 24 and 119) following transfer of embryos derived from the same 3 groups (Perenyi et al., cited in Taverne et al., 2002); overall about one-third of all transfers failed before Day 24, while slightly more than one-fifth resulted in losses between Days 24 and 119.

Following somatic cell cloning a very limited percentage (0.5-5%) of the reconstructed embryos result in full term development. This is mainly due to a high frequency of postimplantation developmental arrest. Such losses are predominantly during the first trimester of pregnancy but can occur much later (Heyman et al., 2002). Heyman et al. (2002) monitored the evolution of pregnancy following the transfer of embryos derived from somatic cell cloning, embryonic cloning and IVP in order to detect the occurrence of late gestation losses and their frequency. On the basis of progesterone concentrations on Day 21, there were no significant differences in the percentages of initiated pregnancies between the groups (55.6-62.7%). Confirmed pregnancy rate by Day 35 using ultrasound scanning was significantly lower in the two somatic cloned groups (27.5-33.8%) compared with the embryonic clones (49.2%) and IVF embryos (52.9%). This pattern was maintained at Days 50, 70 and 90. The incidence of loss between Day 90 of gestation and calving was 43.7% for adult somatic clones and 33.3% for foetal somatic clones compared with 4.3% after embryonic cloning and 0% after IVP.

Conclusions

Reproductive efficiency is comprised of multifactorial components and efforts to enhance reproductive function will require more than a single approach. It is hoped that a better understanding will be achieved with developments in genomics and proteomics. The results of the experiments reviewed demonstrate ways in which dietary intake and composition can affect the reproductive system of cows and other ruminants at several levels. In general, the results are consistent with the conclusion that high dietary intake or high metabolic load is deleterious to oocyte development and the subsequent establishment of pregnancy. This highlights the importance of avoidance of extreme changes in the amount or type of diet around the time of mating. The early post-partum period coincides with a period when cows are in negative energy balance following calving, and are producing at or near peak milk yield, the diet in some management systems is switched from winter feed to grazed grass, and the cow is approaching the breeding season. The challenge remains to modify nutritional and management strategies to maintain the levels of production that can now be achieved as a consequence of genetic selection and still maintain an acceptable level of fertility.

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SHORT COMMUNICATIONS

MACROSCOPIC AND HISTOLOGICAL CONSEQUENCES OF REPEATED TRANSVAGINAL ULTRASOUND-GUIDED BIOPSY OF THE BOVINE OVARY

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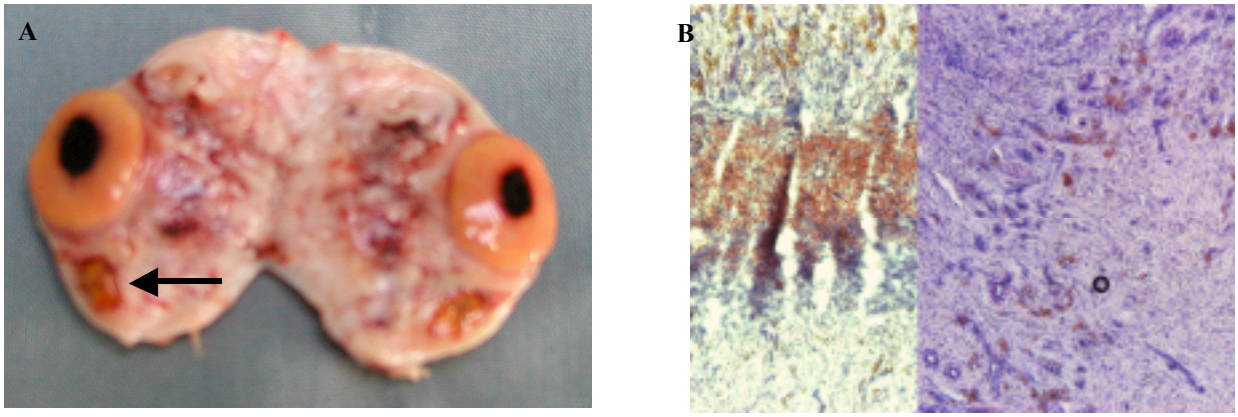
Recently, we developed and tested a new method for repeated prelevation of ovarian biopsies in living donor cattle through ultrasound-guided transvaginal puncture of the ovary (Aerts et al., Reprod Fert Dev 2004, 16 :229-230). Briefly, the procedure involves a modification of a commercially available ovum pick-up (OPU) instrument. The needle carrier has been equipped with a 14-gauge disposable needle, which acts as a trocar and is inserted through the vaginal wall. After conventional OPU- aspiration of the large follicles, a 60 cm long disposable biopsy needle is pushed transvaginally through the trocar needle into the ovarian stroma, under ultrasound control. The biopsy needle has a 20 mm long specimen notch, an outer cannula (diameter 1.2 mm) with a cutting edge, and an automated spring-loaded handle with trigger (Cook, Australia). The biopsy cutting mechanism is released when the biopsy needlepoint is positioned in the ovary.

A total of 10 healthy donor cows with normal reproductive tracts upon rectal examination, were subjected to repeated transvaginal biopsy prelevation. Nine out of 10 cows were biopsied twice per week over a four-week period. Ultrasonical aberrations of the normal ovarian image were seen in some cows: fluid filled cavities and dense, apparently luteinised tissue fragments. To assess the consequences of repeated biopsy, two of the donor cows were slaughtered on Day 2 after the last biopsy session. Following slaughter, the ovaries were identified and transported to the lab. They were examined macroscopically and subsequently fixed in 4% paraformaldehyde for 2 hours at room temperature. After overnight incubation in a 15% sucrose solution for cryoprotection, 14-_m cryostat sections were conventionally stained with hematoxylin. Macroscopical and histological findings are represented in Figure 1.



(Fig 1-A) Postmortem analysis of the ovaries revealed no adhesions and, in accordance with the ultrasound image, CL(-like) structures could be identified (arrow). Macroscopically, bloodclots were apparent throughout the ovary. (Fig1-B) Histological examination showed increased infiltration of red blood cells in the ovarian stroma.

To our knowledge, only one report was previously published on a comparable technique for transvaginal corpus luteum biopsy (Kot et al., Theriogenology 52, 987-993). In this study multiple biopsy prelevation did not seem to interfere with subsequent in vivo luteal function. A follow-up study will reveal whether repeated biopsy prelevation has adverse effects on bovine reproductive efficiency.

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Notes

DETECTION OF CAPRINE ARTHRITIS-ENCEPHALITIS VIRUS (CAEV) IN THE EARLY EMBRYONIC CELLS FROM IN VIVO-PRODUCED GOAT EMBRYOS

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The aim of this study was to examine the susceptibility of early goat embryo cells to caprine arthritis-encephalitis virus (CAEV) infection and replication in vitro.

Zona pellucida (ZP)-free 8–16 cell embryos were delicately loaded on a membrane of culture inserts. The embryo-containing inserts were placed on top of a mixed cell culture monolayer - 80% of COEC (Caprine Oviduct Epithelial Cells) and 20% GSM (Goat Synovial Membrane) cells - infected with CAEV-pBSCA at a multiplicity of infection of 1 (moi=1 in B2 medium supplemented with 10% Fetal Calf Serum (FCS). Co-cultures were incubated at 38.5°C, 5% CO2 in a humidified atmosphere for 6 days. Negative controls were prepared in similar conditions, except the monolayer co-culture cells were not infected with CAEV.

After 6 days of co-culture of early embryo cells in the inserts with non infected and infected monolayers, embryo cells were harvested, washed successively in ten separate wells containing 2 ml of MEM (Minimum Essential Medium) and then transferred into a new insert on the top of a GSM cell monolayer for 6 h. The presence of CAEV was examined by RT PCR in the washing fluids while GSM cells were cultured and observed for 30 days, (with a trypsination each 6 days) for cytopathic effects on development (CPE).

The embryo cells were washed and separated into two batches: The first batch was cultured in a simple medium (B2 medium) for 24 h to examine the production of the virus. The medium was filtered on 0.22 µm and used for virus titration on GSM cells. The second batch was trypsined (trypsin to 0.25%) and cultured for 4 to 8 days. After an additional trypsin treatment, half of the cells was used to look for the provirus DNA by PCR and the viral RNA by RT-PCR and the second half was examined by immunocytochemistry to detect the major viral p28 gag protein expression.

CPE were detected on GSM cells and confirmed as specific from the 4th trypsination at Day 24. The first three washings as well as the simple culture medium were found to be positive by RT-PCR. The embryonic cells were found PCR positive and the culture media RT-PCR positive. The CAEV-p28 was detected by immunocytochemistry. All the control samples were found to be negative.

From these results, we conclude that early embryo cells of ZP-free 8–16 cell embryos are fully susceptible to infection and do replicate CAEV.

Notes

VARIABILITY OF THE DIFFERENT TIME COMPONENTS BETWEEN FLUSHING AND TRANSFER OR FREEZING OF CATTLE EMBRYOS

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Previous studies have shown that the time interval between flushing and freezing or transfer of fresh embryos can influence pregnancy rates following embryo transfer. The aim of this study was to determine which time components are the most variable and can influence embryo survival following transfer. The different time components between flushing of a superovulated donor cow and transfer or freezing of the collected embryos were investigated under field conditions. For each embryo, the total time interval spent in vitro (TIV) was recorded. During January 2003, ET technicians (Embryo Top) recorded systematically times corresponding to each step composing the TIV : end of uterus flushing; beginning and end of search /evaluation of embryos; first and last transfer of fresh embryos (if different groups of recipients were transferred, times were recorded for each group); beginning of equilibration with 1.5M EG and seeding for frozen embryos. Numbers of donor cows and ET technicians were noted for each recovery session (RS). Data from 263 collections (187 RS) were analyzed and means and SD calculated with SAS (PROC Means). The coefficient of variation (CV) was calculated for each time component. A total of 1515 transferable embryos (805 fresh, 710 frozen) were collected. The TIV averaged 200 minutes with a linear distribution and ranged from 50 to 510 minutes (min). The TIV was higher for frozen than for fresh embryos (210 vs. 190 minutes, $P<0.01$, Student's t-test). The TIV did not differ with embryo quality (evaluated according to the IETS criteria).

Table 1. Mean, standard deviation (SD), coefficient of variation (CV) of each component of the interval between flushing and transfer or freezing of embryos (minutes.)

Time interval	Duration of Flushing	End of flushing to search	Duration of search, evaluation	End of evaluation to transfer or freezing	First to last transfer	Equilibration
Mean	31	31	44	81	24	16
SD	8	27	25	64	18	4
CV	25.8%	87.1%	56.8%	79.0%	75.0%	25.0%

The greatest variability was observed for the interval between end of flushing and beginning of searching. This period was mainly influenced by the number of donor cows per ET technician (1 donor cow = 19± vs > 1 donor cow = 45 min, $P<0.01$). The number of donors per ET technician also significantly influenced the interval between the end of embryo evaluation and transfer or freezing, together with the freezing of embryos (at least 1 embryo frozen, 85 min vs 53 min. no embryo frozen, $P<0.05$). The interval between the first and the last transfer varied mainly with the number of herds per RS in which the embryos had to be transferred. The duration of search and evaluation were influenced by the number of collected embryos per RS (1 to 18 embryos = 39 min vs > 18 = 62 min, $P<0.05$).

These results show a strong variability of the TIV and the important influence of the number of donor cows per ET technician on this variability. Complementary studies are running to investigate the influence of TIV and its different components on pregnancy rates.

Notes

COMPARISON OF PROTEIN EXPRESSION AND PROTEIN PHOSPHORYLATION DURING IN VITRO MATURATION OF BOVINE OOCYTES

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Phosphorylation of proteins by specific protein kinases controls basic cellular processes, such as cellular growth and differentiation, cell cycle regulation and meiosis. During meiotic maturation (transition from prophase I to metaphase II) of oocytes, the M-phase kinases, cdc2k or MAPK play a predominant role. Using a proteomic approach, we have made an attempt to uncover some of the targets of these kinases, which are not yet completely identified. 2D-gel electrophoresis was performed with bovine oocytes matured in TCM 199 containing 3% BSA, with 400 oocytes each at the GV stage (0-h maturation), GVBD/M I (10-h maturation) or M II (24-h maturation) and GV-arrested oocytes [24-h maturation with Cyclin-dependent kinase (Cdk) inhibitor, Butyrolactone I, BL I]. The use of protein-specific stain Sypro Ruby allowed the separated proteins to be visualized, whereas phosphoproteins could be stained by the phosphoprotein-specific stain Pro-Q Diamond. The activity of the kinases, Cdc2 and MAPK, was confirmed by Western Blotting and in vitro kinase assay. Gel-overlay analysis by 2D Decodon software program allowed the quantification of protein spots differently expressed or phosphorylated. A minimum of two-fold increase or decrease in the expression or phosphorylation of proteins was considered a significant change. We were able to detect approximately 350 protein spots per gel. 64 of them were picked, digested by trypsin and identified by MALDI-TOF-TOF mass spectrometric analysis. From these peptide mass fingerprints the identity of 30 proteins was certain, while the rest could be identified through homology with other species in databases. Proteins involved in energy transduction, metabolism, cytoskeletal proteins and protein folding and chaperone related proteins could be identified. The overall expression of the protein families was found to be not significantly changed, although the phosphorylation status of distinct proteins showed changes during the maturation process. On the other hand, BL I not only influenced the phosphorylation of some proteins like HSP 70, but also repressed the expression of several metabolism related proteins. A detailed analysis of expression and phosphorylation patterns will be presented and the biological relevance of the obtained results will be discussed.

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Notes

VITRIFICATION-SURVIVAL OF OVINE BLASTOCYSTS IS IMPROVED BY CULTURE WITH ALBUMIN AND HYALURONAN

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The aim of this paper was to improve the survival rate of vitrified in vitro produced blastocysts by improving culture media with the addition of bovine serum albumin (BSA) and hyaluronan (HA). In fact, an improved culture media, enhancing the quality of embryos, may increase the cryotolerance, in turn improving the cryopreservation efficiency.

Embryos were produced by in vitro maturation, fertilisation and culture (IVM/IVF/IVC) of oocytes from slaughtered ewes. At day three of culture (day 0 day of fertilisation) the culture medium was supplemented with 0.4% BSA (fatty acid free) and 6 mg/ml HA while, in the control group 10% of charcoal treated serum (FBS) was added, as routinely performed in our laboratory**.**

Embryos were cryopreserved after exposure at room temperature for 5 min to 10% ethylene glycol (EG) + 10% dimethyl sulphoxide (DMSO), then for 30 sec to 20% EG + 20% DMSO + 0.5M sucrose. They were then loaded into OPS (Open Pulled Straws) and directly plunged into liquid nitrogen. At warming, the embryos were placed into a 0.5M sucrose solution for three min.

Embryos from all groups were transferred in pairs into synchronised recipient ewes and allowed to go to term. The lambing rate indicated that there was an improved survival rate in the embryos cultured with BSA and HA

Table 1.Lambing rate of vitrified embryos cultured with Bovine Serum Albumin and Hyaluronan.

Culture conditions	N° of Blastocysts transferred	N° of Recipients	Lambs born/Transferred embryos (%)
BSA+Hyaluronan	41	20	14/41 (34.1)
10% FBS (control)	39	19	8/39 (20.5)

Notes

A COMPARISON OF THE OUTCOME OF TRANSVAGINAL OOCYTE RETRIEVAL AND IN VITRO EMBRYO PRODUCTION IN FSH-LH STIMULATED VERSUS NON-STIMULATED DONOR COWS

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There is general agreement on an overall positive effect of FSH pre-stimulation prior to OPU (Merton et al., Theriogenology 2003). The aim of this retrospective study was to compare the number of follicles, COCs and cultured IVP embryos obtained from 1396 non-stimulated OPU sessions on 81 donor animals in a twice-weekly OPU scheme, with 640 sessions on 112 donors following FSH-LH superstimulation, subjected to OPU once every two weeks. The stimulation protocol started with the insertion of an ear implant containing 3 mg norgestomet (Crestar, Intervet, Belgium) 8 days before puncture (d-8). The dominant follicle was ablated by ultrasound-guided follicle puncture on d-6. On d-3 and d-2, cows were injected with 40 _g porcine FSH and 10 _g porcine LH (Stimufol, Ulg, Liege) twice daily (8 am – 8 pm), for a total dose of 160 _g FSH and 40 _g LG per donor per stimulation cycle. Animals were punctured 48 hours following the last FSH injection (d0), with the progesterone implants removed the next day. Stimulated donor cows were treated with this protocol at 14 day intervals. Follicles were visualized with a dynamic imaging ultrasound scanner, equipped with a 6.5 MHz sectorial probe. Follicles were punctured with 55 cm long, 18-g needles at an aspiration pressure corresponding to a flow rate of 15 ml per minute. Cumulus oocyte complexes (COCs) were recovered and processed in a routine IVF set up. Results are summarized in Table 1.

Table 1. OPU-IVF results following unstimulated and stimulated OPU - IVF sessions.

	Unstimulated sessions (1996-1999)		Stimulated sessions (1999-2003)
	Per session (2 sessions/week)	Calculated per 2-week period *	Per session = per 2-week period (1 session every 2 weeks)
Animals	81	71	112
No. sessions or periods	1396	310	640
No. follicles	9968	8981	9531
Mean no. follicles	7.1±3.9[a]	29±11.6[b]	14.9±9.9[c]
No. oocytes	5713	5135	7536
Mean no. oocytes	4.1±3.1[a]	17.0±8.6[b]	11.8±8.2[c]
Recovery rate	57%[a]	57%[a]	79%[b]
No. embryos at d 7	997	927	2190
Mean no. embryos	0.7±1.2[a]	3.0±2.9[b]	3.4±3.9[b]
% development	17%[a]	18%[a]	29%[b]

[a]Different superscripts among columns indicate statistically significant differences, $p < 0.05$, (ANOVA for comparison of means , Chi square for proportions) – * in those cases where data on 2 full weeks (4 sessions) were available

Expressed per session, the results demonstrate that FSH stimulation prior to OPU increases production efficiency with significantly more follicles punctured and oocytes retrieved. However, when overall results during comparable 2-week periods are considered (4 non-stimulated sessions versus 1 stimulated), more folicles are punctured and more oocytes are retrieved using the non-stimulated protocol. No significant differences in the number of cultured embryos could be detected, indicating that FSH-LH stimulation prior to OPU might have a positive effect on in vitro oocyte developmental competence because more embryos are cultured with less, presumably better quality, oocytes.

Notes

INVESTIGATIONS ON PrP AND PrPres IN PORCINE BRAIN AND IN STIMUFOL® PREPARATIONS

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As Follitropin and Lutropin, the active molecules contained in Stimufol®, are extracted and purified from porcine pituitaries, it was interesting to test the behaviour of porcine PrP in the Platelia test BSA (Biorad°) and in the purification procedure.

The aims of this study were (1) to test the capacity of the Platelia-test (Biorad°) to detect the physiological PrP in extracts of porcine brain, (2) to test the presence of the physiological PrP in the Stimufol® preparation and (3) to systematically test all batches of Stimufol® for the absence of PrPres before delivery.

Firstly, two samples of porcine brain were collected in a slaughterhouse and 350 mg of tissue (taken in the obex region of the nucleus of vagus nerve and tractus solitarius where PrPres is repeatedly found in large amounts) were submitted to the test with and without the degradation step by the action of proteinase K. The results in terms of optical density were respectively 0.036 and 0.800. These results show that the Platelia-Test is able to detect the PrP protein in porcine brain tissue and the antibodies recognize the undigested material. Secondly, 2 samples of Stimufol® corresponding to 10 mg of soluble proteins were submitted to the test without any digestion by proteinase K. These samples generated values of 0.034 and 0.032 units of optical density. These negative results show that the PrP protein does not follow follitropin nor lutropin during the purification procedure and does not contaminate the final product.

Finally, as it was not possible to test the behaviour of the PrPres in the purification procedure of follitropin and lutropin, all batches of Stimufol® released after March 2001 were tested for PrP after proteinase K digestion. They gave optical density of 0.033-0.033-0.031-0.031-0.030-0.032-0.031-0.033 versus 0.029-0.029-0.026-0.031 for four negative controls of bovine protein solution.

In conclusion, the inocuity of all batches of Stimufol® in terms of Prion transmission is verified since March 2001.

Notes

COMPARISON OF FOLLICULAR DEVELOPMENT AND OOCYTE QUALITY IN LANDRACE AND MANGALICA GILTS AFTER FEEDING WITH DIFFERENT ENERGY LEVELS

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The aim of the study was to determine how different feeding levels affect follicular development and oocyte maturation in Landrace (L, n=20) and Mangalica (M, n=17) pigs. The animals were divided between two feeding groups with a high (HI – 2.5 kg) or low (LO – 1.25 kg) energy level. Oestrus of gilts was synchronized by 15 days feeding regime of 20 mg Regumate®/animal/day (Roussel-Uclaf). After Regumate treatment, follicular growth was stimulated by application of 1000 IU PMSG (Folligon, Intervet) on Day 16. Ovulation was induced by injection of 750 IU hCG (Choriogonin, Richter Gedeon) on Day 19. Ultrasound investigation (US) was done three times (Day 15, 16 and 18) for observation of follicular development. Oocyte and follicular fluid collection was performed by endoscopic ovum pick-up 34 hours after the hCG injection. Cumulus-oocyte complexes were evaluated under a stereo microscope and their morphology was determined. Thereafter they were fixed and stained for further chromatin evaluation. Oocytes were classified as (1) meiosis resumed – GV breakdown, diakinesis, M-I to A-I; or (2) mature – T-I and M-II..

In L gilts considerable differences were observed in follicular development after feeding with different energy levels, whereas in M the treatment had no affect on the number of preovulatory follicles (Table 1). Follicular growth was not affected by feeding levels, but preovulatory follicle size was larger in M.

Table 1. Mean (± SD) number and size of preovulatory follicles in L and M gilts fed with high and low energy levels

Parameter	Landrace		Mangalica	
	High	Low	High	Low
No. of follicles	32.3 ± 10.5[a]	17.1 ± 12.3[b]	25.3 ± 2.9	28.8 ± 7.3
Follicle diameter (mm)				
US 1	2.2 ± 0.3	2.2 ± 0.5	2.2 ± 0.2	2.1 ± 0.1
US 2	4.0 ± 0.5	4.0 ± 0.4	5.1 ± 0.3	5.0 ± 0.4
US 3	5.0 ± 0.5	5.2 ± 0.7		
34 h post hCG	5.7 ± 0.7[a]	5.5 ± 0.8[a]	7.1 ± 0.9[b]	6.9 ± 1.1[b]

[a,b] $P<0.05$

No differences were observed in relation to chromatin configuration in both breeds. In L gilts 69.8 (HI) and 66.7 % (LO) of the oocytes were classified as mature compared to 66.7 (HI) and 62.5% (LO) in M gilts. A two-fold higher estradiol concentration was detected in the follicular fluid of HI- and LO-M (29.6 ± 6.8 and 30.9 ± 10.3 ng/ml) compared to L (16.9 ± 9.7 and 17.9 ± 3.6 ng/ml, $p<0.01$). The mean progesterone level was nearly 4-5 fold higher in M (2020.4 ± 1056 and 1512.2 ± 1121.8 ng /ml) compared to L (386.2 ± 113.7 and 298.8 ± 125.9 ng/ml, $p<0.01$).

Results indicate an influence of energy feeding on follicular number in modern Landrace but not in native Mangalica breed. Follicular steroid hormone milieu differs as well between Landrace and Mangalica gilts but is not dependent on feeding levels. Oocyte maturation was not affected by feeding levels.

Notes

ARE OOCYTE PRODUCTION AND OOCYTE QUALITY BEFORE BREEDING RELATED TO SUBSEQUENT FERTILITY IN HIGH YIELDING DAIRY COWS?

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This study aimed to measure the relationships between oocyte production (by OPU), IVF efficiency between 50 and 70 d post-partum and subsequent fertility in high yielding dairy cows.

Fourteen cows were collected twice weekly for 3 weeks before breeding (first OPU session 54.1±0.5 days postpartum). Cumulus oocyte complexes (COC) were morphologically classified into 4 classes from Q1 (good quality) to Q4 (poor quality). COC recovered from OPU sessions 1, 3 and 5 were in vitro matured, fertilized then cultured in SOF medium for 7 d.

After the last OPU session, cows were oestrus synchronized and artificially inseminated on Day 83±0.5 after calving. Pregnancy was assessed by measuring progesterone (P4) on Day 21-24 and by ultrasonography on Day 35 post-AI. If a return to oestrus was observed, cows were re-inseminated. Cows were considered to be fertile (F) if they were pregnant after 2 AI and unfertile (NF) if they needed >2 AI to be pregnant. Relationships between the numbers of punctured follicles, total COC recovered, Q1+Q2 COC recovered and fertility was analysed using multivariate models including fertility, OPU session number, group (3 contemporary groups), operator as a fixed effect and animal as a random effect (proc mixed of SAS). Global efficiency (blastocysts+morula/collected), cleavage rate (cleaved/collected) and development rate (blastocyts+morula/cleaved) were compared between F and NF cows using the Chi-Square Test.

BCS at calving and AI were not different between F (n=6) and NF (n=8) cows (2.1±0.4 vs 1.8±0.3 and 1.2±0.3 vs 0.7±0.1 respectively, p>0.10). Milk production and fat content at AI were not different between F and NF cows (33.9±3.1 vs 36.2±1.5 kg/d and 38.1±1.7 vs 35.3±1.7 g/kg, p>0.10). However there was a trend for higher milk protein content in F than in NF cows at AI (28.1±1.2 vs 25.7±1.0 g/kg; p=0.06). Follicle number, COC production, good quality COC production, development rate and global IVF efficiency were not different between F and NF, however, cleavage rate was higher in F than in NF cows (Table 1).

Table 1: Oocyte production and IVF efficiency in fertile and unfertile high yielding dairy cows.

	Fertile (n=6)	Unfertile (n=8)	P-value
Aspirated follicles	9.0±1.4	8.7±1.3	0.87
Oocytes recovered	2.3±0.4	3.0±0.4	0.27
Q1+Q2	0.7±0.2	1.2±0.2	0.11
% Q1+Q2	31.2%	41.2%	0.12
% cleaved	**63.4%**	**44.4%**	**0.05**
% developped	32.1%	42.9%	0.41
Global efficiency	20.1%	19.1%	0.86

In conclusion, oocyte production was not related to subsequent fertility in high yielding dairy cow. However, cleavage rate after IVF was higher in fertile than in unfertile cows.

Notes

IN VIVO SURVIVAL RATE OF VITRIFIED OVINE EMBRYOS AFTER BIOPSY

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Due to the time required for multiplex PCR analysis of the embryonic genome, biopsied embryos should be cryopreserved before transfer. The aim of our study was to test the survival rate, after transfer to recipients, of in vivo produced ovine blastocysts after biopsy and vitrification, either immediately after biopsy or after 24 h of in vitro culture.

After a pretreatment with a GnRH antagonist (Antarelix), Ile-de-France ewes were superovulated with pFSH (32 Armour Units) and inseminated. The embryos were collected 7 days after oestrus. Biopsy was carried out with a microblade. Biopsies were kept to perform sex determination by PCR amplification of ZFY/ZFX and SRY sequences and biopsied embryos were vitrified (glycerol 10%, glycerol 10% and EG 20%, glycerol 25% and EG 25% ; 3 steps of 5 min each, Mermillod et al., 1997, 13th AETE Meeting, Lyon, p 182) either just after biopsy (group 0 h), either after 24 h of in vitro culture in 500 µL of SOF medium (group 24h). Control embryos were vitrified in an identical fashion. After thawing, two female embryos were directly transferred into each synchronized recipient. Pregnancy rate was assessed at Day 17 (progesterone) after oestrus and confirmed at Day 42 by ultrasound. A second experiment was carried out on Lacaune (farm conditions) and Ile-de-France embryos, without culture before vitrification. Female Ile de France embryos were transferred whereas male embryos from the Lacaune breed were transferred.

Table 1. Pregnancy and embryo survival rate after thawing and transfer of control and biopsied ovine embryos.

	Experiment 1		Experiment 2			
	Biopsied embryos		Control embryos		biopsied embryos	
	0 h	24 h	Lacaune	Ile	Lacaune	Ile
Recipients	14	13	13	16	13	16
Embryos transferred	28	26	26	32	26	32
Pregnancies : Day 17	78%	61%	64%	56%	54%	44%
Day 42	71%	54%	54%	50%	31%	37%
lambing	46%	36%	54%	50%	31%	25%
Embryo survival	27%	32%	38.5%	31.3%	15.4%	18.8%

In experiment 1, 2-3 weeks before lambing one recipient died in group 1, and 2 in group 24 h. Lambing and embryo survival rates were not significantly different between the two groups ($P > 0.05$). Biopsied embryos could be vitrified immediately after biopsy or after 24h of in vitro culture without any difference on embryo survival rate after transfer into recipients. The same result was obtained with control embryos (not shown).

In experiment 2, the ewe breed had no significant effect on lambing and embryo survival rates. A mean lambing rate of 52% and 27.6%, and a mean embryo survival rate of 34.5% and 17.2% were obtained, respectively with control and biopsied embryos. A sex prediction error of 4% (1/24) was obtained in both experiments. Biopsy induced a 20% decrease in embryo survival rate in the two breeds tested. Similar results have been obtained previously after transfer of fresh biopsied ewe embryos (Guignot et al, ESDAR 2003, abstract P70, 352). The vitrification technique should be further adapted to improve survival of sensitive biopsied ovine embryos.

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Notes

COMPARATIVE INTRACYTOPLASMIC SPERM INJECTION (ICSI) AND IN VITRO FERTILIZATION IN PREPUBERTAL GOAT OOCYTES.

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Intracytoplasmic sperm injection (ICSI) has been introduced as an alternative to in vitro fertilization (IVF). As the rates of in vitro fertilization in prepubertal goats oocytes are very limited, the present study incorporates the ICSI technique as a alternative to IVF. Prepubertal goat oocytes were recovered from a local slaugtherhouse and matured in a conventional IVM medium (TCM199 with serum, hormones and cysteamine) for 27h. Spermatozoa from fresh ejaculates were selected by swim-up and capacitated with heparin plus ionomycin. IVM-oocytes were randomly allocated into 5 groups: a) ICSI group was oocytes with visible first polar body and injected with 1 spermatozoa; b) SHAM group was oocytes with visible first polar body and injected without spermatozoa. c) ICSI+ activation group was ICSI oocytes activated by sequential treatment with 5 µM ionomycin for 5 min and 2mM 6-DMAP for 4 h. d) SHAM + activation group was SHAM oocytes activated as group 3. e) IVF group was IVM-oocytes fertilized by conventional IVF methodology. Presumptive zygotes and activated oocytes were cultured in TALP fertilization medium for 17 hours. After culture oocytes and zygotes were stained with lacmoid to analyze their nuclear stage. These results are shown at Table 1.

Table1. Nuclear stage of ICSI, SHAM and IVF oocytes after 17 hours of culture.

Nuclear morphology	TREATMENTS				
	ICSI	SHAM	ICSI+ activation	SHAM+ activation	IVF
Nº oocytes examined	67	54	73	61	195
Nº (%) not activated (MII)	64 (95.5)	54 (100)	30 (41.1)	29 (47.5)	145 (74.3)
Nº (%) fertilized (2PN)	1 (1.5)c	-	27 (37.0)a	-	47 (24.1)b
Nº total (%)zygotes non correctly fertilized	2 (3.0)c	0	13 (17.8)b	28 (45.9)a	3 (1.5)c
With intact sperm head +	2	-	12	-	0
In 2PN stage	0	-	7	4	0
In ana-telophase II stage	0	-	0	15	0
In 3PN stage	0	-	2	4	0
In 1 PN stage	2	-	4	5	0
With swelling sperm head + 1PN(%)	0	-	1	-	3

Values with different superscripts within each row differ significantly ($P< 0.05$)

In our study with prepubertal goat oocytes the injection stimulus (ICSI and SHAM groups) was not sufficient to activate the oocyte and to form male and female pronuclei. Ionomycin and DMAP must be used to activate oocytes after ICSI treatment. The percentage of oocytes not activated after ICSI and chemical activation was 41.1%. The percentage of IVF oocytes was 25.6%. Significant differences were found in oocytes correctly fertilized (2PN) between ICSI and IVF (37.0 vs. 24.1% respectively). In conclusion, oocyte activation after ICSI could be a good procedure to obtain embryos from prepubertal goat oocytes.

Notes

DEVELOPMENT OF EMBRYOS AFTER IVF OF BOVINE OOCYTES WITH FLOW CYTOMETRICALLY SEXED, FRESH AND FROZEN SPERM

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There is a great interest in application of sex preselected, X and Y chromosome-bearing sperm (Johnson, 1995: Reprod. Fertil. Dev. 7:893-903; Zhang et al., 2003: Theriogenology 60: 1657-1663).

The aim of our investigations was to develop technology of in vitro embryo production in cattle using fresh and frozen-thawed, flow cytometrically-sorted, spermatozoa for IVF. Immature oocytes, recovered from slaughtered ovaries, were matured in vitro (IVM) for 22 to 23 h in modified TCM 199 and were fertilized in vitro with fresh or frozen-thawed, cytometrically-sorted X and Y fractions of sperm of bulls with proven fertility. Sperm was sorted in a MoFloSX cytometer (Cytomation) using the method of XY Inc., Colorado, USA (Research Collaboration Agreement). Simultaneously, control (unsorted) fresh or frozen-thawed sperm of the same ejaculate as sorted spermatozoa was used for IVF. The protocol of sperm capacitation was related to the source of spermatozoa. For control sperm, both fresh and frozen-thawed, and for fresh fractions of sexed sperm the standard procedure of capacitation was applied (K_tska et al., 1998: J. Anim. Feed Sci. 7:353-362). Briefly, sperm was separated by Percoll gradient (90%:45%) centrifugation (500 g for 30 min), washed and placed in 40 µl fertilization drops (TALP-IVF medium containing 10 µg heparin/ml and mixture of penicillamine, hypotaurine and epinephrine) at concentration 1 to 1.5 x 10^6 spermatozoa/ml medium. For frozen-thawed fractions of sexed sperm separation in Percoll gradient was omitted; immediately after thawing X and Y samples were centrifuged (500 g for 10 min) and placed in fertilization drops at concentration 2 to 3 x 10^6 spermatozoa/ml TALP-IVF medium. After IVF, the zygotes were cultured for 24 h in medium B_2. The resulting embryos were co-cultured with Vero cells for another 8 days, i.e. up to the hatched blastocyst stage.

Up to now a total number of 1572 in vitro matured oocytes were fertilized with sexed and control sperm of four bulls. The following results were obtained: for fresh, control sperm 46.2% (86/186) and 25.6% (22/86); for frozen-thawed, control sperm 50.2% (118/235) and 16.9% (20/118); for X fresh fractions 24.5% (50/204) and 12.0% (6/50); for Y fresh fractions 23.8% (40/168) and 30.0% (12/40); for X frozen-thawed fractions 27.6% (104/377) and 13.5% (14/104); and for Y frozen-thawed fractions 25.4% (102/402) and 16.7% (17/102), respectively for cleavage rates and blastocyst rates. The preliminary results suggest that there are differences due to bull effect and sperm conservation and no differences in efficiency between X and Y fractions of sperm. The investigations are continuing.

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Notes

EFFECTS OF IN VITRO VS IN VIVO CULTURE ON EXPRESSION OF EMBRYO DERIVED XIAP TRANSCRIPTS IN SINGLE BOVINE EMBRYOS

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Earlier studies reported that the level of apoptosis in *in vitro* produced bovine blastocysts is higher then in *in vivo* developed blastocysts (Gjørret et al., 2003: Biol Reprod 69, 1193-1200). The molecular basis for this difference has not yet been studied. The regulation and execution of apoptosis is depending on a cascade of events in which many proteins are involved. One of the proteins involved is X-chromosome-linked Inhibitor of Apoptosis (XIAP).

The aim of the present study was to analyze expression of XIAP transcripts and determine if inactivation of the X-chromosome (i.e. dosage compensation) occurred to a similar extend in *in vitro* cultured female embryos as *in vivo* at the early blastocysts (eb), blastocysts (b) and expanded blastocysts (xb) stage, respectively. Furthermore, to verify if this transcript detected in the blastocysts stages was newly expressed from the embryonic genome, transcription was inhibited with a RNA polymerase II specific inhibitor (_-amanitin) to the culture medium from the zygote stage onwards. Oocytes were obtained from abattoir ovaries and after IVM/IVF and IVC in SOF medium, eb, b and xb were collected (*In vitro* group). For the *In vivo* group, embryos were collected from normally cyclic cows, superovulated with 3000 IU eCG (Intergonan; Intervet, Tönisvorst, Germany) at day 7 po by non-surgical uterine flushing. The developmental stage of the embryos was determined by stereomicroscopy and eb, b and xb were collected and stored at -80°C. For the transcription inhibition experiment, embryos were cultured *in vitro* as for the *In vitro* group until the 8-16 cell stage (that is until 100 h after the start of fertilization), one group with 10 mM _-amanitin added to the culture medium (_-amanitin group) and one group without (control group). A highly sensitive semi-quantitative RT-PCR assay (Wrenzycki et al., 1999: Mol Reprod Dev 53, 8-18) was used to determine the relative abundance of gene transcripts in single blastocysts and pools of five 8-16 cell embryos. For sex determination of the embryos DNA was collected from the supernatant of the RNA extraction and prepared using Microcon YM-100 (42412; Millipore, Eschborn, Germany) columns according to the manufacturer's instructions. Embryonic sex was determined by PCR analysis using bovine-specific and Y-chromosome-specific primers.

The relative abundance in 8-16 cell embryos cultured with α-amanitin was 0 (n=6) and in the control group without α-amanitin was 1.62 ± 0.2 (n=6).

Table 1. Relative abundance ± sem (number of replicates) of XIAP transcripts in single male and female eb, b and xb produced *in vitro* and *in vivo*. Different superscripts between male and female within the same developmental stage are significantly different (Anova followed by multiple pairwise comparisons using the Tukey test).

	eb		b		xb	
	male	female	male	female	male	female
In vitro	0.22 ± 0.2^{a} (4)	0.16 ± 0.1^{a} (4)	0.60 ± 0.2^{a} (6)	1.72 ± 0.4^{b} (9)	0.53 ± 0.2^{a} (4)	0.38 ± 0.1^{a} (7)
In vivo	0.30 ± 0.2^{A} (9)	0.38 ± 0.2^{A} (8)	0.62 ± 0.1^{A} (5)	1.31 ± 0.3^{B} (6)	0.44 ± 0.2^{A} (8)	1.38 ± 0.4^{B} (6)

In conclusion, XIAP transcripts in blastocysts are of embryonic origin. No dosage compensation was observed at the blastocysts stage, irrespective of the production method. However, no dosage compensation is observed in *in vivo*-produced expanded blastocysts in contrast to *in vitro*-produced expanded blastocysts. Speculatively, the premature inactivation of the X-chromosome in *in vitro*-produced expanded blastocysts could be related to the increased level of apoptosis in *in vitro*-produced expanded blastocysts.

Notes

STRAIN-DEPENDENT DIFFERENCES IN THE SUPEROVULATORY RESPONSE, EMBRYO DEVELOPMENT AND EFFICIENCY OF TRANSGENESIS IN RATS

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The possible effect of the genotype of immature rats on superovulation response, in vitro and in vivo development of embryos and overall efficiency of transgenesis was studied. The protocols for induction of superovulation using a single injection of 15 IU PMSG or minipump with 10 mg FSH followed by the injection of 30 IU hCG were compared in LEW, WKY, SHRSP or SD and Wistar as representatives of inbred or outbred strains, respectively. In the case of in vitro culture of rat zygotes to the blastocyst stage we cultured the ova overnight in M16 medium and than transferred them into mR1ECM medium. Ova were cultured (10-20 embryos per 700 µl of the medium) in 4-well culture dishes (Nunc) under 5% CO_2 in air at 37°C. The comparisons for multigroup and multifactorial analyses were done with a two-way ANOVA and one-way ANOVA on ranks for multiple group comparisons. A value of $P<0.05$ was chosen as an indication of statistical significance.

After hCG administration females were mated with males of the same strain and sacrificed on Day 1 of pregnancy. The percentage of mated animals was similar in all groups (60.0-100%) independent of the animal strain used. The mean number of ova per donor was not dependent on the hormonal treatment used within each rat strain. At the same time SD and Wistar female rats were more effective to hormonal treatments than animals from inbred strains. SHRSP rats produced significantly more ova per female in response to superovulation compared to other inbred strains.

Significantly lower rates of in vitro development in mR1ECM medium was observed for intact zygotes from WKY rats. However, embryos recovered from SD, SD x Wistar and WKY animals at the 2-cell stage showed no difference in competence to reach the blastocyst stage in vitro (77.6-84.6%). The proportion of offspring developed after oviduct transfer of intact zygotes derived from superovulated donors was slightly lower for WKY rats.

SD and SHRSP zygotes survived after microinjection significantly better than the zygotes from WKY and Lewis rats (~75% v.s. ~55%). Lewis and SD animals showed significantly higher rates of development to term of microinjected zygotes after transfer to recipients compared to the other strains. At the same time we found no significant differences in efficiency of transgene integration per newborn (5.3-22.2%) or per injected zygotes (0.9-2.1%) produced from studied rat strains.

The results of this study demonstrate strain-dependent differences in the response to superovulation protocols, in the sensitivity to the microinjection and in the capability of zygotes to develop in vitro until morula and blastocyst stages or in vivo to term after transfer to the foster mothers. However, despite these genotype-dependent differences all studied strains can be successfully used for transgenic rat production.

Notes

EVALUATION OF THE FUNCTIONAL STATUS OF BOVINE OOCYTES AND EMBRYOS CULTURED IN VITRO

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In order to increase the quality of in vitro derived and cloned bovine embryos, the basic fundamentals of in vitro cytoplasmic maturation of donor oocytes needs to be completely understood. The precise roles of calcium and some co-enzymes, such as NADH and FAD, which participate in oxidizing and reducing reactions within the developing oocyte needs to be determined as well as its role during IVF and IVC. The features of calcium and NADH/FAD metabolism in normal and degenerative oocytes and embryos need to be understood. On other hand, when effective methods are found to evaluate quality of donor oocytes before and after IVM, the success to obtaining viable embryos will increase dramatically. The aim of this study was to evaluate the functional status of bovine oocytes, zygotes and 2-cell embryos cultured in vitro. A morphological classification (cumulus, ooplasm, blastomeres, chromatin status) was used for in vitro matured bovine oocytes, zygotes and 2 cells embryos to determine: 1) density of fluorescence NADH, FAD and the ratio NADH/FAD (Excitation - 365 nm for NADH and 436 nm for FAD, Emission- 465 nm for NADH and 530nm for FAD), and 2) concentration of the calcium of intracellular stores (fluorescence of complex membrane-bound calcium-chlortetracycline was fixed with a fluorescent microscope – Excitation: 390 nm, Emission: 530nm, using 40μM chlortetracycline). The results were expressed in arbitrary units of fluorescence. A total of 378 normal (N) and degenerative (D) oocytes, zygotes and embryos were evaluated. Oocytes were cultured for 24 h at 38.5° C under 5% CO_2 in TCM 199 with 10% FCS and 10 ng/ml recombinant bovine somatotropin (rbGH-"Monsanto"). After IVM, oocytes were fertilized using frozen bovine sperm and zygotes were cultured in TCM 199 with 20% FCS and 10 ng/ml rbGH. Data were analysed using the Student's test. Inhibition of $^{2+}$ exit from intracellular stores was detected in degenerative oocytes at metaphase-II (1.95±0.12-D compared to 1.47±0.10 – N, $P<0.01$) and 2-cell embryos (3.47±0.40-D compared to 1.78±0.14 – N, $P<0.001$). The intensity of a fluorescence NADH also increased in degenerative oocytes at metaphase-II (1.58±0.10-D compared to 1.20±0.12 – N, $P<0.05$) and in 2-cell embryos (1.48±0.25-D compared to 1.18±0.05 – N). We did not find an influence of degeneration on the intensity of NADH fluorescence and $^{2+}$ exit from intracellular stores in zygotes. These data demonstrate that in cattle: 1) NADH concentration and calcium from intracellular stores in oocytes and 2-cell embryos are interconnected; 2) NADH concentration and calcium stores are related to morphological quality and chromatin status in oocytes and 2-cell embryos. The results suggest that these parameters (NADH concentration and the intracytoplasmic calcium stores) may be considered in further optimization of the conditions for oocyte and embryonic development in vitro.

Notes

EFFECT OF TRICHOSTATIN A (TSA) TREATMENT OF DONOR CELLS ON DEVELOPMENT OF BOVINE NUCLEAR TRANSFER (NT) EMBRYOS

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Nuclear reprogramming is the process by which a differentiated nucleus returns to a totipotent stage. It is possible that in somatic cell cloning, donor cells with less epigenetic modifications - lower levels of methylated DNA and/or higher levels of histone acetylation - may be better reprogrammed. To investigate the effect of histone acetylation of the donor nucleus on NT-embryo development we treated adult bovine fibroblasts with trichostatin A (TSA) – a potent histone deacetylase inhibitor - during serum starvation (0.2 mM TSA) or in complete medium (0.08 mM TSA) 24 h prior to nuclear transfer. NT-embryos were constructed by the zona-free method (Oback et al. 2003). Cell couplets were fused by a single DC-pulse of 1.2 Kv/cm applied for 30 μsec. NT-embryos were activated 1.5 to 3.5 h after fusion with 5 μM ionomycin for 4 min followed by 4 h culture in 2 mM 6-DMAP in m-SOF. Zona-free NT-embryos were cultured individually in 3 μl drops under mineral oil at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2. The rates of cleavage, morula compaction D6 (MCD6) and blastocyst formation D7 (BLD7) were recorded. The data were compared by Chi-square test.

The results of the preliminary experiments showed that TSA-treatment of donor cells during serum starvation did not affect the developmental ability of NT-embryos to the stage of morula compaction but significantly decreased the rate of BLD7 (28.7 vs 42.1%, $P<0.05$).

In the second series of experiments we evaluated the development of NT-embryos derived from TSA (complete media)-treated fibroblasts using two different time between fusion and activation that is considered to be the time of active remodelling and reprogramming of the donor nucleus (Table 1).

Table 1. Effect of TSA-treatment in relation to different fusion-activation timing.

Group	Time *, h	N	Cleavage(%)	MCD6 (%)	BLD7 (%)
control	2 - 2.5	69	61 (88.4)	24 (34.8) a	14 (20.3) c
control	3 - 3.5	121	118 (97.5)	58 (47.9) ab	41 (33.9) d
TSA	1.5 - 2	83	78 (94)	43 (51.8) b	29 (34.9) d
TSA	3 - 3.5	112	107 (95.5)	57 (50.9) b	42 (37.5) d

Chi-square test, $P<0.05$. Time* : time between fusion and activation.

Our results indicate that TSA-treatment of donor cells allows NT-embryos to undergo reprogramming faster than in control (TSA 1.5-2 h vs control 3-3.5 h).

In conclusion we demonstrate that there is a significant decrease of blastocyst rate after TSA-treatment of donor cells during serum starvation but not during normal culture. In addition we show that TSA treated donor cell nuclei acquire the ability to be reprogrammed faster. This work was supported by FIRB.

Notes

THE EFFECT OF NEGATIVE ENERGY BALANCE ASSOCIATED NON-ESTERIFIED FATTY ACID LEVELS DURING IN VITRO MATURATION OF BOVINE OOCYTES ON EMBRYO PRODUCTION

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Dairy cow fertility has declined drastically during the last decades. This subfertility is a multifactorial problem. The negative energy balance (NEB) in high-yielding dairy cattle early post partum may affect ovarian function and oocyte quality through elevated levels of non-esterified fatty acids (NEFA) in serum as well as in follicular fluid (FF). The composition of the FF in dairy cows during a period of NEB has been studied thoroughly in our laboratory. Due to increased lipolysis, the NEFA levels in FF also rose but remained significantly lower (± 40%) compared to serum concentrations and never exceeded 0.6 mmol/l. Also the qualitative composition of the NEFA fraction differed significantly between serum and FF for C18:1 (oleic acid: 27.8±2.6% vs. 33.1±1.46%), C18:0 (stearic acid: 25.6±1.7% vs. 12.7±0.8%), C16:0 (palmitic acid: 20.0±1.0% vs. 23.0±0.6%) and C18:2 (linoleic acid: 3.4±0.4% vs. 7.4±0.7%) ($P < 0.05$). The aim of this study was to investigate in an in vitro maturation model whether elevated NEFA levels during in vitro maturation are detrimental to the fertilisation rate, cleavage and subsequent blastocyst formation. The preliminary results are presented.

A total of 3429 oocytes were matured in serum free maturation medium (TCM 199, 20 ng/ml mEGF). According to treatment, palimitic acid (PA), stearic acid (SA) or oleic acid (OA) in ethanol were added at final test concentrations of 0.133, 0.067 and 0.200 mM respectively corresponding to 23%, 13% and 33% of a total NEFA concentration of 0.6 mmol/l (cfr. supra). In contrast to the negative control groups (no ethanol or NEFA), positive controls received equal volumes of ethanol (but no NEFA) as the test groups. Oocytes were matured per 60 for 22-24 hours at 38.5°C and 5% CO_2. After maturation, all groups were coincubated with $1x10^6$ sperm/ml for 18-20 hours. Half of the presumptive zygotes was fixed and stained with Hoechst to determine fertilisation rate while the other half was cultured per 25 for 7 days in 50 _l drops of SOF medium with 5% FCS under mineral oil (5% CO_2, 5% O_2 and 90% N_2). At respectively 48 h and 8 days post insemination, the number of cleaved zygotes and the number of blastocyst were counted. Preliminary data were analysed with a two-way ANOVA in which "replicate" was inserted as random effect. Results are expressed as mean % (±SD) of total number of oocytes.

No significant effect was observed for ethanol or OA on the fertilisation rate, cleavage or blastocyst development. The presence of 67 _M SA during oocyte maturation, however, resulted in significant lower fertilisation rates (64±3.1% vs. 74±2.1% in positive control), cleavage (59±7.4% vs. 77±6.0%) and blastocyst formation (21±10.1% vs. 35±5.2%) ($P < 0.05$). Preliminary results with PA also suggest a negative effect of this fatty acid on the three parameters studied but additional replicates are needed for full confirmation.

Based on these results we can suggest that high levels of NEFA during a period of NEB may influence fertility in high-yielding dairy cows by hampering oocyte maturation as expressed in lower fertilisation rates and subsequent lower cleavage and blastocyst development.

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Notes

THE INFLUENCE OF THE FOLLICLE ON EMBRYONIC/FETAL MORTALITY IN CATTLE

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Impaired reproductive performance is a major problem in dairy cattle, e.g. high embryonic/fetal mortality leading to low pregnancy rates. These problems can originate in the ovulating follicle itself, as it is responsible for providing the adequate environment for oocyte development and is the basis for subsequent formation of the corpus luteum. Previous studies have demonstrated that pregnancy rates were influenced by follicular size at ovulation (e.g. Vasconcelos et al. 1999, Theriogenology 52:1067-1078), but the conclusions have been inconsistent. Heat stress, considered a major cause of low fertility worldwide, is also likely to contribute to the high embryonic/fetal mortality.

In our present study, the ovulatory follicle was described in terms of size and function (plasma oestradiol), as well as its basis for subsequent CL formation and function (plasma progesterone). The effect of these parameters was related to the subsequent pregnancy rates on day 28 and on day 63 after AI. Finally, an effect of environment (season) on these parameters was investigated.

In 2003, lactating cows at the New York Station were studied during December-May (cold season, n=73) or July-September (warm season, n=71), all starting approx. 80 days after calving. Ovulation time was synchronized and one fixed time AI was used (= day -1), and on this day the diameter of the largest follicle (only follicles from 8 to 20 mm were considered as ovulatory) was measured by ultrasound. Blood samples were collected daily from day –1 until day +7, and then on days 14, 21, 28, 35 and 60. Plasma concentrations of progesterone (P4) and oestradiol (E2) were quantified by RIA. Pregnancy diagnosis was determined on day 28 and 63 using ultrasound, and on day 42 using rectal palpation. Data were analysed statistically using F tests for comparison of means and a mixed linear model (Mixed Procedure, SAS Institute Inc.) for the full data analysis.

Overall, 48 of the 144 cows (33%) were pregnant on day 28; of these, 11 (24%) aborted before day 63. None of these rates differed between seasons. The average diameter of the ovulatory follicle was larger in cows which became pregnant than non-pregnant cows on day 28 (15.9±2.4 vs 14.3±3.6 mm, n=48 and 96, respectively; $p<0.001$), but not for the cows aborting between day 28 and 63. No difference was observed between seasons. Follicular function measured as E2 in plasma on day of AI was higher in cows pregnant on day 28 than in non-pregnant cows, but only in the cold season. For non-pregnant cows, the E2 value was highest in the cold season. When comparing cows still pregnant on day 63 with cows aborting between day 28 and 63, the E2 value was higher for the first group, but only in the cold season. The P4 values increased gradually after AI, reaching a plateau from around day 14 in cows still pregnant on day 63. For those cows aborting between day 28 and 63, the P4 started to be lower already from day 7. In both groups, the levels were lower in the warm season. None of these patterns were related to diameter of the ovulatory follicle.

The results illustrate that, in this group of cows, no predictive information regarding embryo survival is obtained from simply measuring diameters of ovulatory follicles. On the other hand, other measures of follicular function (e.g. hormonal profiles) seem to contain predictive information, possibly because they also reflect oocyte quality. Furthermore, the CL function (P4 levels throughout pregnancy) is associated with embryonic mortality already from maternal recognition of pregnancy. The effect of season during the year 2003 did not result in differences; however, the difference in E2 and P4 levels observed between seasons could indicate that follicular and luteal steroidogenesis can be disturbed during warm season.

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Notes

MATURATION OF BOVINE OOCYTES DEFINED BY FOLLICLE SIZE AND THE PHASE OF FOLLICULAR WAVE IN VITRO : EFFECT OF GONADOTROPINS OR EPIDERMAL GROWTH FACTOR ON EMBRYO DEVELOPMENT

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In a previously study we have documented a relationship between the developmental competence of oocytes collected from different-sized follicles in the growth or the dominant phase of follicular wave and embryo production (Machatkova et al., Theriogenology 2004; 61:329-335). The aim of the present study was to compare the effect of gonadotropins or epidermal growth factor (EGF) during maturation of oocytes defined by follicle size and the phase of folicular wave on embryo development. A total of 3476 oocytes collected from small (2-5 mm) or medium follicles (6-10 mm) in the growth/stagnation or the dominance/regression phase of follicular wave were recovered from slaughtered cows. Subsequently, the ovaries of each donor were characterized in terms of morphology. Oocytes were matured in TCM-199 supplemented with 5% oestrus cow serum (ECS) and either FSH/LH (P.G.600, 15 iu/mL) or EGF (50µ /mL). They were fertilized and cultured by standard methods. The developmental competence of oocytes was expressed as the percentages of D7 and D8 embryos developed from fertilized oocytes, and as the percentage of advanced or expanded blastocysts from D7 or D8 embryos. The data was analyzed by the Chi-square test. Both the development rate of oocytes into embryos and the percentage of advanced or expanded blastocysts from embryos were higher for the oocytes collected from small follicles in the growth/stagnation phase and matured in medium with FSH/LH than for those matured in medium with EGF (33.4 vs 27.0 %, $P < 0.05$, and 32.6 vs 25.0 %, $P < 0.01$ for D7 and D8 embryos; 38.4 vs 30.8 % and 69.5 vs 62.6 % for advanced or expanded blastocysts). On the other hand, the oocytes collected from medium follicles in the growth/stagnation phase showed both the highest development rate of oocytes into embryos and the highest percentage of advanced or expanded blastocysts when matured in the presence of EGF as compared with FSH/LH (49.8 vs 47.1 % and 40.0 vs 38.7 % for D7 and D 8 embryos; 54.8 vs 37.9 %, $P < 0.01$ or 77.0 vs 65.4 %, $P < 0.05$ for advanced or expanded blastocysts). When oocytes were collected from small or medium follicles in the dominance/regression phase and matured in medium with either FSH/LH or EGF, the development rates of oocytes into embryos were similar. But in oocytes collected from medium follicles and matured in the presence of EGF, the percentage of advanced blastocysts from D7 embryos was significantly higher than in those matured in the presence FSH/LH (43.3 vs 28.4 %, $P < 0.05$). In conclusion, the expression of developmental competence of oocytes was influenced by the presence of gonadotropins or EGF in maturation medium. In the growth/stagnation phase, oocytes from small follicles, with lesser developmental competence, prefered FSH/LH to EGF but oocytes from medium follicles, with greater developmental competence, prefered EGF to FSH/LH during maturation. In the dominance/regression phase, the two oocyte subpopulations tolerated both FSH/LH and EGF but in oocytes from medium follicles blastocyst formation was accelerated in the presence of EGF compared with FSH/LH as it was in the growth/stagnation phase.

Notes

IMPROVING THE MODE OF FSH APPLICATION FOR SUPEROVULATION TREATMENTS: CLINICAL DATA AND ENDOCRINOLOGY

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Internationally, the use of Follicle-Stimulating Hormone (FSH) to induce superovulation is favoured in a regime which uses eight injections over four days. However, this conventional regime of treatment has some disadvantages: a) Multiple applications given intra-muscularly every 12 hours entail a lot of work for the person who is performing the treatment and b) it stresses the animal to be treated. Therefore, a reduction of injection frequency would have obvious benefits. The number of studies published is, however, low and the detected results are inconsistent.

The aim of our studies was to optimise both the dose and frequency of FSH administration using two commercial preparations (Pluset® and Folltropin®). As a result of the first joint study performed by the Institutes named above, the results were evaluated which were found after the treatment of a total of 810 Fleckvieh donors of the Bavarian breed according to various treatment protocols (Fig. 1).

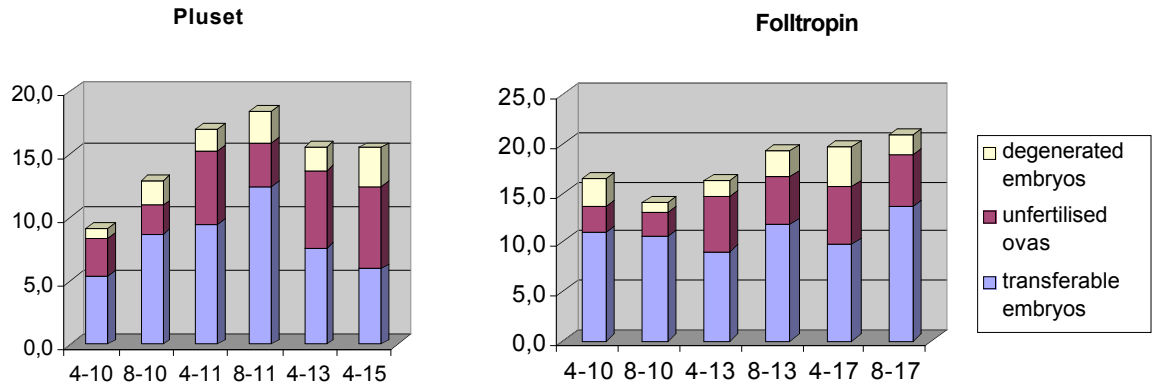
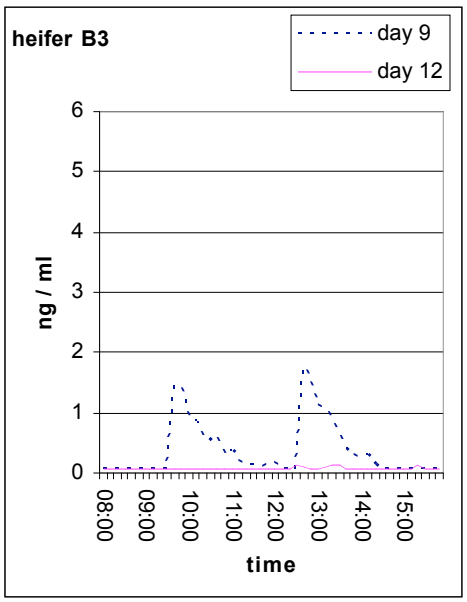


Figure 1. Results of embryo collection depending upon the type of FSH preparation (Pluset® or Folltropin®), the frequency of injection (left number below columns), and dose (ml; right number)



With the aim of characterizing plasma concentrations of reproductive hormones, using three different variants of the Pluset® regime, a total of 12 heifers were bled throughout 8 hours on days 9 and 12 (before and during stimulation by FSH), respectively. The samples were analysed for steroid hormones, LH (Fig. 2 Example), and porcine FSH which had been administrated.

Figure 2. LH concentrations (ng/ml) on experimental days 9 and 12 (beginning of FSH administration: day 10). Sampling was performed every 10 min through 8 h.

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Notes

PARENTERAL _-CAROTENE ADMINSTRATION ON BOVINE EMBRYO DONORS. A PRELIMINARY APPROACH

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The number of viable embryos produced after each superovulation treatment (SOV) and variability of response to exogenous gonadotropins are limiting factors to the largescale use of ET on farms. Despite hormonal methods, micronutrients have been considerated fundamental for reproduction. The aim of this study was to examine the effect of i.m. injection of _-carotene on the yield of viable embryos. 32 adult Holstein cows of top genetic herds were selected for SOV. All received the same SOV protocol with intra vaginal progesterone device (PRID) plus 5 mg oestradiol benzoate, plus 100 mg progesterone on Day 0 without oestrus control and after gynaecological exploration to eliminate cows with any disorders. 16 cows received 600 mg of _-carotene (DALMAVITAL, Fatro-Uriach) 15 days before PRID administration. The same batch of FSH (PLUSET, Calier) was used to treat animals on Day 4 after inserting PRID in a decreasing dose regime during 4 days. Two doses of cloprostenol (Dalmazin, Fatro-Uriach) were administered on Day 3 and PRID was removed with the second prostaglandin dose. Artificial insemination was performed two times, 48 and 60 hours after PRID removal. Embryos were recovered 7 days post-insemination and evaluated according to IETS standards. Data were analysed with GLM (General Linear Model) of one factor. Results are shown in the table below.

Table 1. Mean±standard deviation of total embryos, viable embryos, degenerated embryos and ova in function of _-carotene administration.

GROUP	n	TOTAL EMBRYOS	VIABLE EMBRYOS	DEGENERATED EMBRYOS	OVA
Control	16	9.75±8.43	4.56±4.88	1.75±2.26	3.44±5.45
_-carotene	16	14.25±10.80	6.50±8.40	1.94±2.20	5.81±5.94

The total embryos and viable embryos were higher in the _-carotene group although the difference was not significant owing to large variation in data. The number of corpora lutea evaluated by hand on day of recovery did not differ significantly between groups (12.18±6.22 control vs 13.78±5.17 _-carotene treated). Studying treated cows, 8 of 16 had prior recovery data in our records and comparing results from before treatment and after we find an average of viable embryos of 6.4 vs 10.8 after treatment. In addition, the control group reached the average viable embryo production reported for our team in the last two years of over 290 SOV. If we look at the quality of the embryos produced we find no difference although there was a higher number of quality 1 embryos in the treated group vs control 95/106 (89.6%) vs 64/73 (87.7%) while quality 2 embryos are higher in control group 9/73 (12,30%) vs 9/106 (8,5%) in treated cows. Evaluating all data altogether, results suggest that parenteral administration of _-carotene 30 days before embryo recovery may have some beneficial effects on viable embryo production although statistical difference could not be demonstrated in the present study. Further, more investigation is needed to determinate the effect of this treatment and the best approach and protocol of administration to enhance embryo production.

Notes

EFFECT OF CYSTEAMINE DURING IN VITRO MATURATION OF SLAUGHTERHOUSE DERIVED AND OPU DERIVED BOVINE OOCYTES ON FURTHER EMBRYONIC DEVELOPMENT

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Glutathione (GSH) plays an important protective role in relation to reactive oxygen species generated by normal oxidative metabolism in the cell. The presence of cysteamine during in vitro maturation may facilitate the synthesis of GSH by immature oocytes. In a previous study we showed a positive effect of the presence of cysteamine during in vitro maturation of slaughterhouse derived bovine oocytes on subsequent in vitro embryonic development (Merton et al., 2004, Reprod. Fert. Dev. 16: 279). This report shows the first results of an ongoing field trial with OPU derived oocytes, in order to confirm our previous results obtained with slaughterhouse derived oocytes .

Immature cumulus-oocyte-complexes (COCs) were recovered from ovaries 6 to 8 h upon slaughter (SL) or obtained twice weekly by ultrasound guided transvaginal oocyte collection (OPU) at two collection centres. Both sources of COCs were matured in vitro in TCM199/FCS/LH/FSH supplemented either with or without cysteamine (0.1 mM). Subsequently, matured oocytes were fertilised with frozen-thawed gradient-separated semen and further cultured for 7 days in SOFaaBSA. In case of OPU derived COCs, the experimental design was a 2X2 factorial. Results were analysed by Chi-square analyses.

The results show that the presence of cysteamine during in vitro maturation significantly affected embryo production from OPU derived COCs (31.8% and 39.2% Morula + Blastocyst rate at Day 7 for control and cysteamine, respectively). This higher embryo production rate was totally due to an increased number of Blastocysts. Also the proportion of grade 3 embryos (IETS) was significantly reduced in the cysteamine group. The number of transferable embryos (Morula grade 1 and early-, exp-Blast grade 1 and 2) per session was 1.38 and 2.07 (50 % relative increase) for control and cysteamine, respectively.

Table 1. Effect of cysteamine during in vitro maturation of slaughterhouse- and OPU-derived bovine oocytes on subsequent in vitro embryonic development.

Oocytes	Group	Repl./sess. n	Oocytes n	Cleavage n (%)	Embryo production Day 7		
					Mor+ Blast n (%)	Relative increase	Transferable n (per session)
Sl.*	Control	5	1056	586 (55.5)	205 (19.4)[a]		
	Cyst.	5	1070	634 (59.3)	257 (24.0)[b]	23.7 %	
OPU	Control	181	1365	908 (66.5)	434 (31.8)[a]		249 (1.38)[a]
	Cyst.	167	1259	881 (70,0)	493 (39.2)[b]	23.3 %	346 (2.07)[b]

[a,b]: For each source of oocytes, values in columns with different superscript are significantly different, $P<0.05$.
* data adapted from Merton et al., 2004, Reprod. Fert. Dev. 16, 279 (abstract).

These results show that the presence of cysteamine during in vitro maturation also affects further in vitro embryonic development (both embryo quality and developmental speed) of OPU derived COCs, resulting in a higher embryo production rate. The relative increase in the total Morula and Blastocyst rate at Day 7 seems to be equal for both slaughterhouse- and OPU-derived oocytes. This field trial will be continued in order to investigate the effect of cysteamine on pregnancy rate and offspring born.

Notes

ALTERATIONS AND REVERSIBILITY IN THE CHROMATIN AND CYTOSKELETON OF CALF OOCYTES TREATED WITH ROSCOVITINE

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Improvement of the ability to maintain germinal vesicle (GV) stage oocytes in vitro is important for the acquisition of developmental competence. Maintaining oocytes at this stage without damaging their quality would synchronize meiotic resumption and would allow all collected oocytes to complete nuclear maturation in order to maximize embryo production. GV breakdown in mammalian oocytes is regulated by activation of maturation promoting factor (MPF). Roscovitine (ROS), a specific cdc2 kinase inhibitor, has been shown to reversibly inhibit meiotic resumption in cattle oocytes for 24 h without having a negative affect on subsequent development to blastocyst stage. Oocytes from calf oocytes are less competent to develop than those from cow ovaries. Although fertilization and cleavage rates do not differ greatly between calf and cow oocytes, blastocyst yields are significantly reduced with calf oocytes. This lack of developmental competence may be due to the failure or inability of the calf oocytes to complete cytoplasmic maturation. It is hypothesized that if oocytes can be cultured in vitro under conditions that maintain meiotic arrest at the GV stage, then they may have the opportunity to acquire greater developmental competence. The aims of the present study were to study the efficacy of ROS to maintain calf oocytes at the GV stage and to describe changes occurring in the distribution of chromosomes, microtubules and actin microfilaments following exposure to different concentrations of ROS.

Cumulus-oocyte complexes (COCs) were aspirated from slaughterhouse calf ovaries and cultured for 24 h in TCM199 containing different levels of ROS (0, 12.5, 25, 50 and 100 μM). They were either fixed immediately or cultured for a further 24 h in conditions permissive to maturation and subsequently fixed and processed using specific fluorescent probes in order to evaluate chromosome, microtubule and microfilament distributions by visualization in a confocal microscope.

COCs cultured with any of the concentrations of ROS were significantly blocked at the GV stage. The inhibitory effect varied according to the dose, with 50 μM ROS being the most efficient, with 83.5 % oocytes being blocked in the GV stage when compared to 0 μM (3.3%). When oocytes were released from the inhibitor, similar proportions of oocytes (74.5 and 75.5%) were observed in the metaphase II stage for 0 and 50μM, respectively. The meiotic spindle exhibited a typical MII morphology in 62.7% and 62.5% of oocytes for 0 and 50μM ROS, respectively. A normal distribution of actin filaments was observed in 93.2% and 85% of oocytes exposed to 0 and 50 μM ROS, respectively.

This study showed that 50 μM roscovitine-treated oocytes resumed meiosis after removal of the inhibitor and no significant influence of the inhibitor was detected on chromosome, microtubule and microfilament distributions during roscovitine treatment (50 μM).

Notes

EFFICIENT GENERATION OF TRANGENIC MICE WITH INTACT YEAST ARTIFICIAL CHROMOSOMES BY ICSI

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The production of animals with large transgenes is an increasingly valuable tool for genetic studies, including the characterization and manipulation of large genes and polygenic traits. Intracytoplasmic sperm injection (ICSI)-mediated transgenesis has been suggested to be a valuable tool to generate complete integration and phenotypic expression of large DNA constructs; however, such capability was never clearly shown. By co-injecting spermatozoa and Yeast Artificial Chromosomes (YACs), which are capable of mobilizing genomic fragments of several megabases in length, into metaphase II (MII) oocytes, we were able to produce founders exhibiting germline transmission of an intact and functional transgene of submegabase magnitude. More than 35 % transgenicity was obtained for a YAC transgene (YRT2_LCR) of 250 kb. When compared with the efficiency (1%) of YAC transgenesis by pronuclear microinjection (PI), the efficiency of ICSI-Mediated YAC Transfer (IMYT) was significantly greater, and no different than that obtained after ICSI-Mediated Transfer of EGFP (45%), a construct 50 times smaller than YRT2_LCR. All 3 IMYT founders crossed with non-transgenic mice, transmitted transgene DNA to progeny following a Mendelian pattern. Besides the huge difference in construct size, this rate of YRT2_LCR germline transmission is comparable to that previously reported for EGFP by an analogous method, attesting the stability of the YAC transgene integrants. The efficiency of the applied ICSI methodology in generating transgenics for large DNA constructs was verified when, of the IMYT-derived transgenic offspring, 1/12 (8%) harboured an intact copy of the 250 kb YAC. This was genomically confirmed by Southern Blot Analysis of three transgene markers, and functionally, by a co-inherited pigmented coat color characteristic of YRT2_LCR expression, which was able to rescue the albino background of the host. These data clearly demonstrate the potential of IMYT to generate transgenics. In addition, to our knowledge, this is the first report of stable incorporation and phenotypic expression of large DNA constructs of submegabase magnitude mediated by ICSI.

Table 1. Embryo Cleavage and Transgenesis obtained with 5.4 kb (standard plasmid) and 250 kb (YAC) constructs

Technique Employed	Construct (Size in kb)	Injected Oocytes (No. Sessions)	Embryos Transferred (No. Recipients)	Live Animals (%)	Live Transgenic Animals (%)
ICSI	EGFP (5.4)	219 (6)	163 (8)	22 (13)	10 (45)
IMYT	YRT2_LCR(250)	367 (6)	218 (13)	34 (16)	12 (35)
PI	YRT2_LCR(250)	1733 (12)	959 (44)	93 (10)	1 (1)

The integrity of genomic integrants was evaluated by PCR and Southern blot analysis for YRT2_LCR, and by PCR for EGFP.

Notes

CORRELATION BETWEEN THE NUMBER OF CELLS AND THE DIAMETER OF EQUINE EMBRYOS BETWEEN 6.5 AND 8 DAYS

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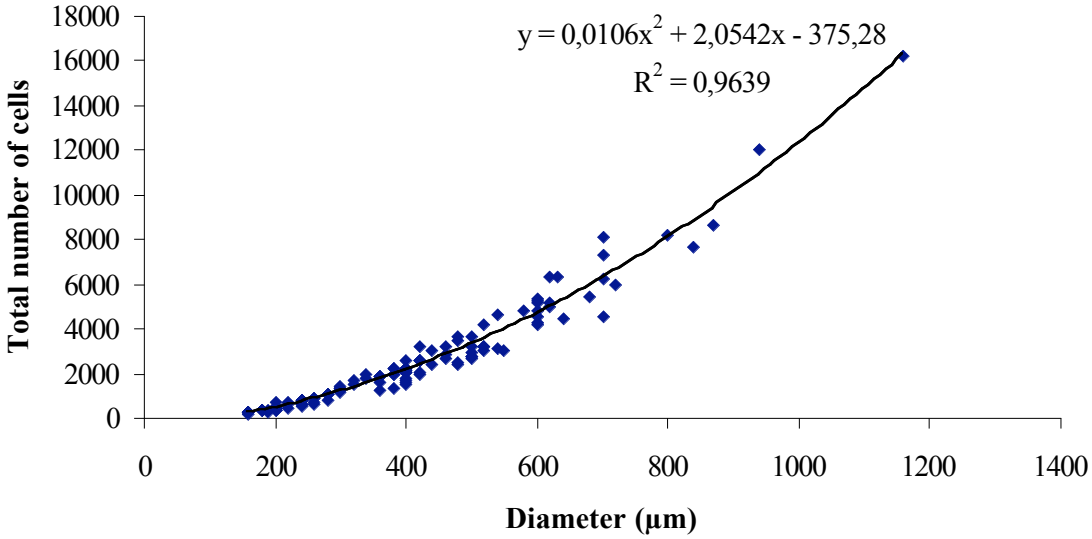
The present study was conducted to examine the correlation between the cell number and diameter of 124 equine embryos collected between 6.5 and 8 days after ovulation. The total number of cells was evaluated by counting DAPI-stained nuclei. After fixation in 4% paraformaldehyde, embryos were incubated in PBS with 1µg/ml DAPI (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. The total number of cells (DAPI labelled) were determined using a counting grid mounted on the eyepiece. Each embryo was placed on a microscope slide and flattened with a cover slip. The correlation between the total number of cells and the diameter were analysed using test correlation coefficient. The diameter of embryos varied from 160 – 260 µm, 160 – 840 µm and 380 - 1640 for 6.5, 7 and 8-day old embryos, respectively. The total number of cells varied between 226 – 726, 265 – 7652 and 2251 – 20322 cells per embryos for embryos aged 6.5, 7 and 8-day old embryos, respectively. One embryo (1640 – 20322) was consideried an outlier and removed from analysis.



The overall trend indicated that the total number of cells and embryo diameter are highly correlated. The correlation coefficient between the total number of cells and the diameter was ($r = 0.98$; $P < 0.001$). There are striking differences in cell numbers between 7 and 8 days old embryos. The cell number in 8-day old embryos was three times higher in comparison with embryos collected 7 days after ovulation. In conclusion, the present study demonstrates that the cell number of equine embryos is correlated with the embryonic diameter and provides a formula to calculate the number of cells based on embryo diameter (µm) ($y = 0.0106x^2 + 2.0542x - 375.28$).

Notes

COMPARISON OF THE VIABILITY OF EQUINE EMBRYOS USING TWO TRANSPORT SYSTEMS : EFFECT OF EMBRYO AGE ON VIABILITY AFTER COLD STORAGE

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The ability to store equine embryos and transport them to other locations provides many benefits. The aim of this study was to compare the viability of 7 and 8-day embryos after 6 h and 24 h cold-storage using two storage systems. Equine embryos (n=100) were recovered on day 7 and 8 after ovulation using 1.5 L warm embryo flushing solution with albumin (0.1% w/v) and kanamycin sulphate (25 mg/L) (Emcare Flushing Solution, ICP, Auckland, NZ). Embryo score ranged between 1 to 4 and diameter ranged from 200 µm to 1700 µm. Embryos were assigned to 10 treatment groups (n=10/group). Embryos recovered on day 7 (n=10) and 8 (n=10) were evaluated immediately after collection (Group-0 h) or stored for 6 h in a 500 ml of Emcare Flushing Solution (EFS) (Group-B-6 h) in a refrigerator at 5°C; for 24h in a 500 ml of EFS (Group-B-24 h) in a refrigerator at 5°C; in 5ml Emcare Holding Solution (EHS) (Group-E-24 h) in an Equitainer (Hamilton-Thorn, S. Hamilton, MA); or in 5ml Ham's F-10 (Group-H-24 h) in an Equitainer. After storage, embryos were washed in EHS then incubated in EHS with 1 µg/ml DAPI (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. Embryos were fixed in 4% paraformaldehyde and maintained at 4°C. To determine the total number of cells, embryos were re-stained with DAPI 0.1 µg/ml in PBS. The percentage of dead cells (DAPI positive, fluorescent cells) per embryo was determined using an inverted fluorescence microscope. Differences between groups were analysed using Kruskal Wallis test (significance at $P \leq 0.05$). The mean diameter of embryos for all groups and the mean (± SEM) percentage of fluorescent dead cells per embryo are summarised in Table 1.

Table 1. Mean embryo diameter and percentage dead cells per embryo.

	Diameter of embryos		Percentage of dead cells	
	Day 7	Day 8	Day 7	Day 8
Group-0 h	387 ± 56[a]	940 ± 79 [b]	0.4 ± 0.3[c]	0.6 ± 0.4[c]
Group-B-6 h	388 ± 33 [a]	966 ± 92 [b]	0.3 ± 0.1 [c]	0.7 ± 0.3 [c]
Group-B-24 h	374 ± 38 [a]	863 ± 130 [b]	4.2 ± 1.3[d]	5.1 ± 1.2[d]
Group-E-24 h	418 ± 41 [a]	1018 ± 124 [b]	1.6 ± 0.6[e]	1.7 ± 0.7 [e]
Group-H-24 h	390 ± 43 [a]	1080 ± 83 [b]	1.7 ± 0.6 [e]	1.8 ± 0.4 [e]

[a, b, c, d, e] Different superscripts indicate significant difference ($P \leq 0.05$).

The percentage of dead cells in Group-0 h and B-6 h was similar and was significantly lower than for embryos stored for 24 h in Groups B-24 h, E-24 h and H-24 h. The percentage of dead cells was similar for Groups E-24 h and H-24 h, but was significantly lower than for embryos stored in a 500 ml EFS at 5°C for 24 h. Within each storage system (0 h, B-6 h, B-24 h, E-24 h and H-24 h) no significant difference in the percentage of dead cells between embryos collected on Day 7 and 8 was observed. These results suggest that there is no significant difference in storage capacity between 7 and 8-day old embryos. Storage in 500 ml of EFS at 5°C for 6 h resulted in embryos of better quality then when using the Equitainer system (E-24 h and H-24 h) and this appears to offer a good alternative for short term storage.

Notes

COMPARISON OF TWO METHODS FOR EQUINE EMBRYOS CRYOPRESERVATION : SLOW FREEZING AND OPS VITRIFICATION

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Slow freezing methods have been applied with relatively low success to equine embryo transfer programs. Recently, an ultra rapid vitrification (Open Pulled Straw method) has been developed and provided encouraging results with embryos from other species. The aim of this study was to compare the survival of embryos after OPS vitrification and slow freezing.

Embryos aged 6.5 days after ovulation were frozen as reported by Lagneaux et al. (J. Reprod. Fert., Suppl 2000; 56 : 561-568). Embryos (n=19) were placed in F1 (PBS + 0.4% BSA) + 100 mM glutamine with increasing glycerol concentration (2.5, 5, 7.5 and 10% (v/v) 5 min each). At thawing, cryoprotectant was removed by 5 steps with decreasing glycerol and sucrose concentrations. Embryos (n=20) were vitrified using the OPS method as described by Berthelot et al. (Reprod Nutr Dev 2001; 41: 267–72). Embryos were equilibrated in TCM199 hepes + 20% NBCS (newborn calf serum) with 7.5% DMSO + 7.5% ethylene glycol (EG) for 3 min and with 18% DMSO + 18% EG + 0.4 M sucrose for 1 min, loaded in OPS and plunged into lN_2. At thawing, embryos were transferred in TCM199-NBCS containing decreasing sucrose concentrations. Embryos were cultured in SOF medium during 3 h and evaluated using DAPI staining. The percentage of cells entering in S-phase was evaluated by incorporation of BrdU. Results were analysed using Kruskal Wallis test.

There were no significant differences ($P > 0.05$) in the mean diameter (175 ± 7 *vs* 178 ± 6), morphological grade (2.3 ± 0.2 *vs* 2.7 ± 0.3) and the percentage of degenerated embryos after 3h of culture (42% *vs* 45%) respectively for slow freezing and OPS methods. The percentage (±SEM) of fluorescent dead cells per embryo for all embryos was 11 ± 2 *vs* 20 ± 6. The percentage of cells with BrdU incorporated into newly synthesised DNA strands did not differ significantly between the two procedures (40 ± 4 *vs* 34 ± 5).

In conclusion, OPS vitrification may be as efficient as slow freezing for the cryopreservation of equine embryos. However, these results should be confirmed by the transfer of OPS vitrified embryos to recipients.

Notes

TRIALS ON EQUINE OOCYTES IN VITRO MATURATION

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The procedures of assisted reproduction (ET-Embryo Transfer, GIFT-gamete Intra Fallopian Transfer, ICSI-Intra Cytoplasmic Sperm Injection, OPU-Ovum Pick Up) are finding interest in the equine reproduction. Currently, they are not used routinely in the clinic, but could be profitable in selected infertility cases. With the aim of establishing the ICSI procedure, between November 2003 and March 2004, 14 series of equine oocyte in vitro maturation (IVM) were performed The ovaries were recovered from mares of unknown reproductive anamnesis, at a commercial abattoir. The ovaries were transported in plastic pouches containing 0.9% NaCl and submitted to manipulation as soon as possible. In the laboratory, after further washings, dissection of the follicles took place under a laminar airflow hood with sterile surgical tools. The gonads of pre pubertal animals or those in deep anoestrous were excluded from the experiment. After removal of the albuginea, each follicle was opened with a scalpel blade and emptied in a Petri dish containing the dissection medium (modified Medium 199). The inside wall of each follicle was scraped with a bone curette and washed with physiological solution. Under the microscope, the compact cumulus oocyte complexes (COC) were been separate from the expanded or nude ones, submitted to three washings in dissection medium and located in groups (maximum 20 oocytes in 300 μm drop of maturation media (modified DMEM-F12, added of SR, EGF, Cystine, Cysteamine, Lactate, FSH, LH, Myoinositol, Glutamine, Ascorbic Acid, Na piruvate, ITS), and incubated for 24h, at 38.5°C and 5%CO_2. After this time, the COCs have been washed in H-SOF media supplemented with Hyaluronidase and Trypsin 1:250 through delicate passages with decreasing diameter glass capillary the cumulus was completely removed and it was possible to detect the presence of the polar body (PB). The oocytes that showed the extruded polar body were considered mature, reaching the metaphase II. The obtained data are express in the underlying table.

Season	Ovary	Follicles (foll/ovary)	COC (COC/ovary)	Recovery %	Compact COC (%)	Expanded COC (%)	Damaged COC (%)	Selected COC (%)	Mature COC (%)
Anoestrus	95	390 (4.11)	227 (2.39)	58.21	96 (42.29)	126 (55.51)	5 (2.20)	176 (77.53)	59 (33.33)
Transition	82	372 (5.17)	260 (3.61)	69.89	93 (35.77)	142 (54.62)	25 (9.62)	227 (87.31)	76 (41.08)
Total	167	762 (4.56)	487 (2.92)	63.91	189 (38.81)	268 (55.03)	30 (6.16)	403 (82.75)	158 (39.18)

Randomly, some maturation control was performed on collected COC that had been immediately denuded in order to verify the incidence to underline to maturation independently from the procedure: the presence of the PB has never been detected. The data derived from November-January (seasonal anoestrus) and from February-March (transition) have been analysed separately and submitted to statistic analysis (*t-test*). Any value of the two period showed a statistically significant difference ($p>0.05$). Nevertheless, in the transition period the recovered COCs and selected ones for the IVM, are more numerous and easy to collect. These preliminary data will be compared with those obtainable in full reproductive season. We can affirm that it is possible to recover normal and useful COC for the IVM and, subsequently, for the ICSI, during any period of the year, without statistically difference. Nevertheless, with respect to the reproductive physiology of the mare, during the reproductive season the number of follicles per ovary could be superior and the recovery of COCs easier.

Notes

RELATIONSHIP BETWEEN FEMALE EARLY EMBRYO SURVIVAL ADVANTAGE AND THE X CHROMOSOME

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During preimplantation in mammalian embryos, although male show higher metabolic rate and growths faster than female embryos, it has been suggested that they are more vulnerable than females to environmental stress (Cagnacci et al., 2003, Hum Reprod 18:885); however, the biological basis for this fragility of male embryos is poorly understood. It has been reported that glucose may alter sex ratio of embryos produced in vitro (Gutiérrez-Adán et al., 1993. AETE Meeting Proceedings, p:206), and that mRNA expression of some important components of energetic metabolism, also involved in controlling the amount of oxygen radicals (i.e., glucose 6-phosphate dehydrogenase, G6PD) are expressed at higher levels in female bovine embryos than in male embryos (Gutiérrez-Adán et al., 2000. Mol Reprod Devel 55:146). We have analyzed in mice, how male and female embryos respond under different stress situations (exposure to dioxins or heat), and how the inhibition of G6PD reduces the difference in developmental speed between male and female embryos.

In a first experiment, mouse embryos were obtained from superovulated females at the 1-cell stage and cultured in vitro until the morula stage. At that point embryos were cultured for 24 h either in presence of dioxin (TCDD) or at 41ºC of temperature versus a control group. Table 1 shows that heat stress but not dioxin affected survival rate of male embryos. This difference could be due to an adaptive mechanism that has only evolved under heat stress conditions. In a second experiment, the effect of dehydroepiandrosterone (DHEA), an inhibitor of G6PD, on the sexual dimorphic behavior of mouse embryos was analyzed. Culture of morulae for 24 h in the presence of DHEA supressed differences in developmental speed between male and female (in the control group transfer of faster expanding Bl produced 72% males vs 38% males resulting from transfers of slow expanding Bl. After culture in presence of DHEA, only 43% males were born form the transfer of faster BL vs 48% males born from the slow Bl). In conclusion, male embryos are more vulnerable to some environmental stress agents than females and the differential mRNA expression of G6PD (a developmentally critical cytoprotective enzyme for endogenous and xenobiotic-initiated embryopathic oxidative stress and DNA damage) contribute to early differences between male and female embryos.

Table 1. Effect of both, dioxin or 24h culture at 41ºC, in the sex ratio of murine fetus

	Morulae in culture	Blastocysts (% of dev.)	Blastocysts transferred	Fetus Male/female	Males %
Control	184	171 (93 [a])	162 (7)	45/37	54.9[a]
TCDD	322	198 (61,5 [b])	184 (9)	21/24	46.7[a]
41ºC	366	234 (63.9 [b])	225 (9)	11/28	28.2[b]

Morulae were collected on the third day after coitus and cultured for 24 h with or without TCDD or for 24 h at 41ºC. [a,b]Significantly different in the same column, Chi-square analysis ($P<0.05$).

Notes

EFFECT OF PRONUCLEAR MICROINJECTION AND OVERNIGHT IN VITRO CUTURE ON DEVELOPMENT OF RAT ZYGOTES

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The aim of the present work was to study the possible effects of pronuclear microinjection of Tris-EDTA (TE) buffer or different DNA-constructs on survival and development of rat zygotes in vitro and in vivo as well as the influence of overnight culture of these embryos before transfer into pseudopregnant foster mothers.

One-cell rat embryos were recovered from immature Sprague Dawley rats treated with PMSG and hCG injection in M2 medium containing 0.1% (w/v) hyaluronidase to remove cumulus cells. Solutions (3 µg/ml) of different linear DNA constructs (size range: 5-8 kb) or TE-buffer alone were microinjected into the male or female pronucleus of zygotes. After microinjection, eggs were cultured 2 h in M16 medium and than transferred into pseudopregnant recipients. In case of in vitro culture of rat zygotes to the blastocyt stage we cultured the ova overnight in M16 medium and than transferred them into mR1ECM medium. Ova were cultured (10-20 embryos per 700 µl of the medium) in 4-well culture dishes (Nunc) under 5% CO2 in air at 37°C. Integration of the transgene was determined by transgene-specific PCRs and Southern blots. The comparisons for multigroup and multifactorial analyses were done with a two-way ANOVA and one-way ANOVA on ranks for multiple group comparisons. A value of $P<0.05$ was chosen as an indication of statistical significance.

The survival rate of zygotes immediately after pronuclear injection of various DNA-constructs or TE-buffer was the same in all groups examined, ranging from 50.0 to 69.2%, thus showing no significant difference. The competence of injected embryos to reach the two-cell stage was similar to non-injected zygotes (~80%). But microinjection, whether with DNA-constructs or TE-buffer alone, impaired in vitro development of injected zygotes to the blastocyst stage to ~10 % v.s. ~35% in control non-injected group. Nevertheless, there was no difference in blastocyst development between zygotes injected with DNA-constructs or with buffer. Also the cell number of in-vitro developed blastocysts was not altered by pronuclear microinjection of the corresponding zygotes. The survival rate to term was about 30% irrespective whether microinjected embryos were transferred immediately after microinjection or after overnight culture in vitro. The percentage of transgenic rats developed from microinjected zygotes was similar in all groups regardless of the DNA-construct used (2.7 to 10.0%).

In conclusion, the main detrimental factor in the microinjection of rat zygotes is the introduction of solution in the pronucleus. Overnight culture of zygotes between microinjection and oviduct transfer does not decrease the efficiency of transgenic rat generation.

Notes

UTILIZATION METHODS OF EMBRYOTRANSFER FOR CHANGING OF THE PRODUCTION EFFICIENCY OF CATTLE HERDS

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The aim of this study was to evaluate alternative methods of formation and extension of beef cattle herds by means of ET. The following variables were studied:

(1) Embryo import aimed at the formation of a purebred herd or sires; recipient conception rate varied from 48.1 to 80.0 % (Table 1).

Table 1. Formation or extension of the herd by transfer of imported frozen embryos, production of the sire

BREED	Number of ET	Conception rate - n	%
Aberdeen Angus	60	30	50.0
Hereford	52	25	48.1
Simmental	10	8	80.0
Highland	37	19	51.4
Galloway	40	32	80.0

(2) Transfer of fresh and frozen embryos produced in the owner´s herd aimed at the imported herd extension; recipient conception rate ranged from 50.8 to 71.2 % (Table 2)

(3) Extension of the herd by transfer of unfreezable embryos (morphological grade 3); conception rate varied is 46.9 % (Table 2)

Table 2. Transfer of recovered good-quality embryos – extension of owner´s imported herd

BREED	Number of ET	Conception rate - n	%
Aberdeen Angus	118	60	50.8
Hereford	80	51	63.8
Simmental	66	47	71.2
Piemontese	94	52	54.4
Charolais	36	20	55.6
Blonde d´Aquitaine	18	12	66.7
Salers	36	19	52.8
Limousine	77	44	52.1
Piemontese – grade 3	128	60	46.9

(4) Formation of the cattle herd by purchase of fresh and frozen embryos produced in Czech Republic; conception rate amounted for fresh embryos from 55.9 to 66.7 %; for frozen embryos from 46.4 to 63.9 % (Table 3).

Table 3. Formation or extension of the herd by purchase of fresh and frozen embryos

BREED	Fresh embryos			Frozen embryos		
	Number of ET	Conception rate - n	%	Number of ET	Conception rate - n	%
Simmental	33	22	66,7	72	46	63.9
Limousine	129	78	60,5	61	31	50.8
Charolais	22	16	72,7	10	6	60.0
Piemontese 1	118	66	55,9	153	71	46.4
Piemontese 2	71	44	62	65	39	60.0

The price of turning out heifers aged 15 months by embryo transfer in the benefit of the embryo price or of the embryo transfer price was 33 – 66 % of the buying price of imported heifer – same age and breed. This research was supported form Ministry of Agriculture by projects NAZV QF 3024 and Ministry of education MSM 2678846201.

Notes

ANALYSIS OF DOWNREGULATION OF mTert DURING DIFFERENTIATION OF ES CELLS

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Telomerase is the enzyme that maintains the ends of linear chromosomes in eukaryotic cells. Recently it has been characterized a segment of the promoter sequence of the reverse transcriptase of murine telomerase (mTert). mTert is expressed with greatest abundance during embryogenesis and becomes widely expressed in adults' tissues at low levels. This low expression level in adult tissues may be due to the presence of pluripotent stem cells present in those tissues.

To examine the relationship between mTert expression and its regulation during differentiation, two constructs comprising different segments of the mTert promoter sequence coupled to the coding sequence of the green fluorescent protein (GFP), were electroporated into R1 ES cells, as well as, into B6D2-derived ES cells established in our laboratory. Transfected ES cell clones were obtained for the two constructs. These constructs were able to mimic the mTert expression, which was associated to green fluorescence. The mTert-GFP transfected ES cells were initially maintained in medium supplemented with LIF, which was subsequently removed to allow differentiation of embryoid bodies (EBs) and other cell types. GFP expression was higher during the first 2 days after LIF removal (period of enhanced cell proliferation), decreasing in the following days in consequence of embryod body differentiation. Both ES cell lines showed reduced GFP expression upon differentiation, suggesting that mTert is the principal determinant of telomerase activity, moreover, different degree of expression and down regulation were reported with the different construct. Based on these results, we believe mTert-GFP is an excellent reporter system for pluripotency on adult tissue, and to identify the promoter elements that down regulate mTert activity through differentiation.

At the present we are generating transgenic mice for these constructs, from which we expect to select fluorescent stem cells from adult tissue. It will be interesting to see if different levels of mTert-GFP expression are associated with different levels of pluripotency.

Notes

CRYOPRESERVATION OF PORCINE EMBRYOS

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The aim of this study was to compare two vitrification methods of porcine perihatching blastocysts with regard to the success of transfer of these embryos to the recipients.

Expanded, hatching, or hatched blastocysts were recovered post mortem from superovulated donors gilts at 5.5 to 6.0 days after artificial insemination with homospermic doses.

In protocol VSI, embryos at the perihatching developmental stage were equilibrated in a culture medium H-MEMD with 10% v/v of glycerol (1.37 M solution of glycerol in medium) for 10 min. and placed in a vitrification medium for 1.5 min. max. (vitrification medium contained 50% v/v 2 M sucrose in triple-distilled water, 30% v/v of glycerol, and 20% v/v of foetal calf serum - FCS). Then they were dropped with a micropipette and stored in liquid nitrogen vapor.

For protocol VSII, we used H-MEMD culture medium supplemented with 20% v/v FCS, 25% v/v ethylene glycol, and 25% v/v dimethyl sulfoxide (DMSO). Embryos were equilibrated for 10 min. in a mixture of the vitrification medium and culture medium (1 : 1), and were kept in the vitrification medium for 1.5 min. Then they were dropped with a micropipette and stored in liquid nitrogen vapor.

Embryos were thawed by immersing a drop containing the embryo in H-MEMD culture medium with 0.8 M sucrose for 10 min. After thawing and washing in the medium with sucrose, all embryos were washed three times in a fresh medium and prepared for transfer.

Recipients were synchronized either using Regumate-feeding followed by treatment with PMSG and HCG (gilts) or using piglet weaning (sows – 1st and 2nd parity). Recipients showing standing heat at the time of donor insemination were used for laparoscopic and non-surgical ET on Day 5.5-6.0 of the cycle.

The fraction of viable embryo vitrified under VSI or VSII protocol was 85% and 80%, compared to 95% in control fresh embryos ($P>0.05$). Pregnancy of recipients was 57.3% (5/7), 67.0% (4/6) for VSI or VSII group and 42.7% (10/23) for control ($P<0.001$).

In conclusion, on the basis of our data we can conclude that the both vitrification protocols yielded similar results and can be used for cryopreservation of porcine embryos.

The research was supported form MZe by projects NAZV QD 0085, NAZV QD 0100, NAZV QF 3218 and MSM 2678846201.

Notes

USING OF CATETHER FOR INTRAUTERINE AND INTRACERVICAL INSEMINATION FOR NON-SURGICAL TRANSFER OF PORCINE EMBRYOS

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The aim of study to evaluate the use of two catheters for artificial insemination (catheter for intracervical insemination – IC, and catheter type Verona for deep cornual insemination –CI) for non-surgical transfer of porcine embryos. Embryos were transfer as fresh and frozen. For transfer were using embryos in development stadium Bl, ExBl and HBl. Post mortem method was used for recovery embryos at 5.5 days of estrus cycle from discarded superovulated breeding gilts.In 72% of cases was recipient calm in the course of transfer and reflux of medium was till 30% from total content. Non-surgical transfer of embryos was realize off 40 clinical healthy gilts and sows by Czech super meat breed and crossbreds Czech Large White x Landrace. Number of transferred embryos off IC was at average 12,71 ± 3,405 embryos and off IU 21,92 ± 3,561 embryos.

The attained results informed about first effective non-surgical transfers of porcine embryos. Count increase in cca 40% had positive influence on conception after transfer of embryos by IU transfer (fresh embryos 40,00% IU vs. 31,81 IC; frozen embryos 66,68% IU vs. 40,00% IC) and on natality; came increas in number of natal piglets about 12,84%, it is about 0,90 piglet/farrow. It was detect cca 20% difference in the rate of embryonal mortality after IU embryotransfer, which we can clear up so, that: more transferred embryos result in bigger ussing of biological reduction of potential progeny number. Better results for conception were get from using of frozen embryos by both methods. Again the natality had increasing tendency for transfer of fresh embryos. The sex ratio of piglets from transferred embryos (frozen and fresh) by both proving methods didn´t show swing from normal a sex parity.

Table 1. Results of embryo transfer by two different non-surgical methods (IU and IC) :

Method of ET	Method of embryonal conservation	Number of recipients [n]	Number of transferred embryos [_± s /_]	Pregnant recipients after ET [%]	Duration of pregnancy [days]
	Fresh	22	12.90 ± 3.890	31.81	118.75
IC	Frozen	5	12.40 ± 3.361	40.00	118.50
	∑	27	12.71 ± 3.405	33.31	118.63
	Fresh	10	22.90 ± 4.840	40.00	121.16
IU	Frozen	3	18.66 ± 2.301	66.68	119.50
	∑	13	21.92 ± 3.561	46.15	120.34

_ ..... average; s ..... standard deviation; IC … intacervical embryotransfer; IU … intrauterine embryotransfer

Table 2. Birth after embryo transfer by two different non-surgical methods (IU and IC) :

Method of ET	Method of embryonal conservation	Natality of piglets		Weaned piglets	Birth : Transferred	Sex ratio _ : _
		Total [_ ± s]	Born alive [_ ± s]	[_ ± s]	[%]	[%]
	Fresh	6.50 ± 1.73	5.82 ± 1.35	5.25 ± 0.95	52.67	67.85
IC	Frozen	6.00 ± 0.75	5.50 ± 0.70	5.50 ± 0.70	55.30	52.63
	∑	6.27 ± 1.25	5.70 ± 1.08	5.38 ± 0.80	53.98	61.24
	Fresh	7.45 ± 2.60	7.00 ± 1.60	6.33 ± 1.21	71.63	54.33
IU	Frozen	7.00 ± 2.11	6.76 ± 1.83	6.76 ± 1.83	75.00	64.28
	∑	7.26 ± 2.34	6.78 ± 1.35	6.51 ± 1.40	73.11	59.30

_ ..... average; s ..... standard deviation; IC … intacervical embryotransfer; IU … intrauterine embryotransfer

The research was supported form MZe by projects NAZV QD 0085, QD 0100, QF 3218 and MSM 2678846201.

Notes

COMPARISONS BETWEEN HEIFERS AND COWS AS OOCYTE DONORS FOR EMBRYO PRODUCTION IN VITRO

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The aims of the present study were to compare different age categories of crossbred beef heifers versus cows, in terms of oocyte yield, morphological quality and developmental competence. Three experiments were designed to test the associated hypotheses. In Experiment 1 oocytes were obtained by manual aspiration from the ovaries of slaughtered crossbred beef heifers (under 30 months old, n=1241) and cows (over 4 years old, n=1125) and processed to in vitro maturation, fertilization and culture. Statistical analysis was done using one-way ANOVA in SAS (Version 6.12, Cary, NC) following arc sine transformation. No significant difference was observed between the two groups in terms of the number of aspirated follicles (14.5 vs 16.4 for heifers and cows respectively) or oocytes recovered (6.0 vs 6.2 for heifers and cows, respectively). A significantly higher proportion ($P<0.01$) of cow oocytes than heifer oocytes reached the blastocyst stage (Day 8: 46.5 vs 33.4%). On the basis of the results from Experiment 1, in Experiment 2 heifers were separated into three age groups to answer the question of whether a higher proportion of younger animals could have contributed to the lower blastocyst development rates compared to cow oocytes. Ovaries were separated according to age of heifer into three groups (1) 12-18 months, (2) 19-24 months, and (3) 25-30 months and compared with cow oocytes. There was no significant difference in the blastocyst yield between the different age groups of heifers (Table 1). Irrespective of heifer age, the blastocyst yield on Day 8 was significantly lower than that from cow oocytes (Table 1). The aim of Experiment 3 was to examine whether the difference in developmental rates observed between heifer and cow oocytes in Experiments 1 and 2 would be replicated following culture in vivo. Grade 1-2 oocytes from crossbred heifers and cows were matured and fertilized in vitro and cultured in vivo in to the ewe oviduct. A significantly higher proportion ($P<0.001$) of presumptive zygotes derived from cow oocytes reached the blastocyst stage following culture in vivo in the ewe oviduct than those derived from heifer oocytes (Day 8: 53.1 vs 25.2% for cow and heifer oocytes, respectively). In conclusion, the origin of the oocyte has a significant impact on its subsequent developmental potential. These results would suggest that in an in vitro production system, cow oocytes should be preferentially used over those from heifers in order to maximize blastocyst development.

Table 1. Effect of age of heifer on oocyte developmental competence after in vitro maturation, fertilization and culture.

Animal Type	No. oocytes	Cleaved (%)	Blastocyst Yield (%)		
			Day 6	Day 7	Day 8
Heifers (12-18 m)	708	84.9^{ab}	15.4	32.3^{ab}	35.0^{a}
Heifers (19-24 m)	804	81.2^{a}	14.2	30.9^{a}	35.2^{a}
Heifers (25-30 m)	682	78.5^{a}	15.5	32.0^{ab}	36.5^{a}
Cows	588	86.7^{b}	15.8	41.0^{b}	48.3^{b}
Overall P value		0.055	0.822	0.12	0.028

$^{a, b}$Values in the same column with different superscripts differ significantly ($P<0.05$).

Notes

A ROLE FOR ENDOGENOUS RETINOID DURING BOVINE MORULA TO BLASTOCYST TRANSITION IN VITRO.

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All-trans retinoic acid (ATRA), a vitamin A (all-trans-retinol; ROH) metabolite, exerts a role on cell growth and differentiation and embryonic development. A number of cell types contain ROH sterified to fatty acids, being retinyl-palmitate the preferential form of storage. Bovine oocytes and embryos show high triglyceride contents (Majerus et al, Mol Reprod Dev, 2000, 57:346-352; Ferguson and Leese, J Reprod Fert, 1999, 116:373-378) and palmitate is the most abundant fatty acid in these oocytes (McEvoy et al, 2000 J. Reprod. Fertil. 118:163-70; Kim et al, 2001, Reproduction,122:131-138). Therefore, ROH might be stored in the bovine embryo and to exert a role during transition to the blastocyst stage. We investigated this by using citral, an ALDH generic competitive antagonist that interferes with retinoic acid synthesis.

Cumulus-oocyte complexes from slaughterhouse ovaries were matured and fertilized in vitro. Presumptive zygotes were cultured in B2 medium with Vero cells until 139 h post-insemination, time at which embryos (morulae [~80%] + early blastocysts) were cultured for 24 h in 400 μL SOFaaci + 5% FCS containing: 1) ATRA 0.6 μM; 2) Citral 130 μM; 3) ATRA 0.6 μM + Citral 130 μM; and 4) no additives. Embryos were allowed to reexpand and/or to hatch for 24 h in SOF+aaci+5% FCS (50 μL droplets). Development was recorded (after treatment -24h- and after a recovery period -48h-), and inner cell mass (ICM) and trophectoderm (TE) cells were counted in Day 8 expanded and hatched blastocysts. Data (5 replicates) were analyzed by CATMOD for effects, processed by GLM and Duncan´s test, and expressed as LSM±SE (^{x,y} $p<0.03$; ^{a,b} $p<0.05$).

Table 1. Development of bovine embryos following treatment with all-trans retinoic acid and/or citral.

Treatment	Day 6 Embryos	% Blastocysts 24h		% Blastocysts 48h	
		Total	Expanded	Total	Expanded
ATRA 0.6 μM	93	36.0±4.0	23.1±3.0	40.3±3.7	34.6±3.8
Citral 130 μM	95	28.9±4.0^{x}	16.9±3.0	40.0±3.7	27.2±3.8^{x}
ATRA 0.6 μM +Citral 130 μM	95	45.0±4.0^{y}	24.7±3.0	48.2±3.7	41.3±3.8^{y}
Control [-]	94	38.5±4.0	19.0±3.0	49.1±3.7	41.1±3.8^{y}

Citral gave blastocysts with more cells in ICM than ATRA and controls (37.3±3.2^{a} [n=13], 32.5±3.3^{b} [n=12] and 29.0±3.1^{b} [N=13] respectively; $p<0.05$), while no differences were observed in embryos cultured in ATRA+citral (32.7±3.3 [N=13]). Differences could not be observed between TE cells and total cells (data not shown); more embryos need to be counted to clarify this. ICM/Total cell percentages were a reflection of differences in ICM for ATRA and citral (28.6±2.5 ^{a} and 32.1±2.4^{b}, respectively). Embryonic cells did contain endogenous retinoid, as RA starvation by citral could be reversed by the presence of exogenous RA. The amount of stored ROH seems to be sufficient to ensure embryonic development, which is in part under the RA control. Embryos deprived of RA showed proliferation in their ICM, which could represent a mechanism to control the undifferentiated cell pool in the bovine. Work is in progress to complete cell counts and to analyze apoptosis, ploidy and gene expression in these blastocysts.

Notes

EFFECT OF DILUENT ON POSTTHAWED *IN VITRO* VIABILITY OF SPANISH IBEX (*CAPRA PYRENAICA HISPANICA*) EPIDIDYMAL SPERMATOZOA

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The Spanish Ibex (*Capra pyrenaica hispanica*) is a wild caprine originating exclusively in the mountains of Spain. Despite its value as a hunting species, the Spanish Ibex is currently considered as a vulnerable species by the IUCN because of the loss of heterozygosity by inbreeding in small isolated populations formed by habitat fragmentation, and because the high risk of certain epizootic diseases in some areas. Long-term storage of spermatozoa by cryopreservation, with high post-thaw survival rates, is essential for the establishment of genetic resource bank in vulnerable and endangered species. The purpose of the present study was to evaluate the effect of two diluents for the cryopreservation of spermatozoa in the Spanish Ibex. Testicles were obtained from 10 mature Spanish Ibex males that were legally culled and hunted during the rutting season in the Almijara y Tejeda National Game Reserve (Málaga, Spain). The collection of epididymal spermatozoa was made across a variable range of time after death (2 h - 32 h) by mean small longitudinal and transverse cuts made with a surgical scalpel. The sperm mass was diluted to a sperm concentration of 800 x 10^6 spz/ml with a commercial medium containing 6% glycerol (Triladyl™-20% egg yolk) for the right epididymis and with a Tris-6% egg yolk medium containing 5% glycerol for the left epididymis. Samples were assessed fresh and after freezing-thawing. No significant differences were seen between diluents (Triladyl™-20% egg yolk *vs* Tris-6% egg yolk) for post-thaw percentage of motile spermatozoa (66.70 *vs* 65.40), the motility rate (3.0 *vs* 3.1), the percentage of acrosome integrity (71.23 *vs* 79.47) and the percentage of sperm viability assessed by staining with nigrosin-eosin (67.46 vs 64.73). The plasma membrane integrity, assessed by means of the hypo-osmotic swelling test, was slightly higher in Triladyl™-20% egg yolk than in the Tris-6% egg yolk medium (74.74 vs 65.75, P = 0.05). In conclusion, both diluents appear to be appropriate for cryopreservation of from epididymal Ibex spermatozoa.

Notes

PRODUCTION OF BOVINE DAY 12-14 EMBRYOS BY INTRAUTERINE CULTURE OF IN VITRO PRODUCED EMBRYOS

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Bovine embryos, which are further developed than the usual in vitro production age of 7-8 days are usable for several purposes, such as detailed morphological analysis and stem cell production. However, it has proved to be difficult to culture the bovine embryos in vitro for a period of 12-14 days. The present study investigated the possibility of culturing D 6-7 in vitro produced embryos (IVP) for a period of 5-7 days in the uterine horns of synchronized heifers.

The IVP embryos were produced by standard procedures from abattoir ovaries and frozen thawed semen from one bull. Briefly, IVM took place in DMEM medium supplemented with 5 % serum, EGF and eCG/hCG, and IVF in TALP medium, both under 5 % CO_2 in humidified air and 38.5 °C, whereas embryo culture took place in SOFaaci supplemented with 10 % serum under 5% CO_2, 5 % O_2 and 90 % N_2.

The embryos were cultured in vitro to D6 or D7 post insemination, when morulae and blastocysts of excellent quality were loaded in groups of 10-30 into 0.25 ml straws in hepes-buffered TCM199 with 10% serum and were transported to the place of transfer in a portable incubator at 38.5°C. The embryos were transferred non-surgically to the distal part of each uterine horn of 2-2_ year-old dairy heifers (n=28) which had been synchronized with injections of cloprostenol (Estrumat Vet, Mallinckrodt Vet, Frederiksberg, Denmark) to a cycle stage of D7 after heat for transfer of D6 embryos and D 8 for transfer of D7 embryos. Four of the heifers were used for transfer twice, 4-6 weeks apart and the remaining ones were used once. After 5-7 days, the heifers were flushed non-surgically by standard method, using a flushing catheter of large caliber (Rüsch 18") and gently and slowly retraction of the fluid. During the flushing, it was possible to notice the largest of the embryos in the fluid as light filaments. The difference in recovering rate among horns was identified by Fishers Exact test. Data are given as LS means ± SEM values and statistical differences assigned at the $p<0.05$ level.

No embryos were obtained in 6 flushings and neither the transferred embryos, the heifers nor the flushing procedures differed in any obvious way from the successful flushings, which were 22 (79%). The overall mean embryo recovery rate was 40 ± 3% with a variation from 7% to 73%. There was a minor, but not significant difference between the recovery rate of embryos cultured in the horn ipsi- versus contra- lateral to the CL, respectively (44 ± 5 vs. 38 ± 6 %). The ovoid or elongated embryos, with their dark bag-like appearance, were very easy to isolate under a stereo-microscope. The oldest elongated embryos were in one occasion damaged and in another tangled, making it difficult to isolate the individual embryo, but apart from that, all the embryos seemed of very excellent healthy quality.

In conclusion, our study have documented that it is possible to culture in vitro produced D 6 and D7 bovine blastocysts in the uterus of synchronized heifers and to achieve an acceptable recovery of high quality embryos 5-7 days later.

Notes

SOMATIC CLONING IN PIGS; THE USE OF LIVE-DNA FLUORESCENT DYE YO-PRO-1 FOR DETECTION OF APOPTOSIS IN NUCLEAR DONOR CELLS

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One of the most important factors which determines the efficiency of mammalian somatic cloning is structuro-functional quality of nuclear donor cells. Morphological criteria, which have been used for qualitative evaluation of somatic cells so far, may be insufficient for practical application in cloning. Biochemical changes which are one of the earliest symptoms the signal transduction of physiological cell death (apoptosis) are not reflected in morphological features of somatic cells. Therefore, appropriate systems of cell selection would enable the sorting donor nuclei with high morphological and biochemical susceptibility in somatic cloning. In this study the effect of the live-fluorescent labelling of cumulus cells on *in vitro* developmental competence of porcine nuclear-transferred (NT) embryos was investigated. To detect the early-apoptotic changes in the cumulus cells, which had been isolated from *in vitro* matured cumulus-oocyte complexes (COCs), single nuclear donor cell suspension was subjected to dying with live-DNA green fluorochrome YO-PRO-1 (Vybrant(T)M Apoptosis Assay Kit 4; Molecular Probes). The source of recipient cells were *in vitro* matured oocytes. Maternal chromosomes were removed by chemically assisted microsurgical technique (Yin *et al.*, Zygote 11: 167-74, 2003). Reconstruction of embryos was achieved by direct microinjection of cumulus cell karyoplasts into a cytoplasm of previously enucleated oocytes. After 1 h incubation in NCSU-23 medium with demecolcine, reconstituted oocytes were artificially activated by application of three consecutive DC pulses of 1.0 kV cm^{-1} for 80 _sec and left in the medium supplemented with cytochalasin B for 2 h. Then, NT embryos were *in vitro* cultured in 50-µL drops of NCSU-23 supplemented with 0.4% BSA-V for 6-7 days at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air. The rates of cleavage and development to morula/blastocyst stages were examined on Days 2 and 6/7, respectively, after activation. After fluorescent analysis of approximately 50 different random samples collected from the population of non-cultured follicular cells, which had been labeled with YO-PRO-1 dye, it was found that only minimal proportion of cumulus cells revealed ultrastructural apoptotic changes. On the basis of morphological features of the cells, it can be ascertained that these ones are the symptoms of late destruction phase of apoptosis. The percentage of late-apoptotic cells with advanced morphological transformations did not exceed 10-15% of a total pool of the follicular cells isolated from *in vitro* matured COCs. A total of 740/892 (82.9%) enucleated oocytes were successfully injected with karyoplasts derived from cumulus cells, which had been previously analyzed on apoptosis (i.e. classified as non-apoptotic). Following activation, 740/892 (82.9%) reconstituted oocytes were selected for *in vitro* culture. 398/740 (53.8%) cultured NT embryos were cleaved. The frequencies of cloned embryos, that reached the morula and blastocyst stages, yielded 171/398 (43.0%) and 32/398 (8.0%), respectively. In conclusion, sufficient selection factor for detection of apoptosis in the cumulus cells are morphological criteria of their classification to somatic cloning. Intensity of the green fluorescence, emitted by the cells, is inversely proportional to exposition time of the cells to UV light.

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Notes

SUCCESFULL TRIAL OF PIEMONTESE EMBRYO TRANSFER IN COSTA RICA

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In order to improve the profitability of beef cattle breeding, Piemontese breed germplasm has been introduce in different farms in Costa Rica. Piemontese x Brahman (PIEMBRA®) F1 or F2 cross breds were produced by artificial insemination; purebred Piemontese embryos were transferred to synchronized Zebu recipients in order to produce males for natural mating in extensive breeding farms and females to serve as future donors for embryo production.

The fertility rate at first service, calculated on 517 animals, was 62.9% in cows, rising to 79.7% in heifers. Natural oestrus was detected using teaser bulls and inseminations were carried out by a trained technician.

As the fertility results were satisfactory it was promising to introduce the embryo transfer procedure. The proposal was to test different sources of embryos in order to verify the efficiency and the applicability of: A) in Vitro Fertilized (IVF) frozen/thawed, B) direct transfer after superovulation, C) frozen/thawed embryos derived from superovulation.

A) 47 IVF embryos, coming from 11 production lots, resulting from Ovum Pick Up (OPU) of 9 donors cows were fertilized with 8 different sires. The same operator transferred them in single injected prostaglandin synchronized Zebu recipients, in two different farms. Total pregnancy percentage results 40.4% but attempted 50% in one farm and 34.9% in the other. Concerning the production lot a considerable difference was registered from one sire that produced the 52.4% of pregnancies. Comparing 4 lots of the same two donors cows inseminated with the same two sires is possible to observe that the first one has determined 47.05% of pregnancies and the second only 28.6%. The total abortion rate was 15.8% (1 at fourth month, 2 at eighth months) and the same rate was registered on newborn mortality. Four females and nine males were born. Two females were inseminated at 20/22 month and showed normal fertility and pregnancy.

B) The morphologically two best heifers became donors in a superovulation program at 16/18 month of age. The stimulation was repeated after 5 months. Cycles have been synchronized by hear implant, (Crestar®) and superovulation induced by p-FSH (Folltropin®). Total dose of 32mg/35mg, divided in 8 decreasing doses was utilized and PgF2_ was injected at 6^{th} administration. The flushings were executed at day 6.5/7 and the recovered embryos were transferred immediately in recipients, Zebu Brahman synchronized with PgF2_ on Corpus Luteum diagnosis. For each section about 20 females were prepared. During the first trial 12 embryos were produced, 8 of them viable, and transferred have produced 6 pregnancies, actually over the 7^{th} month. During the second trial the same procedure was actuated and on 7 recovered embryos, 6 were viable and transferred. Five recipients are positive to the palpation diagnosis on day 45.

C) 15 excellent or good embryos produced by superovulation in two donors cows have been frozen for direct transfer. By now only two of them have been transferred in synchronized Zebu recipient but they were negative to the pregnancy diagnosis. Further transfers will go on in the next future.

In sub-tropical environment, Piemontese sperm doses have demonstrated a suitable fertility on field conditions; the pure breed has demonstrated good adaptability and adequate performances of embryo production and satisfactory result have been obtained with embryo transfer in term of achieved pregnancy.

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Notes

IMPACT OF THE ACCURACY OF THE TIME INTERVAL BETWEEN oFSH INJECTIONS ON FOLLICULAR QUALITY IN SUPEROVULATED COWS

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During superovulation (SO), the variability in ovarian response and number of transferable embryos to gonadotrophic stimulation using FSH or eCG within and/or between donor cows has been related to many interacting, exogenous and endogenous, factors. In this respect, the use of different SO schemes including the duration and timing of the total dose of injected gonadotrophins have a major impact on the final embryo outcome. Therefore, to validate the impact of the accuracy of the time interval between oFSH injections on follicular quality we investigated whether one delayed FSH injection may influence the follicular quality in oFSH-stimulated cows using a controlled LH surge SO-procedure.

Synchronized, cyclic HF non-lactating cows (n= 13) were treated for SO. Briefly, a norgestomet ear implant without accompanying injection (Crestar, Intervet International BV, Boxmeer, The Netherlands) was inserted at Day 9 (oestrus = Day 0) to inhibit the endogenous release of the LH surge, and SO was started at Day 10 using oFSH im (Ovagen ICP, Auckland, New Zealand) twice daily during 4 d (total dose 17 ml). Prostaglandin (22.5 mg PG; Prosolvin, Intervet International BV) was administered concomitant with the fifth dose of FSH. Four cows (Experimental group) received a 6.5 h delayed second FSH injection; nine cows served as controls (Control group). One hour after removal of the ear implants (at 50 h after PG, ie. shortly before the preovulatory LH surge) the ovaries were collected by laparotomy and all preovulatory-sized follicles (diameter ≥10 and ≤15 mm) were counted and immediately thereafter punctured individually to collect follicular fluid (FF; stored at -25ºC) and their concomitant COCs. Moreover, all follicles having a diameter between ≥8 and <10 mm were counted. Concentrations of oestradiol-17_ (E) and progesterone (P) in FF were estimated by RIA. Follicles were qualified as 'normal' or 'deviant' on the basis of steroid hormone concentrations ('normal', 'estrogen active' follicles: > 250 ng/ml); COCs were analysed by light microscopy ('good' quality: transparency and more than four layers of cumulus investment, 'bad quality: bold oocytes, complete or partial and no layers of cumulus cells).

None of the animals showed an increase of LH during the norgestomet treatment. The average number of follicles between ≥8 and <10 mm (3.8 follicles per cow) and the total number of preovulatory-sized follicles studied were not significantly different between groups (9 ± 1.4 and 12 ± 1.9, respectively; mean ± SEM). Based on the steroid concentrations estimated in follicular fluid (Table 1), significantly more follicles were classified as 'deviant' in the experimental than in the control group (82.0% and 34.6%, respectively; RR = 2.4, confidence limits 1.7-3.3, Chi Square P< 0.001). No morphological differences between groups were observed for the number of 'good' quality COCs (77.3 and 82.1%, respectively).

Table 1. Steroid concentrations in FF (ng/ml) of preovulatory-sized follicles in oFSH superovulated cows

Steroid	oestradiol-17_		Progesterone	
Quality of follicle	'good'	'deviant'	'good'	'deviant'
C group (9)	541.3 ± 41.2^{a} (51)	141.3 ± 15.8^{b} (27)	41.6 ± 2.3^{a} (51)	63.9 ± 16.0^{a} (27)
E group (4)	365.2 ± 12.5^{a} (7)	75 ± 11.5^{b} (32)	49.1 ± 3.9^{a} (7)	33.3 ± 1.9^{b} (32)

Values are mean ± SEM; values within a row with different superscripts are significantly different; (number)

A significant decrease in oestradiol concentration was observed in follicles derived from the E-group indicating a dramatic effect on follicular function. It is ikely that follicles need continuous gonadotrophin support throughout the period of follicular superstimulation until the period of final maturation. Since no morphological differences for COCs between groups were observed one could speculate whether COCs collected from 'deviated' follicles lost their potency to develop into a blastocyst.

In conclusion, the inaccuracy of one delayed FSH injection severely suppresses the functionality of the majority of the gonadotrophin superstimulated follicles. The possible effects of these deviated follicles on the potential to ovulate or on the developmental competence of the COCs need to be elucidated.

Notes

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