18ème COLLOQUE SCIENTIFIQUE

18th SCIENTIFIC MEETING

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Professor A.E. WRATHALL

Special Celebration

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Rolduc 06th and 07th September 2002

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Professor A.E. WRATHALL

A.E.T.E. Pioneer Award 2002

A.E. (Tony) WRATHALL
A.E.T.E. Medalist 2002

Tony was born in 1937 in an area considered by many to be the most beautiful part of England, The English Lake District.

His formative years were spend within an agricultural community in a remote valley surrounded by animals, his pets and the horses he rode. His family briefly emigrated to Australia when he was twelve years old but were soon to return. The Boyhood adventure of the long sea crossings to and from Australia stimulated Tony's dreams of becoming a sailor. Tony modestly says that he was poor at mathematics which was vital for navigation and therefore a life at sea was ruled out. This is a surprising admission as Tony was later to calculate disease risk assessment.

His secondary education was at Windermere Grammar School and his career objective, veterinary science. Like most young men at that time, Tony entered military service for two years after leaving school. He had hoped to join a mounted cavalry regiment but mechanisation had preceded him, although he was still able to take his beloved horse with him and keep it in a rented field behind the barracks. Tony describes two life changing events which consumed him whilst in the Army. Firstly, Tony became a practising Christian and later met Anthea, his wife-to-be.

Tony attended the Royal (Dick) Veterinary School in Edinburgh from 1957 to 1962 where his initial understanding of lectures suffered because of his deafness. He married Anthea in his final year at University and on qualifying returned to practice in his native Windermere for three years.

Tony and family, now expanded by two, moved to Bangor where Tony attended and M.Sc.Course on Reproduction and Embryology under the direction of Professor Roberts-Bramble for "one exciting year".

In 1966 he joined the staff of the Central Veterinary Laboratory, Weybridge (now the Veterinary Laboratories Agency) where he was trained as a pathologist. He was involved, with Dr. Jack Done and others, in the terminal phases of classical swine fever (CSF) eradication, in particular, the effects of CSF and other terratogenic agents, on the foetus.

After completing a Ph. D on foetal pathology, he branched out into other aspects of reproductive failure, using embryo transfer (E.T.) as a research technique and as a safe way to move genetic material. Nor did he neglect his own practical efforts for by this time his family had grown to four children.

Over the next thirty years, Tony worked with pre-implantation embryos, trans-placental infections, congenital diseases and seasonal infertility problems (eg. Porcine Autumn Abortion Syndrome). Tony was also involved in the development of a porcine parvovirus vaccine and attributes any success he had in all these areas to the good collaboration he has enjoyed with colleagues in the UK and world-wide.

Later, Tony was to head the laboratory side of the U.K. Brucellosis Eradication Programme. He investigated false positive brucellosis reactions in pigs, which at that time threatened to halt U.K. exports.

In 1985 he became a member of the International Embryo Transfer Society (I.E.T.S.) and its import/export committee. He chaired the Research Sub-Committee and then, for a time, the import/export committee itself. He helped to develop the sanitary protocols for E.T. and other reproductive technologies. He was also involved in drafting part of the O.I.E. Animal Health Code and the European Commission's Directive on E.T..

Since 1989, Tony has been largely involved with B.S.E., scrapie and other "slow" diseases, including the risks of their being transmitted by E.T.. To this Society this aspect of his work is undoubtedly of enormous importance.

Tony retired in the late 1990's and moved back to his native county. Tony continues to do some work part-time but during the recent Foot & Mouth epidemic he worked in a full-time capacity.

Whilst deeply-honoured that the A.E.T.E. should consider him worthy of its pioneer award, Tony insists on attributing any success he has had to his wonderfully supportive wife and to his professional colleagues. To me, Tony has always been self-effacing, unstintingly helpful, a highly valued colleague with a wealth of knowledge and, most importantly, one of "Nature's Gentlemen".

Raymond NEWCOMB

DISEASE CONTROL ASPECTS OF EMBRYO TRANSFER.

A.E. WRATHALL

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Recent epidemics of foot-and-mouth disease and classical swine fever have had severe economic, social and animal welfare effects in the United Kingdom (UK) and other countries. The same may be said about bovine spongiform encephalopathy (BSE) which is still causing much disruption of world trade. These catastrophes have reminded everyone that livestock diseases are not just a problem for farmers but, directly or indirectly, they affect us all,. They have also emphasised the importance of effective control measures to prevent such diseases from entering our national livestock populations. Embryo transfer has a valuable role to play in these control measures.

Dawning awareness of advantages of embryo transfer

Thirty five years ago, just after the last big epidemic of foot-and-mouth disease in the UK, we began to look at the possibility of reducing international movement of live animals, with its attendant disease risks, by use of embryo transfer. The benefits of moving frozen semen for livestock improvement were already well known, but semen carries only half the genetic material and the potential for disease transmission by artificial insemination (AI) is another disadvantage. Our early objectives were, first, to ascertain whether live pig embryos could be moved across the Atlantic from Canada to the UK, and, second, to ensure that any diseases carried by the embryos were detected before releasing the offspring into the national swine population. In those days, by using methallibure, oestrus synchronisation of donor and recipient gilts was relatively easy, but that useful drug was later withdrawn due to its teratogenicity (Vente, Wrathall and Done, 1971). Techniques developed by Polge and others (e.g. Dziuk, Polge and Rowson, 1964) also enabled us to collect and transfer the embryos, but freezing the embryos was (and still is) a major problem, so long distance transport was difficult. Thus although our colleagues from the Canadian Animal Disease Research Institute (ADRI) sent us 34 embryos, which were all transferred into a single gilt, only three piglets were born. In retrospect it is likely that the embryos had been exposed to, and damaged by, low temperatures during their aeroplane journey across the Atlantic.

Despite our disappointment about the small number of offspring, we were able to show by blood testing the embryo donors, the recipients which were held in quarantine before and after embryo transfer, and the three embryo transfer piglets, that no detectable disease transmission had occurred via the embryos. In our conclusion from that study (Wrathall *et al*., 1970) we wrote “The high degree of freedom from infectious disease demonstrated in this trial is most satisfactory, and may confer considerable advantage on the conceptus [i.e. embryo] transfer technique as a means of importing genetic material”.

‘Specific pathogen-free’ and ‘minimal disease’ herds

All herds (and flocks), whether owned by individual farmers, or nationally by countries, are free from at least some infectious diseases, and could be termed ‘specific pathogen-free’ (SPF). Most countries, and some owners, take additional steps to exclude or remove diseases and pathogens which they consider to be especially important, and sometimes this objective is pursued to an extreme, particularly by ‘seedstock’ producers wishing to avoid inadvertently transmitting infections to their customers. Absence of intercurrent infections is also a major consideration when rapid genetic selection is practised in so-called ‘genetic nucleus herds’.

Creation of primary SPF or 'minimal disease' (MD) herds by hysterectomy and removal of the piglets, then rearing the piglets artificially under strict sanitary conditions on isolated premises, was in vogue around the time of our early embryo transfer work. Although initially expensive, this is highly effective for controlling diseases such as pneumonia and dysentery, and was especially favoured by pig breeding companies. Once created, however, keeping the MD status intact is difficult, and getting new genes into the MD herd without introducing diseases from the general population is especially challenging. Pigs from other MD herds ought to be safe, but their freedom from all relevant infections, known and unknown, cannot be guaranteed. Neither can it be guaranteed that further batches of hysterectomy-derived offspring would not carry infections which had crossed the placenta prior to hysterectomy. Artificial insemination (AI), although relatively safe, carries a degree of risk too, as was seen in the Netherlands in 1997-98 when swine fever virus was spread by AI with disastrous consequences (Hennecken *et al*., 2000). Another example occurred in our own Laboratory MD pig herd in the UK when, having purchased semen from an SPF boar stud to inseminate some gilts, we inadvertently brought in porcine parvovirus (PPV) and Talfan virus (an enterovirus) which caused a major outbreak of foetal mortality (Parker *et al*., 1981).

Many years ago I suggested (Wrathall, 1975) that embryo transfer might be an ideal way to safely introduce new genes into MD pig herds, but the idea was too radical at that time. Consequently we devoted more of our efforts to understanding the epidemiology and pathology of repoductive diseases (Wrathall, 1980; 1982; 1986), and to developing alternative disease control strategies such as vaccination (Wrathall, 1988). Nevertheless the MD herd does illustrate in microcosm a challenge facing all livestock-producing countries; how to avoid disease risks when importing animals or their genetic material? This question prompted us to think further about the role of embryo transfer.

Importance of the zona pellucida

Whilst working at the National Animal Disease Centre in the USA we did some work on interactions between PPV and early pig embryos (Wrathall and Mengeling, 1979a,b). The experiments were simple**:** freshly collected embryos were exposed *in vitro* to PPV, then washed, and examined by fluorescent microscopy. This revealed the virus had become adherent to the outer surface of the zona pellucida. Other PPV-exposed embryos were transferred into seronegative recipient gilts and resulted in transmission of infection and death of the embryos. Also, when killed a few days after the embryos were transferred, the recipients showed endometritis and seroconversion. A key observation from this work was that because the virus had remained on the outer surface of the zona pellucida the unhatched embryo was initially protected. When it was transferred, however, the virus was carried into the recipient, and later, when hatching took place, the living embryonic cells succumbed to the infection.

Unbeknown to us at the time, the workers at the ADRI in Canada who collaborated with us on the transatlantic pig embryo project in 1970 had intensified their research on disease transmission by embryos during the 1970s and 1980s. Douglas Hare, Elizabeth Singh and several others were involved, and diseases they studied included enzootic bovine leukosis, foot-and-mouth disease (FMD), infectious bovine rhinotracheitis (IBR), bovine viral diarrhoea (BVD), bluetongue and rinderpest. Similar research was undertaken on Aujeszky's disease and other pig virus diseases by Steve Bolin in the USA, and David Stringfellow, also in the USA, was working on bovine and ovine brucellosis, and on vesicular stomatitis. Michel Thibier and his team in France were involved too, and soon other groups around the world were either doing embryo-pathogen research or were exploring the use of embryo transfer for international trade. The late 1970s and the 1980s were the heyday of embryo-pathogen research, and good progress was made. The pivotal role of the zona pellucida in protecting the early embryo from infection was clarified, and procedures were developed for decontaminating the zona by multiple washes, with or without application of trypsin. Realisation was dawning that, at least for some key bovine diseases, frozen embryos could be moved between countries (or between livestock units) with a negligible risk of disease transmission. Regulatory changes to implement these new ideas followed quite quickly.

Important milestones involving the IETS and the OIE

The International Embryo Transfer Society (IETS), started in Denver, USA in 1974, had formed an Import/Export Committee in 1982 to work on sanitary aspects of embryo transfer. This committee, chaired by Reuben Mapletoft, another Canadian veterinarian, began to lobby governments to relax the strict regulations then used for international movement of livestock embryos. In place of the expensive test procedures on embryo donors and recipients, which were often accompanied by isolation in quarantine, they campaigned for much simpler sanitary protocols based largely on microscopical examination, washing and certification of the embryos themselves.

An important milestone occurred in June 1984 in Illinois, USA. Here, at the 10th International Congress on Animal Reproduction, Doug Hare of the ADRI chaired a symposium on disease transmission by embryo transfer at which many of those working on the subject were brought together for the first time. Subsequently they formed the Research Subcommittee of the IETS Import/Export Committee. Another milestone took place in December 1985 when Reuben Mapletoft led a delegation from the Import/Export Committee to the Office International des Epizooties (OIE) to promote an understanding of the safety and welfare advantages of embryo transfer. Also known as the World Organisation for Animal Health, the OIE is a veterinary organisation with over 150 member countries working together to promote the health of farmed livestock. The early contact between the IETS and OIE laid foundations for international acceptance of the IETS and its Import/Export Committee. Acceptance by the OIE of the IETS' expertise was crucial because the OIE is the designated 'scientific body of referral' (an arbitrator) to the World Trade Organisation on safe trading of animals and animal products, including embryos. The OIE's '*International Animal Health Code'* (the OIE *Code*) contains recommended rules for international trade in animals and animal products, and its recommendations for livestock embryos, which are set out in a series of Appendices in the OIE *Code*, are based on inputs from the IETS. The Appendices are periodically reviewed and updated by the IETS Import/Export Committee (recently re-named the 'Health and Safety Advisory Committee').

Appendices in the OIE *Code* cover *in vivo*-derived embryos of cattle, sheep/goats, pigs, horses, deer, South American camelids and laboratory rodents. Two others cover *in vitro*-produced and micromanipulated bovine embryos. Underpinning the Appendices is the IETS Manual (IETS, 1998) describing procedures for embryo processing, including washing, and a requirement central to them all is accountability of the 'Officially Approved Embryo Collection/Production Team'. This Team should operate under the leadership of a 'Team Veterinarian' who is responsible for certifying that all the official protocols for collection, sanitary processing and identification of the embryos have been followed. Under these circumstances the risk of embryos carrying disease is extremely low, and consequently the OIE *Code* Appendices have been adopted by many countries as a basis for international trade. Their principles have also been incorporated into legally binding Directives for trade in embryos within the European Union (EU), and for imports into the EU from non-EU countries. Strict control of embryo teams by national regulatory authorities is essential to ensure that all the stipulated codes of practice are followed even under the pressures of commercial operation.

Categorising diseases re. their transmission risk

The objective of embryo processing, which includes evaluation of the zona pellucida, washing (sometimes with trypsin treatment) and use of antibiotics in the media, is to 'disinfect', i.e. remove or inactivate any potential pathogens that might be present, but without affecting embryo viability. The efficacy of such processing is well documented, and published data have enabled the Research Subcommittee of the IETS Import/Export Committee to categorise many infectious diseases apropos their risk of being transmitted through *in vivo*-derived embryos. Categorisation is reviewed annually by the Sub-committee and, when appropriate, it is updated. The latest position is shown in Table 1.

Table 1: Categorisation of infectious diseases apropos the risk of their transmission through *in vivo*-derived embryos. Based on the 2001 review of available information by the Research Subcommittee of the IETS Import/Export (now Health and Safety Advisory) Committee.

Category 1. Diseases or disease agents for which sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled* between collection and transfer:
Aujeszky's disease (pseudorabies) (swine) - trypsin treatment required; Bluetongue (cattle); *Brucella*
abortus (cattle); Enzootic bovine leukosis; Foot-and-mouth disease (cattle); Infectious bovine
rhinotracheitis - trypsin treatment required.

Category 2. Diseases or disease agents for which substantial evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled* between collection and transfer, but for which additional experimental data are required to verify existing data:
Bluetongue (sheep); Classical swine fever (hog cholera); Scrapie (sheep) .

Category 3. Diseases or disease agents for which preliminary evidence indicates that the risk of transmission is negligible provided that the embryos are properly handled* between collection and transfer, but for which additional *in vitro* and *in vivo* experimental data are required to substantiate the preliminary findings:
Bovine immunodeficiency virus; Bovine spongiform encephalopathy (goats);; Bovine viral diarrhoea;
Campylobacter fetus (sheep); Foot-and-mouth disease (swine, sheep, goats); *Haemophilus somnus*
(cattle); Sheep pulmonary adenomatosis; Rinderpest (cattle); Swine vesicular disease.

Category 4. Diseases or disease agents on which preliminary work has been conducted or is in progress:
African swine fever; Akabane (cattle); Bluetongue (goats); Border disease (sheep); Bovine anaplasmosis;
Bovine herpesvirus-4; Bovine spongiform encephalopathy; *Brucella ovis* (sheep) ; Caprine arthritis-encephalitis;
Chlamydophila psittaci (cattle, sheep); Enterovirus (cattle and swine); *Escherichia coli* 09:K99 (cattle);
Leptospira spp. (cattle, swine); Maedi/visna (sheep); *Mycobacterium bovis* (cattle); *Mycobacterium*
paratuberculosis (cattle); *Neospora caninum* (cattle); Parainfluenza-3 virus (cattle); Porcine
parvovirus; Porcine reproductive and respiratory syndrome; Scrapie (goats); *Ureaplasma/Mycoplasma*
spp. (cattle, goats); Vesicular stomatitis (cattle, swine).

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*Manual of the IETS (recommendations for sanitary handling)

Assessing disease risks quantitatively

Categorisation, as in Table 1, is helpful for making decisions on importing embryos, but it is not the complete answer. For example, both FMD and IBR are listed in Category 1 (negligible risk), but for veterinary officials the term 'negligible' applied to catastrophic diseases like FMD has a very different meaning to that for endemic diseases like IBR. Another deficit is that not all pathogens have been studied apropos their interactions with embryos, and, for some, there is little chance that they ever will be, so it is unlikely that all relevant diseases will be categorised. Additional approaches are

needed as a basis for regulatory actions, therefore, and one that is increasingly used in the veterinary field is quantitative risk assessment. This entails a mathematical assessment of the probability that, under the predicted circumstances, a specific disease event will take place.

In the case of embryo transfer, knowledge of epidemiology, microbiology, pathology and statistics, and a detailed understanding of embryo technologies, are needed to gauge risk scenarios on each step of the pathway between collection in the exporting country and transfer into recipients in the importing country. This method has been used to quantify hypothetical risks associated with the importation of bovine embryos from an area of South America with high risks of FMD, bluetongue and vesicular stomatitis (Sutmoller and Wrathall, 1997). Despite these three diseases being very dissimilar, their transmission risks were found to be extremely low: less than 10^{-6} in most circumstances.

Dealing with different kinds of infectious disease

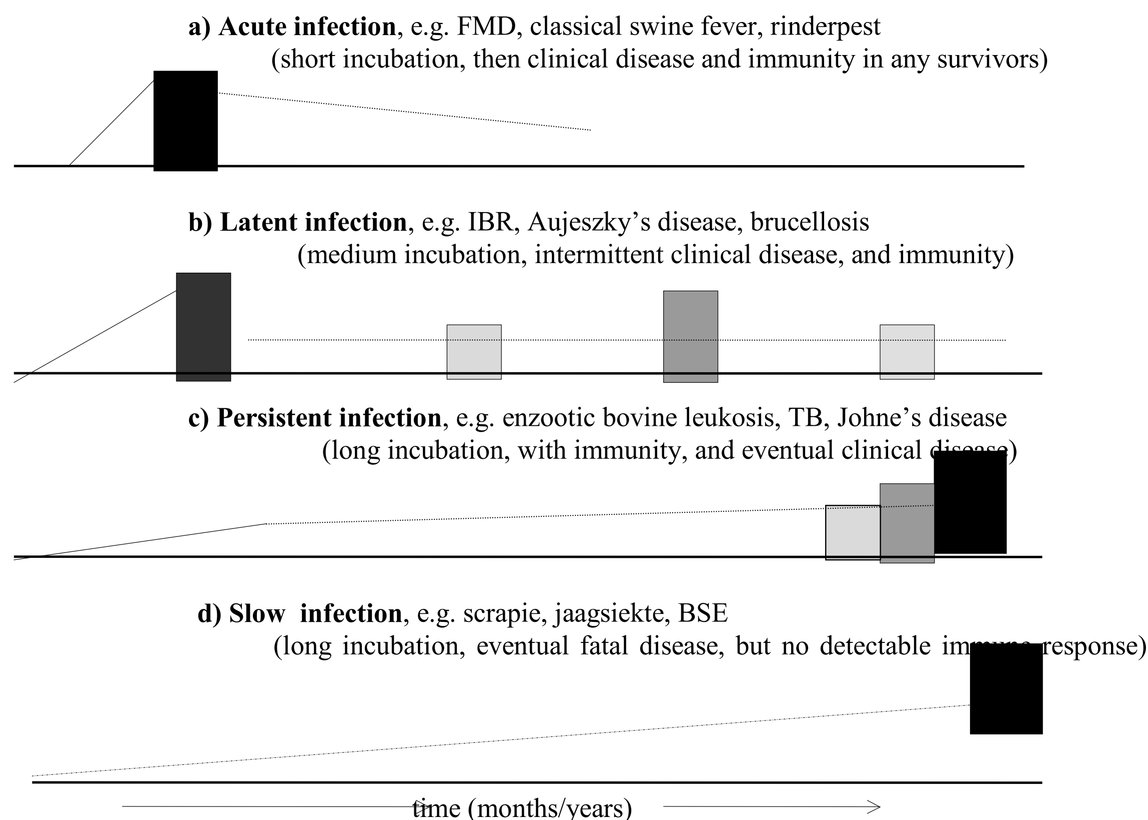

Fig. 1. Illustration of some types of infectious disease.
Key: solid line = pathogen in blood; dotted line = pathogen not readily detectable;
solid box = severe clinical disease; non-solid box = clinical/subclinical disease.
Antibody is usually detectable in a), b) and c).

As shown in Fig. 1, characteristics of different infectious diseases can vary greatly. Some, like FMD and classical swine fever, are highly contagious with rapid onset of clinical manifestations, whereas others progress slowly, taking weeks, months or even years before clinical signs are seen. With yet other diseases periodic recrudescence of the pathogen occurs, giving rise to recurrent bouts of illness. In a few instances some infected animals may never show clinical illness at all, but nevertheless may shed the pathogen and transmit to other animals. With regard to the risk of transmission by embryo transfer, several of each type in Fig. 1 have been studied to a greater or lesser degree, and, as can be seen in Table 1, results have generally been encouraging. Reviews can be found

elsewhere (e.g. Wrathall & Sutmoller, 1998), but it seems appropriate at this juncture to consider some recent work with 'slow' infections of the type illustrated in Fig. 1d.

Studies on 'slow virus diseases'

Diseases which require special attention in the context of embryo transfer are those formerly referred to as 'slow virus diseases'. They include scrapie, jaagsiekte and bovine spongiform encephalopathy (BSE) which have been of much concern recently, not least in the context of international trade. Not all are caused by viruses in the true sense, but their pathogens tend to be poorly defined and cannot be cultured *in vitro*. This, together with their long incubation periods, lack of detectable serological reactions and inability to identify infected animals before clinical onset, makes them difficult to work with. For embryo transfer studies the absence of reliable diagnostic tests makes it is hard to identify infected donors. Likewise finding disease-free recipients, and keeping them free from infection for long periods, is problematic and very expensive, but this is essential to get valid results. In the UK we seem to have had more than our fair share of the 'slow virus' diseases, and have consequently expended a lot of effort studying them, including work to evaluate the risks of their transmission by embryo transfer.

Scrapie is one of the group of fatal brain diseases known as transmissible spongiform encephalopathies (TSEs). It occurs in sheep, and occasionally goats, in many countries, but a few, such as Australia and New Zealand, are free, so the risks of international movement of sheep and goats, and of their genetic material, are of considerable concern.

The possibility that transmission of scrapie could be prevented by the use of embryo transfer received early attention, but with mixed results. Two groups of researchers were involved and controversy arose because one group (Foote *et al*., 1993), working in USA, found no evidence for scrapie transmission via sheep embryos, whereas the other group (Foster *et al*., 1992; 1994; 1996) working in Scotland, did report transmission via embryos. Both groups obtained their embryos from pre-clinical phase scrapie-inoculated donor ewes rather than from ewes with natural, clinical scrapie. Also, in the Scottish case, both donors and recipients came from flocks of sheep with a high incidence of natural scrapie, so evidence that the scrapie in their embryo transfer offspring originated via the embryos is unconvincing (see Wrathall 1997; 2000). More recent work by the USA group (Wang, Foote *et al*., 2000) in which embryos were collected and transferred from donors with natural scrapie once again yielded no evidence for transmission. Consequently the IETS has now designated scrapie as a Category 2 disease.

Jaagsiekte, also called 'sheep pulmonary adenomatosis', is another slow and fatal disease of sheep, and is characterised by progressive carcinoma of the lungs, respiratory distress and loss of body condition. The pathogen is probably a retrovirus (oncovirus subgroup), but, in the absence of reliable culture methods or serological tests, diagnosis is not possible in pre-clinical infected animals. As with scrapie, jaagsiekte occurs in some but not all sheep-producing countries, and can cause substantial losses, especially if sheep are intensively housed, so international movements must be strictly controlled. Ability to control its transmission by using embryo transfer would be a valuable option.

Transfer studies from clinically affected and suspect ewes started in 1985 at the UK Veterinary Laboratories Agency. Most of these donor ewes were unfit for surgery, so their embryos were collected after euthanasia, and this was followed by examination for jaagsiekte lesions. The embryos were processed using IETS Manual procedures. Thirty eight embryo transfer offspring from confirmed jaagsiekte-positive donor ewes, and 55 from suspect (but unconfirmed) ewes, survived for at least five years without clinical or post mortem evidence of the disease. No evidence of disease was found in the surviving recipients either. Thus, while the numbers were not quite enough to put jaagsiekte into one of the higher IETS categories (see Table 1), Parker *et al*. (1998) concluded that embryo transfer is a valuable way to prevent its transmission from infected sheep flocks.

BSE. Studies with embryos from BSE-affected cattle began in 1990, soon after the disease emerged in the UK. Their purpose was both to facilitate resumption of embryo exports and to ascertain whether salvage of genetic material from infected animals would be feasible, if this ever became

necessary. It was already known by that time that the major route of BSE transmission was via food contaminated with infected bovine-derived protein, but later, from a cohort study in which calves from BSE-affected and unaffected dams were monitored for seven years (Wilesmith *et al*., 1997), it was found that a small but significant number of BSE cases arose from maternal transmission. The level of maternal transmission was also found to increase as the period between birth of the calf and onset of clinical signs in the dam decreased. Thus, if a cow gives birth within six months of, or after onset of clinical signs, the probability of her calf acquiring the disease rises to at least ten per cent. This enhanced risk in late incubation suggests that maternal tissues are most infectious to the embryo, foetus or calf during the clinical phase. In our studies, therefore, embryos were collected from clinically affected cows, many of which had been inseminated with semen from clinically affected bulls.

All of the embryos were processed according to the IETS Manual, then frozen. Later, the viable ones were transferred singly into 347 recipient heifers imported to UK from New Zealand, and 266 live offspring were born of which 54% had a BSE positive sire as well as a BSE positive dam. Recipients were monitored for clinical signs of BSE for seven years after transfer, and the offspring likewise for seven years after birth. At the end of that time their brains, plus those of casualties which died before seven years, were examined for BSE by histopathology, PrP immunohistochemistry, and by electron microscopy for scrapie-associated fibrils (SAF). All were negative. In addition, 1020 non-viable embryos were sonicated and injected intracerebrally into susceptible mice (20 embryos per mouse) which were monitored for up to 700 days after injection, and their brains were then examined for spongiform lesions. Again, all were negative. It can be concluded, therefore, that embryos are unlikely to carry BSE infectivity even if collected at the end-stage of the disease when the risk of maternal transmission is highest.

Although the extreme resistance of the BSE agent to physical and chemical inactivation means that disinfection is extremely difficult, it is emphasised that sanitary codes of practice recommended in the OIE Code (1992), and in the IETS Manual (1998), were used in the study to guard against possible disease transmission risks, including any risks associated with contaminated media and equipment. Protein constituents in the embryo collection and processing media were obtained from New Zealand, and, wherever possible, disposable equipment was used. A full report on the work has recently been published (Wrathall *et al*., 2002) but the IETS has not yet had the opportunity to decide whether BSE should be moved up from its current Category 4 position (Table 1).

Conclusions

A dawning awareness in the early 1970's of the animal health advantages of embryo transfer led to an expansion of research on embryo-pathogen interactions, and, in 1982, to formation of the IETS Import/Export Committee. In 1985 a working relationship was forged between the IETS and the OIE which gave credibility to proposals for simplified sanitary codes of practice for embryo movements, and these codes are now widely accepted by regulatory authorities as a basis for international trade. Quantitative risk assessments have demonstrated that even with high profile diseases like FMD and bluetongue the risks of importing embryos from infected countries are exceedingly small when the OIE/IETS codes are used. Other studies, particularly in the UK, have shown that despite major diagnostic difficulties, the risks of transmitting slow virus diseases like jaagsiekte in sheep and BSE in cattle are also very low when the codes are applied.

This paper deals mainly with transfer of *in vivo*-derived embryos for which the disease control advantages are well researched. While less is known about embryos produced *in vitro*, or by genetic manipulation and cloning, it is evident that some risks do exist with these, and they are under close scrutiny in the IETS committees. Fortunately the basic sanitary principles set out in the IETS Manual for *in vivo*-derived embryos can still be applied to good advantage in these other situations, and Appendices setting out codes of practice for *in vitro*-produced and micromanipulated embyos have been published in the latest editions of the OIE *Code*.

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National Statistical Data of Bovine Embryo Transfer Activity in Europe (2001).

TABLE : 1 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **AUSTRIA**

A.E.T.E 2002
Data collected by
Dr. Fischerleitner Franz

Total number of approved E.T. teams in the country	10
Number of teams providing data	8

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	289	B/A=	11.46
	Embryos collected	**B**	3312	C/A=	5.83
	Embryos transferable	**C**	1685	C/B=	50.8%
In vitro	Nb of oocyte donors				
(OPU)	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total *in vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	1685	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	298		
In vivo	Frozen	**I**	866		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1164	H+I+J+K=	
Number of frozen stored embryos		**M**	1071		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	74.4%	(I+K)/L=	%

Number of E.T. calves born (2001)

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

* data not available

TABLE : 2 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **BELGIUM**

A.E.T.E 2002
Data collected by
Dr. Beckers Jean-François

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	1150	B/A= 7.62
	Embryos collected	**B**	8768	C/A= 5.65
	Embryos transferable	**C**	6507	C/B= 74.2%
In vitro	Nb of oocyte donors		374	
(OPU)	Nb of OPU sessions			
	Nb of transferable embryos	**D**	1839	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**	1839	=(D+E)
Total number of transferable embryos		**G**	8346	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	2075	
In vivo	Frozen	**I**	5037	
In vitro	Fresh	**J**	874	
In vitro	Frozen	**K**		
Total embryos transferred		**L**	7986	H+I+J+K=
Number of frozen stored embryos		**M**	3863	
% of *in vitro* embryos transferred		**N**	10.9%	(J+K)/L=
% of frozen embryos transferred		**O**	63.1%	(I+K)/L= %

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	*
Number of calves born from *in vitro* embryos	372
Total	*

* data not available

TABLE : 3 EMBRYO TRANSFER ACTIVITY IN 2001

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

Data collected by
Dr. Petelikova Jirina

Total number of approved E.T. teams in the country	5
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1036	B/A=	8.94
	Embryos collected	**B**	9265	C/A=	4.68
	Embryos transferable	**C**	4857	C/B=	52.4%
In vitro	Nb of oocyte donors		2		
(OPU)	Nb of OPU sessions		11		
	Nb of transferable embryos	**D**	55		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	108		
Total *in vitro* embryos		**F**	163	=(D+E)	
Total number of transferable embryos		**G**	5010	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2546		
In vivo	Frozen	**I**	2192		
In vitro	Fresh	**J**	55		
In vitro	Frozen	**K**	137		
Total embryos transferred		**L**	4930	H+I+J+K=	
Number of frozen stored embryos		**M**	2495		
% of *in vitro* embryos transferred		**N**	3.89%	(J+K)/L=	
% of frozen embryos transferred		**O**	47.2%	(I+K)/L=	%

Number of E.T. calves born (2001)

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

* data not available

TABLE : 4 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **DENMARK**

A.E.T.E 2002
Data collected by
Dr. Callesen Henrik

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	784	B/A= 10.24
	Embryos collected	**B**	8033	C/A= 6.91
	Embryos transferable	**C**	5422	C/B= 67.5%
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**		
Total *in vitro* embryos		**F**	0	=(D+E)
Total number of transferable embryos		**G**	5422	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	2070	
In vivo	Frozen	**I**	2055	
In vitro	Fresh	**J**	0	
In vitro	Frozen	**K**	0	
Total embryos transferred		**L**	4125	H+I+J+K=
Number of frozen stored embryos		**M**	3272	
% of *in vitro* embryos transferred		**N**	0%	(J+K)/L=
% of frozen embryos transferred		**O**	49.8%	(I+K)/L= %

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	1533
Number of calves born from *in vitro* embryos	0
Total	1533

* data not available

TABLE : 5 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **ESTONIA**

A.E.T.E 2002
Data collected by
Dr. Jaakma Y.

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	12	B/A= 5.2
	Embryos collected	**B**	62	C/A= 3.4
	Embryos transferable	**C**	41	C/B= 66.1
In vitro	Nb of oocyte donors		0	
(OPU)	Nb of OPU sessions		0	
	Nb of transferable embryos	**D**	0	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0	
Total ***in vitro*** **embryos**		**F**	0	=(D+E)
Total number of transferable embryos		**G**	41	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	31	
In vivo	Frozen	**I**	10	
In vitro	Fresh	**J**	0	
In vitro	Frozen	**K**	0	
Total embryos transferred		**L**	41	H+I+J+K=
Number of frozen stored embryos		**M**	10	
% of *in vitro* embryos transferred		**N**	0%	(J+K)/L=
% of frozen embryos transferred		**O**	24%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 6 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **FINLAND**

A.E.T.E 2002
Data collected by
Dr. Mikkola Marja

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	308	B/A=	8.0
	Embryos collected	**B**	2464	C/A=	5.42
	Embryos transferable	**C**	1671	C/B=	67.8%
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**	20		
Total *in vitro* embryos		**F**	20	=(D+E)	
Total number of transferable embryos		**G**	1691	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	755		
In vivo	Frozen	**I**	843		
In vitro	Fresh	**J**	18		
In vitro	Frozen	**K**	23		
Total embryos transferred		**L**	1639	H+I+J+K=	
Number of frozen stored embryos		**M**	776		
% of *in vitro* embryos transferred		**N**	2.5%	(J+K)/L=	
% of frozen embryos transferred		**O**	52.8%	(I+K)/L= %	

Number of E.T. calves born (2001)

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

* data not available

TABLE : 7 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **FRANCE**

A.E.T.E 2002
Data collected by
Dr. Guérin Bernard

Total number of approved E.T. teams in the country	27
Number of teams providing data	23

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	6880	B/A= 9.13
	Embryos collected	**B**	62840	C/A= 5.38
	Embryos transferable	**C**	37071	C/B= 59%
In vitro *(OPU)*	Nb of oocyte donors		199	
	Nb of OPU sessions		245	
	Nb of transferable embryos	**D**	819	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	0	
Total *in vitro* embryos		**F**	819	=(D+E)
Total number of transferable embryos		**G**	37890	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	17786	
In vivo	Frozen	**I**	15136	
In vitro	Fresh	**J**	601	
In vitro	Frozen	**K**	163	
Total embryos transferred		**L**	33686	H+I+J+K=
Number of frozen stored embryos		**M**	13844	
% of *in vitro* embryos transferred		**N**	2.3%	(J+K)/L=
% of frozen embryos transferred		**O**	45.4%	(I+K)/L= %

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	3546**
Number of calves born from *in vitro* embryos	79
Total	*

** Partial data corresponding to 6608 embryo transfers

* data not available

TABLE : 8 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: GERMANY

A.E.T.E 2002
Data collected by
Dr. Clauss Karin

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	3229	B/A= 10.38
	Embryos collected	**B**	33535	C/A= 6.13
	Embryos transferable	**C**	19790	C/B= 59%
In vitro	Nb of oocyte donors		*	
(OPU)	Nb of OPU sessions		1297	
	Nb of transferable embryos	**D**	3564	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	1132	
Total *in vitro* embryos		**F**	4696	=(D+E)
Total number of transferable embryos		**G**	24486	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	7477	
In vivo	Frozen	**I**	4500	
In vitro	Fresh	**J**	1132	
In vitro	Frozen	**K**		
Total embryos transferred		**L**	13109	H+I+J+K=
Number of frozen stored embryos		**M**	12577	
% of *in vitro* embryos transferred		**N**	8.6%	(J+K)/L=
% of frozen embryos transferred		**O**	34.3%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 9 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **GREECE**

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

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

EMBRYO PRODUCTION				
In vivo	Flushed donors Embryos collected Embryos transferable	**A** **B** **C**	12 62 45	B/A= 5.16 C/A= 3.75 C/B= 72.5%
In vitro *(OPU)*	Nb of oocyte donors Nb of OPU sessions Nb of transferable embryos	 **D**	5 11	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**	11	=(D+E)
Total number of transferable embryos		**G**	56	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	36	
In vivo	Frozen	**I**		
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	36	H+I+J+K=
Number of frozen stored embryos		**M**	20	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 10 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **HUNGARY**

A.E.T.E 2002
Data collected by
Dr. Solti Laszlo

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	306	B/A= 7.89
	Embryos collected	**B**	2416	C/A= 4.40
	Embryos transferable	**C**	1346	C/B= 55.7%
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**	164	
Total *in vitro* embryos		**F**	164	=(D+E)
Total number of transferable embryos		**G**	1510	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	597	
In vivo	Frozen	**I**	200	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	797	H+I+J+K=
Number of frozen stored embryos		**M**	1166	
% of *in vitro* embryos transferred		**N**	-	(J+K)/L=
% of frozen embryos transferred		**O**	25.1%	(I+K)/L= %

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	187
Number of calves born from *in vitro* embryos	-
Total	187

* data not available

TABLE : 11 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **IRELAND**

A.E.T.E 2002
Data collected by
Dr. Lonergan Pat

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	742	B/A=	8.30
	Embryos collected	**B**	6160	C/A=	4.38
	Embryos transferable	**C**	3266	C/B=	53%
In vitro	Nb of oocyte donors		68		
(OPU)	Nb of OPU sessions		350		
	Nb of transferable embryos	**D**	822		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	41		
Total *in vitro* embryos		**F**	863	=(D+E)	
Total number of transferable embryos		**G**	4129	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1706		
In vivo	Frozen	**I**	1401		
In vitro	Fresh	**J**	743		
In vitro	Frozen	**K**	37		
Total embryos transferred		**L**	3887	H+I+J+K=	
Number of frozen stored embryos		**M**	2643		
% of *in vitro* embryos transferred		**N**	20.0%	(J+K)/L=	
% of frozen embryos transferred		**O**	37%	(I+K)/L=	%

Number of E.T. calves born (2001)

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

* data not available

TABLE : 12 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **ITALY**

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

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	1086	B/A= 14.65
	Embryos collected	**B**	15917	C/A= 7.18
	Embryos transferable	**C**	7806	C/B= 49.0%
In vitro	Nb of oocyte donors		207	
(OPU)	Nb of OPU sessions		486	
	Nb of transferable embryos	**D**	1204	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	1255	
Total *in vitro* embryos		**F**	2459	=(D+E)
Total number of transferable embryos		**G**	10265	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	3780	
In vivo	Frozen	**I**	3465	
In vitro	Fresh	**J**	100	
In vitro	Frozen	**K**	3505	
Total embryos transferred		**L**	10850	H+I+J+K=
Number of frozen stored embryos		**M**	7461	
% of *in vitro* embryos transferred		**N**	33.2%	(J+K)/L=
% of frozen embryos transferred		**O**	64.2%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 13 EMBRYO TRANSFER ACTIVITY IN 2001

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

Data collected by
Dr. de Ruigh Lisette

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2330	B/A=	5.85
	Embryos collected	**B**	13650	C/A=	5.20
	Embryos transferable	**C**	12120	C/B=	88.8%
In vitro	Nb of oocyte donors		165		
(OPU)	Nb of OPU sessions		1532		
	Nb of transferable embryos	**D**	1514		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	35		
Total *in vitro* embryos		**F**	1549	=(D+E)	
Total number of transferable embryos		**G**	13669	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2755		
In vivo	Frozen	**I**	9847		
In vitro	Fresh	**J**	611		
In vitro	Frozen	**K**	808		
Total embryos transferred		**L**	14021	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**	10.1%	(J+K)/L=	
% of frozen embryos transferred		**O**	76%	(I+K)/L=	%

Number of E.T. calves born (2001)

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

* data not available

TABLE : 14 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **NORWAY**

A.E.T.E 2002
Data collected by
Dr. Kommisrüd Elisabeth

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	79	B/A= 8.68
	Embryos collected	**B**	686	C/A= 5.41
	Embryos transferable	**C**	428	C/B= 62.4
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**	*	=(D+E)
Total number of transferable embryos		**G**	428	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	30	
In vivo	Frozen	**I**	303	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	333	H+I+J+K=
Number of frozen stored embryos		**M**	430	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	90.1%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2001

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

Data collected by
Dr. das Chagas e Silva Joao Nestor

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	27	B/A=	6.15
	Embryos collected	**B**	166	C/A=	4.30
	Embryos transferable	**C**	116	C/B=	69.8%
In vitro	Nb of oocyte donors		11		
(OPU)	Nb of OPU sessions		10		
	Nb of transferable embryos	**D**	*		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	175		
Total *in vitro* embryos		**F**	175	=(D+E)	
Total number of transferable embryos		**G**	291	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	48		
In vivo	Frozen	**I**	72		
In vitro	Fresh	**J**	-		
In vitro	Frozen	**K**	-		
Total embryos transferred		**L**	120	H+I+J+K=	
Number of frozen stored embryos		**M**	88		
% of *in vitro* embryos transferred		**N**	-	(J+K)/L=	
% of frozen embryos transferred		**O**	60%	(I+K)/L=	%

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	62
Number of calves born from *in vitro* embryos	0
Total	62

* data not available

TABLE : 16 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **ROMANIA**

A.E.T.E 2002
Data collected by
Dr. Zamfirescu Stela

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	11	B/A= 4.45
	Embryos collected	**B**	49	C/A= 3.36
	Embryos transferable	**C**	37	C/B= 75.5%
In vitro	Nb of oocyte donors			
(OPU)	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**	37	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	28	
In vivo	Frozen	**I**	9	
In vitro	Fresh	**J**	0	
In vitro	Frozen	**K**	0	
Total embryos transferred		**L**	37	H+I+J+K=
Number of frozen stored embryos		**M**	-	
% of *in vitro* embryos transferred		**N**	-	(J+K)/L=
% of frozen embryos transferred		**O**	24.3%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 17 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: SLOVAKIA

A.E.T.E 2002
Data collected by
Dr. Cesual P.

Total number of approved E.T. teams in the country	1
Number of teams providing data	3

EMBRYO PRODUCTION				
In vivo	Flushed donors Embryos collected Embryos transferable	**A** **B** **C**	403 3458 2036	B/A= 8.58 C/A= 5.05 C/B= 58.9%
In vitro *(OPU)*	Nb of oocyte donors Nb of OPU sessions Nb of transferable embryos	 **D**	60 15	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	36	
Total *in vitro* embryos		**F**	51	=(D+E)
Total number of transferable embryos		**G**	2087	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	1147	
In vivo	Frozen	**I**	443	
In vitro	Fresh	**J**	-	
In vitro	Frozen	**K**	-	
Total embryos transferred		**L**	1590	H+I+J+K=
Number of frozen stored embryos		**M**	542	
% of *in vitro* embryos transferred		**N**	-	(J+K)/L=
% of frozen embryos transferred		**O**	27.9%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 18 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: SPAIN

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

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	352	B/A= 9.38
	Embryos collected	**B**	3303	C/A= 4.69
	Embryos transferable	**C**	1650	C/B= 50%
In vitro *(OPU)*	Nb of oocyte donors		16	
	Nb of OPU sessions		217	
	Nb of transferable embryos	**D**	93	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**	93	=(D+E)
Total number of transferable embryos		**G**	1743	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	525	
In vivo	Frozen	**I**	991	
In vitro	Fresh	**J**	8	
In vitro	Frozen	**K**	22	
Total embryos transferred		**L**	1546	H+I+J+K=
Number of frozen stored embryos		**M**	1575	
% of *in vitro* embryos transferred		**N**	1.9%	(J+K)/L=
% of frozen embryos transferred		**O**	65.5%	(I+K)/L= %

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	371
Number of calves born from *in vitro* embryos	4
Total	375

* data not available

TABLE : 19 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **SWEDEN**

A.E.T.E 2002
Data collected by
Dr. Gustafsson Hans

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	294	B/A= 8.56
	Embryos collected	**B**	2518	C/A= 5.60
	Embryos transferable	**C**	1647	C/B= 65.4%
In vitro	Nb of oocyte donors		-	
(OPU)	Nb of OPU sessions		-	
	Nb of transferable embryos	**D**	-	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	-	
Total *in vitro* embryos		**F**	-	=(D+E)
Total number of transferable embryos		**G**	1647	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	754	
In vivo	Frozen	**I**	800	
In vitro	Fresh	**J**	-	
In vitro	Frozen	**K**	-	
Total embryos transferred		**L**	1554	H+I+J+K=
Number of frozen stored embryos		**M**	889	
% of *in vitro* embryos transferred		**N**	-	(J+K)/L=
% of frozen embryos transferred		**O**	51.5%	(I+K)/L= %

Number of E.T. calves born (2001)

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

* data not available

TABLE : 20 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **SWITZERLAND**

A.E.T.E 2002
Data collected by
Dr. Rainer Saner

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	264	B/A= 13.24
	Embryos collected	**B**	3495	C/A= 8.17
	Embryos transferable	**C**	2157	C/B= 61.7%
In vitro	Nb of oocyte donors		31	
(OPU)	Nb of OPU sessions		331	
	Nb of transferable embryos	**D**	129	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total *in vitro* embryos		**F**	129	=(D+E)
Total number of transferable embryos		**G**	2286	=(C+F)
EMBRYO TRANSFER				
In vivo	Fresh	**H**	446	
In vivo	Frozen	**I**	1543	
In vitro	Fresh	**J**	0	
In vitro	Frozen	**K**	135	
Total embryos transferred		**L**	2124	H+I+J+K=
Number of frozen stored embryos		**M**	1845	
% of *in vitro* embryos transferred		**N**	6.35%	(J+K)/L=
% of frozen embryos transferred		**O**	79.0%	(I+K)/L= %

Number of E.T. calves born (2001)

Number of calves born from superovulated embryos	1100
Number of calves born from *in vitro* embryos	45
Total	1145

* data not available

TABLE : 21 EMBRYO TRANSFER ACTIVITY IN 2001

COUNTRY: **UNITED KINGDOM**

A.E.T.E 2002
Data collected by
Dr. Breslin Lydia

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

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	*	B/A=
	Embryos collected	**B**	*	C/A=
	Embryos transferable	**C**	*	C/B=
In vitro *(OPU)*	Nb of oocyte donors		*	
	Nb of OPU sessions		*	
	Nb of transferable embryos	**D**	*	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	*	
Total *in 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**	3767	H+I+J+K=
Number of frozen stored embryos		**M**	*	
% of *in vitro* embryos transferred		**N**	*	(J+K)/L=
% of frozen embryos transferred		**O**	*	(I+K)/L= %

Number of E.T. calves born (2001)

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 2001

I. EMBRYO PRODUCTION

(Data collected from 21 countries)

***In vivo* produced embryos (superovulation)**	
- number of flushed donors	**19 594**
- number of transferable embryos	**109 698**
- mean number per flushed donor	**X = 5.60**
***In vitro* produced embryos:**	
From OPU	
- number of oocyte donors	**1 138**
- number of OPU sessions	**4 479**
- number of blastocysts produced	**10 065**
From slaughterhouse collected ovaries	
- number of blastocysts produced	**2 966**
Total *in vitro*	**13 031***
Total number of transferable embryos	**122 725**

* Data for i*n vitro* embryo production from 14 countries

(Y.HEYMAN, AETE Rolduc 2002)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2001

II. EMBRYO TRANSFERS

(Data collected from 21 countries)

	Number of recipients transferred
- **From *In* vivo produced embryos**	**94 603***
- **From *In vitro* produced embryos**	**8 972***
Total number of embryo transfers	**107 342**
Proportion of IVF embryos transferred	**8.66 %**
Proportion of frozen embryos transferred	**52.7 %**

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

(Y.HEYMAN, AETE Rolduc 2002)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES*

EUROPE year 2001

Species	Embryo Production	Embryo Transfers	Countries
Sheep	**613**	**441**	Czech rep France Greece Hungary Portugal Romania
Swine	**302**	**184**	Czech Rep Hungary
Goat	**198**	**103**	Denmark France
Horse	**309**	**260**	Austria Czech Rep Finland France Italy

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

(Y. HEYMAN, AETE Rolduc, 2002)

INVITED LECTURES

THE USE OF GENETIC TESTING IN VETERINARY DIAGNOSTICS - SOME PRACTICAL ASPECTS

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Many research papers and reviews have been written on the use of genetic markers for the selection of animals with particular traits or for the identification of carriers of various genetic defects. Rather than adding a new one to the list I will try to focus the attention on some practical aspects of genetic testing.

Within the last couple of years my laboratory have mapped two recessive genetic disorders in cattle, including the gene for Complex Vertebral Malformation (CVM) in Holstein calves (Agerholm *et al.*, 2001) and Bovine Spinal Dysmyelination (BSD) in crossbred American Brown Swiss cattle (Nissen *et al.*, 2001). For CVM we initially developed a marker-based genetic test, which was soon followed by a gene-based test, both of which are being protected by patenting. The rights to perform the CVM test have been licensed to more than 10 laboratories all over the world. In the case of BSD we have developed a marker-based test which are freely available (Nissen *et al.*, 2001).

Since the establishment of detailed genetic maps of the genomes of farm animals, the development of new marker-based genetic test are becoming scientifically trivial and mainly dependent upon research funding and availability of DNA from animals segregating the traits of interest. Gene-based tests are still difficult to develop, but with the progress, which are being made in the sequencing of the bovine and porcine genomes, this will soon improve and a large number of new tests will be developed in the next couple of years. However, the experiences from our CVM and BSD projects shows that there are limitations at a more practical level that need to be considered before the emergence of a great number of new genetic tests, these are related to decisions about what animals should be tested?, what consequences should be taken from the result of the test? And importantly, how is it possible to ensure that the proprietary rights of the inventors of the genetic tests and associated funding agencies are protected.

When to use a genetic test and what to do with the result.

Before the emergence of gene technology, breeders have been limited to the removal of animals, which are expected carriers of genetic disease, through the analysis of pedigrees of affected animals. This strategy is merely useful for limiting the amount of damage done, since it will only be effective for genetic disorders where the frequency of the disease allele is relatively high. Testcrosses, where bulls are used on a panel of known carrier cows, represent a costly but efficient alternative. This approach largely prevents the birth of affected calves, but it does not permanently eliminate the disease allele from the population as well as long term costs are significant.

With the introduction of DNA-based detection of carriers of recessive defect or of carriers of advantageous QTL-alleles, breeders have obtained a new and very efficient tool.

Though seemingly simple to use when only a few genetic tests are available, it becomes progressively more difficult when trying to use a multitude of genetic tests as the basis for selection. At present, almost all animals, which are tested positive for either BLAD or CVM, are eliminated from breeding. This might be an appropriate action in the situation where the allele frequency of both disease-genes are relatively high and where the cost of genetic testing prevents the testing of most of the cows in the population to prevent the births of affected calves. However, when more test come available, the breeder will have to get used to the fact that nobody is perfect, every animal will have its flaws. In a near future it can be envisioned that top-bulls will be subjected to an extensive analysis of their genome, either by massive SNP typing or by partial sequencing of coding regions of the genome. Such an analysis will almost invariably lead to the discovery of recessive genetic variation of undesired character. When confronted with a battery of genetic tests the breeders will have to establish the principles for deciding which tests to apply on which animals and importantly, what consequences should be taken from the results of the test. The practice that will develop will be strongly influenced by the cost of genotyping, since the genotyping of the cow population will allow for "informed" matings where combinations of animals which doesn't carry similar disease alleles are chosen.

Protection of intellectual ownership.

Many research laboratories involved in the mapping of genes underlying heritable diseases and economic traits depend upon funding from commercial sources like breeding organisations etc. In order to attract funding for a particular project it is becoming a frequent demand that the project at least has a potential of generating revenue from the patenting of discoveries.

However, it is an increasing problem that in the real life of animal genetics, patenting does not guaranty protection of a genetic test. It seems to be much to simple for any small private or academic research laboratory to increase their income by doing genetic testing for local breeders or breeding associations, without considering the issue of proprietary rights. The size of the operation of these criminal activities is often to small to warrant legal action to be taken. Another potential leak in the protection of proprietary rights, is the fact that closely linked markers can be identified and often haplotypes can be found, which are in almost complete disequilibrium with the genetic trait of interest and therefore are almost equally suited for use in genetic test procedures. When these haplotypes has been established it is very hard to prove that they were identified with the help of information protected by the patent.

At present, the only realistic way to protect the rights to genetic tests in farm animals seem to be by withholding the molecular information like the gene name, chromosomal position etc. This however, only provides protection on a short term since its should be followed up by patent protection, and thereby disclosure, because of the risk of others patenting the same discovery. For many reasons this is an unacceptable approach. For the scientists, it hinders the publication of research papers, which is an essential part of the academic accreditation system and for the end-users of the genetic testing; it prevents the assessment of the validity and scientific quality of the test.

This paper is a small collection of experiences from a relative newcomer to the field of animal genetic testing and it does not pretend to be exhaustive in the coverage of the problems and challenges associated with this field. However, the expectations to the future economic importance of this field calls for a broad discussion of these issues in the relevant organisations of both veterinarians, breeders and scientists as well as the organisations funding and supporting the scientific discoveries in the area of animal genetics.

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SUCCESSFUL PREGNANCIES IN COWS FOLLOWING DOUBLE FREEZING OF A LARGE VOLUME OF SEMEN

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Bull semen Cryobanking

Cryobanking of semen has had a major impact on dairy cattle genetic breeding

In addition to its role in young bull genetic breeding, cryobanking of bull semen is an important backup for sufficient insemination doses in cases of disease, infertility or mortality.

Freezing and storage of semen is done regularly using mini (¼cc) or midi (½ cc) straws. However, cryobanking of a large number of straws is time consuming, expensive and requires a lot of storage space and liquid nitrogen. An alternative procedure which will reduce these expenses could be the freezing of a whole ejaculate in one test tube (12ml) and only when needed (when the bull is a "proven bull") the test tube will be thawed and then be refrozen in regular mini straws. We describe here the use of a new technology for large volume (whole ejaculate) freezing/thawing and refreezing in mini-straws.

MTG technology

Our novel freezing technology is based on "Multi-thermal gradient (MTG^R,IMT, Israel) (1)" directional solidification and is used mainly for freezing sperm and large tissue. The semen in the test tube is moved at a constant velocity (V) through a linear temperature gradient (G) so the cooling rate (G x V) and ice front propagation are precisely controlled (Fig. 1).

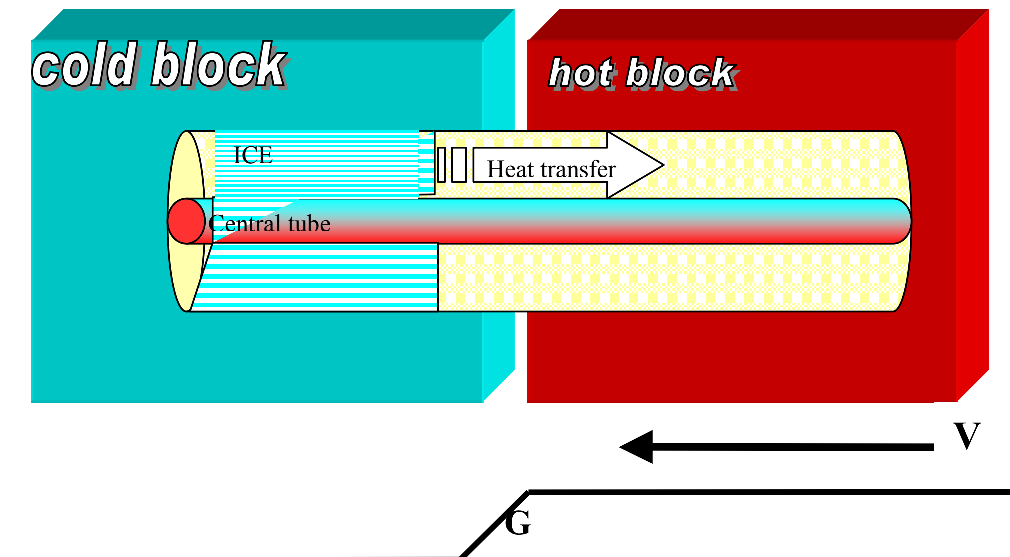

Fig. 1 Schematic design of the MTG freezing

This method also enables the incorporation of controlled seeding into the freezing process. When any liquid is cooled below its freezing point, it remains a liquid, in an unstable super-cooled state, until freezing starts at randomly distributed nucleation sites and spreads throughout the entire volume of the liquid. As discussed above, in the conventional equiaxed method of freezing, ice grows with uncontrolled velocity and morphology, and may disrupt and kill the cells of the samples. Ideally, the velocity of the freezing front should be such that the ice morphology does not disrupt the cells or tissue. However, the rate of cooling appropriate for favorable ice morphology may not be appropriate for other desired outcomes of a sample's freezing protocol. The laterally varying gradient used in our technology allows cooling to proceed at differing rates under varied temperature regimes, thereby facilitating full control over nucleation and ice crystal morphology. This technique allows very precise control of the cooling rate (0.01 to 1000°C/minute) within a large volume.

The freezing apparatus can control ice crystal propagation by changing the thermal gradient (G) or the liquid-ice interface velocity (V) and so optimizing the ice crystal morphology during freezing of cells and tissue. The rate of cooling also affects the morphology of the intercellular ice crystals (3): morphologies such as closely packed needles kill cells by external mechanical damage (unpublished observation). Thus, maximizing the survival rate of cells subjected to freezing and thawing requires careful control of the freezing process i.e. interface velocity. Using a cryomicroscopy observation we found that survival of sperm shows biphasic curve where at a very slow velocity ice will grow in a planner form which will kill all cells. At higher velocity ice crystals will form secondary branches and survival will increase, however at higher velocity (i.e. 300µm /sec) ice will start to form "needle-like" ice crystals which will decrease PTM, but in a higher velocity will permit very high survival (fig. 2) depending on the space between the ice crystals (4). Finally, at very high velocity (i.e. >3000µm /sec), directional solidification will not occur and survival will decrease.

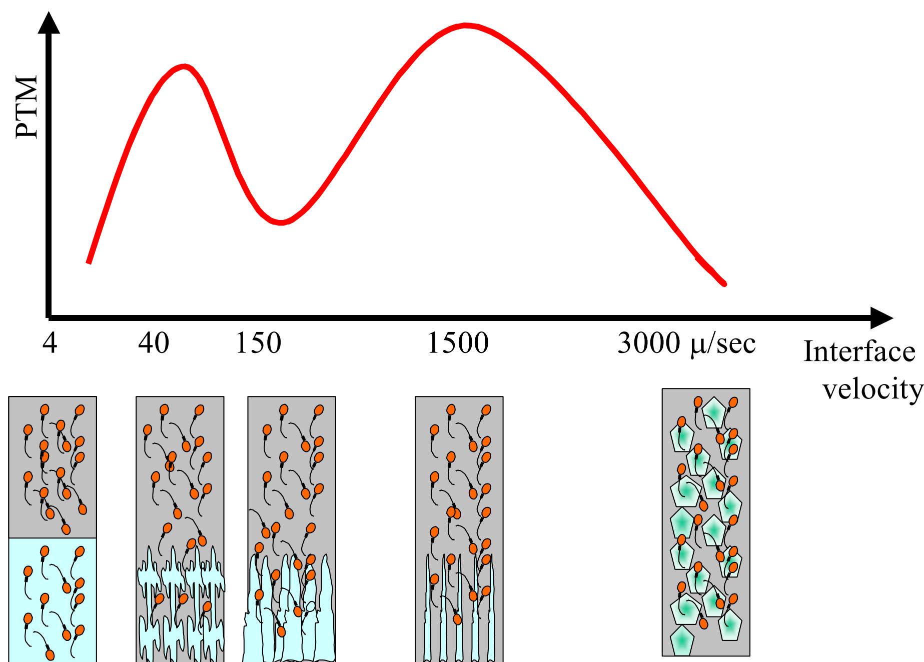

Fig. 2 Effect of interface velocity (V) on ice crystals morphology and sperm post thaw motility (PTM)

Heat transfer problems associated with large volume freezing

In a conventional slow-freezing method, temperature of the chamber is dropped in a controlled stepwise manner. This method is based on using multidirectional (equiaxed) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity and geometrical shape of the container and of the biological material within it (5). The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable. Furthermore, the ambient temperature gradients within the freezing chamber and the unreliability of temperature recording measurements (6) add to the difficulty of achieving the optimal cooling rate in a large volume sample.

Cryobanking of large volume semen

Each of the ejaculate was tested for semen concentration and motility (>70%) before dilution. We used AndroMed® (minitub, Hauptstrabe, Germany) for the semen dilution to have a final concentration of $15x10^6$ sperm/ml.

Freezing of a whole ejaculate was done in a special test tube (12ml) in which the central part is a hollow channel. Heat transfer is opposite to the test tube movement and is parallel to the tube length axis (fig. 1). The empty channel in the middle of the large test tube facilitate directional freezing and rapid thawing in the inner side of the test tube.

Sperm PTM after freezing in a large volume was very high. We found a survival rate of 90-100% (normalized PTM) in the two bulls we cryopreserved in the MTG technique. These results were superior to MTG freezing using mini straws (data not shown), which suggest the benefit of using MTG freezing of large volume for sperm cryopreservation. Results shows a 75±5% post thaw motility after freezing a 12ml test tube and 50±5% after second freezing/thawing in a mini-straws, respectively. Controlled vapour freezing showed a 60±10% post thaw motility which were lower then the results after MTG freezing of mini straws.

The large volume freezing may be very useful for cryobanking of bull semen, for example, AI centre that have a bank of 10,000 straws which are made from 25 ejaculates (400 straws /ejaculate). We calculated that these 10,000 straws will fit into **13 goblets** (750 straw/goblet). In comparison, when we freeze a large volume (12ml test tube) the 25 ejaculates will be frozen in 25 test tubes which will be stored only in **2 goblets**. This means that we need 6.5 time more goblets using straws in comparision to test tube freezing. In this case, the present method gives a capability to have a bank of "waiting bulls" in some of the AI centers which presently do not use a semen cryobanking. In addition this method will save money in labour and consumables (filling, printing, LN for freezing and for storage etc.).

In conclusion, the MTG technique could be very useful for large volume cryopreservation and double freezing for sperm cryobanking.

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THE MONITORING OF BOVINE PREGNANCIES DERIVED FROM TRANSPLANTATION OF *IN VITRO* PRODUCED EMBRYOS

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Introduction

In cattle breeding, the interest to collect data on the course of a pregnancy in individual females generally subsides, once a positive pregnancy diagnosis has been made within two months after mating or artificial insemination. The same is true for pregnancies obtained after embryo transfer, despite the fact that sometimes considerable efforts and costs had to be made to reach a pregnancy on the first place. Attention for the pregnant females is only resumed at the time of calving, when it needs to be judged if delivery can take place spontaneously or should be accomplished by artificial means to optimize the chances for survival of the calf. Observations and data connecting (pathophysiological) events during (early) pregnancy and parturition with the morbidity and performance of calves at later life, are not routinely available. However, such an approach appears to become more relevant against the background of the recently pronounced concept (Barker, 1994, 2000; Barker and Clark, 1997; Ravelli *et al*., 1998) that several diseases encountered during adult life might have their origin during the period of foetal development. The same might be true for physical performance and (re)production in domestic species (Maxfield *et al*., 1998a,b; Maltin *et al*., 2001; Rhind *et al*., 2001). In this respect one seems to have forgotten that, within the context of research on growth and production of farm animals, Grahm Everitt already warned in 1968 that "The extent to which events of later life may be modified by factors operating during the formative stages appears insufficiently appreciated" (see Bell, 1992). The so-called "Large Calf Syndrome" (Young *et al.*, 1998; Boerjan *et al*., 2000; Greve and Jacobson, 2001) might be an undiserable example of such types of long term effects.

Early reports on increased embryonic and foetal losses and the variable occurrence of deviant foetal development during pregnancies obtained from *in vitro* produced embryos (reviewed by Kruip en den Daas, 1997), have stimulated the interest for research on prenatal development of farm animal species, especially in ruminants. Besides many (ongoing) studies to unravel the cellular and molecular mechanisms of deviant development of IVF-derived and cloned embryos (Young *et al.*, 1998; Sinclair *et al*., 2000; Cross, 2001, Niemann and Wrenzycki, 2000), several authors have reported on placental and foetal growth and pathophysiological events during the perinatal period (Farin *et al.*, 1995, 2001a, Garry *et al*., 1996; Schmidt *et al*., 1996; van Wagtendonk-de Leeuw *et al*., 1998, 2000; Jacobson *et al*., 2000a,b; Sangild *et al*., 2000; Heyman *et al*., 2002; Renard *et al*., 2002). The bovine foetus and placenta are not easely accessible for (on farm) investigations, especially during the second and third trimester of gestation.Yet, there is a need for more intensive monitoring of pregnancies, not only to collect more accurate information on the exact timing of prenatal losses, but also to document or predict deviations in prenatal development. Such approaches will contribute to the understanding of the mechanism of disturbed development and will allow timely clinical interventions, either during pregnancy or at parturition. This contribution will therefore concentrate on late embryonic, foetal and

perinatal losses resulting from pregnancies derived from transfers of IVF-derived and cloned embryos, with a special focus on methods by which such pregnancies can be more intensively monitored. Own data from a field study, in which we compared the course and outcome of IVP and MOET pregnancies, will be presented as well.

The embryonic and early foetal period

Pregnancies after transfer of IVP-embryos

When recipients show a regular return to oestrus (between 19 and 22 days after their first oestrus), it can be assumed that early embryonic mortality occurred, i.e. before maternal recognition of pregnancy took place. Both after A.I. and embryo transfer of *in vivo* or *in vitro* produced embryos, high rates of such early embryonic losses have been reported (Stubbings and Walton, 1986; Markette *et al.*, 1985; Reichenbach *et al.*, 1992; Farin and Farin, 1995, Hasler *et al.*, 1995), especially when quality grade 2 embryos had been used for transfer. Recently, Heyman *et al* (2002) concluded on the basis of plasma progesterone levels, that on day 21 no significant differences in the percentages of presumed pregnancies existed between groups of recipients to which either cloned or IVF embryos had been transplanted.

Immediately after this period with regular returns, the presence of a pregnancy can be presumed (on the basis of plasma progesterone profiles or irregular returns) or diagnosed for the first time (by means of ultrasonography or measurements of plasma pregnancy proteins). When a positive early pregnancy diagnosis is followed by a return to oestrus between days 23 and 42, late embryonic mortality has occurred. Under field conditions, however, the first pregnancy diagnosis usually takes place only during the foetal period, to avoid any interference with a possibly pregnant animal. This means that it is usually very difficult to derive accurate figures on early and late embryonic mortality from published data on pregnancy rates. In their world-wide retrospective review on results from transfers of IVP embryos, Kruip and den Daas (1998) reported that while 70% of the embryonic losses after AI or ET occurred within the first 21 days of fertilization, this was only 58% for IVP embryos. This might be associated with a more strict selection of good quality IVP blastocysts. A more detailed picture can be obtained from the data published by van Wagtendonk *et al.* (2000). These authors compared return rates and pregnancy outcome after (single) transfers of either a MOET, IVP-coculture or IVP-SOF embryo (table 1):

Table 1. Returns to oestrus after transfer of a single MOET, IVP-Coculture or IVP-SOF embryo to recipients of the herd of Holland Genetics (after van Wagtendonk-de Leeuw *et al.*, 2000).

	MOET (n = 465)	IVP-co-culture (n = 157)	IVP-SOF (n = 101)
Total return (%)	54.4	51.5	46.1
Returns between days 0-31 as % of total returns:	80.6	68.2	80.9
Returns between days 24-31 as % of day 0-31 returns:	17.2	31.0	13.2
Returns between days 32-52 as % of total returns:	13.1	20.0	10.6
Returns between beyond Day 52 as % of total returns:	6.3	11.8	8.5

Total return rates were not significantly different between the three groups, but return rate between 0 and 31 days was higher in recipients with a MOET or 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 52 were also higher in this group (though not significantly different). In a recent paper (Lopez-Gatius *et al.*, 2002) pregnancy losses occurring between days 38 and 90 were found to be 11.2% and 9.9% for lactating dairy cows, inseminated after spontaneous or synchronized oestrus respectively.

In a recent field study, we monitored groups of recipients with similar types of embryos (for details of production: see van Wagtendonk-de Leeuw *et al.*, 2000) by regularly taking blood samples between day 7 and day 119 after embryo transfer. Around day 35 transrectal ultrasonography took place for pregnancy diagnosis and in 65 of these recipients ultrasonography was repeated at weekly intervals for foetometry and measurements of foetal heart rate (see below). Data are presented in table 2.

Table 2. Early and late returns to oestrus of recipients to which either a MOET, IVP-coculture or IVP-SOF embryo had been transferred (Perényi *et al*, in preparation)

	MOET (n = 118)	IVP-co-culture (n = 44)	IVP-SOF (n = 106)
Total % pregnancy failures:	55.1	52.3	63.2
Returns before day 24 as % of total pregnancy failures:	54.8	56.5	58.2
Returns between days 24 and 119 as % of total pregnancy failures:	38.7	34.8	41.8
Returns beyond day 119 as % of total pregnancy failures:	6.5	4.5	0

Apart from the fact that no late foetal losses occurred in recipients with an IVP-SOF embryo, there were no differences between the three groups as to the relative proportions of early embryonic losses (before day 24) and late embryonic+early fetal losses (between days 24 and 119). Overall, about one third of all transfers failed before day 24, while slighly more than one fifth of the transfers resulted in losses between days 24 and 119.

On the basis of plasma progesterone (P4: see Dieleman and Bevers, 1987) and PAG1 (see Zoli *et al.*, 1992) profiles of these animals, we were able to analyse the pregnancy failures between day 24 and 119 in more detail. By using plasma levels from ongoing pregnancies with calvings at term as reference values, decreases of P4 and PAG1 levels below one tailed 95% confidence intervals resulted in the attribution of each case of pregnancy failure with a complete data file (n = 56) to one of the following three groups:

A: recipients in which a drop in P4 occured before the plasma PAG1 level decreased;

B: recipients in which a decrease of the PAG1 level occurred before a decline of P4;

C: recipients in which decreases occurred at (about) the same time.

Typical examples of plasma profiles from recipients of group A and B are presented in figures 1A and B.

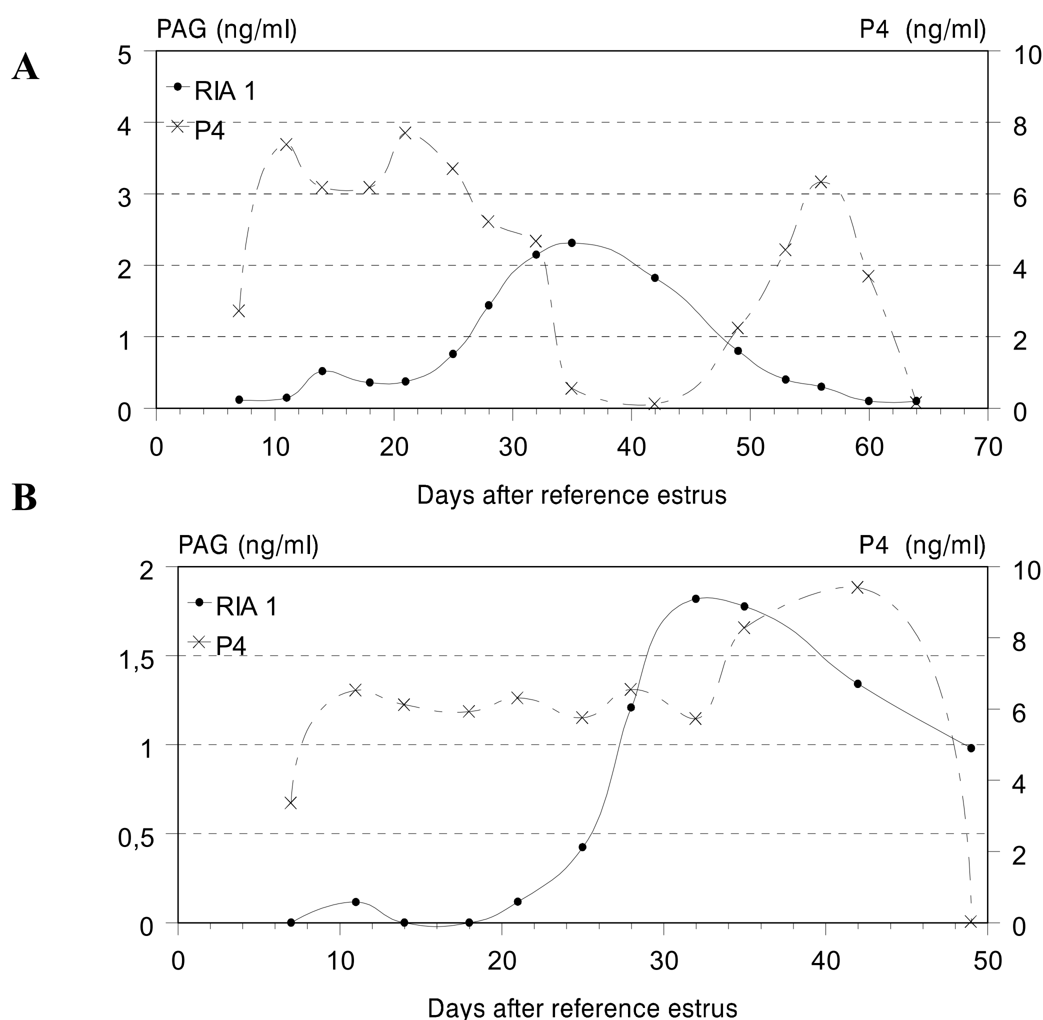

Figure 1. Plasma progesterone (P4) and PAG1 levels in two cases with an early pregnancy loss. **A:** a recipient, pregnant after transfer of a MOETembryo but returning to oestrus on day 42 after the reference oestrus; a decline of plasma P4 preceeds a decrease in PAG1 levels; **B**: a recipient, pregnant after transfer of a IVP-SOF embryo but returning to oestrus on day 49 after reference oestrus; a decline in the PAG1 level preceeds the drop in P4 (Perényi, 2002).

Table 3 presents the distribution of these three types of pregnancy failures, occurring between day 24 and 119, over the three groups of recipients:

Table 3: Distribution of three types of pregnancy losses, occuring between days 24 and 119, amongst three categories of recipients

	MOET	IVP-coculture	IVP-SOF
Type A pregnancy loss (n = 36)	19 (53%)	3 (8%)	14 (39%)
Type B pregnancy loss (n = 16)	5 (31%)	2 (13%)	9 (56%)
Type C pregnancy loss (n = 4)	0	1	3

These data indicate that while type A occurred at an almost equal rate in recipients with MOET (53%) and IVP-embryos (47%), type B losses occurred two times more frequently in recipients with IVP embryos (69% versus 31%). If one accepts that PAG1 profiles reflect the viability of the trophoblast/placenta (Szenci *et al.*, 1998;2000), this would implicate that in pregnancies which have already passed the stage of maternal recognition of pregnancy, failures after transfer of IVP embryos are more often the result of disturbed conceptus development than failures occuring after transfer of MOET embryos. In this respect it is interesting to note that a lack of vascularization of the allantois has been more often associated with conceptuses derived from *in vitro* produced ruminant embryos (Peterson *et al.*, 2000; De Sousa *et al.*, 2001).

We were not able to detect significant differences in plasma P4 and PAG1 levels between the three groups of recipients during the first 4 months of ongoing pregnancies (Vos *et al.*, 2001), but we neither found differences between the mean birth weights of calves from these three groups. Yet, an increased foetal weight (Farin *et al.*, 1995) and increased placental weight (Farin *et al.*, 1995, Bertolini and Anderson, 2002) have been reported already during midgestation in recipients with IVP embryos. It appears that the dramatically increased plasma levels of PAG during the final weeks of gestation are far more better correlated with foetal weight (Schmidt *et al.*, 1996) than the levels during early pregnancy. However, it remains to be investigated to what extend the reduced numbers of placentomes, but with a much larger individual size, which have been found in IVP pregnancies by Bertolini and Anderson (2002), do affect plasma PAG levels. Compared to placentas from foetuses derived from *in vivo* embryos, relative abundance of binucleate cells, by which the pregnancy proteins are produced, have been found in the placentas from *in vitro* embryos on day 63 (Farin *et al.* 2001b), although the same authors had previously reported a lower volume densities of binucleate cells in placentas of IVP foetuses at day 222 (Farin *et al.*, 2000). There is clearly a need for more detailed studies on possible relationships between gross morphology (number and size of placentomes), vascularization, cellular differentiation and production of proteins and hormones of the bovine placenta during normal A.I., MOET and IVP pregnancies (see also below).

The observations on early losses reported above, urgue for an even more intensive monitoring of early IVP-pregnancies. While it has been described (Curran *et al.*, 1986a; Boyd *et al.* 1988) that transrectal ultrasonography can be used to visualize details of bovine conceptus at very early stages, reliable quantification of ultrasonographic details of conceptus development is only possible at a later stage (Curran, 1986b; Kahn, 1994; Ginther, 1998). Especially measurements of foetal body structures (CRL:crown rump length; BPD:biparietal diameter of the cranium; CAU: cross section of the abdomen) can be expected to reflect both retardation or enhancement of foetal growth. Because it had been frequently reported that pregnancies derived from IVP embryos had a higher incidence of both prenatal losses and resulted more often in heavy foetuses and offspring, we performed repeated ultrasonographic measurements of foetuses between days 35 and 119 to compare foetal development in pregnancies resulting from transfer of MOET, IVP-coculture and IVP-SOF embryos. Reports from early pregnancies in women suggested that deviations from normal foetal heart rate (FHR) may be predictive for future foetal losses or for foetuses with chromosomal abnormaliities (Schats *et al.*, 1990; Tannirandom *et al.*, 2000). Reports on abnormal vascularization of the allantois during early pregnancy (Peterson *et al.*, 2000; De Sousa *et al.*, 2001) and deviant cardiac structure and function, reported in some foetal and newborn ruminants resulting from IVP-embryos (Sinclair *et al.*, 1999; Hill *et al.*, 1999; Van Wagtendonk-de Leeuw *et al.*, 2000) ask for more studies on prenatal cardiovascular function. We therefore also repeatedly measured FHR in foetuses of our three groups of bovine pregnancies (Breukelman *et al.*, 2001). At weekly intervals, transrectal scans were video-taped and measurements of BPD and CAU (at the insertion of the umbilical cord) and calculations of FHR were performed afterwards. It appeared that repeated and reliable measurements of CRL of the same foetus were less often possible with transrectal scannnig than the other two types of foetometry. Results

demonstrated that no significant differences in BPD, CAU and FHR existed between the three groups of foetuses during months 2-4 of pregnancy. Final birth weights of the calves appeared to have no effect when included in the analysis of the last measurements that had been performed on these foetuses.

FHR data are presented in figure 2.

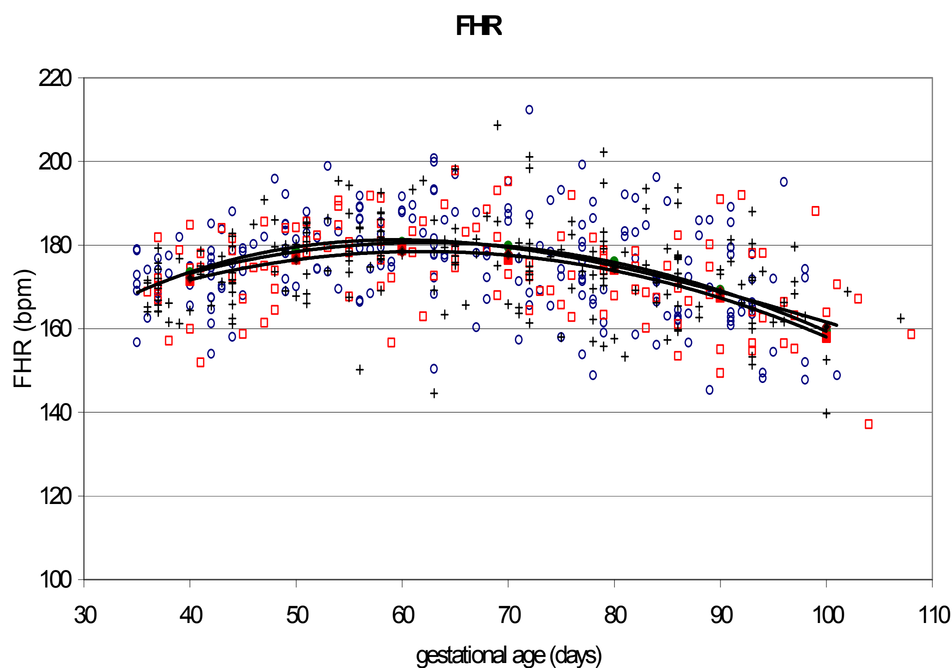

Figure 2: Plots of individual (open symbols) calculated Fetal Heart Rates and LS-means (closed symbols and with lines of best fit) for three groups of foetuses; circles: MOETfoetuses (n = 25; squares: IVP-coculture foetuses (n = 14); rhombs: IVP-SOF foetuses (n = 22) (Breukelman, 2000)

Changes in FHR followed a parabolic curve, different from initial data published by Curran *et al* (1986b), but comparable to the curve that was based on a limited group of A.I. foetuses (Ginther, 1998). Plots of trend lines for FHR of our individual foetuses demonstrated that in some of the pregnancies that did not proceed until term, FHR was outside the 95% confidence intervals. This indicates that it might be useful to further explore FHR, based on more frequently performed measurements, as an early indicator of future foetal death in the bovine.

Pregnancies after transfer of cloned embryos

Because embryonic and foetal losses are considerably higher and abnormal foetal development and increased birth weight occur more often in pregnancies after transfer of cloned embryos (Kruip and den Daas, 1997; Wilson *et al*, 1995; Renard *et al.*, 2002.), it appears even more appropriate to use intensive ultrasonographic and biochemical monitoring in recipients of cloned embryos. In a recent paper from France (Heyman *et al*., 2002) such screening was performed during pregnancies obtained

after transfer of cloned (somatic adult, somatic foetal or embryonic) and IVF-coculture embryos. As already indicated above, early embryonic losses did not appear to be increased after transfer of cloned embryos, as judged by plasma progesterone levels on day 21. However, at the time of the first and the second transrectal ultrasound scan, significantly less recipients with somatic clones appeared pregnant. Data on the percentage of pregnant animals at different stages of the first trimester and the final calving rates are summarized in table 4:

Table 4. Data on pregnancies of recipients to which either a cloned embryo (3 different types) or an IVF-coculture embryo had been transferred (after Heyman *et al.*, 2002).

	Embryos cloned from somatic adult cells (n = 133)	Embryos cloned from somatic fetal cells (n = 40)	Embryos cloned from embryonic cells (n = 67)	Control IVF-cocult embryos (n = 51)
% presumed pregnant D21	55.6	57.5	62.6	62.7
% found pregnant D35	33.8	27.5	49.2	52.9
% found pregnant D50	27.1	22.5	41.8	50.9
% found pregnant D70	14.3	22.5	37.3	49.0
% found pregnant D90	12.0	22.5	34.3	47.0
% calves at term	6.8	15.0	34.3	49.0

Foetal survival decreased dramatically, especially in the recipients who received an embryo cloned from adult somatic cells. Foetal deaths were not observed beyond day 90 in recipients of the control IVF or embryonic cloned groups. Similar to our own findings on PAG1 levels during ongoing pregnancies of IVP and MOET, no significant differences in plasma PSP60 levels were found between the 4 groups of pregnancies that produced live, full term calves. Interestingly, plasma PSP60 levels on day 50 in recipients with somatic clones which lost their conceptus between days 50 and 90, were already significantly higher than in recipients with surviving somatic and subsequently nonsurving embryonic clones. This would mean that the trophoblast of somatic clones is more likely to demonstrate aberrant early cellular differentiation, leading to increased numbers of binucleate cells. If this is directly associated with a less developed early vascularization, resulting in subsequent placental insufficiency and foetal death, remains to be investigated. At later stages (days 150, 180 and 210), recipients of somatic clones which had developed a severe hydroallantois, also had significantly elevated PSP60 levels as compared to nonpathological pregnancies of the control or cloned groups. Compared with IVF controls, increased size of placentomes (ultrasonographically measured at intervals of 2 weeks) was found in recipients with somatic clones, but this was not reflected in altered foetal functions like counted FHR and growth of diameter of the foetal aorta.

The late foetal period

Direct monitoring of foetal functions is very difficult during the second half of gestation in the bovine. Due to the large size of the foetus, its variable intrabdominal position and the limited penetration depth of ultrasound transducers, access to foetal body structures becomes rather limited, especially during the final two months of gestation. This means that growth curves based on ultrasonographic measurements of foetal structures usually finish around the end of the second trimester (for details see: Kähn, 1994; Ginther, 1998). Although measurements of the diameter of the

foetal eye can be used to estimate foetal age, there is no evidence as to whether this parameter accurately reflects differences in foetal growth. So conclusions on deviations of foetal growth during later stages of development have to be based on either transversal observations of collected foetuses or on measurements in newborns. Under farm conditions, prenatal identification of abnormal intrauterine development is more likely indicated by the recognition (by external features, ultrasound observation and transrectal examination) of recipients with hydroallantois, a pathological which has been reported more often in cows pregnant from IVP embryos (Kruip and den Daas, 1997; Heyman *et al*., 2002). Additional monitoring of the course of pregnancy and placental and foetal well being has to rely on measurements of proteins (different PAG's, PSPB's) and hormones (progesteron, conjugated and unconjugated oestrogens, prostaglandin metabolites, cortisol, placental lactogen) in maternal plasma (Kindahl *et al*., 2002), although their value for the prediction of abnormal foetal development or foetal distress needs further exploration.

Prenatal, ultrasound-guided puncture of foetal fluids has been applied during early stages of bovine pregnancies (Vos *et al*., 1990; Garcia and Salaheddine, 1997), but reports on fluid aspiration during late gestation are not available and might be judged to be too risky. Under experimental conditions a surgical method (installing catheters in foetal umbilical vessels) was used to investigate differences between IVP and A.I. control foetus with respect to blood chemistry and hormone levels during the final days before delivery (Sangild *et al*., 2000). While IVP foetuses had elevated hemoglobin and (as a consequence a higher oxygen content) and lowered lactate levels compared to A.I. foetuses in arterial samples, there were no differences between the two groups in arterial oxygen saturation, foetal glucose tolerance, blood cortisol levels and the response to ACTH. In fact none of the observed differences pointed to a poor preparation of IVP foetuses to extrauterine life, in contrast to the clinical findings reported earlier for several newborn cloned calves by Garry *et al.* (1996). Because both an increased limb length (Jacobson *et al*., 2000b) and a high incidence of flexural deformities of the limbs (Garry *et al*., 1996) have been observed in newborn calves derived from *in vitro* produced embryos, it appears very relevant to explore foetal motility (Ginther, 1998) during IVP pregnancies. Prenatal restriction of articular mobility is associated with flexural abnormalities in the cow (van Huffel, 1990) and disturbed foetal mobility around calving could contribute (through abnormal foetal posture and/or position) to the higher rates of dystocia reported for IVP calvings. Also prolonged measurements of foetal heart rate (FHR) might be useful in this respect, because different FHR patterns have been associated with different so-called behavioural states, both in human and animal foetuses (for reviews see: Nijhuis, 1992). Significant differences in characteristics of FHR between acidotic and nonacidotic calves have been found during calving (Jonker *et al*., 1996), and continuous, transcutaneous Doppler measurements of prenatal FHR appeared feasible in the cow (Jonker *et al*., 1998). Preliminary data showed no differences in late gestational FHR characteristics between healthy A.I. and IVP foetuses (Breukelman, 2000), but the value of such measurements for the prediction of prenatal distress still needs to be assessed in the bovine.

Summarizing conclusions

While pregnancies obtained from transplantation of *in vitro* produced embryos suffer from increased (though variable) rates of embryonic, foetal and perinatal losses and developmental disturbances, it appears rather difficult, especially under farm conditions, to identify conceptuses with deviations in development at an early stage. Transversal studies, collecting conceptuses at different stages of gestation, demonstrated that significant differences of foetal and placentome weight and size may occur rather early in gestation, but noninvasive longitudinal ultrasonographic foetometry and countings of FHR did not reveal significant differences between ongoing pregnancies of MOET and IVP embryos during the first 4 months. Determinations of maternal plasma levels of placentally derived pregnancy proteins seem to be more valuable for a timely detection of abnormal development. For pregnancy losses between days 24 and 119, a decrease of protein levels preceeding rather than

following luteolysis, occurred more frequently in IVP than in MOET recipients of the same herd. This indicates that pregnancy failures after transfer of IVP embryos are more often the result of a failure of conceptus development. On the other hand, abnormally elevated levels of these proteins around day 50 were found to precede foetal losses in pregnancies from somatic clones, pointing to a disturbance of placental cellular differentiation. Limited access to the uterine contents restrict direct measurements of foetal and placental growth during the last trimester of gestation. Besides a close and regular clinical inspection of the dams and blood sampling for the monitoring of hormones and pregnancy proteins, transabdominal studies of foetal movements and FHR might improve prenatal diagnosis of abberrant foetal life in cows.

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SUPEROVULATION IN CATTLE: PRACTICAL ASPECTS OF GONADOTROPIN TREATMENT AND INSEMINATION

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Dose-response relations

Ovulation number and following number of recovered oocytes and embryos show dose-dependent pattern. This was partly or fully shown for hMG [47], eCG [25, 46, 56] and FSH [17, 21, 24, 42, 44, 48, 63]. Kanitz *et al.* [32] showed for FSH that with increasing doses the number of ovulations increased significantly until a plateau was reached for the parameter. It was not possible to increase ovulation number with further increasing FSH doses. Quite the reverse, very high FSH doses were followed by a depression in ovulation number (figure 1) as it was also shown for eCG [56]. The mechanisms responsible for the decrease in ovulation number are not well understood. Saumande and Chupin [57] speculate, that the depression of ovulations is more dependent on type of gonadotropin than on FSH/LH ratio. In our experiments we found, that high FSH doses can disturb ovulation process at two levels, at the level of the pituitary and at ovarian level. On the one hand the portion of animals with a LH surge decreased. On the other hand the number of follicles which ovulate after a LH surge decreased (table I). Increased progesterone concentrations during oestrus are involved, at least in the depression of LH surge by inhibiting the development of the positive estrogen feedback. Another reason for a reduced ovulation number may consist in a down-regulation of follicular FSH receptors by high doses of the homologous ligand. Results of a field trial with an uniform application and insemination schedule allow the conclusion that each FSH product has an optimal dose range (figure 2)

Bioactivity of FSH products

Different pharmaceutical products with gonadotropins as their active substance and moreover batches of one product can differ in their bioactivity and/or immunoactivity [4, 6, 18, 43]. The reasons consist in differences according to the specific protein content and in different bioactivities of the gonadotropin in dependence on degree of glycosilation of the molecules and on occurrence of isoforms [9, 15, 45, 62, 68, 80]. Although such differences are well known for many years no data have been available regarding their consequences for results of superovulation. We examined the bioactivity of four different batches of one commercially available FSH product with an *in vivo* bioassay [4]. The relative bioactivity was in the range from 78.9 % to 127.8 %. The four batches were used for superovulation. Dairy cows (n = 525) from day 60 to 90 after calving received eight injections of FSH (total amount 24 mg) in decreasing dose starting day 10 to 12 of the oestrous cycle. $PGF_{2\alpha}$ was given 48 and 60 h after the first FSH injection. Thereafter all cattle were time-oriented inseminated. FSH batches were used independent of the relative bioactivity. Results of superovulatory treatment were significantly influenced by the bioactivities of the batches (table II). The mean fertilisation rates did not differ significantly between groups.

FSH/LH ratio

FSH preparations produced from pituitaries contain beside FSH also other biological active substances like LH. The amounts of FSH and LH and the activities can be different between different batches of one product and between different products [19, 43, 55]. Determination of the amounts or activities is done with assays which recognise either the structure or the function of the hormones [49]. The results of determination of FSH and LH content in different FSH products or about the activities of the hormones can not be compared between products, because different assays and different standard hormones are often used. The more important question is the possible biological effect of the so called FSH/LH ratio. Donaldson *et al.* [18], Beckers [3], Chupin *et al.* [11], Gonzales *et al.* [24], Tribulo *et al.* [67], Yamamoto *et al.* [79] showed that FSH products with reduced LH content gave superior results according to ovulation number, fertilisation rate and embryo quality. It was speculated [29] that "high" LH activities cause luteinisation of follicles, receptor down regulation and premature ovulations. "Low" LH activities should cause deviation in oocyte maturation, fertilisation and early embryonic development [29]. Moreover, the "right" FSH/LH ratio should be able to reduce variability of superovulatory response. This was the background to demand FSH products with reduced LH content or highly purified FSH. But the above cited results were not uncontradicted [52, 71]. Because the effects of FSH/LH ratios were usually assessed in terms of the number or quality of recovered oocytes or embryos after superovulatory treatment we determined effects of gonadotropin preparations with different FSH/LH ratios on follicular growth and FSH binding of granulosa cells.

Crossbred heifers (n = 78) were randomly divided in 4 groups. Animals received purified porcine pituitary hormone preparations (Ovaset®, Sanofi) in a total amount (according NIH-P1 standard) of 0.29 mg FSH (FSH group), 0.29 mg FSH and 0.19 mg LH (FSH/LH group) or 0.19 mg LH (LH group) in a treatment of 6 injections on 3 consecutive days starting on day 10 of oestrous cycle. Untreated animals (buffered saline, 6 injections) with the same stage of cycle were used as control. Ovaries were recovered 76 h after the first injection. Follicles of all ovaries were counted and classified according to size. Altogether 1668 follicles from 49 animals were classified as atretic or nonatretic by a macroscopic method [40]. Granulosa cells from individual follicles (>2mm diameter) were analysed by flow cytometry for the detection of FSH and LH binding using an indirect quantitative immunofluorescence technique [FITC-labelled anti-hormone-antibodies (UCB) were used for staining after incubation of cells with ligands]. Statistical analysis were done by EPICS ELITE (Coulter) or by means of the Statistical Analysis Package SAS® [33]. The results are summarised below.

FSH and FSH/LH treatments increased significantly the number of medium and large follicles. LH treatment alone was not able to induce follicular growth (table III). FSH binding capacity on granulosa cells was significantly stimulated by the homologous ligand (Figure 3) whereas LH binding capacity was not significantly different between groups. Finally the portion of nonatretic follicles was significantly influenced by hormone treatment (table IV). LH is not necessary for superovulatory treatment, but it is also tolerated to a certain amount without negative effects on superovulatory response (table IV).

In the light of discussion about the effects of LH in FSH products the results of superovulation after application of recombinant FSH (rFSH) are interesting. Although the first report about results [72] was very encouraging following data [73] showed, that it was neither possible to increase the number of transferable embryos nor possible to decrease variance of results through application of rFSH. More recently Takagi *et al.* [66] investigated whether recombinant human FSH (rhFSH) without contaminating LH can exert a normal superovulation response in cows. One group of heifers (n = 9) was stimulated with rhFSH and another group (n = 9) was treated with equine chorionic gonadotrophin (eCG). Daily transrectal ultrasonography showed that eCG- and rhFSH-stimulated heifers had the same follicular growth characteristics and equal numbers of follicles >8 mm in

diameter after 3 days of stimulation. But there were considerable differences in steroid production between groups. rhFSH-treated heifers had lower oestradiol concentrations during the first days of stimulation and lower progesterone concentrations in the period after the LH surge than did eCG-stimulated animals. Moreover rhFSH-treated heifers had fewer LH pulses than did eCG-treated heifers and LH surge occurred later. Multiple ovulations occurred in only three of six rhFSH-treated heifers, but in all four eCG-treated heifers with an LH surge. At 24 h after the LH surge, the percentage of metaphase II stage oocytes with cortical granules distributed close to the oolemma was significantly lower in the rhFSH group (7.3%) than in the eCG group (55.9%). The authors concluded, that the final follicular maturation is impaired in heifers treated with rhFSH, which might be due to the combination of a lack of LH activity in the gonadotrophin preparation and the severe suppression of LH pulsatility. But it has to be considered, that the dose of rhFSH for superovulation of cattle was based on reported doses of human menopausal gonadotropin and rhFSH for human treatment. Moreover Gosselin *et al.* [26] found, that there was no significant correlation between estradiol or progesterone and LH pulse frequency, amplitude and mean concentrations at any time in control or superovulated animals. Their study demonstrates that superovulation treatment in the cow causes a rapid decrease in pulsatile release of LH and suggests that this effect is not mediated through the negative feedback actions of estradiol and progesterone.

Ovulation time and time-oriented insemination

To determine the ovulation period (i.e. first ovulation, last ovulation) different direct and indirect methods can be used. The most uncertain direct method is the rectal palpation. It allows only to recognize if the animal is in the process of ovulation. With some experience number of ovulations can be estimated roughly. The method of endoscopy is much better and allows the sure determination of the first ovulation. Unfortunately it is not possible to detect all ovulations, especially in the cases of high numbers of ovulations when blood is present on the surface of the ovaries during ovulation period. The use of ultrasound is the most less invasive method. But the first and moreover the last ovulation are not easy detectable. LH determination can be used as an indirect method because the time interval between LH surge and start of ovulation is relatively constant and shows in most of the papers no large variation. When FSH was used for stimulation of follicular growth the mean time intervals from induction of luteolysis to LH surge ranged from 44 to 48 h. The standard deviation for the parameter was between 2 and 12 h [27, 41, 75, 76]. We investigated also the time interval from induction of luteolysis to LH surge.

Therefore, altogether 39 heifers and cows were treated 8 times with FSH for 4 days starting between day 10 to 12 of oestrous cycle. $PGF_{2\alpha}$ was given 48 and 60 hours after the first FSH injection. All animals received jugular catheters. Blood samples were drawn via catheters every second hour for LH determination. The mean time interval form $PGF_{2\alpha}$ to LH surge was $46,5 \pm 4,4$ hours. It was independent of the factor 'heifer' or 'cow'. Based on a 6 hourly endoscopic observation of the ovaries of these animals we detected the start of ovulations 62 to 68 hours after induction of luteolysis. This is in agreement with observation done by others [1, 50]. The authors observed start of ovulations by means of ultrasound diagnosis 68 to 69 hours after induction of luteolysis. The standard deviation for the parameter was very small. In our investigations and in those of others [14, 41] it tooks 24 – 30 h from LH surge until start of ovulations. The time interval from the first to the last ovulation varied from 1.2 to 12 hours [1, 41, 50, 77].

Based on these data we developed the following scheme for a time-oriented insemination in heifers and cows (figure 4). The time necessary for capacitation of sperm cells from bulls (approximately 6 hours) is considered in the scheme. We investigated fertilisation rate after superovulatory treatment with FSH and time-oriented insemination. Altogether 180 cows were flushed one time after a single superovulatory treatment and 43 cows were flushed 145 times after repeated superovulatory treatment. All cows were inseminated according to data in figure 4. For each

insemination one straw with 20 million sperm cells was used. After thawing in water bath (38 ^{0}C, 15 seconds) insemination was done into the uterus. Flushing of the uterus was performed on day 7. Using the time-oriented insemination after superovulation of cows with FSH fertilisation rates were above 85 % after first or repeated superovulatory treatment (table V). The fertilisation rates obtained with time-oriented insemination were higher than those between 60 and 75 % normally reached under routine conditions [52, 69]. They are comparable with recent results from Dalton *et al.* [13]. The last authors reached 81 % fertilisation rate under experimental conditions in a relative small group of animals (n = 10 cows) with one insemination 24 h after standing heat. In contrast to our experiments 50 million sperm cells were used for one insemination.

Frequency of FSH application

In most cases FSH is given two times per day for four days [16, 36]. But there have been attempts to decrease the number of injections. Purwantara *et al.* [51] concluded from their experiments, that treatment with Folltropin once daily for 3 days gives a folliculogenic and superovulatory response similar to a treatment regimen where it is given twice daily for 4 days. Other data [36, 58, 61, 65, 78] suggest that the multiple intramuscularly injections of FSH can be replaced by a single injection, given subcutaneously. But data from Callejas [10] show, that a single injection of FSH-P was not sufficient enough to give the same results reached with eight injections of the same product. Superovulation can also be induced by a single injection of hMG [64]. Kelly *et al.* [36] investigated superovulatory response, estradiol concentrations and follicular populations in regard to single or multiple injections of FSH using two different FSH products. The authors found, that estradiol concentrations can be significantly influenced by frequency of FSH application and FSH product.

In our own experiments FSH application (Folltropin®, Vetrepharm, Canada) twice daily for 4 days was compared with application once a day. Superovulatory treatment was started in Simmental cows between day 8 to 13 of the oestrous cycle. Control animals (group 1) received 8 injections of FSH (total dose: 400 mg). Animals in group 2 and 3 received only one injection per day for four days. The total dose for FSH was 360 mg in group 2 and 260 mg in group 3. $PGF_{2\alpha}$ was given 72 and 82 hours after the first FSH injection. Insemination was done in the same way in all animals. Results indicate that FSH application once a day can be as efficient as twice daily injection (table VI).

Follicular population at the time of treatment

Response to superovulatory treatment is characterised by a high degree of variation. A review about various factors, which have been reported to influence the response after treatment, is given by Kafi and McGowan [31]. Several authors [28, 30, 48, 70] reported, that the presence of a dominant follicle on the day of initiation of superstimulation decreases the superovulatory response. Whereas ablation of the largest/dominant follicle prior to superovulatory treatment can increase superovulatory response [7, 37, 38, 59], but under field conditions results could not be repeated (39). The ablation of the two largest follicles was as efficient as the ablation of all follicles >5 mm in diameter [2]. In contrast to that Bergfelt *et al.* [5] and Diaz *et al.* [16] did not find a positive effect of follicle ablation on superovulatory response. From these results it was concluded, that exogenous FSH treatment appears to override any systemic inhibitory effect that a persistent dominant follicle may be exerting at the pituitary and possibly the ovary. The opposing results from the different studies can be partly explained by different time intervals between follicle ablation and superstimulation, by different time points for ablation (growing phase, static phase or decline phase of the dominant follicle) and by different criteria for evaluation of the results.

Several authors [8, 54] found that high number of potential recruitable follicles was associated with better superovulatory response. Kawamata [35] determined relationships between the number of

small follicles prior to superovulatory treatment and superovulatory response. A total of 55 superovulations were induced in Holstein cows. The ovaries were examined ultrasonographically once 0 - 1.5 days before the initiation of superovulatory treatment. The number of small follicles 3 – 6 mm in diameter on both ovaries before superovulatory treatment was found to be significantly correlated with the numbers of corpora lutea after superovulation ($r = 0.440$, $P<0.001$), total ova recovered ($r = 0.503$, $P<0.001$) and transferable embryos recovered ($r = 0.482$, $P<0.001$). More recently (Cushmann *et al.* [12] found, that the number of CL was correlated positively with total number of primordial, tertiary, and medium surface follicles. Number of CL was related to number of tertiary follicles in a positive linear manner and the number of medium follicles in a positive quadratic manner. In contrast to that Singh *et al.* [60] did not find any correlation between the number of follicles observed at the start of FSH treatment and the number of corpora lutea. Also Fernandez *et al.* [22] found no significant correlation between follicular population at the beginning of the treatment and number of ovulations and embryo yield. Drawing a conclusion from the above-cited results and from the knowledge about follicular dynamic in cattle [14, 20, 34, 53, 74] it can be expected that the ovulation number after superovulatory treatment depends on the number of follicles which respond to treatment with proliferation, differentiation and ovulation in a period of 120 hours. The quantity and quality of follicles in this pool (figure 5) is the main reason for variability of superovulatory response. Different results in the literature can be caused by methods for counting and classifiing the follicles (i.e. resolution limit of ultrasound).

Conclusions for practise

1. To maximize the number of transferable embryos the applied FSH dose has to be in a product specific optimal range. Comparisons between different FSH products according to this range are not useful because different standards and different assays for estimation of the FSH content or bioactivity of the product are used.
2. A reduced bioactivity of FSH should be considered as one possible reason for insufficient results of superovulatory treatment. Other factors have to be excluded before.
3. LH contaminations in FSH products are not necessary for successful superovulatory treatments, but contaminations are undoubtedly tolerated to a certain amount without negative effects on results.
4. Recombinant FSH has no advantage in regard to the mean number of transferable embryos and variance of superovulatory results in comparison to purified FSH from pituitaries. Other factors like BSE or other transmissible diseases are not considered in this conclusion.
5. The knowledge about ovulation period in superovulated cattle allows successful time-oriented insemination in heifers and cows. Two inseminations are sufficient for high fertilisation rates. It has to be considered, that the recommended time for time-oriented A.I. in figure 4 is valid, when luteolysis is induced 48 hours after start of gonadotropin treatment.
6. Ablation of the dominant follicle prior to superovulatory treatment can increase the number of transferable embryos. But this treatment cannot reduce variability after superstimulation, because the main reason for variation of ovulation number consist in the number of follicles, which respond to gonadotropins with proliferation, differentiation and ovulation in a period of 120 hours.
7. FSH application once a day can be as efficient as twice daily application. The application regime has to be tested for each product.

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Figure legends:

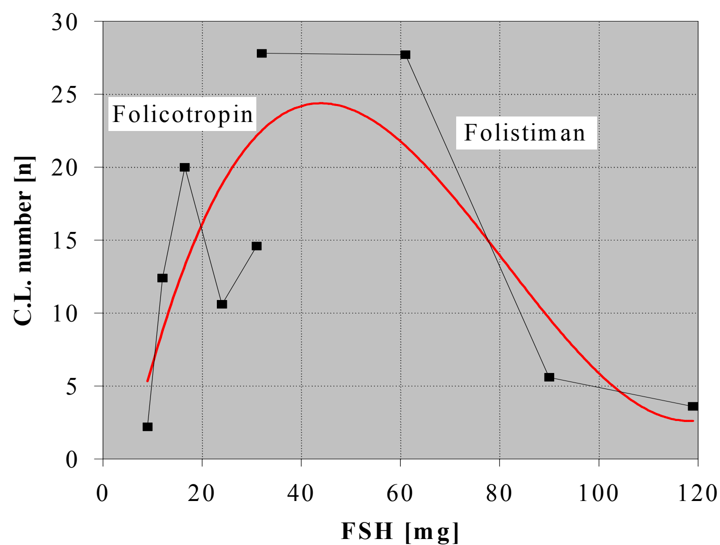
Figure 1: Polynom regression of 3rd degree for dose-response relations after FSH application in model studies (n = 5 animals per point)

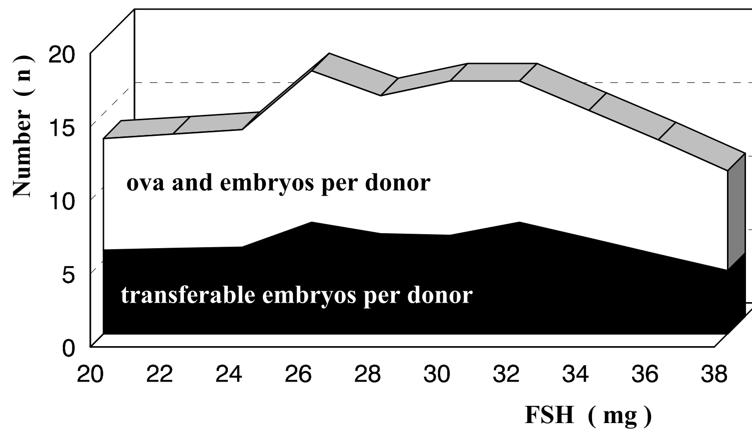
Figure 2: Dose-response relations after FSH application in a clinical study (n = 584 cows)

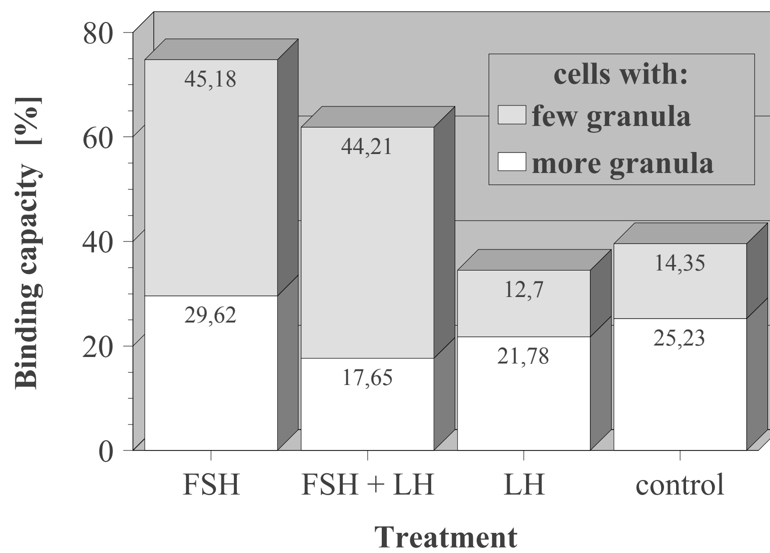
Figure 3: FSH binding capacity on granulosa cells after treatment of cattle with FSH, FSH/LH, LH or saline

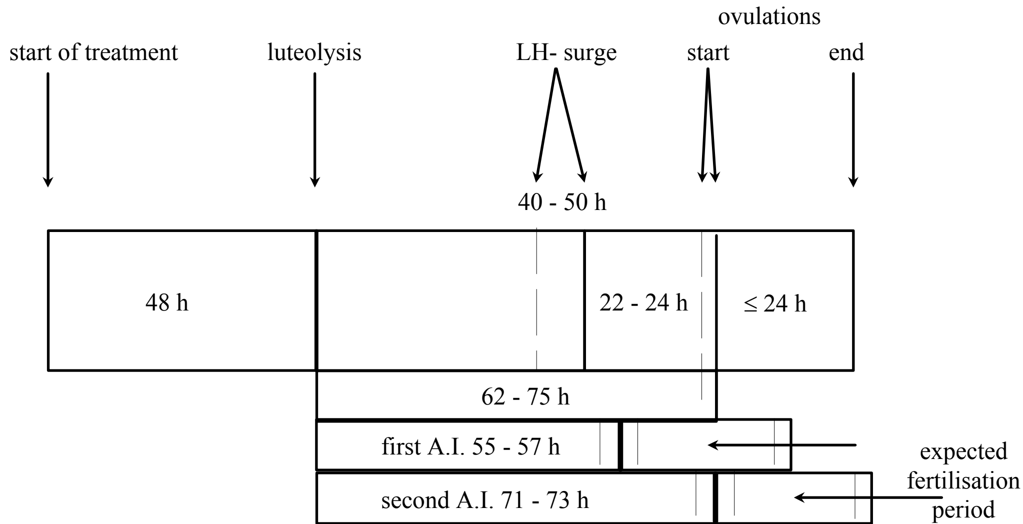
Figure 4: Scheme for time-oriented insemination in superovulated cattle

Figure 5: Follicular population in cattle




Number (n)
20
15
10
5
0
ova and embryos per donor
transferable embryos per donor
20
22
24
26
28
30
32
34
36
38
FSH (mg)


Binding capacity [%]
80
60
40
20
0
45,18
29,62
44,21
17,65
12,7
21,78
14,35
25,23
cells with:
few granula
more granula
FSH
FSH + LH
LH
control
Treatment


ovulations
start of treatment
luteolysis
LH- surge
start
end
40 - 50 h
48 h
22 - 24 h
≤ 24 h
62 - 75 h
first A.I. 55 - 57 h
second A.I. 71 - 73 h
expected fertilisation period

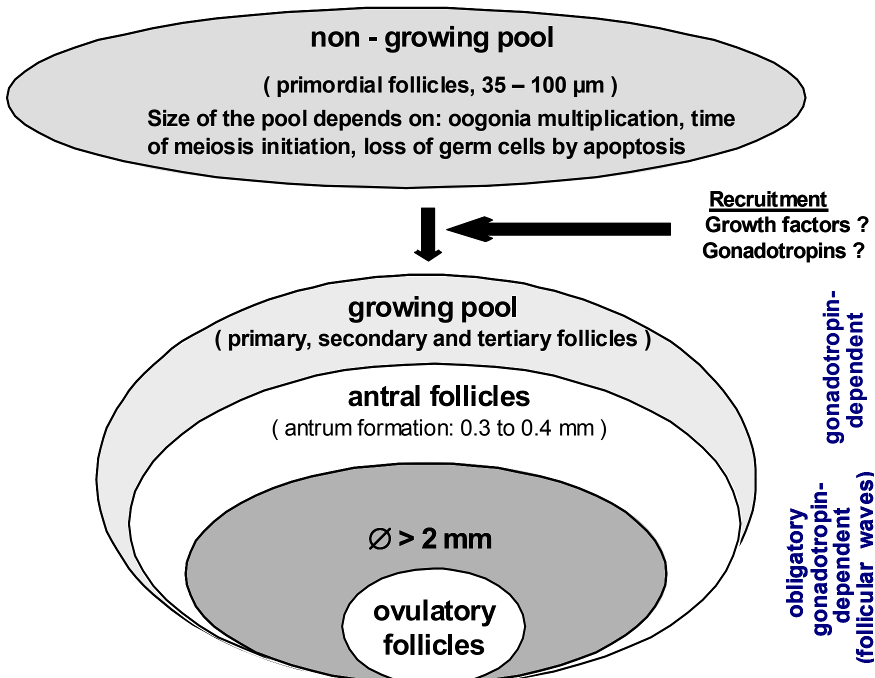

non - growing pool
(primordial follicles, 35 – 100 µm)
Size of the pool depends on: oogonia multiplication, time of meiosis initiation, loss of germ cells by apoptosis
Recruitment
Growth factors ?
Gonadotropins ?
growing pool
(primary, secondary and tertiary follicles)
antral follicles
(antrum formation: 0.3 to 0.4 mm)
∅ > 2 mm
ovulatory follicles
gonadotropin-dependent
obligatory gonadotropin-dependent (follicular waves)

Table I: Estradiol and progesterone concentrations, number of animalc with LH surge and ovulation number in dependence on FSH dose

FSH (mg)	Animals (n)	E2-Maximum during estrus (pg/ml) x ± s	P4-Concentration during estrus (ng/ml) x ± s	Animals with LH surge (n)	Corpora lutea (n) x ± s
32	5	74.6[a] ± 29.7	1.2[a] ± 0.3	5	27.8[a] ± 16.4
61	5	67.2[a] ± 15.4	1.2[a] ± 0.4	5	27.7[a] ± 18.7
90	5	64.0[a] ± 13.8	1.6[b] ± 0.7	3	5.6[b] ± 5.1
119	5	44.8[b] ± 14.9	1.7[b] ± 0.3	2	3.6[b] ± 7.2

[a : b] $p < 0.05$

Table II: Relative bioactivity in four batches of one FSH product and results of superovulation and embryo recovery after application of 24 mg FSH per donor independent of bioactivity (n = 525 cows)

Batch	Relative bioactivity (%)	Recovered eggs and embryos per donor (n) x ± s	Transferable embryos per donor (n) x ± s
1	127.8	10.3[a] ± 7.2	7.4[a] ± 5.3
2	106.0	10.2 ± 7.3	6.8 ± 5.5
3	89.0	8.9[c] ± 5.7	6.4 ± 4.6
4	78.9	8.1[b] ± 6.4	6.1[c] ± 5.5

[a : b] $p < 0.01$ [a : c] $p < 0.05$

Table III: Follicular populations after treatment of heifers with FSH, FSH/LH, LH or aCl

			FSH 0.29 mg	FSH/LH 0.29/0.19 mg	LH 0.19 mg	Control NaCl
animals	(n)		21	20	18	19
follicles 1-3,9 mm	(n)	x ± s	$14.3^{a} \pm 10.2$	$11.6^{a} \pm 10.4$	$23.5^{b} \pm 15.0$	$28.0^{b} \pm 17.2$
follicles 4-8 mm	(n)	x ± s	$9.1^{c} \pm 5.2$	$8.7^{c} \pm 5.3$	$5.0^{d} \pm 2.9$	$4.7^{d} \pm 3.4$
follicles > 8 mm	(n)	x ± s	$15.6^{e} \pm 12.1$	$12.7^{e} \pm 9.7$	$1.9^{f} \pm 1.1$	$1.9^{f} \pm 1.4$
total	(n)	x ± s	39.0 ± 17.3	33.1 ± 16.7	31.0 ± 15.8	33.3 ± 17.1
min. – max.	(n)		17 - 73	6 - 71	12 - 67	11 - 79

a : b, c : d, e : f $p<0,05$

Table IV: Portion of intact follicles and results of superovulation and embryo recovery in dependence on treatment with FSH, FSH/LH or LH

Treatment	Ovaries (n)	Portion of intact follicles (n)	Animals (n)	Corpora lutea (n)	Transferable embryos (n)	Fertilisation rate (%)
FSH	4	46^{a}	5	$18.0^{d} \pm 1.2$	$6.6^{f} \pm 4.9$	78
FSH/LH	4	37^{a}	5	$13.2^{d} \pm 5.2$	$8.8^{f} \pm 4.0$	98
LH	4	6^{b}	5	$1.2^{e} \pm 0.4$	$0.2^{g} \pm 0.4$	
Control	4	18^{c}	5			

a : b $p < 0.01$ b : c, d : e., f : g $p< 0.05$

Table V: Results of time-oriented insemination in superovulated cows stimulated once or repeatedly

Donor number (n)	Recoveries (n)	Ova and embryos per recovery (n) x	Embryos per recovery (n) x	Fertilisation rate (%)	Transferable embryos per recovery (n) x
180	180	9.0	7.7	85.6	6.2
43	145	9.8	8.4	86.2	5.4

Table VI: Results of embryo recovery and evaluation of embryos in dependence on frequency and dose of FSH treatment in Simmental cows

		Group 1 (control)	Group 2	Group 3
FSH dose	(mg)	400	360	260
Number of injections	(n)	8	4	4
Animals	(n)	11	24	31
Ova and embryos per donor	(n) x	16.9	20.5	16.0
Transferable embryos per donor	(n) x	10.9	9.5	11.1
Fertilisation rate	(%) x	76.3	65.6	79.4
Portion of transferable embryos	(%) x	64.5	46.5	67.4

SUPEROVULATION IN PERSPECTIVE

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1. Introduction

The objective of superstimulatory treatments in the cow is to obtain the maximum number of fertilized and transferable embryos with a high probability of producing pregnancies [6]. Wide ranges in superovulatory response and embryo yield have been detailed in several reviews of commercial embryo transfer records. In a report of 2048 beef donor collections, a mean of 11.5 ova/embryos with 6.2 transferable embryos were collected from each cow [27]. However, variability was great in both the superovulatory response and embryo quality; 24% of the collections did not produce viable embryos, 64% produced fewer than average numbers of transferable embryos and 30% yielded 70% of the embryos. Embryo recovery from 987 dairy cows yielded slightly fewer ova/embryos and there was similar variability in response among animals [25]. The high degree of unpredictability in superovulatory response creates problems affecting both the efficiency and profitability of embryo transfer programs [24].

Variability in ovarian response has been related to differences in superovulatory treatments, such as gonadotrophin preparation, batch and total dose, duration and timing of treatment, and the use of additional hormones in the superovulatory scheme. Additional factors, which may be more important sources of variability, are inherent to the animal and its environment. These factors may include nutritional status, reproductive history, age, season, breed, ovarian status at the time of treatment and the effects of repeated superovulation. While considerable recent progress has been made in the field of bovine reproductive physiology, factors inherent to the donor animal which affect superovulatory response are only partially understood. The purpose of this review is to address practical aspects of bovine superovulation with a view to simplifying superstimulatory procedures, improving responses and reducing variability; in the interest of space, reference to review articles will be done as much as possible.

2. Gonadotropins and Superovulation

Factors associated with the administration of exogenous gonadotrophins affecting superovulatory response include source, batch and biological activity of the gonadotrophin [36]. We have investigated the biological activity of gonadotrophins and the effect that FSH and LH activities in gonadotrophin preparations have on the superovulatory response in the cow. We have also investigated the use of the highly purified porcine pituitary extract (Folltropin®-V; Vetrepharm Canada Inc, Bellville, ON, Canada) [7, 20]. Pertinent research on the biological activity of gonadotrophins and how this affects superovulatory response in the cow will be reviewed.

Three different types of gonadotrophins have been used to induce superovulation in the cow; gonadotrophins from extracts of porcine or other domestic animal pituitaries, equine chorionic gonadotrophin (eCG) and human menopausal gonadotrophin (hMG) [5, 36]. Prostaglandin (PGF) or

its analogues have been used for the induction of luteolysis in a superstimulatory regimen, to allow for precise timing of onset of estrus and of ovulation. The biological half-life of FSH in the cow has been estimated to be 5 h or less so it must be injected twice a day to successfully induce superovulation [33]. The usual regimen has been 4 or 5 days, twice daily treatments of FSH with a total dose of 28 to 50 mg (Armour) of a crude pituitary extract (FSH-P) or 400 mg NIH-FSH-PI of the purified pituitary extract, Folltropin®-V. Forty-eight or 72 h after initiation of treatment, PGF is injected to induce luteolysis. Estrus occurs in 36 to 48 h, with ovulation 24 to 36 h later.

Equine chorionic gonadotrophin is a complex glycoprotein with both FSH and LH activity [35]. It has been shown to have a half-life of 40 h in the cow and persists for up to 10 days in the bovine circulation; thus it is normally injected once followed by a PGF injection, 48 h later [18]. The long half-life of PMSG causes continued ovarian stimulation, unovulated follicles, abnormal endocrine profiles and reduced embryo quality [32, 34, 41]. These problems have been largely overcome by the intravenous injection of antibodies to eCG at the time of the first insemination, 12 to 18 h after the onset of estrus [18, 21]. Recommended doses of eCG range from 1500 to 3000 IU, with 2500 IU by intramuscular injection commonly chosen.

Monniaux *et al.* [33] treated a group of cows with 2500 IU eCG and another with 50 mg (Armour) FSH-P and observed that ovulation rate and the percentage of cows with more than 3 transferable embryos was slightly higher with FSH-P than eCG. Although these results were in agreement with those of Elsden *et al.* [19] others have found no differences between pituitary FSH extracts and eCG [5, 22, 30]. Endocrine studies have revealed that eCG-treated animals more frequently had abnormal LH and progesterone profiles than did the FSH-treated cows [23, 32]. These were associated with reductions in both ovulation and fertilization rate [15]. In a study of cows repeatedly superstimulated at 60 to 90 day intervals over 1 year, we found no differences in superovulatory response between two different pituitary extracts (Folltropin®-V or FSH-P) and eCG with or without a monoclonal antibody to eCG (Neutra-PMSG; Intervet, Boxmeer, Holland) administered at the time of the first insemination [30]. However, numbers favored Folltropin®-V and eCG with with Neutra-PMSG. Others have made similar observations [22].

Although folliculogenesis in mammals requires both FSH and LH, there is considerable variability in FSH and LH content of crude gonadotrophin preparations. Radioreceptor assays and *in vitro* bioassays have revealed variability in both the FSH and LH activity of eCG, not only among pregnant mares, but also between bleedings in the same mare at different times during gestation [35]. We have also examined the effects of the FSH/LH ratio of eCG on superovulatory responses with immature rats and found that there was a positive correlation between the ratio of FSH/LH activity and superovulatory response. Lower ratios of FSH/LH activity appeared to reduce ovulatory response in rats and additional LH, when added to eCG reduced superovulatory response in cows [35, 36].

Purified pituitary extracts with low LH contamination have been reported to improve superovulatory response in cattle. Chupin *et al.* [16] superstimulated three groups of dairy cows with an equivalent amount of 450 µg pure pFSH and varying amounts of LH, and showed that the mean ovulation rate and the number of recovered and transferable embryos increased as the dose of LH decreased. They observed that as LH activity increased, the dose of FSH required to induce an acceptable response also increased. It has been suggested that embryo quality may be adversely influenced by high LH levels during superstimulation due to premature activation of the oocyte [34].

We have completed several experiments with the LH-reduced Folltropin®-V utilizing several different total doses, ranging from 100 to 900 mg of NIH-FSH-P1 activity [5, 20]. There was no evidence of detrimental effects of dose on embryo quality. Ovulation rates continued to increase to 400 mg NIH-FSH-P1 (40 mg Armour) and did not increase beyond that dose. At the same time fertilization rate and transferable embryo rate remained constant throughout the dose range used. On the other hand, doubling the dose of LH-rich preparations (FSH-P or hCG) resulted in significantly reduced fertilization rates and percentages of transferable embryos [5]. Collectively, data support the hypothesis that the detrimental effects of high doses of pituitary gonadotrophins on ova/embryo quality is due to an excess of LH.

Recently, we investigated the long-term safety of Folltropin®-V in a retrospective study involving 1949 donor cows and their offspring i.e., second and third generation donor cows which were a result of superovulation and embryo transfer. Reproductive safety was examined by calculating the number of viable embryos collected from each cow and the number of normal calves born to cows that had been previously superstimulated with Folltropin®-V. Embryological safety was measured by the number of live calves produced from superovulation and embryo transfer using Folltropin®-V. The main data set examined all available records with respect to treatment number, number treatments in a sequence, the status of mother/donors and whether they were a product of embryo transfer, the number of known calves produced from embryo transfer and the number of calves born naturally to embryo donors. A smaller data set was based on known family relationships from four generations for the same end-points. Statistical analyses, based on analyses of variance, revealed no significant difference among the observed variables (numbers of embryo recovery, calves by embryo transfer, natural born calves etc.) as a consequence of the independent variables. We concluded that there was no evidence of adverse effects of treatment, or repeated treatment of donor cows with Folltropin®-V, on reproductive performance, embryo production or resulting offspring.

Although it is generally believed that some LH is required for successful superovulation, endogenous LH levels may be adequate. Looney *et al.* [28] reported that recombinantly produced bFSH induced high superovulatory responses without the addition of exogenous LH. In addition, fertilization rates exceeded 95% and viable embryos rates exceeded 85%. These data suggest that LH is not needed in superovulatory preparations and that embryo quality may be superior with pure FSH. The very high fertilization rates and transferable embryo rates in the absence of exogenous LH tend to suggest that administration of LH, at any dose, may be detrimental to embryo quality.

An experiment was designed to determine the effects of exogenously administered LH on superovulatory response in *Bos taurus* cattle [43]. Cross-breed beef cows were randomly placed into one of four treatment groups to be superstimulated with a total dose of pFSH equivalent to 400 mg NIH-FSH-P1 over 4 days. Cows in Group I received a standard porcine pituitary extract much like FSH-P (100% LH), whereas cows in Group II received a preparation with approximately 68% LH removed (32% LH), cows in Group III received a preparation with approximately 84% LH removed (16% LH - equivalent to Folltropin®-V), and cows in Group IV received a preparation with 98% LH removed (Pure FSH). Superovulatory responses clearly divided these cows into two distinct groups (Table I); those with high LH (Groups I and II) and those with low LH (Groups III and IV). Overall, there were more ovulations, ova/embryos collected ($P<0.05$), and there tended to be more fertilized ova ($P<0.07$) in the two groups with the least LH (Groups III and IV). With the doses used in this experiment, there was no affect of LH on ova/embryo quality. Results demonstrate that LH within FSH preparations affects superovulatory response and that the maximum acceptable level of LH would appear to be between 15 and 20%.

Table I. Superovulatory response of *Bos taurus* cows superstimulated with FSH (400 mg NIH-FSH-P1) and varying amounts of LH [43].

			Ova/embryos				
Group	n	CL	Total	Fert	(%)	Trans	(%)
I (100% LH)	21	10.2^a	7.3^a	5.3^c	(73)	4.0	(55)
II (32% LH)	20	11.1^a	6.4^a	4.6^c	(72)	3.9	(61)
III (16% LH)	20	15.6^b	13.6^b	9.7^d	(71)	7.7	(57)
IV (Pure FSH)	20	17.2^b	13.2^b	8.3^d	(63)	5.5	(42)

Means with different superscripts are different (ab - $P<0.05$; cd - $P<0.07$).

In yet another experiment involving Brahman-cross (*Bos indicus*) heifers superstimulated with 400 mg NIH-FSH-P1 containing 100%, 16% or 2% LH, Tribulo *et al.* [42] reported that the more purified preparations caused the higher superovulatory response (Table II). Overall, the most purified

preparation (Group III) induced more CL and tended to result in more ova/embryos and fertilized ova when compared to the least purified preparation (Group I). The intermediate preparation (16% LH; group II) induced an intermediate response. However, there were obvious seasonal effects. Responses with pure FSH and 16% LH were superior to the crude extract (100% LH) during summer months, but only the pure FSH was more efficacious during winter months.

Table II. Superovulatory responses of *Bos indicus* heifers, superstimulated with FSH (400 mg NIH-FSH-P1) and varying amounts of LH [42].

	Summer				Winter				Overall			
Group	n	CL	TO/E	FO	n	CL	TO/E	FO	n	CL	TO/E	FO
I (100% LH)	13	8.5	4.7	4.2	14	3.7[a]	4.4	3.4	27	6.0[a]	4.6	3.9
II (16% LH)*	12	19.2	9.6	7.0	15	5.9[a]	1.6	0.8	27	11.7[ab]	5.8	4.0
III (Pure FSH)	14	16.5	7.0	5.7	15	19.4[b]	10.6	8.3	29	18.1[b]	8.5	6.8

Ab - Means within a column with superscripts not in common are different ($P<0.05$)
* Group II differed between summer and winter ($P<0.05$).

These results would appear to contradict the findings of Page *et al.* [39] who reported that superovulation and embryo quality in Holstein heifers was not affected by LH levels in cool weather; whereas a low LH preparation (Folltropin®-V) yielded more CL and significantly more fertilized ova and transferable embryos during heat stress. It becomes apparent that stress is the common factor. *Bos taurus* breeds likely find summer heat stressful, whereas *Bos indicus* breeds likely find winter temperatures stressful. In either case, the more purified extracts resulted in greater superovulatory responses during conditions of stress.

We have also investigated the use of Folltropin®-V as a single bolus injection for superstimulation of cattle. A single subcutaneous injection of Folltropin®-V at a dose equivalent to 400 mg NIH-FSH-P1 resulted in a superovulatory response equivalent to that of a 4 day, twice daily intramuscular treatment regimen [10]. During the course of these studies, it was found that a more consistently high superovulatory response occurred when the subcutaneous injection was made behind the shoulder as opposed to in the neck region. We have since found that splitting the single subcutaneous dose (Day 0 – 75%; Day 2 – 25%) improved results in cows with little subcutaneous fat [29], and we have preliminary results suggesting that the ischiorectal fossa may be an alternative site for a single injection of FSH [17]. In fact, anything that results in increased absorption of FSH (eg. intramuscular injection or injection in the neck region of lean cows) resulted in a reduced superovulatory response. Although Folltropin®-V has been reported to have more than 80% of LH removed, there may be sufficient LH remaining to result in an over-dose when administered in a single bolus injection or when absorption rate is increased.

A single bolus subcutaneous injection of Folltropin®-V has much to offer superstimulatory treatment protocols, especially when twice daily treatments may result in stress which may suppress superovulatory response. In one study involving *Bos indicus* heifers, a single subcutaneous injection of Folltropin®-V resulted in a significantly greater superovulatory response than a twice daily, four-day treatment schedule [10]. We attributed the difference to the stress associated with twice daily treatments and handling.

When comparing experiments, route of administration must also be considered [reviewed in 5]. We have observed that twice daily intramuscular injections of Folltropin®-V resulted in a significantly higher superovulatory response than twice daily subcutaneous injections. We have also demonstrated that a single intramuscular injection resulted in higher circulating FSH levels than did a single subcutaneous injection [5]. However, the subcutaneous injection resulted in a more prolonged increase in FSH levels and a significantly improved superovulatory response [10].

Individual studies often show little or no difference in results among the various gonadotrophins used for superovulation in the cow. It is also obvious that breed, environment, nutrition and the individual animal response are factors which complicate ovarian stimulation. The role of stress has not been well documented, and more studies are required to understand the stress-cortisol-cytokine-hormone effects on reproductive performance and superovulation.

3. ANIMAL INFLUENCES ON SUPEROVULATION

With a better understanding of ovarian function has come a greater capability of controlling it. Our expanding knowledge of the roles of the CL and follicular waves in the bovine estrous cycle has resulted in renewed enthusiasm about the prospects of precise synchronization of estrus and ovulation. The intention of the following discourse is to provide an overview of normal ovarian events in cattle, and to discuss how these events impact on the effectiveness of superstimulation regimens. We hypothesized that ovarian response to exogenous stimulation is contingent upon the physiologic status of the ovaries at the time of superstimulation.

3.1. Ovarian Follicular Wave Dynamics

It has been shown that greater than 95% of bovine estrous cycles are composed of either two or three follicular waves [reported by Adams, Fortune, Ginther, Roche and Boland, and others; reviewed by Adams in 2]. Single-wave cycles have been reported in heifers at the time of puberty and in mature cows during the first interovulatory interval after calving. Four-wave cycles are observed occasionally in Bos indicus cattle [40]. The proportion of animals with two- versus three-wave cycles varies among reports; some report a majority of two-wave cycles and others report a majority of three-wave cycles while others have observed a more even distribution [reviewed in 2]. Although the subject has not been systematically studied, there does not appear to be a clear breed- or age-specific preference for one follicular wave pattern over the other, nor is there any apparent difference in fertility [4]. In a study of the effects of nutrition on follicular dynamics, cattle fed a low energy ration had a greater proportion of three-wave cycles than those fed higher energy rations [37]. Preliminary data collected from 9 heifers during their first 2 years suggest that the pattern is repeatable within individuals (Adams, unpublished). In another study in Bos indicus cattle, four of 25 cows had four follicular waves per cycle; one cow changed from four waves in the spring to three waves in the autumn. The evolutionary reason for a two- or a three-wave cycle, or indeed for the wave-like pattern itself, is unclear; however, the differences in wave patterns are distinct and they have clear implications regarding ovarian synchronization and superstimulation.

Simply put, the wave pattern of follicular development refers to periodic, synchronous growth of a group of antral follicles. In cattle, follicle wave emergence is characterized by the sudden (within 1 to 2 days) growth of more than 20 small follicles that are initially detected by ultrasonography at a diameter of 3 to 4 mm [2]. For about 2 days, growth rate is similar among follicles of the wave, then one follicle is selected to continue growth (dominant follicle) while the remainder become atretic. In both two- and three-wave estrous cycles, emergence of the first follicular wave occurs consistently on the day of ovulation (day 0). Emergence of the second wave occurs on day 9 or 10 for two-wave cycles, and on day 8 or 9 for three-wave cycles. In three-wave cycles, a third wave emerges on day 15 or 16. Successive follicular waves will remain anovulatory until luteolysis occurs. The dominant follicle present at the onset of luteolysis will become the ovulatory follicle, and emergence of the next wave is delayed until the day of ovulation. The CL begins to regress earlier in two-wave cycles (day 16) than in three-wave cycles (day 19) resulting in a correspondingly shorter estrous cycle (20 days vs 23 days, respectively). Hence, estrous cycle length may provide a clue to numbers of follicular waves that a given cow has within each cycle.

3.2. Role of gonadotropins in follicular wave development

The mechanism involved with follicular wave dynamics is based on differential responsiveness of the ovary to FSH and LH [2]. Periodic surges in circulating concentrations of FSH are responsible for eliciting follicular wave emergence; hence, cows with two-wave cycles have two FSH surges and three-wave cycles have three surges [3]. Circulating FSH is subsequently suppressed by negative

feedback by estradiol and inhibin from the emerging follicles and the following nadir in FSH effectively prevents new wave emergence. The transient rise in FSH permits sufficient follicular growth so that some follicles acquire LH responsiveness which allows survival without FSH. At the time of follicle selection, 2 or 3 days after wave emergence, FSH is declining rapidly. The follicle destined to become dominant apparently acquires receptors for LH and has the competitive advantage over follicles destined to become subordinate. However, LH responsiveness and the ability to become a dominant follicle likely represents a quantitative rather than an absolute difference between follicles in a wave. Subordinate follicles can become dominant if the original dominant follicle is removed or if exogenous FSH is supplied [2]. Further, the competition for LH among multiple dominant follicles (i.e., superstimulated with FSH) is apparent by the smaller maximum diameter attained compared to single dominant follicles. Continued suppression of LH as a consequence of luteal-phase progesterone secretion causes atresia of the dominant follicle, and FSH is again allowed to surge. This surge has no effect on the dying dominant follicle, but is responsible for eliciting the emergence of the next wave. The ovarian cycle then repeats itself. Relief from progestational suppression (i.e., luteolysis) allows LH pulse frequency to increase, permitting further growth of the dominant follicle and dramatically higher circulating concentrations of estradiol, which results in a surge of LH followed by ovulation.

The conventional protocol of initiating ovarian superstimulation during mid-cycle (8 to 12 days after estrus) was arrived at empirically, but studies in which a lesser response to superstimulatory treatments initiated early in the estrous cycle (2 to 6 days after estrus) vs later (9 to 11 days after estrus) validated the convention [22, 26]. The reason for the relative success of the conventional approach may be explained by what we now understand about follicular dynamics.

We hypothesized that superstimulatory response would be greater if treatment was initiated before selection of a dominant follicle. In an initial study, recombinant bFSH given to heifers before the time of selection (day 1, ovulation = day 0) resulted in more ovulations than that given after the time of selection (day 5) of the dominant follicle of Wave 1 [1]. A subsequent study was done to determine if exogenous FSH given at the expected time of the endogenous wave-eliciting FSH surge had a positive effect on the superstimulatory response [38]. The endogenous surge in FSH was expected to peak 1 day before wave emergence, so superstimulatory treatments were initiated on the day before, the day of, or 1 or 2 days after wave emergence. Significantly more follicles were recruited and more ovulations occurred when treatment began on the day of, or the day before, follicular wave emergence.

In a direct comparison between waves, results of another study did not reveal any difference in the number of large follicles recruited, the number of ovulations induced, or the number of ova/embryos recovered in heifers in which superstimulation was initiated on the day of emergence of Wave 1 or Wave 2 [reviewed in 1]. Consistent with the previous study [38], when treatment was initiated ≥1 day after wave emergence, the superstimulatory response was reduced. These data suggest that superovulation may be induced with equal efficacy when treatment is initiated during the first or second follicular waves, and that the superstimulatory response is enhanced if treatment is initiated at the time of wave emergence.

Based on duration of the developmental phases of the dominant follicle in two-wave and three-wave interovulatory intervals, the probability at any given time that the dominant follicle is not functionally dominant is approximately 30% (6 of 20 days) for two-wave heifers and 35% (8 of 23 days) for three-wave heifers. More importantly, only 20% (4 or 5 days) of the estrous cycle is available for initiating treatment at the time of follicular wave emergence. Therefore, 80% of the cycle is not conducive to an optimal superovulatory response. To obviate these problems, studies have been done to determine if superstimulation subsequent to elective induction of follicular wave emergence could be used with equal efficacy to the conventional protocol.

One approach involved transvaginal ultrasound-guided follicle ablation to synchronize wave emergence among heifers at random stages of the cycle followed by the insertion of a progestogen implant and treatment with Folltropin®-V 1 day after ablation, and PGF 48 and 60 h later [9]. Non-ablated control heifers were given Folltropin®-V 8 to 12 days after estrus. Combined over two

experiments (Table III), there was no difference in the superovulatory response between the ablated and non-ablated groups. In another study, Bungartz and Niemann [13] obtained a significantly higher superovulatory response when the dominant follicle was ablated 2 days before initiating gondadotrophin treatments. More recently, we have shown that ablation of the two largest follicles at random stages of the cycle will ensure that the dominant follicle is removed and a new wave will emerge 1 to 2 days later [8].

Another approach to the synchronization of follicular wave emergence for superovulation involves an injection of 5 mg estradiol-17β after the insertion of a progestogen implant, followed by the administration of Folltropin®-V beginning 4 days after estradiol treatment [11, 12]. PGF was given 48 h after Folltropin®-V treatment was initiated and the progestogen implant was removed 12 h after PGF treatment. Control heifers were given the same dose of Folltropin®-V between 8 and 12 days after estrus. Combined over two experiments (Table III), the superovulatory response in the estradiol-treated groups was equivalent to that of the control groups.

Table III. Response in control heifers superstimulated between days 8 and 12 of the cycle compared to synchronization of wave emergence by follicle ablation or progestogen + estradiol (P+E) [9, 11].

	Ablation-induced wave synchrony		Steroid-induced wave synchrony	
	Control	Ablation	Control	P+E
No. of heifers	35	60	52	56
CL	22.9	18.6	23.7	24.3
Total ova/embryos	10.1	9.8	12.3	12.4
Fertilized ova	7.3	7.8	7.9	9.3
Transferable embryos	5.4	5.6	4.9	5.2

Our preferred approach to the synchronization of follicular wave emergence for superstimulation involves an injection of 5 mg estradiol-17β plus 100 mg progesterone at the time of CIDR-B (Vetrepharm Canada Inc) insertion followed by Folltropin®-V given as a single or multiple dose beginning 4 days after estradiol treatment [11, 12]. PGF is given 48 h after Folltropin®-V treatment is initiated and the CIDR-B is removed 12 h later. Combined over several experiments, the superovulatory response in the estradiol-treated groups has been equivalent to or greater than that of control groups superstimulated on days 8 to 12 of the cycle. In a more recent experiment, we compared synchrony of follicular wave emergence and superovulatory response after treatment of norgestomet-implanted cows with estradiol-17β or estradiol valerate [31]. Follicular wave emergence occurred on days 3 or 4 (mean = 3.6 days) in all 37 cows treated with estradiol-17β, while follicular wave emergence occurred between days 3 and 6 (mean = 5.7 days) in 68% of estradiol valerate-treated cows. Superovulatory response and total ova/embryos collected were also greater in the estradiol-17β-treated group. Data suggest that the greater synchrony of follicular wave emergence following treatment with estradiol-17β and progesterone provided an advantage for the elective induction of superovulation. In another study, a dose of 1 mg of estradiol benzoate was as efficacious as 5 mg estradiol-17β in synchronizing follicular wave emergence on day 4, whereas a dose of 5 mg estradiol benzoate resulted in a mean of 5 days with more variability [14]. Unfortunately, we have not investigated synchrony of follicular wave emergence following treatment with a reduced dose of estradiol valerate. In any case, these studies demonstrate that elective induction of follicle wave emergence offers the advantage of initiating superstimulatory treatment at a time that is optimal for follicle recruitment. Thus, the full extent of the estrous cycle is available for superstimulation and the need for detecting estrus or ovulation and waiting 8 to 12 days to initiate gonadotropin treatments is eliminated.

It is noteworthy that in studies involving superstimulation coincident with wave emergence, the response to a single bolus injection of Folltropin®-V was as good or better than the response to a multiple injection scheme. The nadir between FSH surges is responsible for preventing the emergence of a new wave; provision of exogenous FSH during the period of the FSH nadir may result in "break through" growth of small follicles prior to the time of expected new wave emergence (i.e., effects of dominant follicle suppression were overcome by FSH) [reviewed in 1, 2]. This may explain how large doses of exogenous FSH in conventional superstimulation schemes can overwhelm the endogenous rhythm and mask the wave effect. If superstimulatory treatment is given for a long enough period, follicle recruitment will become apparent, regardless of follicular wave status at the time of gonadotropin treatment. However, asynchronous recruitment may result in more variability in ovarian follicular response, and in the quantity and quality of oocytes and embryos collected.

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SHORT COMMUNICATIONS

OOCYTE RETRIEVAL AFTER REPEATED OVUM PICK-UP IN UNSTIMULATED SHEEP AND GOAT

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Ovum pick-up (OPU) allows to retrieve oocytes *in vivo* to be used for *in vitro* techniques. The aim of this work was to study the oocyte yield in sheep and goat after consecutive OPU sessions performed once a week. Follicles from halothane-anesthetized animals were aspirated with a 23 G needle and –25 mmHg.

Experience 1: Rasa Aragonesa ewes were synchronized during the breeding season by 40 mg fluorogestone acetate sponges (Chronogest, Intervet, Spain) for 13 days plus 125 μg cloprostenol (Estrumate, Schering, Spain) at sponge insertion. Animals were then randomly distributed into Control group (sponges removed at first OPU session, n=6) or Sponge group (sponges maintained and replaced every week, n=6). The progestative treatment was intended to avoid ovulations and preovulatory follicles. OPU was performed weekly on 5 consecutive sessions by laparoscopy. Oocytes were collected and evaluated as: COC (cumulus-oocyte complex); 3ly (oocytes with at least three layers of cumulus cells) and D (denuded oocytes). We considered COC and 3ly categories as adequate for *in vitro* maturation.

Experience 2: Mixed-breed goats were synchronized during the anoestrus season by 45 mg fluorogestone acetate sponges (Chronogest, Intervet, Spain) for 12 days plus 125 μg cloprostenol 48 hours before sponge withdrawal. Animals were then randomly distributed into Control group (n=6) or Sponge group (n=7). OPU was performed weekly on 6 consecutive sessions by laparoscopy and oocytes were collected and classified following the same procedure as in sheep.

Results: From 1200 punctured sheep follicles 547 oocytes were recovered, from which 437 were adequate for *in vitro* maturation. Mean recovery rate was 45.6% although there was a significant difference between groups (Table 1). Progestative treatment showed statistical differences in oocyte yield concerning 2nd and 3rd OPU sessions but not in the overall per group yield.

In goats, from 1480 punctured follicles 732 oocytes were recovered, giving a recovery rate of 49.4% and 4.9 good oocytes per goat per session. No significant differences were found between groups (Table 2), probably due to that experience 2 was carried out during anoestrus season.

In conclusion, repeated OPU in unstimulated small ruminants gives enough number of oocytes for *in vitro* techniques while maintaining donors alive and harmless.

Table 1- Data of oocyte recovery in sheep after 5 consecutive OPU sessions

Group	Punctured follicles	Recovered oocytes	Recovery	COC+3ly	Oocyte yield**
Control	533	214	40.2%	167 (78%)	5.6±1.1
Sponge	667	333	49.9%*	270 (81%)	9.0±1.5

*P<0.001; **Oocyte yield: Mean No.of good oocytes/ewe/session

Table 2- Data of oocyte recovery in goat after 6 consecutive OPU sessions

Group	Punctured follicles	Recovered oocytes	Recovery	COC+3ly	Oocyte yield*
Control	655	352	53.7%	186 (53%)	5.2±0.6
Sponge	825	380	46.1%	191 (50%)	4.6±0.6

*Oocyte yield: Mean No.of good oocytes/goat/session

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Notes

THE STIMULATORY EFFECT OF SOW FOLLICULAR FLUID ON IN VITRO OOCYTE DEVELOPMENTAL COMPETENCE DEPENDS ON FOLLICULAR SIZE

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The data about the effect of follicular fluid (FF) from different sized follicles on nuclear and developmental competence is rather conflicting. Tao *et al* (Theriogenology 1995;43:334 abst) found no differences between pFF either collected from small (2-5 mm) or large (>5 mm) follicles on nuclear and blastocyst formation, while others (Yoon *et al* Reprod Fertil Dev 2000;12:133-139) reported higher blastocyst formation when oocytes were cultured in maturation medium containing FF obtained from large follicles. These experiments used COCs and FF from prepubertal gilts. Recently, it has been shown that oocytes from adult pigs have a greater developmental competence than those from prebubertal pigs (Marchal *et al*, Theriogenology 2001;56:17-29).

Most *in vitro* maturation protocols currently utilize pFF from small and medium size (2-6 mm) follicles. The aim of this study was to evaluate the effect of sow FF from small (2-4 mm, SFF) and large (5-8 mm, LFF) follicles during *in vitro* maturation on nuclear maturation of sow oocytes and subsequent fertilization and embryo development. In two experiments, COCs aspirated from 2-6 mm follicles sow ovaries, were cultured for the first 22 h in 500 µl of TCM-199 supplemented with 2.2 mg/ml $NaHCO_3$, 0.1% PVA and 100 µM cysteamine, with or without 10% pFF and/or 0.05 IU/ml recombinant hFSH (Organon, Oss, The Netherlands). For the next 22 h, the COCs were cultured in the same medium, but without follicular fluid and FSH. *In vitro* maturation, fertilization and culture took place at 38.5°C in a humidified atmosphere of 5 % CO_2 in air. After culture, cumulus cells were removed and the oocytes were either fixed and stained with DAPI to evaluate the nuclear stages or fertilized in mTBM with 0.1% BSA and 1 mM caffeine for 24 h with 5×10^5 sperm/ml fresh sperm. The presumptive zygotes were then cultured in NCSU23 with 0.4 % BSA for 6 days. Subsequently, embryos were evaluated morphologically and the blastocysts were fixed and stained with DAPI to determine cell numbers. Data were analyzed by Chi-square test, and the blastocyst cell numbers (mean ± SEM) were analyzed by T-test.

Both FF stimulated nuclear maturation in the presence of FSH. However, only LFF was active in the absence of FSH. Cleavage and blastocyst formation rates significantly increased in the presence of LFF compared to SFF. The mean number of cells per blastocyst did not differ significantly between treatments (Table 1).

In conclusion, follicular fluid from large (5-8 mm) follicles should be added to the IVM medium of sow COCs, in order to increase nuclear maturation and blastocyst formation.

Table 1. Nuclear maturation, cleavage and blastocyst formation rates of sow COCs matured in TCM-199 supplemented with follicular fluid and/or FSH.

Maturation medium	No. of ocytes	MII (%)	No. of oocytes	Cleaved (%)	Blast. (%)	Blast./Cl (%)	Number of cells (mean±SEM)
TCM	289	53^a	309	34^a	8^a	28^a	36 ± 16^a
SFF	248	57^a	257	38^a	9^a	23^a	38 ± 16^a
LFF	329	73^b	293	47^c	17^b	35^b	38 ± 11^a
TCM	304	47^a	549	26^a	7^a	27^a	36 ± 16^a
FSH	309	59^b	532	37^b	19^b	52^b	42 ± 14^a
SFF+FSH	312	84^c	525	41^b	13^c	32^a	37 ± 12^a
LFF+FSH	411	89^c	493	51^c	22^b	43^b	42 ± 12^a

[a,b,c] Different superscripts in the same column differ significantly ($P<0.05$).
Each experiment repeated at least three times.

Notes

IN VITRO EMBRYO DEVELOPMENT FROM BOVINE OOCYTES MAINTAINED IN FOLLICULAR FLUID OR TCM-HEPES

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The media and pH changes during retrieval of the oocytes and the beginning of maturation *in vitro* (IVM) are limiting factors, not yet completely understood when using OPU-PIV. In order to evaluate the effect of the transport medium on *in vitro* embryo development of bovine oocytes, 1381 Cumulus-oocyte complexes (COCs) were obtained by aspiration of 2-8mm follicles from ovaries collected at a slaughterhouse. All oocytes obtained were randomly divided in 4 groups: in the Control group, the oocytes were matured *in vitro* in modified TCM-199 for 24h, added rFSH-h, Estrus Mare Serum (EMS) and Na + pyruvate, incubated at 39ºC, 5% CO_2 and saturated humidity; in the WB group, the oocytes were kept in a water bath at 39ºC for 24h, inside of 1.0mL Corning® tubes (430324) with TCM-HEPES (5.95mg/mL), and the same chemicals used in the Control group, except bicarbonate; in the group bFF6C, the oocytes were maintained in bovine follicular fluid (bFF) from 2-8 mm follicles for 6h at 30ºC, followed by maturation in TCM-199 for 18h with 5% CO_2 and saturated humidity; in the bFF6WB group, the oocytes were kept in bFF for 6h, followed by maturation in TCM-HEPES in a water bath for 18h. Fertilization was accomplished in FERT-TALP medium + heparin (30 µg/mL) and PHE under the same conditions of humidity and temperature as for the IVM, for 18h. The sperm, selected by the swim-up method, were used at concentration of 1 x 10^6/mL. Zygote culture was performed in SOFaaci (Holm P *et al*. Theriogenology 52, 683-700, 1999)+5% EMS in a four-well dish, under mineral oil, kept inside sealed bags containing 5% O_2, 5% CO_2 and 90% N_2. Data from 14 replicates were analyzed by GLM procedure (SAS 6.12 release, 1998) and means were compared using Tukey's test.

Table 1: *In vitro* embryo development from bovine oocytes maintained in follicular fluid or TCM-HEPES

MATURATION	Oocytes n	Cleavage n (%)	D7 embryos n (%)	D9 embryos n (%)	D9 Hatched n (%)
Control 24h	362	331 (91.4)[a]	131 (36.2)[a]	82 (22.7)[a]	54/131 (41.2)[a]
Water bath 24h	344	304 (88.4)[ab]	132 (38.4)[a]	83 (24.1)[a]	57/132 (43.2)[a]
bFF6C 18h	343	308 (89.8)[ab]	115 (33.6)[a]	71 (20.7)[a]	41/115 (35.7)[a]
bFF6WB 18h	332	278 (83.7)[b]	111 (33.4)[a]	76 (22.9)[a]	45/111 (40.5)[a]

a, b Different letters in the same column indicate significant differences (P<0.05).
(bFF6C= oocytes in bFF for 6h and maturation for 18h in TCM-199
bFF6WB= oocytes in bFF for 6h and maturation for 18h in TCM-HEPES, in a water bath).

There were significant differences on cleavage rates between the Control and bFF6WB group. However, there was no difference on the D7 and D9 blastocyst or hatched blastocyst rates. The overall hatched blastocyst cell number was 138.9 ± 3.9 (mean ± SEM) being similar between groups (P>0.05). Results show that it is possible to maintain bovine oocytes in bFF for 6h at 30ºC before being matured for 18h or mature them for 24h in TCM-HEPES in a water bath without impairing their *in vitro* development after fertilization. These alternatives could facilitate the transport and maturation of OPU oocytes.

Notes

DNA REPLICATION DURING THE FIRST CELL CYCLE OF BOVINE NUCLEAR TRANSFER EMBRYOS

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A large part of experiments of cloning by nuclear transfer in domestic animal uses donor cells that are, or have been induced, in a quiescent state referred to as G0 which correspond to an «out of cycle» state of the nucleus. This exit of the cell cycle is reversible, at least in certain cell types, and the nuclei of quiescent cells can re-enter the cell cycle when cultured in the favorable conditions or when transferred into an enucleated oocyte. It is known that, in bovine species, the onset and the duration of DNA replication during the first cell cycle in the one-cell stage is correlated with the developmental potential of the IVF embryo. In the case of somatic nuclear transfer embryo, the timing of replication is not well characterized and may depend on the state of the donor cell and of the recipient cytoplasm. In this study, we have used bovine enucleated oocytes to investigate the onset and the length of DNA synthesis in quiescent somatic nuclei after they have been transferred into these recipient cytoplasts.

Cumulus-oocyte complexes obtained from slaughterhouse ovaries were matured *in vitro* for 20 h, then enucleated at 22h. The donor cells were induced in G0 by cultured in starvation condition (0.5% FCS) for 4-10 days. The enucleated oocytes were fused with donor cells by applying 2 DC pulses of 2.2 kV/cm for 30 µs and activated by incubation in 10 µg/ml cycloheximide and 5 µg/ml cytochalasin B for 5h, then cultured in B_2 medium supplemented with 2.5% fetal calf serum. At every hour from 5h to 18h after electrical pulses, reconstructed embryos were exposed to 5'-bromo-2'-deoxy-uridine (BrdU) for 30 min and fixed in 2.5% paraformaldehyde. The BrdU incorporation was then assessed by immunocytochemical procedure. Parthenogenetic experiments were run in parallel with the same manipulation but without removal of metaphase plate. At least two replicates at each time point were carried out for all experiments.

DNA synthesis started at 6 h post fusion in nuclear transfer (NT-MII) embryos (10 of 53 embryos) and at 7h (3 of 28) in parthenotes (P-MII). In both groups, the DNA replication ended at 18h after electrical pulses. The replication period was 12h and 11h in NT-MII group and P-MII group respectively. The duration of S-phase (difference between the time at which half of NT-MII or P-MII entered/exited the S-phase) was the same for NT-MII and P-MII group (6.5h and 6.7h respectively, P>0.05). In NT-MII group, 100% of nuclei were in S-phase at 8-9h post fusion, and thenafter the proportion of replicating nuclei decreased until 18h post-fusion. When donor cells cultured in low-serum medium were switched to normal medium (10% FCS), the mean time of onset of first DNA replication was 18h after switching. These results suggest that the S-phase of a nucleus previously induced in G0 is totally governed by the recepient cytoplasm after transfer. In comparison with the S-phase in IVF embryos (Comizzoli *et al*, *Biol Reprod* 2000; 62: 1677-1684), the replication period in nuclear transfer embryo is particularly short. We are now trying to determine if this duration can be modulated by the timing of activation of the recipient oocyte or by the type of donor cell.

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Notes

OOCYTES DEPENDS ON THE TIMING OF EXPOSURE THE EFFECT OF ESTRADIOL ON *IN VITRO* NUCLEAR MATURATION OF BOVINE

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Addition of 1 μg/ml 17ß-Estradiol (E2) during IVM of bovine oocytes, negatively affects nuclear maturation as reflected by a decrease in the proportion of metaphase II stage (MII) oocytes, and by an increased incidence of nuclear aberrations, after 22 h of culture (1). Whether these effects are the result of long exposure (22 h) to estradiol during culture or more dependent on the timing of exposure (before or after germinal vesicle breakdown (GVBD)) is not clear. The objective of this study was to investigate the effects of different periods of 17ß-Estradiol exposure on IVM of bovine oocytes.

Bovine cumulus-oocyte complexes, aspirated from 2-8 mm follicles of slaughterhouse ovaries, were denuded by vortexing for 3 min. The denuded oocytes (DO) were cultured in: 1) TCM 199 for 22 h (Control); 2) TCM 199 + 1 μg/mL E2 during 22 h; 3) TCM 199 + 1 μg/mL E2 from 0-8 h and TCM 199 without E2 from 8-22 h or 4) TCM 199 without E2 from 0-8 h and TCM 199 + 1 μg/mL E2 from 8-22 h. The DO were incubated at 39ºC in a humidified atmosphere of 5% CO_2 in air and, after 22 h of maturation, they were fixed in 2.5% glutaraldehyde. The nuclear stage was assessed following DAPI staining. The experiment consisted of 3 replicates and the results were analysed by Chi-square tests.

Table 1. Effect of different periods of exposure to 17ß-Estradiol (1 μg/mL), on *in vitro* nuclear maturation of bovine oocytes.

Group	n	Aberrations (%)	GV (%)	MI (%)	MII (%)
1 (Control)	266	19 (7.1) [a]	6 (2.3) [a]	75 (28.2) [a]	166 (62.4) [a]
2 (E2; 0-22 h)	248	32 (12.9) [b]	16 (6.5) [b]	123 (49.6) [b]	77 (31.0) [b]
3 (E2; 0-8 h)	323	15 (4.6) [a]	12 (3.7) [ab]	174 (53.9) [b]	122 (37.8) [b]
4 (E2; 8-22 h)	307	42 (13.7) [b]	20 (6.5) [b]	103 (33.6) [a]	142 (46.2) [c]

GV=germinal vesicle; MI=metaphase I; MII= metaphase II
[a,b,c] Values within column with different superscripts differ significantly ($P<0.05$)

The presence of E2 in the culture media throughout the 22 h of culture (group 2) had not only a negative effect on the percentage of nuclear aberrations, but also on the percentage of MII oocytes. When estradiol was present only for the first 8 h of the culture (group 3), there was no effect on the percentage of nuclear aberrations. However, there was a reduction of the percentage of MII oocytes, indicating a block between the MI and MII phase. Presence of E2 during 8-22 h of culture (after GVBD) (group 4) clearly resulted in an increase of the percentage of oocytes showing nuclear aberrations. Since the transcriptional nuclear activity strongly declines after the GVBD (2), these results indicate that the metaphase spindle formation is effected by estradiol in a non-genomic pathway.

1 - Beker, ARCL *et al*., Theriogenology, 2002 (in press)
2 -Tomek *et al*., Reprod Dom Anim 2002 37:86-91

Notes

IN VITRO AND *IN VIVO* DEVELOPMENT OF ZONA PELLUCIDA-INTACT MORULA STAGE PORCINE EMBRYOS WITH OPS METHOD AND AN APPROPRIATE CONCENTRATION OF CRYOPROTECTANTS

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Cryopreservation of zona pellucida-intact porcine embryos would be a major advantage to ensure minimal risk of disease transmission during embryo transfer of genetic material. The objective of our study is to investigate *in vitro* and *in vivo* survival rates using the Open Pulled Straw (OPS) method for vitrification of morulae (Vajta *et al*., 1997, Cryo-letters, 18, 191-195). *In vitro* and *in vivo* studies will be performed with 2 different concentrations of cryoprotectants.

Embryos donors were Large White hyperprolific (LWh) cyclic gilts which were inseminated twice (12h to 16h apart) with Pietrain semen after the beginning of oestrus. The gilts were slaughtered and the embryos recovered by flushing uterine horns 5 days after the 1st insemination. Only morulae were selected for vitrification. A comparison has been done between two different concentrations of cryoprotectants for vitrification, using OPS method. The dilution medium of cryoprotectants was a standard Hepes buffered TCM199 as previously described (Berthelot *et al*., 2001 Reprod. Nutr.Dev., 41, 267-272) and supplemented with 20% of calf serum. After a 2-step equilibration in 18% ethylene glycol (EG), 18% dimethylsulphoxide (DMSO) and sucrose for the first concentration (1) and 20% DMSO+ 20% EG and sucrose for the second (2), 4-6 embryos in a 2 µl droplet were loaded into the end of the straw using the capillary effect and then plunged into liquid nitrogen. Straws were thawed as described by Vajta *et al*., (1997, Acta Vet. Scand., 38, 349-352). Then, vitrified/warmed morulae were either cultured *in vitro* for 1 to 3 days in TCM medium + 10% of foetal calf serum to record the number of developed embryos, either 10 groups of 20 vitrified/warmed morulae were surgically transferred in each of 10 Meishan recipients at day 4 of the cycle. Pairwise comparison was made with S-plus proportion test. Since a constant of morulae number was transferred to recipient, the number of piglets born was analysed with the wilcoxon rank sum test; the number of piglets was set to zero when recipient was open.

Table 1*:* *In vitro* or *in vivo* development of vitrified/warmed LWh morulae with hepes TCM199 containing two different cryoprotectant concentration

	In vitro results		*In vivo* results after transfer of 20 vitrified morulae per Meishan recipient	
Concentration of cryoprotectant	Control	Vitrified	Farrowing rate	Number of born piglets per recipient
1)- 18% DMSO + 18% EG + Sucrose	90% (81)	31%* (61)	70%** [10]	6-0-0-5-0-9-6-1-4-4-#
2)- 20% DMSO + 20% EG + Sucrose		8%* (77)	0%** [10]	0-0-0-0-0-0-0-0-0-0-#

( ) Total number of cultured morulae *p = 0.0009 *;* [ ] total number of recipients **p=0.0025; #p= 0,.0013

Vitrification was followed by a low *in vitro* survival rate with the higher concentration of cryoprotectant (2). A significant difference was observed between concentrations 1 and 2 (31% versus 8% p = 0.0009). *In vivo* , no piglets were obtained with the higher concentration (2) for the other, 7 recipients farrowed 1 to 9 born piglets (p=0.0013) (table 1).Overall 35 piglets were born and 2 of them were stillbirths The survival rate of total transferred morulae was 16.5%.

These data suggest that OPS method can be useful to cryopreserve LWh zona pellucida-intact morula stage. But our study demonstrated also the important effect of the cryoprotectant concentrations.

Notes

A COMPARISON OF A MECHANICAL SECTOR AND A LINEAR ARRAY TRANSDUCER FOR ULTRASOUND-GUIDED TRANSVAGINAL OOCYTE RETRIEVAL (OPU) IN THE COW: PRELIMINARY RESULTS

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A comparison was made between a linear array and a mechanical sector transducer for the use in ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. Five dairy cows with normal reproductive tracts upon ultrasonal examination, were used in a biweekly OPU program lasting for 4 weeks. The cows were punctured on Mondays and Thursdays, using 2 OPU systems: a 5.0 MHz Multiple Angle mechanical sector transducer (Pie Medical; Bols *et al*., Theriogenology 1995), and a 5.0 MHz linear array transducer, mounted in a new OPU handle (Nutricell / Pie Medical, Campinas, Brazil). Both systems were used in combination with the same disposable needle guidance system. Cows received epidural anaesthesia (5 cc Procain) to prevent them from straining. They did not receive hormonal stimulation prior to OPU. Both ovaries were visualized with each transducer before any follicle was punctured. The number of follicles with a diameter of ≥ 5 mm was recorded with both the mechanical sector and the linear array transducer. Subsequently, one ovary was punctured with the system containing the mechanical sector transducer, while the other was punctured with the device that held the linear array transducer, using 55 mm long disposable, 19-g short beveled, sterile hypodermic needles. An aspiration pressure corresponding to a flow rate of 15-20 ml H_2O per minute was used. During the next puncture session within the same week, both systems were switched and used on the corresponding ovary. All manipulations were performed by the same operator. Oocytes were processed in a routine IVF laboratory immediately following retrieval. Parameters assessed were the number of follicles with a diameter of ≥ 5 mm, visualized with both systems in each cow, and the total number of oocytes retrieved with each system. The results are tabulated below.

Table 1. Total number of visualized follicles with a diameter of ≥ 5 mm and the total number of oocytes retrieved with the mechanical sector and the linear array OPU set up, during a 4 week OPU program.

Cow ID	Total number of visualized follicles ≥ 5 mm		Total number of oocytes retrieved per cow	
	Mech. sector transducer	Linear array transducer	Mech. sector transducer	Linear array transducer
37	30	27	25	17
09	30	27	13	1
60	13	15	5	4
67	18	20	13	9
68	14	14	15	6
TOTALS	105	103	71	37
Mean ± SEM	21.0 ± 3.8	20.6 ± 2.8	14.2 ± 3.2	7.4 ± 3.2

Results indicate that in terms of follicle visualization, no differences were found between the two types of transducers (paired t-test, $p>0.05$). A significantly higher number of oocytes was recovered when using the mechanical sector transducer (paired t-test, $p<0.05$), which is partly caused by the higher mobility of the ovary during transrectal palpation. However, an 'experience' factor concerning the manipulation of the new holder for the linear array transducer, can not totally be excluded.

In conclusion, both systems can be used for transvaginal ultrasound-guided oocyte retrieval in the cow, although the system using the sector probe seems to yield a higher number of oocytes.

Notes

RELATIONSHIP BETWEEN CUMULUS MORPHOLOGY AND *IN VITRO* MATURATION RATE OF EQUINE OOCYTES

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The objective of this preliminary study was to investigate if gross observation of cumulus morphology of equine oocytes could be related to *in vitro* maturation rate.
A total of 149 oocytes (in 11 replicates) were recovered by means of follicular aspiration (follicles diameter 5-30 mm; aspiration pressure 140-150 mm Hg; needle diameter 18 G) from 120 ovaries of slaughtered mares, from January to March 2002. Oocytes were grouped into three categories according to their cumulus morphology: compact (A), expanded (B), corona radiata (C). Each group was separately matured in TCM 199 with Earle's salts, supplemented with 20% Foetal Calf Serum, 10 µg/ml ovine FSH, 10 µg/ml ovine LH, 4 µg/ml estradiol-17β (Dell'Aquila *et al*., 1999), at 38.5°C, in a humidified atmosphere with 5% CO_2 under paraffin oil, for 30 hours. Denuded oocytes were observed under microscope to detect the presence of the first polar body and the increased peri-vitellinum space, as indexes of maturity.

The overall recovery rate was 1.25 oocytes per ovary and the percentages of oocytes in groups A, B and C were 47.0, 22.1 and 30.9 % respectively. Maturation rate (Mean ± S.E.M) for the three categories of oocytes was the following: A: 34.3 ± 6.5; B: 33.3 ± 8.4; C: 43.5 ± 9.3. The analysis of variance did not show any significant correlation between cumulus morphology and maturation rate.

On the whole, our results show a recovery rate comparable to previous works where oocytes were collected through aspiration (Dell'Aquila *et al*., 1997; Sosnowski *et al*., 1997). Even if follicular aspiration assures lower recovery rates compared to other techniques and can either damage cumulus-oocyte complexes, we chose this method for its higher similarity to in-vivo ovum pick-up. The mean maturation rate (37.2 ± 4.8) is lower than values previously reported in literature (Cochran *et al*., 1998 ; Dell'Aquila *et al*., 1999) and we suppose that it may be due to the young age (around 2 years) of the slaughtered mares. Relate to maturation results, some previous works showed higher percentages from oocytes with expanded cumulus: cumulus expansion is associated with initial follicular atresia and with the re-assumption of meiotic competence (Hinrichs & Williams, 1997). We hypothesize that in our work at least some of the oocytes bearing only the corona radiata could have lost an expanded cumulus during the aspiration procedure and could have more correctly been assigned to group B.

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Notes

GnRH APPLICATION IN FSH SUPEROVULATED GOATS: EFFECT ON THE NUMBER OF CORPORA LUTEA AND EMBRYO YIELD

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One of the more problematic aspects of the embryo transfer (ET) procedure is the variable response to superovulatory treatment. In order to improve the success of ET, several workers administered GnRH after sponge removal. Walker *et al*. (1989) (Theriogenology, 31: 741-752) notes in ewes that the use of GnRH in ET appears to be justified and is likely to improve embryo yields. Akinlosotu and Wilder (1993) (Theriogenology, 40: 895-904) observed in goats treated with a FSH and GnRH regimen, an increased follicular development, ovulation rate and higher blood progesterone levels; moreover, GnRH treatment improves embryo quality. The aim of this study was to evaluate the effect of GnRH in superovulated goats on the number of corpora lutea (CL) and embryo yields.

During anoestrous, 19 Canary goats were synchronized with an 11-day fluorogestone-acetate (FGA) intravaginal sponge and $PGF_{2\alpha}$ 2 days before FGA withdrawal: 10 goats received 50 µg of GnRH at 24 h after FGA removal and 9 goats saline solution. During reproductive season, 16 goats were synchronized: 8 goats received 50 µg of GnRH at 36 h after FGA removel and 8 goats saline solution. Starting at 48 h before FGA withdrawal, superovulation was induced with 6 doses (4, 4, 2, 2, 2, 2 UA) of porcine FSH (Stimufol, Prof. Beckers, Liège) at 12 h intervals. To determine LH surge blood samples were collected at 4-h intervals from days 1 to 3 after sponge removal. Ovulation rate was determined by endoscopy in anoestrous and by direct observation at time of embryo recovery in reproductive season.

During anoestrous, in both treated and control groups 5 goats (50 and 55%, respectively) experienced premature regression of all CL. In 7 of 10 goats treated with GnRH at 24 h, there was 2 consecutive LH surges at 28.4 ± 0.4 and 40.6 ± 2.5 h after FGA withdrawal. During reproductive season, the goats treated with GnRH at 36 h showed only a LH surge at 37.0 ± 2.0 h after FGA withdrawal and no differences were detected in maximum level neither length of LH wave between GnRH and control groups. In both treated and control groups there were 2 goats (25 %) with premature regression of all CL.

Table 1. Number (mean ± ESM) of normal CL, recovered embryos/oocytes, transferable embryos and unfertilized oocytes in GnRH and control goats with no premature regression of all CL.

	24 h after FGA withdrawal		36 h after FGA withdrawal	
	GnRH Group (n = 5)	Control Group (n = 4)	GnRH Group (n = 6)	Control Group (n = 6)
No. of normal corpora lutea	12.8 ± 2.2	11.0 ± 2.3	26.5 ± 2.6^a	13.0 ± 2.0^b
No. of embryos/oocytes recovered	--	--	21.5 ± 3.4^a	9.0 ± 2.4^b
No. of transferable embryos	--	--	9.6 ± 4.1	5.2 ± 1.4
No. of unfertilized oocytes	--	--	8.8 ± 4.3	2.2 ± 0.7

ab – Within rows, values with different superscripts are significantly different (P<0.05)

Table 1 shows the number of normal CL, recovered embryos/oocytes, transferable embryos and unfertilized oocytes (mean ± SEM) in goats with no premature regression of all CL. The treatment with GnRH at 24 h has no effect on CL number. The treatment with GnRH at 36 h improved all parameters with significant differences (P<0.05) in normal CL and embryos/oocytes recovered.

In superovulated goat a GnRH treatment increases ovulation rate and embryo yield when it is applied 36 h after sponge removal. This treatment has no influence on premature regression of CL.

Notes

PREGNANCY-ASSOCIATED GLYCOPROTEIN (PAG) CONCENTRATIONS IN EMBRYO RECIPIENT GOATS AND MATED GOATS

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In ruminant species, when implantation is initiated, the trophoblast binucleate cells migrate from the trophoblast to fuse with maternal endometrial epithelial cells, and the *pregnancy-associated glycoproteins* (PAG) are released directly into maternal tissues. In the present work, concentrations of PAG were measured throughout pregnancy in embryo recipient goats (n = 10) and mated goats (n = 11). Plasma concentrations of PAG were determined by RIA system previously described in goat species (González *et al*., 1999, Theriogenology, 52: 717-725).

In embryo recipient group, 3 goats kidded single, 5 double and 2 triplets. In mated goat group, 3 goats kidded single, 6 double and 2 triplets. Figure 1 shows the PAG concentrations (mean ± SEM), from Day 17 to 24 of pregnancy. On days 20, 22 and 24, the concentrations of PAG were significantly higher (P<0.05) in embryo recipient goats than in mated goats, with no effect of embryo number. Figure 2 shows the concentrations of PAG (mean ± SEM) from week 4 to parturition: there were no significant differences between embryo recipient goat and mated goat groups.

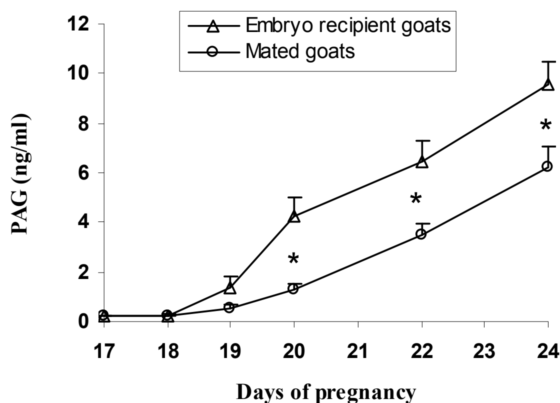

Figure 1. PAG concentrations (mean ± SEM) from day 17 to 24 of pregnancy (* P<0.05).

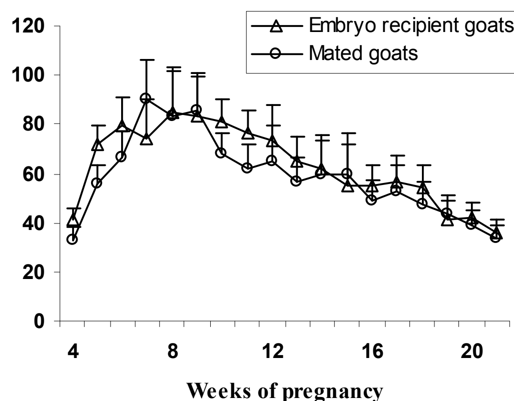

Figure 2. PAG concentrations (mean ± SEM) from week 4 to parturition.

In cattle, the embryo genotype has effect on PAG concentrations (Zoli *et al*. 1992, Biol Reprod, 46: 83-92; Patel *et al*. 1997, Eur J Endocrinol, 137: 423-428). In goat, (Fernández-Arias *et al*. 1999, Theriogenology, 51: 1419-1430) found that PAG concentrations were higher in inter-specific pregnancies (Spanish-ibex goat in domestic goat recipients) than in intra-specific pregnancies (domestic or ibex goats with embryo of the same species). In the present work there was an embryo influence on PAG concentrations which were higher as well as a greater genetic difference between the doe and embryo was present (case of embryo recipients goats), suggesting that PAG could be implicated in the immunological embryo-maternal interactions.

Notes

IN VIVO DEVELOPMENT OF 8-DAY RABBIT EMBRYOS PRODUCED BY PARTHENOGENETIC ACTIVATION OR SOMATIC NUCLEAR TRANSFER

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In a previous experiment (Theriogenology, 2001, vol. 55, 1, p. 260), we have determined that oocytes and nuclear transferred (NT) embryos reconstructed from fresh *cumulus* cells exhibited the highest *in vitro* development up to the blastocyst stage (89.6%, n = 130 and 46.7%, n = 135, respectively) when exposed to a cycloheximide (5µg mL-1) and 6-DMAP (2 mM) mixture for one hour following two sets of electrical pulses applied one hour apart. Under such conditions, we have recently shown that these NT embryos developed to term (Nature Biotechnol. 2002, 20, 366-369).

In the present study, we analysed the cell number in parthenotes at days 3 and 4 (blastocyst stage) and compared it with NT embryos and zygotes. We found 73 ± 16, 52 ± 17, 90 ± 19 and 133 ± 41, 94 ± 19, 193 ± 52, respectively. The slower *in vitro* development up to the blastocyst stage, as previously observed in NT embryos, suggested that implantation could be delayed to allow the *in vivo* development of manipulated oocytes (parthenogenesis or nuclear transfer) after transfer into recipients.

We therefore compared (Table 1) the *in vivo* development at day 8 of NT embryos, parthenotes and zygotes transferred into asynchronous recipients (mated with a vasectomized male 16 hours after donor oocytes) or into synchronous recipients (mated at the same time as donor oocytes).

Table 1. Implantation after transfer into synchronous or asynchronous (-16 h) recipient at day 8.

Type of embryos	Type of recipients	Recipients pregnant at day 8 / total transferred	No of implantation sites (% from embryos transferred into pregnant recipients)	No of implanted blastocysts / No recovered
Control (1-cell)	synchronous	5 / 6	15 / 54 (27.8%) a	9 / 9
Parthenotes (1-cell)	synchronous	8 / 20	17 / 78 (21.8%) a	0 / 1
NT (1-cell)	synchronous	5 / 16	7 / 91 (7.7%) b	0
Parthenotes (4-cell)	asynchronous	5 / 9	15 / 44 (34.1%) a	3 / 3
NT (4-cell)	asynchronous	6 / 13	12 / 59 (20.3%) a	1 / 7

Values with different letters were significantly different ($P<0.05$)

We found that NT embryos exhibited a higher and significant implantation rate in asynchronous transfer. Parthenotes also exhibited a higher but not significant increase of implantation capability ($P<0.2$). They were capable of implantation in contrast to what was observed after synchronous transfer manipulated oocytes (parthenogenesis or nuclear transfer). This asynchrony between donor oocytes and recipient mothers could be a key to overcome the failure of *in vivo* development beyond the implantation stage.

Notes

SOMATIC NUCLEAR TRANSFER IN GOATS: INFLUENCE OF THE CULTURE SYSTEM ON PERI-IMPLANTATION DEVELOPMENT

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This experiment aimed at comparing the potential *in vivo* development of goat embryos following somatic nuclear transfer (NT) into recipients either at an early stage (4-cell) or after *in vitro* culture up to the blastocyst stage in SOF medium or co-culture with Vero cells.

Donor nuclei were obtained from cells isolated from a 44 days old caprine foetus. A foetal fibroblast cell line was frozen, stored and cultured over passage 4. *In vivo* matured oocytes recovered from superovulated goats were used as recipient cytoplasts. Enucleation was performed in M199 supplemented with cytochalasin B (CB) (5 µg.mL $^{-1}$). Somatic cells and cytoplasts were fused (2 DC pulses of 2.0 Kv.cm^{-1} for 50µsec each in 0.3M mannitol) and reconstructed embryos were incubated in M199 for two hours. Activation was then performed by incubation in the presence of ionomycin (5 mM) for 5 min. Activated embryos were incubated in M199 containing 6-DMAP (2 mM) and CB (5 µg.mL^{-1}) for three hours. NT embryos were either cultured *in vitro* in droplets for 35h (Group 1) or for seven days in SOF medium containing 10% FCS (Group 2) or B2 medium containing 2.5% FCS with Vero cells (Group 3).

In group 1, 4-cell stage NT embryos selected at 35 h were surgically transferred into the oviduct of recipients (8 to 15 embryos/recipient). In groups 2 and 3, compacted morulae and blastocysts selected at day 7 were transferred into the uterine horn of recipients under coelioscopy (3 to 7 embryos/recipient). Plasma progesterone levels were measured on day 21 and pregnancy was confirmed by ultrasound scanning on days 35 and 45.

In vitro development to the morula-blastocyst stage in groups 2 and 3 was 36.3% (45/124) and 27.5% (30/109) respectively. *In vivo* development is presented in Table 1.

Table 1. *In vivo* development of goat embryos after somatic NT according to culture system before transfer into recipients.

	Group 1 (4-cell stage)	Group 2 (Blastocysts / SOF)	Group 3 (Blastocysts / Vero cells)
No of recipients	9	9	7
Progesterone test at day 21	5 / 9	7 / 9	6 / 7
Pregnant at day 35	4 / 9	5 / 9	6 / 7
Pregnant at day 45	2 / 9 (2 + 3 foetuses)	3 / 9 3 + 2 + 2 foetuses)	3 / 7 (2 + 2 + 1 foetuses)

As observed in other species after somatic cloning, we noticed a large number of pregnancy losses (>50%) between day 35 and day 45. This underlines the importance of events occuring during the peri-implantation period. Irrespective of the system and timing of transfer into recipients, peri-implantation losses before day 45 occured in the three groups. However, ultrasonography showed apparently normal foetuses with visible heart beats.

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Notes

CYSTEAMINE IMPROVES *IN VITRO* GOAT OOCYTE MATURATION IN DEFINED MEDIUM.

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Addition of glutathione (GSH) precursors, such as cysteamine to the IVM medium increases bovine oocyte GSH content and developmental competence (de Matos and Furnus, Theriogenology,2000,53:761-771).

The aim of this study on goat oocytes was: (1) to evaluate the effects of different concentrations of cysteamine in a defined maturation medium on maturation, fertilization and embryo development and (2) to compare in vitro embryo production after oocyte maturation in defined medium or medium containing 10% follicular fluid and FSH (Cognie and Poulin, ICAR, 2000, vol.2, Abstr.216), both supplemented with the optimal concentration of cysteamine.

Exp.1: Goat oocyte-cumulus complexes were collected from slaughterhouse ovaries and matured at 39°C in 5% CO_2 for 24 hours in M199+ EGF (10 ng/mL) with 0, 25, 50, 100, or 200 µM of cysteamine (Sigma). Oocytes (n = 150/treatment, 4 replicates) were *in vitro* fertilized and cultured in SOF-BSA in the presence of serum to the blastocyst stage as previously described (Cognie *et al.*, 1995, AETE, 146).

Exp.2: Blastocyst production was evaluated (6 replicates) at day 7 post insemination after oocyte maturation in M199 + EGF + 100 µM cysteamine (EGF group, n = 257) or in M199 +10% goat follicular fluid +100 ng/mL oFSH + 100 µM cysteamine (FF group, n = 221). The viability of these blastocysts was tested by transfer to synchronized recipients (2 embryos per recipient). Pregnancy rate was diagnosed by progesterone assay on Day 21, confirmed on Day 41 by ultrasound and at term.

Results:

Exp.1: The cleavage rates were similar in all groups (82 to 88%) but blastocyst rates /total oocytes, increased significantly (P< 0.001) when cysteamine was present in the maturation medium: 8%, 37%, 52%, 53% and 48% for 0, 25, 50, 100 and 200 µM, respectively.

Exp.2: The cleavage rates (81 and 84% at 2dpi) and blastocyst rates (47.1 and 46.6% at 7dpi) were similar in EGF and FF groups, respectively. *In vivo* development is presented in Table 1; gestation length and birth weight of kids were normal in the two groups.

Table 1 : Pregnancy rate and embryo survival of goat embryos after oocyte IVM in cysteamine-containing M199 supplemented with EGF at 10ng/mL (EGF) or 10% follicular fluid and oFSH at 100 ng/mL (FF).

IMV Method	Recipients n (embryos)	Pregnancy rate day 21	day 41	Kidding rate %	(n)	Embryo survival %	(n)
EGF	8 (16)	88	75	75	(6)	50	(8)
FF	10 (20)	80	70	70	(7)	45	(9)

In conclusion, addition of cysteamine to defined IVM medium improved the efficacy of goat in vitro embryo production to the level achieved when goat oocytes were matured in the presence of FF.

Notes

PREGNANCY PROTEIN PSP60 LEVELS IN PLASMA INCREASED IN RECIPIENT COWS WITH CLONED FOETUS WHICH DEVELOPED HYDRALLANTOIS

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Several abnormalities have been associated with somatic clone pregnancies. In cattle, cloned foetuses are larger and recipient cows often develop hydrallantois in the third trimester of pregnancy. Pregnancy Specific Protein 60 (PSP60) is a glycoprotein which belongs to the Pregnancy Associated Glycoprotein family (PAG) and is produced by binucleate cells of the trophoblast. Throughout pregnancy, the binucleate cells migrate and fuse with uterine cells to form syncytial masses from which their secretory products are excreted into the maternal circulation. Thus, PAG are useful markers of gestation. Clone recipients with hydrallantois (CRH) have significantly increased PSP60 levels in plasma until 150 days of pregnancy compared with control pregnancy and clone pregnancy without hydrallantois (Heyman *et al*., 2002, Biol. Reprod., 66 : 6-13). This may be due to (1) oversecretion of PSP60 by binucleate cells, due to specific overexpression or non-specific increased synthetic activity of the cells ; (2) abnormal proliferation of binucleate cells in the cloned conceptus ; or (3) to the hypertrophy of the placentomes associated with cloning in cattle.

When hydrallantois signs appeared at ultrasonographic exam, CRH were slaughtered for collection of placental and foetal tissues. Placentas were grossly examined and all the placentomes were counted and weighed. Placentas from 10 CRH and 4 control cows (AI and IVF) were examined. There was a significantly reduced number of placentomes (68 ± 22 vs. 96 ± 34, $p < 0.05$) of heavier weight (138.2 ± 52.1 g vs. 64.6 ± 36.5 g, $p < 0.05$) in CRH compared to controls. Moreover, total placentome weight was significantly heavier in CRH (9.3 ± 3.8 kg vs. 5.5 ± 2.1 kg, $p < 0.05$). The colocalisation of expression of PSP60 and bovine placental lactogen (bPL), another hormone specifically produced by binucleate cells was studied by immunohistochemistry on these placentomes. There was no difference in the distribution and general appearance of binucleate cells nor in the expression of PSP60 or bPL between CRH and control placentomes. On-going cell counting indicate that the ratio of binucleate/mononucleate trophoblast cells does not differ between groups. Trophoblast development also appeared to be normal.

These results indicate that the most probable cause of increased PSP60 concentrations in abnormal clone pregnancies is due to hypertrophy of placental tissue rather than deregulated proliferation of binucleate cells. Further studies are in progress to confirm these results.

Notes

LAMBING RATE AFTER TRANSFER OF BLASTOCYSTS IN VITRO PRODUCED WITH RECOMBINANT HUMAN GONADOTROPHINS IN THE MATURATION OOCYTE MEDIUM

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The objective of this work was to determine the viability, after transfer, of vitrified and fresh blastocysts in vitro produced derived from oocytes matured in a medium with human recombinant gonadotrophins (r-FSH and r-LH).

Sheep oocytes were collected from slaughterhouse-derived ovaries and divided in two different groups of maturation: the recombinant gonadotrophins group (rG), matured in TCM199 supplemented with 4mg/mL BSA, 100µM cysteamine, and with 0.1 UI/mL r-FSH (Gonal-F® 75, Serono, Italy), 0.1 UI/mL r-LH (Lhadi® 75UI, Serono, Italy), 1 µg/mL estradiol-17β; the control group (cG), cultured, as usually in our laboratory, in TCM199 with 10% serum (FBS), 100 µM cysteamine, 1 µg/mL estradiol-17β, 5 µg/mL FSH, 5 µg/mL LH. After maturation, fertilisation and culture, the embryos that reached expanded blastocyst stage (d 6 to 7) were divided as follows: rG, n 36 vitrified and n 38 fresh-transferred; cG, n 19 vitrified and n 47 fresh-transferred. The embryos for vitrification were exposed at 10% ethylene glycol (ET) + 10% dimethylsulphoxide (DMSO) for 3 min; then, for < 45 sec to 20% EG + 20% DMSO + 0.3 M trehalose (T). They were loaded into open pulled straws (OPS) and immediately plunged into LN_2. Warming was carried out placing the OPS into a Falcon tube with TCM199 + 20% FBS and 0.5M T for 3 min, then the blastocysts were transferred in pairs directly into synchronised ewes.

There were not significant differences on lambing rate between treatments in both vitrified and fresh transferred blastocysts (Tab. 1). However, a slightly improvement in the lambing rate was observed in the recombinant groups. To be able to confirm this result, more observations are needed. In conclusion, these data provide support for the responsiveness of sheep oocytes to recombinant gonadotrophins used for in vitro embryo production.

Table 1. Lambing rate of vitrified and fresh embryos produced with recombinant gonadotrophins in the maturation medium of the oocytes

Treatment		Blastocysts transferred n	Recipients n	Lambs born/ vitrified embryos n (%)	Lambs born/ fresh embryos n (%)
r-FSH + r-LH	Vitrified	36	18	7/36 (19.4)	
	Fresh	38	19		19/38 (50.0)
Control	Vitrified	19	9	3/19 (15.8)	
	Fresh	47	23		18/47 (38.3)

Notes

SUPEROVULATION OF HEIFERS WITHOUT RECTAL CHECK BEFORE FSH APPLICATION

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As standard procedure before the start of superovulation (SO) programs a rectal check of the ovaries is performed, to ensure the presence of a Corpus Luteum (CL). Our hypothese was that in virgin heifers this rectal check is not necessary to improve the flushing results when the program is started after a regularly, normally shown heat. Therefore this study investigated the value of the rectal check on the superovulation results.

Virgin heifers were stabled at two donor stations. They were flushed one, two or three times (at 5 week intervals) in the age of 12-16 months (at first flush the average age was 1 year and 32 days). A normal FSH superovulation program of 8 decreasing dosages of FSH (OVAGEN, 176 IU NIH-FSH-S1, ICP, New Zealand) in 4 days was used. Prostaglandin (PROSOLVIN, Luprostiol, 15 mg, Intervet, The Netherlands) was administrated at the 5th and 6th FSH injection. Firth FSH injection started between 9-14 days after heat. All heifers were checked by rectal palpation and scored as “Good” (presence of CL) or “Bad” (no CL and/or cystic). Normally we should have rejected the last group of donors after this heat. Totally 306 heifers were checked, 294 of them were flushed. Twelve heifers were not flushed because of veterinary or breeding problems (5) or because they did not show heat after superovulation (7). Beside these 12 animals there were 33 heifers we should have rejected for superovulation because of a bad rectal check, but in this study we started the SO program and flushed them. So under normal conditions we should have not flushed 15 % of the animals while now we only did not flush 4 % of the animals. The total study covered 1 ½ year.

Results are shown in table 1. Results were analysed by Chi-square analysis and Student T-tests. No significant differences were found. The good group (261) resulted in a total of 1107 embryos, 16 % flushes without any embryos. The bad group (33) resulted in a total of 151 embryos and 24 % of these flushes did not yield any embryos.

Table 1. Effect of using rectal check, as a parameter to reject animals in a SO program, on embryo yield.

Group (n)	Total embryos	0-flushes	Embryos/flush
Good (261)	1107	43 (16%)	4.2
Bad (33)	151	8 (24%)	4.6

These results show that in our situation, heat control is enough and useful as parameter to start SO program in virgin heifers. There was no additional value of the rectal check. Most important is that in total more embryos are produced by also using the “Bad” animals. Therefore this method is now a standard at our donor stations. We check all animals at the age of 11 months on a normal genital tract by rectal palpation. We have a very severe heat control system and start SO program after heat at the age of 12 months.

Notes

PREMATURATION OF CALF OOCYTES WITH MEIOTIC INHIBITORS : EFFECT ON NUCLEAR MATURATION AND BLASTOCYST DEVELOPMENT

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Oocytes collected from prepubertal calves have a lower competence to development than oocytes from adult animals. Incubating calf oocytes with inhibitors of meiosis before the maturation step could help to increase their developmental potential.

Cumulus-oocyte complexes (COCs) from calves aged 6 to 9 months were collected from abattoir ovaries. After selection on morphological criteria, COCs were prematured and/or matured in TCM199 containing 10 ng/ml EGF and 0.4 mM pyruvate. Prematuration was performed for 24h in the same medium in the presence of 25 µM Roscovitine (Rosco) or in a mixture of 6.25 µM Butyrolactone I (BLI) and 12.5 µM Rosco. The COCs were then washed and matured for 24h or 17h. Part of the oocytes were fixed after prematuration and maturation in order to evaluate the stage of nuclear maturation. The remaining COCs were fertilized and cultured in SOF medium containing 5% FCS in an atmosphere of 5% 0_2, 5% CO_2 and 90% N_2 for 7 days at 39°C. Blastocyst rates were evaluated at Day 8 post-insemination.

Results are summarized in Table 1. Prematuration with both treatments prevented at least partly the resumption of meiosis in a majority of the oocytes. After a further step of maturation, nuclear maturation (metaphase II stage) was achieved in the same proportion as for non prematured (control) oocytes. However, further embryonic development was impaired by comparison with control oocytes without prematuration. As nuclear maturation could be accelerated after prematuration with meiosis inhibitors, the duration of the maturation step was reduced to 17h. Some improvement in embryo development was observed after this reduction but the developmental rates remained lower than for control oocytes without prematuration.

Table 1 – Effect of prematuration of prepubertal calf oocytes with meiotic inhibitors on nuclear maturation and blastocyst development

	Stage of nuclear maturation		
	Before maturation < Metaphase I	After maturation Metaphase II	Blastocysts at Day 8 % (on oocytes)
24h maturation			
Control	100% (N = 17) [a]	58% (N = 76) [a]	14% (61/429) [a]
Roscovitine	74% (N = 69) [b]	60% (N = 48) [a,b]	4% (16/376) [b]
BLI + Rosco	73% (N = 60) [b]	77% (N = 64) [b]	8% (29/372) [c]
17h maturation			
Control	100% (N = 75) [a]	63% (N = 60) [a]	16% (39/239) [a]
Roscovitine	67% (N = 52) [b]	63% (N = 46) [a]	10% (31/307) [c]
BLI + Rosco	66% (N = 64) [b]	67% (N = 64) [a]	8% (22/269) [c]

[a,b,c] Data with different superscripts differ within the same column (Chi square – $P<0.05$). Total of 3 replicates.

In conclusion, prematuration of calf oocytes with meiotic inhibitors allows for a reversible prevention of nuclear maturation. However, oocytes have a lower developmental potential after prematuration which was not observed with oocytes from adult animals submitted to the same treatments (Ponderato *et al*., Mol Reprod Dev, 2001, 60:579-585).

Notes

EMBRYO TRANSFER AS A METHOD TO ELIMINATEPATHOGENIC AGENTS IN A RABBIT COLONY

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Genetically valuable New Zealand White rabbits were contaminated by different types of pathogenic agents such as a parasite (Encephalitozoon cuniculi), a bacteria (Clostridium perfringens) and a virus (rotavirus). To regain the SPF status of this contaminated breeding unit, embryos from the infected does were transferred into SPF recipient females.

Does (n = 32) received ovarian stimulation as previously described (Joly *et al*., 1996). Stimulated females were sacrificed 62 to 70 hours post mating to recover compact morulæ. After laparotomy, uterine horns were excised. Retrograde flush of uterine horns was performed with DPBS (Sigma) added with 10% FCS. After collection, quality of embryos was evaluated to select only embryos suitable for cryopreservation and further transfer. Only zona pellucida intact embryos were washed. All embryos with cellular inclusions inside their mucin coat were discarded. Selected embryos from each donor were washed in 10 successive baths of DPBS to dilute contaminants as much as possible.

Embryos were cryopreserved in 1.5M DMSO in DPBS added with 20% FCS (Techakumphu and Heyman, 1987) in 0.15 ml transparent straws (CBS, Paris, France). All the surgical embryo transfers were performed in SPF conditions. Recipient rabbits (n = 32) received an IV injection of 75 IU hCG (Chorulon®, Intervet,) to induce pseudopregnancy. At 60h after injection, thawed embryos were introduced into the fallopian tube (5 to 9 morulæ per oviduct). Health screenings were performed on sanitised rabbits 24-26 weeks after birth to follow FELASA guidelines (Federation of European Laboratory Animal Science Association).

893 embryos were collected, among which 92% (821) had an intact zona pellucida and were selected for cryopreservation (average of 25.7 embryos per donor, 821/32). To preserve the genetic value of the breed, 40% of frozen embryos were kept in liquid nitrogen. From 478 thawed embryos, 97.5% were recovered (466) and 89.5% showed good quality with intact mucin layer and zona pellucida (417) and were transferred into 36 recipients (10 to 18 embryos per recipient). After transfer, 72% of recipients delivered (26/36) and 24.9% (104/417) of transferred embryos developed to full term into live pups. One year later, health screenings performed on sanitised rabbits confirm the recover of our SPF health status.

In conclusions, we demonstrate ET of washed and cryopreserved embryos is an effective method to eliminate different types of pathogenic agents in the rabbit species. This method is suitable for rabbit embryos which are enclosed in a mucin coat provided embryos with mucin attached cells are eliminated to avoid any possibility of disease transmission.

References:

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Notes

PREGNANCY ASSOCIATED GLYCOPROTEINS ISOLATED FROM EWE PLACENTA

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The Pregnancy-Associated glycoproteins (PAGs) constitute a large family of glycoproteins synthesized in the outer epithelial layer of the Artyodactyl placentas. As part of them are released into the maternal circulation, their measurement is useful for both pregnancy diagnosis and follow-up.

In the sheep, using a biochemical approach, the PAGs were first identified under three names including SBU-3 antigens (Atkinson *et al*., 1993; J Biol Chem 268:26679), oPSPB (Willard *et al*., 1995; J Anim Sci 73:960) and ovPAGs (Xie *et al*.,1997; Proc.Natl. Acad. Sci 94:12809). Today, we know that all these molecules belong to the same family termed ovPAGs. Parallel to these studies, molecular biology techniques identified nine full cDNA sequences including ovPAG-1 to ovPAG-9 and showed that their expression varies in a temporal manner during pregnancy.

The aim of our study was to investigate the ovPAGs isolated from different stages of pregnancy in order to develop new specific and sensitive assays.

Two different stages of pregnancy were used, placentas removed between 66 and 100 days of gestation and after day 100. The protein extract was first fractionated by acidic and ammonium sulfate precipitation. The 40-80% ammonium sulfate immunoreactive fraction was submitted to anion exchange chromatography (DEAE-cellulose). The fractions 0.04 M and 0.08 M NaCl were separately submitted to the CM ceramic column. The main spots transferred onto PVDF were submitted to the Edman degradation.

Our results presented in the table show that the population of PAG is different in the two batches of placenta. A total of seven molecules, all different ''inter se'' and different of SBU-3 antigens or ovPAGs, isolated by Xie *et al.* were isolated and identified. Four of them (from 66 to 100 days placenta) correspond to inferred cDNA sequences already published by Xie *et al*. (1997)

Table 1. N-terminal amino acid sequences of ovPAGs and their consensus and their comparison to SBU-3 and ovPAGs

		Identity (%)						
		SBU-3 antigens (Atkinson *et al*., 1993)			ovPAGs (Xie *et al*., 1997)			
Gestation stages	Sequences	57[a]	62[a]	69[a]	55[a]	60[a]	61[a]	65[a]
≥ 100 days	RGSNLTIHPLRNIRD	56	69	60	33	73	87	87
	ISSRVSXLTIHPLRNIMDML	45	67	53	46	60	65	65
	RGSXLTILPLRNMRDIVY	58	87	78	33	66	83	88
From 66 to 100 days	RVSNLTIHPLRNIILDRYVG	46	56	50	40	53	70	65
	RDSNVTILPLRNMKD	67	77	80	33	66	80	93
	RGSNLIIHPLRNIRDTFYVGKIT	43	61	60	26	73	80	76
	RGSNLTIHPLRNTKDLVYLG	84	61	60	33	73	80	80
Consensus	R--S-----I-- PLRN----------------	[a]: represent molecular weight (kDa)						

The proteins isolated and identified in this study confirm a temporal expression of ovPAG during pregnancy and open the way for new physiological studies on sheep pregnancy.

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Notes

THE EFFECT OF BMP-2 ON IN VITRO PRODUCTION OF BOVINE EMBRYOS AND IT'S LOCALIZATION IN THE OVARIAN FOLLICLES

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily, which are involved in the regulatory events of folliculogenesis. The role of BMPs in the reproduction system is poorly understood. Although some BMPs (BMP-2, 3, 4, 6, 7, and 15) are expressed and type I and II receptors have been demonstrated in the ovary, their importance for the reproduction process is unclear. In large domestic ruminants and humans, it is suggested that BMPs like BMP-2 and BMP-4 are major intraovarian factors that regulate the follicle selection. In present study we investigated the presence of BMP-2 in the bovine ovary and it's effect on bovine *in vitro* maturation (IVM), fertilization (IVF) and embryo development.

Bovine ovaries were collected from the slaughterhouse and cumulus-oocyte complexes (COCs) were aspirated from antral follicles (2-8 mm). COCs were cultured in groups of 35 in 500 µl of IVM medium for 23 h. The IVM medium consisted of M199 supplemented with 10 % FCS +/- 0.05 IU/ml recombinant human FSH and +/-100 ng/ml of BMP-2. IVF took place in TALP medium supplemented with heparin and PHE (D-Penicillamin, Hypotaurine and Epinephrine) using frozen-thawed spermatozoa, centrifuged over a percoll gradient. After 20 h of incubation, the oocytes were freed from cumulus cells and were placed in a co-culture of BRL (Buffalo rat liver) cells. IVM and IVF as well as in vitro embryo culture (IVC) took place at 39°C in a humidified atmosphere of 5 % CO_2 in air. For morphological studies, ovaries were fixed overnight in 4 % paraformaldehyde. They were dehydrated via a graded ethanol series and embedded in paraffin wax, and finally cut into 5 µm-sections. After rehydration, the sections were submitted to boiling citrate buffer for 10 min to retrieve antigen and then incubated with a monoclonal antibody against BMP-2 (1:100) for 1 h at room temperature.

Cumulus cell expansion and nuclear maturation in BMP and/or FSH treated groups did not differ from those in the controls. In addition there was no effect of treatment on the outcome of IVM, IVF and IVC (table 1). Positive immunostaining for the BMP-2 protein was observed in granulosa cells of early-staged follicles and granulosa cells, internal theca cells and occasionally in the oocytes of antral follicles.

Table 1: Results of IVP of cattle embryos in dependence on culture system during maturation

	n	% Cleavage Day 4	% Blastocyst Day 7	% Blastocyst Day 9	% Blastocyst Day 11
M199	593	63	2	13	15
M199+BMP-2	577	54	2	14	15
M199+FSH	332	78	11	25	27
M199+FSH +BMP-2	353	75	10	25	26

It is concluded that despite of the presence of BMP-2 in bovine ovaries, it has no prominent effect on IVM, IVF and embryo development.

Notes

EFFECT OF β-MERCAPTOETHANOL ON THE ICM AND TROPHECTODERM CELLS ALLOCATION IN IVP BOVINE BLASTOCYSTS

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Oxidative stress is increased during *in vitro* culture conditions and may contribute to the reduced efficiency of *in vitro* embryo production. Several authors have reported the beneficial effect of precursors or stimulators of glutathione synthesis on the quality (hatching rate and cell number) of IVP bovine blastocysts. The present work aimed to evaluate whether β-mercaptoethanol (β-ME), a stimulator of gluthatione synthesis could influence the allocation of cells of the inner cell mass (ICM) and trophectoderm (TE) cells.

In vitro-derived bovine embryos were obtained from abattoir oocytes after IVM/IVF and IVC in SOF medium containing 5% fetal calf serum under 5% O_2, 5% CO_2 and 90% N_2. Morulae were collected at Day 5 post-insemination (pi) and cultured in the absence or the presence of β-ME (0.1 mM). Blastocysts from each group were collected at day 8 pi for differential cell staining. We have used the protocol described by Thouas *et al.* (2001), with some minor changes. Briefly, embryos were incubated for 30-60 sec at the lab temperature in TCM-199 containing 0.5% (v/v) Triton X-100 and 50 µg/ml propidium iodide. Embryos were immediately transferred to an iced solution of ethanol (100%) with 25µg/ml bisbenzimide (Hoechst –33342). Embryos were then left overnight to 48 hours at 4°C in dark. Under a fluorescence microscope, ICM nuclei appeared blue while TE nuclei were pink.

The proportion of morulae developing to the blastocyst stage was not affected by β-ME (37/50 - 74% vs 25/35 - 71% in the control; χ^2 - P>0.05). However, the hatching rate was significantly increased by β-ME (23/50 - 46% vs 8/35 - 23%; χ^2 - P<0.05). Results of the differential cell counts are summarized in the Table 1. The presence of β-ME in the culture medium significantly increased the mean cell number (P<0.05; ANOVA 2). The proportion of ICM cells (per total cells) of hatched blastocysts was significantly increased by β-ME (P<0.05; ANOVA 1), whereas it was unaffected in unhatched blastocysts (P>0.05; ANOVA 1).

Table 1: Effect of 0.1 mM β-ME on ICM and trophectoderm cells allocation of IVP bovine blastocysts.

	Hatched blastocysts		Unhatched blastocysts		Total blastocysts	
	- β-ME	+ β-ME	- β-ME	+ β-ME	- β-ME	+ β-ME
N	5	16	16	14	21	30
Total cells	148 ± 17[a]	187 ± 10 [a]	109 ± 7 [a]	131 ± 10 [a]	115 ± 8 [a]	163 ± 7 [b]
ICM/Total cells	28 ± 4 [a]	37 ± 2 [b]	32 ± 2 [a]	34 ± 3 [a]	33 ± 2 [a]	31 ± 2 [a]

[a,b] Values with different superscripts are significantly different within the same line and in the same category of blastocysts (ANOVA – P < 0.05).

Our results suggest that the presence of a stimulator (β-ME) of glutathione synthesis at the time of compaction/blastulation does not modify the blastocyst yield, but improves their blastocyst quality (hatching rate and cell number). Moreover, β-ME could influence cell allocation in hatched blastocysts, by increasing the proportion of ICM cells.

Notes

EFFICACY OF A SUPEROVULATION PROCEDURE WITH DEFINED LH SURGE IN THE BOVINE

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Superovulation (SO) with defined LH surge facilitates collection of preovulatory oocytes or embryos at fixed time after AI. To control the LH surge a progesterone block is used to suppress endogenous release, followed by GnRH administration to induce a defined LH surge. This SO procedure has been applied in experiments during 1999-2002. To validate the SO procedure we analyzed the data for: 1. effectiveness of progesterone block, 2. superovulatory response and 3. embryo yield 7 d after AI.

Synchronized, cyclic animals (HF cows and heifers, n = 202) were used for SO. Briefly, follicles >5 mm were disabled by transvaginal ultrasound-guided puncturing at Day 8 (estrus = Day 0). At Day 9 a Crestar ear implant (3 mg norgestomet; Intervet International BV, Boxmeer, The Netherlands) without accompanying injection was inserted, and SO was started at Day 10 by oFSH im (Ovagen ICP, Auckland, New Zealand) twice daily during 4 d (total dose of 10 and 17 ml for heifers and cows, respectively). Prostaglandin (22.5 mg PG; Prosolvin, Intervet) was administered im concomitant with the fifth dose of FSH. Ear implants were removed 51.7 ± 0.2 h (SEM, n = 202) after PG. GnRH (1.0 mg Fertagyl or 0.021 mg Receptal; Intervet) was administered im immediately after removal of the implant or within 4 h when preovulatory oocytes were collected.

The majority of the animals (n = 184, 91.1%) showed a normal LH profile without increase during norgestomet treatment (Figure 1a; excluding 9 animals ovariectomized before the LH surge). There were no significant differences between cows and heifers (average interval between GnRH and maximum of the LH surge 2.3 ± 0.03 h, and amplitude of the LH surge 27.0 ± 0.7 ng/ml). Of the 18 animals with a deviating LH profile (Figure 1b), 11 animals showed an increase of LH during norgestomet treatment and 7 after that. Significantly ($P<0.05$) more cows (14/75, 18.7%) than heifers (4/127, 3.1%) showed deviation in LH profile.

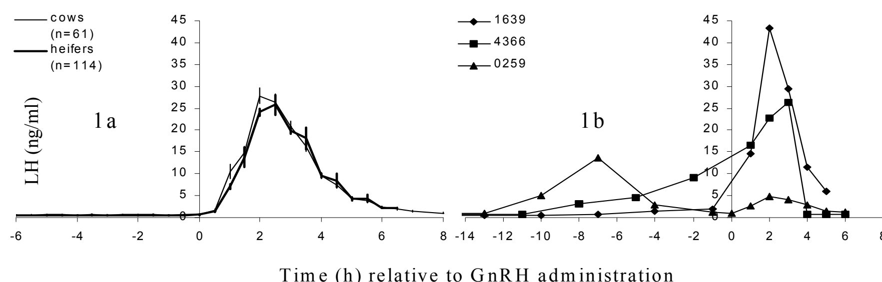

Figure. Plasma LH concentrations in cattle superovulated with defined LH surge.
1a: normal profile (mean ± SEM) in 175 animals; 1b: examples of deviating profiles in 3 animals

The superovulatory response was 23.9 ± 1.5 preovulatory follicles/ animal (n = 72) when oocytes were collected. In the 118 animals used for collection ex vivo of embryos at specific stages, 17.1 ± 1.1 corpora lutea/ animal were counted. Embryo yield at Day 7 after AI (191.4 ± 0.2 h after LH surge) was 5.9 ± 0.7 embryos/animal (n = 26). In total 9.8 embryonic structures per animal were recovered (rate 75.5%), comprising 3.7 degenerated (incl. 1 cell stage), 0.2 2-32 c. stage, 2.7 morulae and 3.2 blastocysts. In conclusion: the progesterone block effectively suppresses the endogenous LH surge. It can be assumed that in only 5.4% (11/202) of the animals the timing of LH was not controlled and may have affected subsequent oocyte and/ or embryo development. In the other 7 animals with deviating LH profile the maximum of the LH surge was within the normal range after GnRH. The superovulatory response and the embryo yield during this SO procedure were normal. Therefore, it can be concluded that this is a reliable procedure to obtain oocytes and embryos at specific stages.

Notes

CRYOSURVIVAL OF SHEEP EARLY EMBRYOS IS INFLUENCED BY INTRINSIC SENSITIVITY TO FREEZING

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Developmental stage of embryos affects their cryosurvival. Frozen-thawed early embryos show lower in vitro developmental rates than morula and blastocyst stages (Whittingham *et al*., 1972. Science 178:411-414). This drop in viability may be caused by a higher sensitivity to freezing or by an inadequacy of the culture system (Wilmut I. 1986. Dev. Biol. NY 1985, 4: 217-247). The objective of this study was to evaluate the sensitivity to freezing of early sheep embryos by comparing *in vitro* developmental rates between frozen-thawed embryos and fresh embryos cultured with the same procedure. A total of 213 embryos, classified as viable by their morphological characteristics, were used. These embryos were obtained 3-3.5 days after oestrus detection from ewes treated with standard superovulatory protocols (Cocero *et al*., 1999. ITEA 20:702-704). Embryos were distributed in three categories: 2-4 cells (n = 72); 4-8 cells (n = 73) and 8-12 cells (n = 68). Embryos in each category were randomly allocated to one of two groups. In the first one, fresh embryos were placed on an ovine oviduct epithelial cells monolayer just after recovery, and cultured to the blastocyst stage. In the second group, embryos were previously frozen using a conventional two-step ethylene-glycol method (Cocero *et al*, 1996. Cryobiology 33: 502-507). Just after thawing and cryoprotectant removal with saccharose, embryos were cultured in the same system described for the first group. Developmental rate was obtained by dividing the number of embryos reaching the blastocyst stage by the total number of embryos in culture. Chi square analysis showed significant differences ($p<0.001$) between frozen-thawed and fresh early embryos in all three stages considered, as shown in Table 1. These results indicate that low developmental rates obtained after freezing-thawing of early embryos are caused by their intrinsic sensitivity to freezing, since the coculture system was capable of supporting development of non frozen embryos.

Table 1. Blastocyst rate of freezing-thawed early embryos vs fresh cultured early embryos.

Embryo stage	Freezing-thawed embryos	Fresh embryos
2-4 cells	17.1% (6/35)[a]	67.5% (25/37)[b]
4-8 cells	31.4% (11/35)[a]	89.4% (34/38)[b]
8-12 cells	22.2% (8/36)[a]	100% (32/32)[b]

Note.a≠b:p<0.001

This work was supported by a grant in the project SC 00-051-C3.1

Notes

MITOCHONDRIAL ACTIVITY IN BOVINE CUMULUS-OOCYTE COMPLEXES FOLLOWING TREATMENT WITH MEIOTIC INHIBITORS.

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Reversible inhibitors of meiosis are used to prevent the spontaneous resumption of meiosis occurring when bovine cumulus-oocyte complexes (COCs) are removed from ovarian follicles. Butyrolactone-I (BLI) was successfully used for meiotic inhibition (Kubelka M *et al.*, Biol Reprod. 2000;62:292-302). However, a detrimental effect of BLI on oocyte mitochondrial morphology has been shown (Fair T., Mol Rep. Dev, 2002, in press). Our objective was to evaluate the effect of BLI on mitochondrial activity in COCs. For this study, we used a cationic dye, JC-1. The accumulation of this dye inside the mitochondria is membrane potential (MMP) dependent. When accumulating inside mitochondria the dye fluorescence shifts from green to red.

COCs were collected from abattoir ovaries and were prematured for 24h in TCM 199 in the presence of 100 µM Butyrolactone I (BLI). Thereafter, they were matured in 199 + 10 ng/ml EGF for a further 24h. MMP was evaluated at the end of the prematuration period and after maturation. Freshly collected COCs (before maturation) and mature COCs without prematuration were used as controls.

COCs were incubated for 1 hour in 7.66 µM JC-1, washed and immediately evaluated by confocal microscopy (excitation at 488 nm and 568 nm emission measured at 522DF32 nm, green and 605DF32 nm, red). The COCs were then incubated for 10 min in 2 mg/ml dinitrophenol (DNP), a mitochondrial uncoupler, and re-evaluated by confocal microscopy. The image analysis was performed with NIH Scion images program. An integration of the total emitted light density was calculated for each wavelength (green and red). The ratio between red and green fluorescence was then established and compared between treatments.

Due to high variations in oocyte staining, analysis was only performed on cumulus cells. COCs that have been prematured in the presence of BLI showed a lower MMP than those that were not prematured (Table 1). The decreased MMP persisted after 24h maturation in absence of BLI.

Table 1: Evaluation of mitochondrial membrane potential in cumulus cells after prematuration with Butyrolactone I

Treatment	Before maturation		After maturation	
	COCs analysed N	Ratio red/green Mean ± SD	COCs analysed N	Ratio red/green Mean ± SD
control	19	3.5 ± 2.06 [a]	22	1.23 ± 0.26 [a]
control + DNP	18	0.5 ± 0.2 [b]	30	0.2 ± 0.11 [b]
Butyrolactone I	19	2.17 ± 0.7 [c]	26	0.53 ± 0.39 [c]
Butyrolactone I + DNP	15	0.3 ±0.09 [b]	18	0.19 ± 0.11 [b]

[a,b,c] Data with different superscripts are different (Student T test $p<0.05$)

In conclusion, mitochondrial activity is reduced in cumulus cells when prematuration is performed with BLI. The decrease in MMP is still visible after maturation. This could impair cytoplasmic maturation and further embryonic development after in vitro fertilisation. We are now investigating the effect of other meiotic inhibitors on the same parameters.

Notes

INFLUENCE OF OVARIAN STATUS ON NUMBER AND VIABILITY OF SHEEP EARLY EMBRYOS

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Presence of a large dominant follicle, (LF, ≥6mm in size), at start of FSH treatments has a negative effect on superovulatory yields in sheep, decreasing the number of embryos recovered from uterus (Gonzalez-Bulnes *et al.* 2002, Theriogenology, 57: 1263-1272). Possible causes can be related with alterations in ovulatory processes or deficiencies in embryo transfer from oviduct to uterus. On the other hand, presence of a Corpus luteum (CL) has a positive effect, decreasing embryo degeneration (Gonzalez-Bulnes *et al.* 2002, Theriogenology, accepted for publication). Possible causes can be related with alterations in oocyte/embryo developmental competence by itself or by changes in oviduct/uterine environment. To determine whether effects are decreasing embryo yields, early embryos were recovered from oviduct in a total of 64 Manchega ewes. Sheep were treated, in breeding season, with 40 mg FGA sponges (Chronogest®) for 14 days plus 125 µg of cloprostenol on Day 12, just before starting the administration of Ovagen™ twice daily for 4 days. Embryos were placed in ovine oviduct epithelial cells monolayer just after recovery and cultured until blastocysts stage to evaluate its viability. A transrectal ultrasonography on Day 12, just prior start the FSH treatment, showed presence of LF in 20 ewes (31.2%) and presence of CL in 31 of the 64 ewes (48.4%). There were not detected any effects derived from the presence of LF or CL (Table 1), or any interaction between both variables. These results suggest that deleterious effect from presence of LF on embryo recovery from uterus may be caused by alterations during transition of embryos from oviduct to uterus. In the other hand, the decrease in embryo viability found in uterine retrieval from ewes with absence of CL may be more related with alterations in the uterine environment than alterations in embryo developmental competence. Both facts remark the key role of the donor genital environment in superovulatory yields.

Table 1. Superovulatory yields in relation with the presence of LF or CL at start ofFSH treatment.

	Large follicle		Corpus luteum	
	Absence	Presence	Absence	Presence
Number of animals	44	20	33	31
Ovulation rate	12.4 ± 1.4	11.6 ± 0.8	11.4 ± 0.8	12.3 ± 1.0
Number of recovered embryos	9.1 ± 1.2	9.4 ± 0.6	8.9 ± 0.7	9.1 ± 0.8
Recovery rate (%)	71.1 ± 6.0	80.5 ± 2.9	75.9 ± 3.5	78.5 ± 3.6
Number of viable embryos	5.9 ± 0.9	7.6 ± 0.8	7.3 ± 0.8	6.9 ± 1.0
Viability rate (%)	76.0 ± 9.1	78.8 ± 5.6	81.4 ± 5.4	72.7 ± 8.5

Notes

PROGESTERONE LEVELS, CORPUS LUTEUM QUALITY AND PREGNANCY RATES IN HEIFERS TREATED WITH PROPYLENE GLYCOL PRIOR TO EMBRYO TRANSFER. A FIELD TRIAL

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Nutritional status of the recipient is an important factor related to pregnancy rates after embryo transfer (ET). Oral administration of propylene glycol (PG), a gluconeogenic precursor, increases the molar percentage of ruminal propionate to be transformed into pyruvate, and eventually glucose, in the liver (Moore *et al*., Vet Med 1997 92: 1061-72). The aim of this study was to determine the effect of the oral administration of PG to recipient heifers on serum levels of progesterone (P4), corpus luteum (CL) quality and pregnancy rates.

Cyclic Holstein heifers (n = 169), aged 13-16 months, from 35 dairy farms were used as potential recipients for embryos. For experimental design, heifers were oestrus synchronized with 2 doses of $PGF_{2\alpha}$ given 11 days apart, and distributed as follows: 1) heifers fed daily with 250 ml PG for 20 days, starting at day of first $PGF_{2\alpha}$ injection, and 2) untreated heifers. Oestrus detection (Day 0) was based on behavioral signs and serum P4 levels by ELISA (Ovucheck Plasma®, Vetoquinol Diagnostics, Spain). According to both parameters, 149 recipients were considered in the study. On Day 7, serum P4 was again analyzed and a quality score from 1 (excellent and good) to 3 (rejected) was applied to the CL of each recipient by agreement of two experienced ET technicians. *In vivo* frozen-thawed embryos (n = 82) were non-surgically transferred into the uterine horn ipsilateral to the CL, irrespective of the recipient allocation to the PG or control group. Data were analysed by one-way ANOVA and results are shown in tables 1 and 2. Data of P4 and CL are mean values ± SEM. Superscripts represent significant differences: ^{a,b} ($p<0.09$); ^{x,y} ($p<0.001$).

Table 1. Serum progesterone concentrations (P4; ng/ml) and corpus luteum (CL) quality values on day 7 of all recipient heifers.

Treatment	N	P4	CL	N selected for ET (%)
Propylene glycol	75	5.87±0.36 ^{x}	1.46±0.08 ^{x}	52 (69)^{x}
Untreated	74	4.47±0.35 ^{y}	2.11±0.10 ^{y}	28 (38)^{y}

The CL quality was positively correlated with P4 levels (0.39; p<0.001). These correlations were higher in the untreated group (0.45; p<0.001) than in the PG group (0.21; p<0.06). In contrast to untreated controls, PG treatment increased P4 levels in all categories of CL (data not shown) and CL quality. As a consequence, administration of PG leads to an optimized use of recipients.

Table 2. Day 7 serum progesterone concentrations (P4; ng/ml) and corpus luteum (CL) quality values, and Day-60 pregnancy rates (PR) in heifers transferred with a frozen embryo.

Treatment	N	P4	CL	PR (%)
Propylene glycol	52	6.16±0.41	1.27±0.06	65±6.6^{a}
Untreated	28	5.76±0.51	1.10±0.06	46±9.6^{b}

Pregnancy rates and CL quality were positively correlated in recipients treated with PG (0.26; p<0.06). Heifers treated with PG gave higher PR than untreated heifers. The gain in pregnancy rates in heifers receiving PG can be explained in part by an increased CL quality. Consequently, and since P4 and CL values did not differ between groups in transferred animals, other factors related to PG should be considered.

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Notes

PRE-IMPLANTATION DEVELOPMENT *IN VIVO* OF BOVINE BLASTOCYSTS PRODUCED *IN VITRO*: MORPHOLOGICAL STANDARDS AND GENE EXPRESSION PATTERNS

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The timing of *in vitro* development of bovine embryos produced by *in vitro* fertilisation (IVF) is generally asynchronous and give rise to early (Day 6) and late (Day 8) forming blastocysts. It is known that after *in vivo* transfer of late forming blastocysts the pregnancy rate is reduced. The two to three weeks occurring between transfer of IVP blastocysts and the earliest possible pregnancy diagnosis corresponds to the late pre-implantation period and is critical for early embryonic losses. However, despite its obvious biological importance, this developmental period has poorly been investigated so far. The aim of this work was to compare the *in vivo* developmental potential of early and late forming blastocysts produced by IVF (Day-6 and Day-8 blastocysts, respectively).

In vitro embryos were produced through IVM, IVF and culture in B2 medium with vero cells as routinely achieved in the laboratory (Menck *et al.* 1997, RND 37:141-150) and resulted in overall blastocyst rate of 65.3% (n = 1024 inseminated oocytes in 8 replicates). Early blastocysts (D6), 23%, and late forming blastocysts (D8), 9%, of similar morphological grading were selected to assess and compare their differentiation status by the distribution of known cellular markers using immunofluorescence techniques. Both groups of blastocysts were nonsurgically transferred on day 6 or 8 to temporary synchronous recipients (n = 8 recipients, 6 blastocysts per recipient). The conceptuses were collected 10 to 12 days after transfer by gentle flushing of the uterus post slaughter to evaluate their stage of development. Both differentiation of the trophoblast (elongation and Interferon τ expression) and of the embryonic disc (gastrulation stages and expression of the gastrulation marker: *brachyury*) were analysed by western blots and compared to that of Day-18 *in vivo* control conceptuses derived from AI.

Immunohistological analysis for cellular differentiation of the inner cell mass and the trophectoderm indicated that Day-6 and Day-8 blastocysts qualified as grade 1 did not differ from each other before transfer However, after transfer we observed that the earlier the blastocysts formed in culture the better they developed *in vivo*. As evidenced by gastrulation staging and *brachyury* expression, embryos originating from Day-8 blastocysts always presented a delayed gastrulation process as compared with development of those from Day-6 blastocysts. Additionally, trophoblast morphology and Interferon-tau western blots indicated that late forming blastocysts developed into conceptuses with functional trophoblasts but smaller embryonic discs than expected from Day-18 *in vivo* controls.

In conclusion, in the bovine species, functional parameters commonly used to define grade 1 IVP blastocysts before transfer did not sufficiently discriminate their developmental potentials. Taken together, the criteria used here to assess the developmental progress of IVP blastocysts 10 to 12 days after *in vivo* transfer, provide a new basis to analyse the late pre-implantation period and the developmental potential of bovine IVP embryos. These criteria may well be suitably applied to embryos derived from somatic nuclear transfer.

Notes

SPERM MOTILITY, HYPO-OSMOTIC RESISTANCE AND FERTILITY AFTER FREEZING OF LOW DOSES OF BULL SEMEN

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The aim of the present pilot study was to evaluate if the freezing of small volumes of semen affects sperm motility and hypo-osmotic resistance post-thaw and the pregnancy rates in heifers after deep uterine insemination (DUI).

Semen was collected from 2 Estonian Holstein bulls whose average field fertility after conventional AI was 50-55% (Bull A) and 60-65% (Bull B). Semen was diluted and frozen using Triladyl® - egg yolk extender so that each conventional insemination dose contained 40×10^6 and deep uterine insemination dose 2×10^6 sperms. The latter was achieved by packing of 1 cm column of diluted semen between two columns of extender separated by the air bubbles in the 0.25 ml French straw.

Post-thaw progressive motility of AI and DUI doses was evaluated subjectively using a microscope. For osmotic resistance test HOT-1 semen was incubated in a fructose-sodium citrate solution (150 mOsm/l) for 60 min. HOT-2, showing the difference in the percentage of swollen sperm after 2 min of incubation in NaCl solutions with an osmolarity of 66 and 130 mOsm/l, was repeated twice, first (1) immediately after thawing and then after 6 h incubation of the semen at 37°C (2).

Heifers were synchronized with 25 mg of prostaglandin $F_{2\alpha}$ (Dinolytic®, Pharmacia&Upjohn) at 14-days interval and inseminated 80-82 h after the 2nd injection into the uterine body (AI) or deep into the uterine horn (DUI) ipsilateral to dominant follicle bearing ovary. Pregnancy was diagnosed by rectal palpation 45-60-days post-insemination.

Table 1. Sperm quality characteristics and the insemination results.

Bull	Sperm concentration	Post-thaw motility, %	HOT-1, %	HOT-2 (1), %	HOT-2 (2), %	Pregnancy % (n)
Bull A	2.4×10^9 (initial)					
AI	40×10^6	55	43	1	-8	50.0[a] (6/12)
DUI	2×10^6	40	34	1	-7	72.2[a] (13/18)
Bull B	1.8×10^9(initial)					
AI	40×10^6	65	28	13	5	70.6[a] (12/17)
DUI	2×10^6	65	30	7	3	70.0[a] (14/20)

[a] *values with the same superscript within the column did not differ significantly, $p>0.05$*

The results of this pilot study indicated that freezing of small volume of semen packed as a small column in the middle of a straw does not affect significantly sperm functional parameters post-thaw and enables to achieve high pregnancy rates after deep uterine insemination in synchronized heifers. Futher studies are needed to evaluate if deep uterine insemination could improve the fertility of bulls whose non-return rates after conventional AI are below the average.

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Notes

HYPERGLYCEMIA-INDUCED APOPTOSIS AFFECTS SEX RATIO OF PREIMPLANTATION EMBRYOS

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We have reported that exposure to high concentrations of glucose (20 mM) before implantation resulted in a decrease in the number of cells per embryo and an increase in the frequency of apoptotic cells. Here we have evaluated the effect of high concentrations of glucose (20 mM) in the medium used during *in vitro* culture on the sex ratio of bovine blastocysts derived from *in vitro*-matured and *in vitro*-fertilized oocytes and on sex ratio of mice foetus. In this study, we also determined whether blastocyst production of the X-linked inhibitor of apoptosis protein (XIAP) differs between the sexes.

Bovine oocytes were matured, inseminated, and cultured *in vitro* in mSOF medium with 10% FCS with or without glucose supplementation. Female B6CBAF1 mice 2-cells embryos were obtained from superovulation and were cultured in KSOM medium with our without glucose and were transferred at blastocysts stage into pseudopregnant recipients. A significantly higher proportion of females bovine embryos and mice foetuses (day 14) were found among those embryos that developed under hyperglycemic conditions *in vitro*. Total mRNA was isolated from two pools (15/pool) of male and female blastocysts sexed by PCR and a quantitative reverse transcription was performed using specific reverse primers to XIAP plus β-actin. The expression of β-actin was used as a reference value to quantify XIAP in the real time-quantitative PCR. Our results show that female blastocysts produce significantly higher amounts of XIAP mRNA than males and this could be central in explaining the higher proportion of female bovine blastocysts and mice foetuses observed following culture *in vitro* under hyperglycemic-induced apoptosis. This mechanism provides an explanation for the significant reduction of male children born to diabetic mothers.

The level of glucose might affect differentially to male or female preimplantation embryos producing a distortion in the sex ratio: low level of glucose (~5 mM; embryos are affected by oxygen radicals due to increased glycolytic activity -i.e. Crabtree-like effect-) produce a preferential loss of female embryos; and high levels of glucose (~20 mM; decreased expression of glucose transporters that led to a drop in intra-embryonic free glucose levels in blastocysts inducing cell death by apoptosis) produce a preferential loss of males embryos.

Notes

CRYOPROTECTIVE EFFECT OF HETEROCYCLIC DERIVATIVES OF CARBOXYLIC ACIDS

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Increasing mammalian sperm, oocyte, and embryo resistance to cryogenic factors is a challenge for reproductive biotechnology. It is known that heterocyclic derivatives of carboxylic acids (HDCA) modulate cell metabolism at extreme conditions. The objective of the present research is to improve survival of thawed bovine sperm after cryopreservation with additives of HDCA.

More than 20 derivatives of N-maleoheterozide as representatives of the HDCA series were tested to prove out their cryoprotective effect. Bovine sperm samples from seven bulls with normal fertility were suspended in cryoprotective solution containing 5% glycerol. Tested substances were added to one part of sperm samples. Another part of bovine sperm samples did not contain the substance additives and was used as a control. The volume of samples was 250 µL with concentration 20000000 cells per mL. The samples were frozen by the method of passive cooling of thermo-block in the neck of Devar vessel X-34-B at freezing rates 25^0C/min and thawed in water bath at 40^0C. The sperm motility was visually determined as a ratio of linearly onward moving cells to their total number (%). Activity of the enzymes succinate dehydrogenase (SDH) which shows mitochondrial energy metabolism and γ-glutamyltransferase (γ-GT), as a marker of protein biosynthesis was measured with a calorimetric method, while the acrosine content was measured with a turbidimetric method in suspension of spermatozoa after thawing. The data were processed using Fisher's variance ratio.

As a result of the experiment, it was established that adding the tested substances at 10 (-7) M final concentrations to the suspension of native spermatozoa increased sperm motility by 15 to 28% (P<.01) vs. the control. Introduction of 7-methyl-N- maleoheterozide ($C_9H_{12}N_2O_3S$) as a representative of the HDCA series into the cryoprotective medium with 10 (-7) M final concentration demonstrated a significantly higher preservation of enzyme activity after a freezing-thawing cycle vs. the control: the SDH activity was 2.79 ± 0.67 mM/mg protein·min vs. 0.61 ± 0.26 mM/mg protein·min (P<.05), the γ-GT activity was 6.48 ± 1.59 µcatal/L vs. 2.76 ± 1.30 µcatal/L (P<.05), the acrosine content was 1.68 ± 0.48 mg/mL vs. 0.70 ± 0.23 mg/mL (P<.01). The motility of the thawed sperm frozen in a cryoprotective medium with additive of the same substance was 33.6% higher (P<.01) vs. the control.

Thus, HDCA have cryoprotective properties and produce a complex effect on the key metabolic pathways in frozen-thawed bovine sperm, which increases survival of the cells after thawing. HDCA as additives to cryoprotective medium may up new direction in solving reproductive biotechnology problems.

Notes

THE EFFECT OF GROWTH HORMONE ON EARLY DEVELOPMENT AND APOPTOSIS OF IN VITRO PRODUCED PREIMPLANTATION SOW EMBRYOS

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In the pig, despite successes, the *in vitro* production of embryos is still challenged by problems such as poor cytoplasmic maturation of the oocyte, delayed embryo development, low blastocyst cell numbers and a high incidence of apoptosis compared with *in vivo* produced embryos. In this study the effect growth hormone (GH), applied during *in vitro* embryo culture, on early embryo development and blastocyst apoptosis in the sow was investigated.

Cumulus-oocyte-complexes from sow follicles, 2-6 mm in diameter, were matured for 24 h in NCSU23 (Petters and Wells, J Reprod Fertil 1993; (Suppl):61-73) with 10% porcine follicular fluid, 0.1 mg/mL cysteine, 25 μM β-mercaptoethanol and 10 IU/mL hCG and eCG (Intervet International BV, The Netherlands). For the next 18 hours maturation took place in the same medium, but without added hormones. *In vitro* fertilization (IVF) took place in modified Tris-buffered medium (113.1 mM NaCl, 3.0 mM KCl, 20.0 mM Tris, 11.0 mM D-glucose, 7.5 mM $CaCl_2.2H_2O$ and 5.0 mM Na-Pyruvate) with 0.1% BSA and 1 mM caffeine for 24 h. The presumptive zygotes were then cultured in NCSU23 with 0.4% BSA, with or without 100 ng/ml bovine GH (NIH-GH-B18) for 6 days. Embryo development was assessed on Days 2 and 6. No difference was found between the Control (n = 1536) and GH (n = 1525) groups regarding the Day 2 cleavage rates (34% for both groups) and Day 6 blastocyst rates (33 vs 35% for Control and GH). Day 6 blastocysts were fixed and processed for total cell number and incidence of apoptotic cells using TUNEL (TdT-mediated dUTP-X nick end labeling) with DAPI counterstain for total cell count. The apoptotic index is defined as: (no. TUNEL-labeled nuclei)/(total no. nuclei) X 100. The results are given in Table 1 and were analyzed using Chi-square.

Table 1. The effect of GH supplementation during *in vitro* embryo culture of IVM/IVF sow embryos on Day 6 blastocyst apoptosis and total cell number.

Treatment	Incidence of apoptosis (%)	Apoptotic Index – only embryos with apoptosis	Apoptotic Index – all embryos	Total Cell Number Apoptotic Blastocysts	Total Cell Number Non-Apoptotic Blastocysts
Control (n = 65)	41.5^a	5.4 ± 0.8	2.2 ± 0.5^a	37.6 ± 2.4^c	41.5 ± 2.0
GH (n = 73)	17.8^b	5.3 ± 0.5	0.9 ± 0.2^b	33.2 ± 3.3^{d}*	40.7 ± 1.4

Within columns, values with different superscripts are significantly different: $^{a,b}p<0.01$, $^{cd}p<0.05$; * value differs significantly from non-apoptotic group: $p<0.05$

Addition of 100 ng/ml GH during embryo culture did not affect embryo up to Day 6 after IVF. However, GH supplementation during embryo culture significantly reduced the percentage of blastocysts with apoptotic cells. Furthermore, a correlation between the number of blastomeres and incidence of apoptosis was indicated in the GH group, with non-apoptotic blastocysts containing significantly more blastomeres than apoptotic blastocysts. The fact that GH did not have an effect on the rate of blastocyst formation may be due to the metabolic needs of the pre-blastocyst stage porcine embryo: GH stimulates glucose uptake in embryos and in porcine embryos a significant increase in glucose utilization is found only at the blastocyst stage.

In conclusion, the apoptosis-reducing effect of GH in Day 6 blastocysts indicates that GH acts as a “survival” factor for blastomeres during *in vitro* preimplantation development of sow embryos.

Notes

DEVELOPMENTAL RATE AND CELL NUMBER OF BOVINE EMBRYOS COLLECTED FROM THE GENITAL TRACT AT 45 H OR 100 H AFTER OVULATION AND CULTURED IN SOF MEDIUM UNTIL DAY 7

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Several studies demonstrated differences in the developmental rate and total cell number of bovine preimplantation embryos cultured *in vitro* vs. developed entirely *in vivo*. We investigated which part of the *in vivo* development after fertilization is responsible for the difference in developmental rate and cell number. Therefore, embryos were collected ex vivo from 59 cows after superovulation with FSH at 3 times: 45 h post ovulation (po) (45 h group), when most embryos are still in the oviduct, at 100 h po (100 h group) and at day 7 po (*in vivo* group), when embryos are in the uterus. The embryos collected at 45 and 100 h po were further cultured in SOF medium until day 7 po. For a control group of morulae and blastocysts, 270 oocytes were obtained from ovaries collected at the abattoir and after IVM/IVF (Izadyar *et al*., 1996, Animal Reproduction Science, 45, 37-45) and IVC in SOF medium, 29% developed into morula or blastocyst at day 7 po (*in vitro* group). The stage of the embryos was determined by light microscopy. The total cell number was determined by staining the embryos with propidium iodide and using confocal laser-scanning microscopy.

At 45 h po 173 structures were collected of which 5% 2-4c, 28% 5-7c, 57% 8c, 2% 9-16c and 9% 1-c or degenerated and at 100 h po 202 structures of which 17% 7-15c, 31% 16-32c, 12% at the morula stage and 40% 1-c or degenerated. For the *in vivo* group 170 structures were collected, 75% was at the morula or blastocyst stage and 25% was degenerated. In the 45 h group 68% of the embryos > 2c that had been put into culture, developed to the morula or blastocyst stage and likewise for the 100 h group 90% of the >6c put into culture developed to the morula or blastocyst stage.

Table: Effects of variable time of development in vivo on developmental rate and cell numbers (± sem (number of embryos)) of embryos of day 7 po

Group	Time po *in vivo*	Developmental rate[1)]					Number of cells per embryo		
		%m	%eb	%b	%xb	%hb	m + eb	b	xb + hb
in vitro	0 h	9^{a}	9^{abc}	20^{a}	61^{a}	1^{ac}	$113±10^{A}$(6)	$114±14^{A}$(5)	$160±8^{A}$(23)
45 h	45 h	9^{a}	15^{ac}	18^{a}	47^{a}	10^{bc}	$92±6^{AB}$(16)	$127+12^{A}$(9)	$175±8^{AB}$(33)
100 h	100 h	0^{b}	6^{ab}	20^{a}	58^{a}	16^{b}	-	$143±14^{A}$(11)	$201±8^{B}$37)
in vivo	165 h	43^{c}	13^{abc}	13^{a}	26^{b}	5^{ac}	$128±9^{B}$ 28)	$166±11^{A}$(18)	$205±21^{B}$(15)

[abc] different superscripts within columns are significantly different ($p<0.05$), analyzed by chi-square test with Yates correction
[AB] different superscripts within columns are significantly different ($p<0.05$), analyzed by ANOVA and Bonferroni test
[1]) relative to total number of morulae and blastocysts

These results suggest that *in vitro* conditions, regardless the time of *in vitro* culture, accelerate development towards blastocyst stages. On the other hand the cell number seems to be lower when embryos are cultured *in vitro* for a longer period of time. Results indicate that between 45 and 100 h po the number of cells at the blastocyst stage is defined, that is when embryos migrate *in vivo* from the oviduct to the uterus.

Notes

IN VIVO PRODUCTION OF BOVINE EMBRYOS USING TWO FEEDING REGIMES DIFFERING IN UREA CONTENT

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High blood urea content of dams and addition of urea in IVM media for cattle oocytes has been associated with altered oocyte maturation, embryo growth and viability (McEvoy *et al.*, 1997; De Wit *et al.*, 2001). The aim of the present study was to assess any effects of different blood urea content of donor cows on number of embryos produced, embryo growth and embryo quality. The embryos were produced as part of a project aimed at identification of genes influencing early embryonic development.

Ten first lactation Norwegian Cattle cows were divided in two groups. The standard diet consisted of grass silage and concentrate, including a total energy content corresponding to the amount of milk produced. Cows in Group 1 were given this diet from parturition. Group 2 cows were fed the same ration, but with an extra supply of urea corresponding to a calculated rise in blood urea content of at least 3 mmol/l during the first month of lactation. When the desired level was obtained, the feeding regimes were maintained for 2.5 months. At the end of this period (Period 1) superovulation and embryo collection were carried out for all cows. After flushing, cows in both groups were fed standard diets for at least one month, before switching the groups and repeating the procedure (Period 2). Semen from the same AI bull was used for all inseminations. The embryos were collected and treated according to IETS recommendations, including classification of developmental stage and quality before freezing in ethylene glycol. Donor cows were controlled concerning protein intake from calving until end of the experimental period. Milk samples were collected morning and evening twice a week for urea analysis. From the first day of superovulation until flushing, milk samples were collected daily for progesterone analysis. Data from the two groups were compared by Student's t-test procedure. Level of significance was set to $p<0.05$.

There were no significant differences in total number of embryos collected or number of good quality embryos, either between periods or between different feeding regimes. The number of embryos recovered, embryo quality and urea blood concentration are given in Table 1. The milk progesterone profile from start of superovulation until flushing corresponded well with the number of good quality embryos collected. Cows giving none, or unfertilised/degenerated embryos only, showed a clearly different milk progesterone profile from cows yielding high quality embryos.

Table 1. Embryo recovery, embryo quality and average milk urea concentration (± SD) with two feeding regimes differing in urea content.

	Group 1		Group 2	
	Period 1	Period 2	Period 1	Period 2
Flushings, N	4*	4*	4**	5
Total no of embryos, X	9	11	10.3	15.4
No good quality embryos, X	2.3	9	7	13
Milk urea mmol/l, (mean±SD)	4.57 ± 0.72	7.81 ± 0.97	8.92 ± 0.54	3.61 ± 0.71

* One cow in Group 1 was excluded
** One cow in Goup 2 was in anestrous at time for superovulation in period 1

References:

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Notes

PARTHENOGENETIC ACTIVATION OF RAT OOCYTES

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Oocyte activation is a crucial step in the cloning procedure. There are a variety of agents that activate mammalian oocytes and that have been used for the cloning of sheep, goat, pig, cattle and mouse. However, activation protocols must be optimized for use in every species, e.g., the rat.

The ability of three different agents to activate rat oocytes was assessed in this study, ethanol, strontium, and electric pulse. Immature female Wistar rats were induced to superovulation by injection of pregnant mare's serum gonadotropin, followed 45-50 hr later by human chorionic gonadotropin (hCG). Oocytes were recovered at different times (12 – 26 hours) after hCG injection. Different types of oocyte activation protocols were used: ethanol (7 min in 8% solution), strontium (2 h in 2 mM) and electric pulse (two direct current pulses 60V, 20 µsec were applied with 100 msec between the pulses). Incubation with cytochalasin B was used for diploidization. Activation rate was estimated as proportion of oocytes with pronucleus and activated ova were cultivated in R1ECM medium in vitro to blastocyst stage or transferred into pseudopregnant females that were sacrificed at day 11 of pregnancy.

Each method was able to induce parthenogenetic activation of postovulatory rat oocytes but activation efficiency depended on age. Young oocytes (12-14 h after hCG) were more difficult to activate than aged ones (more than 22 h after hCG) and activation was only observed with strontium and electric pulse. Aged oocytes were activated by all three methods. At the same time we observed a great inter-individual difference of activation between rats independent of the activation protocol. Oocytes from some rats exhibited a high percentage of activation (70-90%) while others only reached 5-10%. Oocytes could develop to blastocysts *in vitro* after all three activation protocols and implanted after transfer to foster mothers. However, we did not obtain live fetuses.

These results show that further optimization of the activation protocol will be necessary to achieve successful cloning in the rat.

Notes

DIPLOID AND HAPLOID ACTIVATION OF BOVINE OOCYTES WITH A COMBINATION OF CYCLOHEXIMIDE AND 6-DMAP

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Cycloheximide (CHX) and 6-DMAP (DMAP) at concentration of 10 µg/ml and 2mM respectively are used routinely for the activation of bovine oocytes in association with ionomycin. By contrast, little information is available on their effect when used in association.

For this purpose bovine oocytes were matured in medium TCM 199 supplemented with 10% FCS and 0.1 IU LH and 0.1 IU FSH for 24 h. The oocytes with one extruded polar body were activated with 5 µM ionomycin for 4 minutes, rinsed and transferred in microdrops under oil of medium SOF-AA-BSA supplemented with CHX and/or DMAP. After 4 h incubation the oocytes were rinsed twice and transferred in microdrops of medium SOF-AA-BSA without inhibitors. Ten to 14 h later the oocytes were fixed and stained with lacmoid. Oocytes with one or two pronuclei were considered activated. All experiments were done in three replicates.

In a preliminary experiment the two inhibitors where used alone at decreasing doses and that resulted in a progressive reduction of the activation rate. For CHX we obtained an activation rate of 69% (30/44), 47% (21/45) and 46% (19/41) at concentrations of 10 µg/ml, 5 µg/ml and 2.5 µg/ml respectively. Similarly, the activation rate following 6-DMAP treatment was 100% (30/30), 93% (26/28) and 43% (13/30) at concentrations of 2 mM, 1 mM and 0.5 mM respectively. In the following experiment we used the two inhibitors in association at decreasing doses. We found that the activation rate was very high even when the compounds were used at a concentration sixteen times lower (0.6 µg/ml CHX + 0.12 mM DMAP) than usual indicating a clear synergic effect. We also observed that the rate of second polar body extrusion increased progressively with the decreasing doses of inhibitors. (Table 1).

Table 1. Effect of the association cycloheximide + 6-DMAP (CHX+DMAP) at decreasing concentrations on the activation and 2nd polar body extrusion of bovine oocytes

CHX + DMAP	10µg/ml+ 2mM	5µg/ml+ 1mM	2.5µg/ml+ 0.5mM	1.25µg/ml+ 0.25mM	0.6µg/ml+ 0.12mM	0.3µg/ml+ 0.06mM	0.15µg/ml+ 0.03mM
N of oocytes treated	40	39	40	41	40	39	40
N of oocytes activated	38	38	38	39	37	17	15
% activation	95	97.4	95	95.1	92.5	43.6	37.5
% extrusion 2nd PB	0	0	4.5	35	80.7	77.7	92.6

In conclusion this study demonstrates that cycloheximide and 6-DMAP act in a synergic manner to induce diploid and haploid oocyte activation in bovine.

Notes

USE OF BROMEXIN CHLORHYDRATE TO TRANSCERVICAL EMBRYO RECOVERY IN NULLIPAROUS SAANEN GOATS RAISED IN NORTHEAST OF BRAZIL

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Transcervical procedure for embryo recovery in goats can be maximized, among others factors, by use of nulliparous females. This study was conducted to test the use of bromexin chlorhydrate on cervical ripening of nulliparous Saanen goats for transcervical embryo recovery.

For this, the oestrus of five nulliparous Saanen goats were synchronized by placing a intravaginal pessary of 60 mg of medroxyprogesterone acetate (Promone-E, Rhodia-Mérieux Veterinária Ltda., Brazil) for 11 days. Forty-eight hours prior the end of progestagen treatment, the females were treated with 50 µg of cloprostenol (Ciosin, Coopers Ltda., Brazil), intramuscularly. The goats were superovulated with 200 mg of pFSH (Folltropin-V, Vetrepharm, Canada) divided into six decreasing doses (40, 40, 40, 40, 20, 20 mg) at 12 h intervals. Following pessary withdrawal, all females were tested for oestrus at 12 h intervals with the aid of Saanen bucks and naturally mated at the oestrus onset and a second time 24 h after. The goats were treated with 9 mg of bromexin chlorhydrate (Bisolvon, Boehringer Ingelheim Vetmedica S.A., Mexico) 1 hour prior embryo collection. The ovulation rate was verified by laparoscopy 24 hours preceding the transcervical embryo collection which was performed 7 days after mating. The embryos were classified according to their stage of development and quality.

The mean (± S.D.) interval between pessary withdrawal and estrus onset was 21.6 ± 15.1 hours and the oestrus length was 39.2 ± 10.3 h. All females showed a super-ovulatory response with a mean (± S.D.) ovulation rate of 12.6 ± 1.8 corpora lutea. The mean recovery rate was 43.9% (5.6 ± 2.4 structures per goat). After evaluation under a stereo microscope, it was observed morulae and blastocysts resulting in 96.4% of fertilization rate. From the recovered embryos 44.44; 40.74 and 14.81% were of quality I, II and III, respectively. In conclusion, bromexin chlorhydrate can be used in the trancervical procedure for embryo collection in nulliparous Saanen goats.

Notes

TEMPORAL SENSITIVITY OF BOVINE EMBRYOS TO CULTURE ENVIRONMENT

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The quality of *in vitro* produced embryos continually lags behind that of their *in vivo* derived counterparts. We have clearly shown that the culture period post-fertilization (from Day 1 to 7) is the most critical period determining blastocyst quality (Rizos *et al.*, *Mol Reprod Dev* 61:234-248). Several major developmental events occur during this 6-day window including the first cleavage division, the activation of the embryonic genome, morula compaction and blastocyst formation. Any modifications of the culture environment affecting any, or all, of these processes could have a major effect on the quality of the embryo. The objective of this study was to isolate these developmental events, using the ewe oviduct model, and identify the period of post-fertilisation culture that is most critical in determining blastocyst quality. Presumptive zygotes were randomly assigned to treatment and cultured either *in vitro* in synthetic oviduct fluid (SOF) for 6 days, *in vivo* in the ewe oviduct for 6 days, or combinations of both as shown in Table 1. Where possible, cleavage was assessed at 48 h (or at recovery from the ewe oviduct) and blastocyst development was recorded on Days 7 and 8 and expressed as a percentage of total oocytes. Blastocyst quality was assessed by the ability of the embryo to cryopreservation (OPS vitrification); for this purpose a representative number of Day 7 blastocysts were used. Data were analysed by chi-square analysis.

Table 1. Development and survival of bovine embryos produced under various culture conditions.

Treatment	N	% cleaved	Blastocyst yield		% survival 24h after vitrification/warming
			Day 7	Day 8	
SOF 6D	354	82.5	26.5[ab]	30.2[a]	32.3[b]
SOF 2d/Ewe 4d	400	82.2	3.8[c]	8.2[b]	100[a]
SOF 4d/Ewe 2d	334	79.9	0[d]	0[c]	-
Ewe 2d/SOF 4d	400	78.6	20.2[a]	22.6[d]	6.7[c]
Ewe 4d/SOF 2d	400	-	31.0[b]	32.5[a]	40.6[b]
Ewe 6d	400	-	24.2[ab]	28.9[ad]	100[a]
In Vivo		-	-	-	100[a]
Significance		NS	P<0.05	P<0.05	P<0.05

There was no difference in blastocyst yield when culture took place either *in vitro* or *in vivo* for the entire 6-day period (30.2 vs 28.9%). Culture in SOF for either 2 or 4 days, followed by subsequent culture in the ewe oviduct resulted in a significantly lower yield of blastocysts than all other groups, the effect being most marked in SOF 4d group, where all embryos were degenerate on recovery. In terms of cryotolerance, *in vivo* produced (control) blastocysts, those cultured *in vivo* from Day 2 on or those cultured *in vivo* for the entire 6 days had the highest survival rates. Those embryos which spent the longest period in SOF (SOF 6d and Ewe 2d/SOF 4d) had the lowest survival. These results indicate that some windows of embryo development are more pre-disposed to aberrant programming than others.

Notes

EMBRYO TRANSFER IN BOER GOATS RAISED IN NORTHEAST OF BRAZIL: USE OF TRANSCERVICAL PROCEDURE FOR EMBRYO RECOVERY

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The extent by which embryo transfer is used in small ruminants is primarily limited by the complex anatomy of the cervix. However, transcervical embryo collection has repeatedly been attempted in goats and sometimes led a degree of success. This trial was conducted therefore to apply the technique of superovulation and transcervical embryo collection in Boer goats raised in Northeast of Brazil. For this, the oestrus of four multiparous Boer goats (donors) and 10 undefined breed goats (recipients) were synchronized by placing a intravaginal pessary with 45 mg of fluorogestone acetate (Syncro Part, Sanofi, França) for 11 days. Forty-eight hours prior the end of progestagen treatment, the females were treated with 50 µg of cloprostenol (Ciosin, Coopers Ltda., Brazil) and the recipients received 300 IU of eCG (Novormon, Syntex, Argentina), intramuscularly. The donors were superovulated with 200 mg of pFSH (Folltropin-V, Vetrepharm, Canada) divided into six decreasing doses (40, 40, 40, 40, 20, 20 mg) at 12 h intervals. Following pessary withdrawal all females were tested for oestrus at 12 h intervals with the aid of Boer bucks, and the donors were naturally mated twice a day during the oestrus length. The donors were treated with 50 µg of cloprostenol and 1 IU of oxytocin (Prolacton, Tortuga, Brazil) 24 hours prior embryo collection. The ovulation rate was verified by laparoscopy immediately prior transcervical embryo collection which was performed 7 days after mating. Donors were immobilized in a standing position by two persons. A third person inserted the lubrificated speculum into the vagina and grasped the lip of the external os of the cervix with the Allis forceps. The speculum was removed and the external os was carefully pulled caudally until it almost reached the vulvar opening. The cateter was introduced, and the cervical canal was passed by gentle probing and twisting motions. The catheter was introduced into the desired uterine horn, the free end was connected to an infusion tube leading to a flask filled with 150 mL of flushing medium (Phosphate Buffered Saline). It was realized four flushings. The embryos were classified according to their stage of development and quality. The embryo transfer was performed by the semi-laparoscopic method. Sixty days after embryo transfer, recipients were assessed by ultrasonography for pregnancy diagnosis. Oestrus was detected in all treated goats. The mean (± S.D.) interval pessary withdrawal to estrus onset was 48.0 ± 14.7 h and 34.8 ± 10.0 h for donor and recipient goats, respectively. All donors showed a superovulatory response with a mean (± S.D.) ovulation rate of 11.3 ± 2.9. The recovery rate was 77.0% (7.67 ± 3.3 structures per goat). After evaluation, it was observed 100.0% of fertilization rate. From the recovered embryos 74.0; 22.0 and 4% were of I, II and III, respectively. The pregnancy and embryo suvival rates were 60% and 47.8, respectevely. The transcervical embryo collection is an efficient method to be used in Boer goats raised in Northeast of Brazil.

Notes

ULTRASTRUCTURE FEATURES OF IN VITRO BOVINE SPERMATOZOA CONTAMINATED BY MYCOPLASMA MYCOIDES SUBPS MYCOIDES LC

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Notes

EVALUATION OF FROZEN SPERM FROM FIGHTING BULLS BY *IN-VITRO* FERTILIZATION OF *IN-VITRO* MATURED OOCYTES.

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The genetic resources conservation and genetic improvement programs start from the availability of evaluated and tested frozen sperm. In fighting bulls the evaluation of male fertility by means of artificial insemination can not be performed due to the low number of animals in the farms. One way to evaluate these males could be the combination between motility and morphological parameters and in vitro fertilizing capability of frozen sperm. Bavister (Gamete Physiology, ed. Norwell, Massachusetts 1990. Serono Symposia, USA. Pp 77-105), indicated that the only way of providing unequivocal information about sperm fertilizing capability is to include the alive oocytes-sperm interaction in the studies. For this experiment sperm from 3 fighting bull males was collected by electroejaculation. The extender used to freeze the sperm was Tryladil® (minitüb), and the freezing process consisted of 2 hours at 5ºC, after which sperm was packaged in 0,25 ml plastic straws, and then they were frozen in liquid nitrogen vapour (5 cm above liquid nitrogen) for 10 minutes, being plunged later into liquid nitrogen. Ovaries were obtained from a slaughterhouse, and the oocytes were collected by follicle aspiration and catalogued according to Le Guienne (AETE newsletter, 1999; 10:6-8). The oocytes were *in vitro* matured in TCM199 containing FBS (10%), EGF (10 ηg/ml) and gentamycin (50 µg/ml). They were incubated for 24h (38.5ºC, 5% CO_2, in air and saturated humidity). The sperm was thawed in a water bath at 42ºC during 12 seconds, and its quality was evaluated. Several parameters were noted: percentage of normal acrosome (NAR), abnormal spermatozoa (AR) and motile sperm (MO, subjective). Motility was also evaluated with CASA (SCA, Microptic), and several kinetic parameters were noted: VCL, VSL, VAP, LIN and ALH*[1]. The mean values observed for the sperm quality parameters were: 58.3 % (NAR), 7.3 % (AR) and 34.8 % (MO) and for the kinetics parameters: VCL (112 μs^{-1}), VSL (68 μs^{-1}), VAP (78.3 μs^{-1}), LIN (66.3) and ALH (4.8 µ). Thawed sperm was washed with Hepes-buffer Tyrode's, subjected to swim-up (Parrish, J.J and col. Theriogenology 1986; 25:591-600) and lead to co-cultured medium TALP, supplemented with heparin (10 µg/ml). Insemination was performed with a concentration of 2×10^6/ml, for 18h (38.5ºC, in 5% CO_2, in air, in humidified atmosphere). Putative zygotes were cultured in SOF medium (Takahashi y First. Theriogenology 1992; 37:963-978) under the same conditions. 48h after the insemination, we was performed proceed to the evaluation, and the number of embryos which developed to 2-16 cells was noted. In table 1 results observed after *in vitro* fertilization are summarized.

Table 1.: *In-vitro* fertilization rate per male.

	MIV (Nº COC´s)	FIV (Nº COC´s)	CIV (Nº putative zygotes)	Nº Zygotes (%)
PINTO	103	75	62	6 (8.0)[b]
PUCOL	97	68	57	16 (23.5)[a,b]
PERDIGON	138	113	87	38(33.6)[a]

[a,b] Percentages with different superscripts within a column differ significantly ($P<0.05$). - 3 sessions/male.

Significant differences between males were observed in the percentage of embryos obtained at the end of the assay. Results show that it is possible to create fighting bull sperm banks, and to evaluate the quality of sperm post-thawing by IVF techniques.

*[1] *VCL: track speed. VSL: progressive speed. VAP: average pach velocity. LIN: linearity. ALH: amplitude of lateral head displacement.*

Notes

CARBON-ACTIVATED FILTERED AIR DURING IN VITRO CULTURE INCREASED PREGNANCY RATE OF IVP BOVINE EMBRYOS

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Many of the environmental conditions for gametes and embryos in *In Vitro* Production (IVP) systems, e.g. media composition, temperature, pH, water quality and atmospheric composition, have been relatively standardized. However, little attention has been paid to the quality of the ambient laboratory air. Only a few studies looked at the effects of chemical air contamination on IVP outcome. Therefore, this study investigated the effects of an intra-incubator carbon-activated air filtration system (CODA, <genX> International, Inc.) during IVC on embryonic development and subsequent pregnancy rate.

Immature Cumulus-Oocyte-Complexes (COCs) were obtained twice weekly by ultrasonic guided transvaginal oocyte collection. COCs were matured in TCM199/FCS/LH/FSH, fertilised with frozen-thawed Percoll -separated semen and subsequently cultured for 7 days in SOFaaBSA. Day 7 embryos were transferred either fresh or frozen/thawed. The experimental design was a 2 X 2 factorial, whereby the presumptive zygotes were placed either in a normal CO_2-O_2 incubator (Control group) or in an identical CO_2-O_2 incubator with a CODA intra- incubator air purification unit (CODA group). Every five weeks the CODA intra- incubator air purification unit was transferred to the other incubator to eliminate any variables due to the performance of the specific incubators. The total experiment covered one year. Results were analyzed by Chi-square analyses for embryonic development and logistic regression analysis for pregnancy rates.

The embryo production rate at Day 7 was not affected by the CODA air purification unit (23.4% and 24.7% for control and CODA respectively). Also, there was no effect on the distribution of embryos among the different grades or stages. The pregnancy rate however was affected significantly (P = 0.043) for both fresh and frozen/thawed embryos (Table 1).

Table 1: Effect of CODA air purification unit during in vitro culture on pregnancy rate of both fresh and frozen/thawed IVP bovine embryos.

Group	embryo	Embryos n	Pregnancy %
Control	fresh	401	41.0
	frozen	298	35.6
CODA	fresh	381	46.3
	frozen	337	40.8

These results show that ambient air purification by the CODA intra- incubator air purification unit results in a significant increase in pregnancy rate of in vitro produced bovine embryos and therefore is used in our production laboratory ever since. Because embryonic developmental kinetics was not affected, these data suggest an improvement of the intrinsic quality of the embryo. Noteworthy is the fact that this improvement was achieved with ambient air purification during only the six days in culture. The possible effects of air filtration during IVM and IVF remain to be investigated. More fundamental research is needed to identify underlying mechanisms.

Notes

EVALUATION OF VIABILITY AND APOPTOSIS IN HORSE EMBRYOS STORED UNDER DIFFERENT CONDITIONS AT 5°C

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The aim of this study was to evaluate the viability of horse embryos after storage in three different conditions at 5°C for 6 and 24h. To this end, embryos were initially stained with DAPI, to detect dead cells, and subsequently stained with TUNEL assay to differentiate the apoptotic cells.

Fifty embryos, collected non-surgically on day 7 after ovulation, were stored in Emcare (ICP, Auckland, NZ) for 6 and 24h, and in Hams'F10 (Sigma, St. Louis,Mo) or Vigro (AB Technology, Pullman, WA) for 24h at 5°C. One group of embryos was stained immediately after collection (0h) (groups n = 10). To evaluate the viability, embryos were incubated with 1µg/ml of DAPI in PBS for 15 min, followed by evaluation in situ using an inverted fluorescence microscope to determine the % of dead cells (DAPI+). Immediately thereafter, the embryos were fixed in 2.5% paraformaldehyde and maintained at 4°C. To identify the apoptotic cells, a terminal deoxynucleotidyl transferase (TdT) mediated dUTP labeling (TUNEL) was used. Fixed embryos were washed three times in PBS supplemented with 0.1% polyvinylalcohol (PVA:Sigma) and permeabilized with 0.1% Triton X-100 in PBS. Then embryos were washed twice in PBS+0.1% PVA, before incubation in 20 µl of the freshly prepared TUNEL reaction mixture under mineral oil for 1h at 37°C. Positive controls were incubated in DNase for 20min at 37°C and washed in PBS/PVA before TUNEL staining. Negative controls were incubated with the labelling solution (fluorescein-dUTP) in the absence of the TdT enzyme solution. Stained embryos were washed twice in PBS+ 0.1% PVA, mounted on a glass microscope slide with PBS and visualized under an epifluorescence microscope, equipped with DMU filters, to detect the apoptotic cells (TUNEL+) and, by changing filters, the previously stained DAPI+ cells. The number of nuclei positive for TUNEL, DAPI, and TUNEL+DAPI were determined. Finally, to determine the total number of cells, embryos were dismounted, washed twice in PBS+0.1% PVA and stained with DAPI 0.1 µg/ml in PBS. Differences in the proportions of cells positive for both TUNEL and DAPI, or positive only for either DAPI or TUNEL were compared using Mann-Whitney U test.

	DAPI+/TUNEL+	*DAPI+/TUNEL-*	*DAPI-/TUNEL+*	N° cells total
Fresh embryos	0.04 ± 0.03	0	0.12 ± 0.10	2040 ± 404.7
Emcare 6h	0.08 ± 0.03	0.07 ± 0.03	0.15 ± 0.05	1930.5 ± 345.4
Emcare 24h	1.30 ± 0.33	0.47 ± 0.24	0.06 ± 0.02	2775.5 ± 709.6
Ham's 24h	1.37 ± 0.64	0.44 ± 0.18	0.07 ± 0.03	2718.6 ± 774.8
Vigro 24h	1.41 ± 0.71	0.36 ± 0.24	0.13 ± 0.06	2603.9 ± 838.3

The proportion of dead apoptotic cells per embryo ranged from 0% to 7% and increased with storage time, but no differences were detected between the storage mediums. There was a strong correlation between the TUNEL-labelled cells and the DAPI-labelled cells (R = 0.87).

Horse embryos remain highly viable after chilling as only a small proportion of dead cells is detected by DAPI labelling, which is highly correlated with the TUNEL-labelled cells. Therefore, DAPI staining proves to be a quick and reliable method for assessing embryo viability.

Notes

EFFECT OF FSH ADMINISTRATION BEFORE OPU ON OOCYTE, EMBRYO AND CALF QUALITY

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FSH administration prior to OPU is a method to increase the number of embryos per OPU session. In our hands this treatment resulted in a 1.5 to 3.0 times increase in the number of embryos, depending on the FSH schedule used (Wagtendonk and De Ruigh, Theriogenology 1999; 51: 436). This study presents data on the quality of oocytes, embryos and calves obtained after FSH administration.

First parity donor animals received two or four injections of FSH 36 and 48 h or 36, 48, 60 and 72 h before OPU (a total of 10 cc Ovagen, ICP, Auckland, New Zealand; 176 IU NIH-FSH-S1). OPU was performed once a week. As a control, oocytes were collected from the same donor animals without FSH pre-treatment in a twice a week OPU scheme. COCs were matured, fertilized and cultured in SOF or M199/Co-Culture system as described previously (Wagtendonk *et al*., Theriogenology 2000; 53:575-597). Embryos were transferred fresh or frozen to recipient animals. After birth the weight of the calves was measured as well as the gestation length, the sex, the ease of calving, perinatal mortality and the presence of congenital malformations. The calf results were analyzed using the MODEL statement of the Genstat statistical program:

$$y = \mu + \text{donor} + \text{Sex} + \text{culture system} + \text{FSH} + \text{FSH*culture system}$$

No difference was observed in the oocyte quality between the control group and the FSH group. In both groups about 25% of the oocytes were graded as I and 65% as II. In contrast however, the embryo development was significantly effected by the FSH treatment. In the SOF culture system embryos developed faster after FSH pre-treatment resulting in a lower percentage of early blastocysts and higher percentage of expanded blastocysts in the FSH group (see Figure below) as compared to the control. The same tendency was observed with the Co- Culture system.

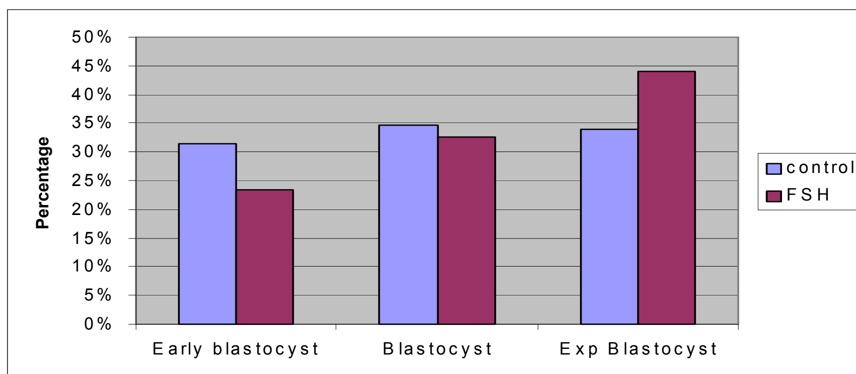

Figure 1. Effect of FSH pre-treatment on embryo development in the SOF culture system

There are also indications that FSH pre-treatment has an effect on the birth weight of the calves. Both with the SOF as well as with the Co-Culture system the birth weight from the FSH-calves was lower (44.7 and 44.6 Kg) than birth weight from the control calves (46.2 and 46.1 Kg) ($p = 0.08$).

It can be concluded that FSH pre-treatment does not influence oocyte quality. Embryo development however, seems to be accelerated after FSH treatment, while the birth weight of the calves seems to be reduced. More data are needed to obtain a definitive proof. (Partly funded by Eureka BTIP EU 1569)

Notes

SOURCES OF VARIATION OF EQUINE EMBRYO MORTALITY AND PREGNANCY RATES AFTER TRANSFER

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The use of equine embryo transfer (ET) is particularly interesting in Selle Français mares which can produce offspring via embryo transfer for later running competitions. Because only few equine embryos are produced at each session and because most of these embryos have a high commercial value, the transfer of the embryos has to be optimised. So the aim of this retrospective study was to investigate the sources of variation of embryo transfer results under field conditions in private stud farms.

A total of 138 transfers were realised between March and June 2001 by AGIRE ET team in 3 stud farms. Embryos were collected on day 7 after ovulation and transferred fresh into recipients by cervical method. Recorded data include stage and size of the embryos, age and parity of donor mares, recipient characteristics such as age, parity, breed, day of oestrous cycle at transfer, rank of transfer as well as following transfer parameters: date, characteristics of cervix passage (easy/difficult) and operator. Pregnancy was diagnosed on days 14 and 45 after ovulation by ultrasonography. Late embryo mortality (LEM) was defined as a negative pregnancy diagnosis on day 45 following a positive diagnosis on day 14. Chi-square tests were performed to identify the potential variation factors of transfer results. Significant factors were further analysed by a multivariate model of logistic regression (SAS, Logistic procedure).

Pregnancy rates averaged 63.8% and 53.8% on days 14 and 45 respectively, corresponding to 12.5% of LEM. Significant effects of embryo stage, age of donor, difficulty of cervix passage and stud farm were observed on pregnancy and/or LEM rates (Table 1). Age of donors seemed to influence LEM rate ($P = 0.07$), increasing from 3.7 % (1/27) in young donors (2 to 5 years) to 21.1 % (8/38) in old donors (>10 years). Embryo stage tended to influence embryo transfer results on day 45 ($P = 0.08$) with pregnancy rates ranging from 34.5 % (10/29) for morulae to 65.2 % (30/46) for young blastocysts. A difficult cervix passage lowered the success rate of transfer by 43 % in comparison with an easy passage (day 14: 68 % vs. 25 %, $P = 0.01$; day 45: 60 % vs. 16.7 %, $P<0.01$). But there was no specific effect on LEM rate ($P>0.2$). The stud farm was also associated with pregnancy rates ranging from 47.6 % in farm A (n = 82) to 82.8 % in farm B (n = 29) on day 45 ($P = 0.05$).

Table 1: Association between variation factors and embryo transfer results after multivariate logistic regression analysis (* P<0.10 ; ** P<0.05, *** P<0.01)

Variable	Pregnancy on D 14	Pregnancy on D 45	Embryo mortality
Age of donor mares	NS	NS	*
Embryo stage	*	NS	NS
Stud farm	*	**	NS
Cervix passage	**	***	NS

To conclude, a difficult cervical passage was found as a major factor influencing the success of ET in the mare. The effects of the stud farm should be further studied with management factors of donor and recipient mares.

Notes

OPTIMIZATION OF SUPEROVULATION IN THE RAT

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The method for the induction of superovulation for the collection of large numbers of rat ova is very important as basic technology for the production transgenic and cloned animals. Superovulation protocols using single injections of pregnant mare's serum gonadotropin (PMSG) or minipumps with follicle stimulating hormone (FSH) were compared for evaluation of the yield and quality of embryos obtained from immature Sprague-Dawley (SD) rats.

We used the following criterions: total number of ova, rate of fertilization, *in vitro* embryo development, sensitivity of zygotes to the microinjection of foreign DNA into the pronucleus and their *in vivo* development after transplantation into the oviduct of a recipient. Female SD rats were stimulated with 15 IU PMSG or 10 mg FSH (NIH-FSH-P1) followed by the injection of human chorionic gonadotropin (hCG) at doses 20 and 30 IU per female ($n = 11\text{-}14$). After hCG administration they were mated with males of the same strain and sacrificed on day 1 of pregnancy. The percentage of mated animals and the fertilization rate was similar in all groups. In the PMSG treatment group the number of ovulated ova was 40 ± 11.4 when 20 IU hCG were given and 66.3 ± 3.9 for 30 IU. In rats given FSH/hCG, the number of ova was 71.3 ± 7.9 and 64.9 ± 19.4 for 20 and 30 IU of hCG, respectively.

The fertilized ova of both groups were cultured to blastocyst stage in the R1ECM medium *in vitro*. The rates of blastocyst development between zygotes of the two groups did not differ significantly (31.3 and 23.3%). Zygotes obtained after various protocols of superovulation were equally resistant to microinjection into the pronucleus. The proportion of offspring developing from microinjected zygotes after oviduct transfer (26.2 and 26.8%, respectively) and rate of transgene integration per newborns (7.3 and 4.9%, respectively) was similar in both experimental groups.

The results from this study demonstrate that superovulation of immature SD rats by PMSG or FSH is equally effective and shows no difference in fertilization rate, sensitivity of fertilized ova to microinjection, *in vitro* and *in vivo* embryo development.

Notes

EMBRYO DEVELOPMENT OF BOVINE OOCYTES HELD IN BOVINE FOLLICULAR FLUID FROM DIFFERENT SIZE FOLLICLES

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In commercial programs of ovum pick-up and bovine *in vitro* production it is important to have the best conditions for the oocytes before maturation. Bovine oocytes have been maintained in the follicular fluid before maturation, keeping them at the germinal vesicle stage. To evaluate the effect of holding the oocytes in bovine follicular fluid (FF) on the rate of embryo development, eight hundred eighty-one (881) oocytes were aspirated from 2-8 mm follicles from bovine slaughterhouse ovaries during seven replicates. The oocytes were randomly distributed between four treatments. The control group (n = 217) was constituted by oocytes matured for 24 hours in modified TCM-199 with Estrus Mare Serum (EMS), pyruvate and recombinant human FSH in incubator with 5% CO_2, 39°C and saturated humidity. In the FF small group (3 to 5 mm follicles; n = 216), the oocytes were held for 6 hours in follicular fluid at 30°C and matured for 18 hours in the same conditions than the control-group. The oocytes from FF medium group (5.1 to 8 mm follicles; n = 226) and from the FF large group (>8 mm follicles; n = 222) were held in follicular fluid and matured as FF small ones. Fertilization was accomplished during 18h, in FERT-TALP + heparin and PHE at the same temperature and gaseous atmosphere used for maturation. Fertilization was performed with semen selected by swim-up method. Zygotes were cultured for 8 days in SOFaaci medium + 5% EMS in incubator at 39°C using gasified bags with 5% CO_2, 5% O_2 and 90% N_2. The data were analyzed by the GLM procedure (SAS 6.12 release-1998) and means were compared with Tukey's test.

Table 1: Development of bovine oocytes held in follicular fluid from different size follicles

	Oocytes n	% Day 2 cleavage	% blastocysts Day 7	% blastocysts Day 9	Day 9*hatched blastocysts n (%)
Control	217	94.5[a]	30.4 [a]	18.9[a]	25/66 (37.9)[a]
Follicular fluid small	216	85.6 [ab]	19.0 [b]	13.9[a]	17/41 (41.5)[a]
Follicular fluid medium	226	83.6 [b]	23.5 [ab]	20.4[a]	31/53 (58.5)[a]
Follicular fluid large	222	82.9 [b]	29.3 [a]	24.8[a]	29/65 (44.6)[a]

*Calculated over day 7 blastocysts. [a,b] Different letters within the same column differ significantly ($P<0.05$).

The culture of bovine oocytes in FF from small follicles reduced the embryo production at day 7, in comparison to control group and to oocytes exposed to FF from >8 mm follicles. Follicular fluid of small follicles tended ($P<0.07$) to decrease blastocyst rate, at day 9, when compared to FF of large follicles. Hatched blastocyst cell numbers were not influenced by treatments (means ± SEM of 129.5 ± 8.1, 163.7 ± 12.4, 161.8 ± 15 and 175 ± 12.4 to the control, FF small, medium and large groups, respectively). In addition, it was observed a better morphological quality in the embryos derived from oocytes held in follicular fluid.

Notes

VIABILITY OF PORCINE EMBRYOS AFTER 24H STORAGE IN DIFFERENT CONDITIONS

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Storage of porcine embryos is required for transportation in Embryo Transfer and breeding programmes. The present study evaluates the effect of short-term storage (24 hours) on the viability of porcine embryos. Since swine embryos are sensitive to cooling, we tested 18°C, 25°C and 38°C as storage temperatures, using three different media: DPBS, TCM199 (T) and Emcare® (E), and two different ages: day 4 (D4) and day 5 (D5) embryos (post ovulation).

Gilts (n = 49), 6-8 months of age, were synchronized and inseminated (Therio. 53 1063-70 2000). After slaughter, the embryos were collected and transported at 25°C to the lab. A total of 412 embryos (morulae/blastocysts) were obtained (D4 n = 233; D5 n = 179) and randomly used as controls or distributed over the 18 experimental groups. Embryos were cultured (5 per 500 µl medium) in closed eppendorfs in a thermobox. Viability was assessed using an embryo quality staining procedure that combines three different dyes: Ethidium Homodimer for membrane integrity, Hoechst 33342, for nuclear morphology, and TUNEL for *in situ* detection of fragmented DNA.

Embryonic parameters were: 1) developmental stage, 2) diameter, 3) number of dead cells, 4) number of condensed nuclei, 5) number of nuclei with fragmented DNA, and, 6) total number of cells (1&2 measured both before and after storage). Hatching of embryos was highest (28 out of 53 = 52%) in D5 embryos at 38°C (no hatching at 18°C). Diameter and temperature were positively correlated in D4 embryos (r = 0.95). Average results of the embryo damage parameters were: 3) 9.2% ± 0.2, 4) 1.3% ± 0.0 and 5) 1.3% ± 0.1 (Mean ± SD).

The proportion of non-intact embryos (≥1% of dead cells, condensed nuclei or fragmented DNA) in D4 and D5 were 35% and 22% respectively. The group with the highest incidence of damaged cells was: D4-Emcare®-25°C, best results were observed in D5-TCM199-38°C. (Table 1).

Table 1: Embryonic evaluation of: Controls (0h), D4, D5, D4-E-25°C, D5-T-38°C.

Groups	(n)	Embryos with ≥1 damaged cells or nuclei (%)	Nr. of cells per Embryo Mean ± SD	Dead Cells (%) Mean ± SD
0h-Control D4	37	3	42 ± 18	3 ± 0.0
0h-Control D5	23	0	60 ± 23	0 ± 0.0
24h - All D4	196	35	47 ± 24	9 ± 0.2
24h - All D5	156	22	70 ± 27	3 ± 0.2
D4-E-25°C	23	65	34 ± 18	31 ± 0.4
D5-T-38°C	15	0	50 ± 14	0 ± 0.0

In conclusion TCM199 and 38°C was the best combination of medium and temperature, and Day 5 embryos the preferable embryonic stage for storage.

Notes

COMPARISON OF DEVELOPMENTAL POTENTIAL OF KERATINOCYTES AND FIBROBLASTS AFTER NUCLEAR TRANSFER IN BOVINE

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Transplantations of nuclei from different somatic cell types into mammalian enucleated oocytes revealed that their genome contains the genes required for the development of viable and fertile animals. In most experiments however the cells used as donor are issued from a pool of cultured cells where their differentiation status is undefined. At the moment, evidence of the genetic totipotency of nuclei from terminally differentiated somatic cells is lacking. The epidermis is a tissue that is maintained through the proliferation of stem cells which produce daughter cells that undergo terminal differentiation. We established primary cultures of ear epidermal cells from adult animals in the bovine species to evaluate the developmental potential of nuclei from fully differentiated cells, and we compared it with the potential of fibroblasts derived from the dermis of the same animal, these cells having previously proved to be fully reprogrammed when used as donor cells in nuclear transfer experiments.

Outgrowth from ear skin biopsies were subcultured in a medium for the proliferation of keratinocytes (adapted from Reynolds A.J., Jahoda C.A.B. (1991), J. Cell Sci., 99, 373-385). Fibroblasts were selectively harvested by a cell detachment solution (Accutase, PAA Laboratories). Keratinocytes were suspended by trypsinisation. Cells suspensions were kept in complete medium overnight at 4°C to restore cytoplasmic membrane. Four hours before nuclear transfer, keratinocytes and fibroblasts suspensions were incubated at room temperature for one hour with primary antibodies anti-desmoplakin I & II (Boehringer-Manheim) then for one hour with biotinylated donkey antimouse antibodies, and finally for 15 minutes with fluorescein avidin. Cells were observed and selected according to their peripheral immuno-labelling intensity with a micropipette under the microscope before being fused with an enucleated IVM oocyte. Reconstructed embryos were activated in 10 µg/ml cycloheximide and 5 µg/ml cytochalasin B for 5h after fusion, then cocultured on Vero cells for 7 days in microdrops of B2 medium supplemented with 2.5% FCS.

The reconstructed embryos cleaved at the same rate with both type of cells, but the development to the blastocyst stage was significantly lower with keratinocyte type as compared to the fibroblast type (Table 1).

Table 1. *In vitro* development of embryos reconstructed with bovine keratinocytes and fibroblasts

Cell Type	Couplets	Fused (%)	Cleaved (%)	Morula (%)	Blastocysts (%)
Fibroblasts	82	71 (86.6)	49 (69.0)	32 (45.0)	32 (45.0)[a]
Keratinocytes	111	85 (76.6)	51 (60.0)	9 (10.6)	8 (9.4)[b]

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

We are using this model to assess whether nuclei issued from differentiated keratinocyte present differences in the remodeling and in the cell cycle characteristics during the first cell cycle after nuclear transfer into an enucleated bovine oocyte.

Notes

BIOENERGETIC INFLUENCE OF CLOPROSTENOL UPON CATTLE LUTEAL HOMOGENATES AND MITOCHONDRIA *IN VITRO*

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The luteolytic activity of prostaglandin $F_{2\text{-}\alpha}$ ($PgF_{2\text{-}\alpha}$) is traditionally explained by luteal vasoconstriction via endothelin (ET-1) release and phagocytosis promotion by induction of monocytic migration to the corpus luteum. Meanwhile the influence of $PgF_{2\text{-}\alpha}$ on mitochondrial processes of oxidation and restoration was also noticed.

The aim of present work was to study the action of $PgF_{2\text{-}\alpha}$ (cloprostenol) on tissue respiration and ATP-ase activity in cattle luteal homogenates (H) and isolated mitochondria (M) during the sexual cycle, pregnancy and functional pathology of the ovary.

We have investigated corpora lutea of 20 cows with various forms of ovarian luteinization. For this purpose, 625 ng of synthetic cloprostenol (oestrophan) were added to each milliliter of reaction medium.

At the beginning of pregnancy the influence of cloprostenol on succinatoxidase activity in corpora lutea is the most prominent. This influence of cloprostenol is the least on pathologic forms of luteinization (Table 1).

Table 1: Influence of oestrophan on succinatoxidase activity in corpora lutea of cows (mcatom O2/ mg protein/ min), M ± m, n =4)

Cycle stage,	Control		Oestrophan		Influence, +%	
ovarian state	M	H	M	H	M	H
Luteal	4.63 ± 0.12	3.21 ± 0.14	7.78 ± 0.27	5.71 ± 0.32	68	78
Pregnancy	13.79 ± 0.40	6.52 ± 0.13	21.65 ± 0.54	13.23 ± 0.59	57	103
Luteal cyst	11.64 ± 0.20	6.84 ± 0.30	14.78 ± 0.33	9.78 ± 0.19	27	43
Persistent corpus luteum	14.88 ± 0.48	7.60 ± 0.31	16.52 ± 0.64	10.49 ± 0.27	11	38

We registered the inhibiting action of oestrophan upon ATP-ase activity of luteal cells and M, which was most prominent in persistent corpora lutea and less noticeable—in tissue of the luteal cyst (Table 2).

Table 2: Influence of oestrophan on ATP-ase activity in corpora lutea of cows (mcg P/ mg protein/ 30min), M ± m, n = 4

Cycle stage,	Control		Oestrophan		Influence, -%	
ovarian state	M	H	M	H	M	H
Luteal	247.7 ± 36.0	159.1 ± 45.2	165.1 ± 52.2	95.3 ± 22.74	50	67
Pregnancy	202.3 ± 55.8	161.2 ± 43.7	170.0 ± 32.5	125.9 ± 45.2	19	28
Luteal cyst	71.4 ± 22.3	60.4 ± 16.7	66.1 ± 19.7	53.4 ± 16.0	8	13
Persistent corpus luteum	125.0 ± 38.2	78.2 ± 15.6	79.1 ± 18.4	44.9 ± 13.5	58	74

The influence of cloprostenol on bioenergetic processes in luteal H is more active in comparison with M. This could be explained by intactness of $PgF_{2\text{-}\alpha}$ receptors on nuclear membranes of luteocytes in H.

The increased quantities of ADP are needed to supply tissue respiration in the presence of $PgF2_{\alpha}$, while the restoration of ADP is suppressed. Therefore, the results we obtained allow us to consider the opposite $PgF_{2\text{-}\alpha}$ influence upon oxidation processes and ATP breakdown as a partial explanation of luteolytic action of $PgF_{2\text{-}\alpha}$.

Notes

SPERM BINDING CHARACTERISTICS TO BOVINE OVIDUCTAL EPITHELIA AROUND OVULATION

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The mammalian oviduct plays a key role in sperm-storage, -capacitation and -selection. Specific oviduct secretions and/or binding to oviductal cells are thought to be responsible for the extension of the functional life span of sperm. Possibly hormonal signaling from the corpus luteum and subsequently the preovulatory follicle will effect oviduct epithelial secretory activity which may influence sperm redistribution and sperm-oviduct binding. The aims of this study were to compare morphology and sperm binding properties of bovine oviduct epithelia around ovulation and to investigate whether primary cultures of these epithelia can be used.

Cows were treated according to a timed insemination protocol (GH, PG, and GnRH) and slaughtered at about 6 h before ovulation or within 5 h after ovulation. Oviducts and ovaries from non-inseminated cows (n = 4) were obtained immediately after slaughter and oviducts were dissected from surrounding tissue. Small pieces from isthmus and ampulla ends were randomly divided into two groups: (1) for immediate use for sperm binding (explants) and (2) for primary cultures of the epithelial cells (until 7 d). Explants and primary oviductal cells after culture were incubated for 2 h with 1 x 10^6 fresh-ejaculated sperm cells in TCM 199 at 38^0C and 5%CO_2. Thereafter, explants and cell cultures were fixed in Karnovsky solution. The morphology and sperm binding properties of the ampulla and isthmus epithelia were visualised using scanning electron microscopy and confocal laser scanning microscopy after staining with Hoechst 33258 (to detect cell nuclei) and fluorescein conjugated peanut agglutinin (to stain acrosomes and epithelial cell surface). Both techniques were employed to distinguish intact functional cells from deteriorated cells. In explants before and after ovulation large numbers of ciliated cells were found in the ampulla and isthmus (50-80% of cells). Increased epithelial secretion was detected in non-ciliated cells at the post-ovulatory phase (most predominantly in the ampulla ipsilateral to the preovulatory follicle). Irrespective of the oviduct region and time around ovulation, very pronounced sperm binding to ciliated cells was observed. The numerous pocket like structures detected in the oviduct do not seem to act like sperm reservoirs since sperm binding outside pockets was equal to that inside. The sperm binding to the post-ovulatory ampulla and isthmus was lower than before ovulation while the number of adhered sperm to these two sites was approx. equal (as was the case for ipsilateral compared to contralateral oviducts). In primary cultured oviductal epithelial cell monolayers, cells loose their typical morphology (loss of cilia, extreme cell flattening) as well as their sperm binding properties. No differences were found in sperm binding characteristics between cell cultures of ipsilateral and contralateral oviducts irrespective of the time of contrary to what has been observed for explants. Nevertheless, we found higher numbers of sperm cells bound to cultured cells of the ampulla than of the isthmus (P = 0.02). Presently we are comparing these results with sperm binding properties from oviducts collected before and after ovulation from inseminated cows.

We conclude that endocrine changes around the ovulation induce a drop in sperm binding which is likely related to the secretory pattern of oviductal epithelial cells. Furthermore, to study the process of sperm binding oviduct explants are superior to their corresponding cultures.

Notes

SANITARY CONTROLS IN BOVINE IVF EMBRYO PRODUCTION

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The aim of this study was: 1) evaluate if bacteria and virus are present during different steps of IVF techniques, 2) determine if the contaminations could reduce the embryo production efficiency, 3) verify if slaughtering procedures could interfere with initial contamination of the ovaries.

Bacteria like *Pseudomonas aeruginosa, Corynebacterium pyogenes, Streptococcus* and *Staphylococcus aureus* are known to cause endometritis as well as BHV_1, BHV_4, BVDV. Those pathogens are supposed to be able to join at the outer COC (Cumulus-Oocyte Complex) and adhere at embryo cells and ZP surface and they can cause reproductive failure or disease transmission [1.2.3.4.6]. During 80 laboratory trials, 660 Piedmontese bovine ovaries were collected from 3 different slaughterhouse (A-B-C). The ovaries, after dissection from the uterine body, were rapidly disinfected with 70° alcohol and carried to the laboratory in PBS at 26-33 °C within 3 hours from removal. Slicing method was utilized for obtaining the COC and routine IVF procedure was performed [5]. Samples of culture media, cellular debris and rejected COCS from each trial were submitted to examination. Enrichment and insemination on selective media were executed and each plate was examined after incubation; colonies were stained with Gram method and the most typical ones for morphology, growing and staining characteristics were identified with specific biochemical method (API gallery). For virological analyses cellular debris fraction was submitted to precipitation with PEG (Polyethylene Glycol), then filtered and placed in culture on renal bovine cells monolayers. Positive samples were submitted to immunofluorescence staining method that allowed identification of the viral agent[7]. Results are summarized in Tab.1. Some identified bacteria are saprophytic, other ones are opportunistic pathogens and further can cause alimentary toss infections in the humans but in any case can be certainly referred to donors pathology. With the same withdrawal ovary procedure in the C slaughterhouse we identified only one positive sample that can demonstrate more respect in hygiene during evisceration. The same tests performed on media and discarded embryos at the end of the IVF procedure was always negative. These results can be determined by infectious agent dilution and by the effect of antibiotic addition. The repeated washing of oocytes and embryos requested by IETS procedures allowed a significant reduction of infection dose. The marked increased production of morulae/blastocysts in negative samples (tab.2) allows to conclude that optimal hygiene conditions enhance the production data.

Table 1: Bacteriological identification in culture media and rejected COCs (*in 3 samples an association of bacteriological agents is observed)

BACTERIOL. exams	Slaughter A	Slaughter B	Slaughter C	VIROL. exams	Slaughter A	Slaughter B	Slaughter C
Polymicrobial	1	1	0	BHV_1	0	0	0
Environmental bacteria	3	0	0	BHV_4	0	0	0
Staphilococcus spp	2	1	0	BVDV	0	1	0
Escherichia hermanii	1	0	0				
Bacillus spp	3	0	0				
Pseudomonas spp.	1	4	0				
Xanthomonas spp.	1	1	0				
Klebsiella oxytoca	1	0	0				
Coliformi	0	2	0				
Enterobacter spp.	0	3	0				
Aeromonas spp	0	1	0				
Pasteurella multocida	0	1	0				
Moraxella lacunata	0	1	0				
Acinetobacter lwoffii	0	0	1				
Comomonas spp.	0	1	0				
Negative	17	20	20		30	32	21
Total	30	33*	21		30	33	21

Table 2: Relationship between microbiological results and IVF

Note: 1, 2, 4, 8 C (1, 2, 4, 8 cells) M/B (Morulae/Blastocyst) D (Degenerate) NF (Not fertilized)

Bacteria and virus	Oocytes N	1 C %	2 C %	4 C %	8 C %	M/B %	D %	NF %
Positive	822	32.12	18.98	10.83	2.80	6.56	19.83	8.88
Negative	1389	33.26	10.73	7.49	3.82	14.54	18.57	11.59

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Notes

ATP CONTENT IN OOCYTES FROM ANIMALS SELECTED FOR *IN VITRO* BLASTOCYST PRODUCTION

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The oocyte stores and conceals all the essential elements necessary for embryonic development. The ATP content of the oocyte is critical for its maturation and has been suggested as an indicator of its potential development capacity [1]. Oocyte developmental competence is also influenced by the maturation conditions. Recently we have demonstrated in the cow, as is well known in the mouse, that oocyte developmental competence is influenced by its maternal origin [2]. The objective of this study was to evaluate the ATP content of oocytes collected from cows whose *in vitro* embryo developmental capacities have been previously characterized.

Six contemporaneous Holstein cows born and raised in the same farm, with the same nutrition and without any common relative for at least two generations were estrous synchronized and OPU was performed twice a week during 10 weeks. Oocytes of quality grade 1, 2 and 3 were frozen for ATP measurement before and after *in vitro* maturation (24 h.) in 50 µl droplets of TCM 199 medium with Vero cell co-culture. Oocytes were freed from the cumulus cells before freezing. Oocytes recovered from slaughterhouse ovaries were used as control. The ATP was measured using a commercial assay kit (based on the luciferin-luciferase reaction, Bioluminescent Somatic Cell Assay Kit, FL-ASC; Sigma, St. Louis, MO) and a luminometer (Bioluminat Junior; Berthold, Wildbad, Germany). Oocyte quality and ATP content for each cow was recorded and statistically analyzed using SPSS software.

ATP content was measured in 531 oocytes (257 before and 245 after maturation). Mean overall ATP content was 2.28 ± 0.95 pmol/oocyte and maturation did not significantly change their values (2.46 ± 0.83 pmol/oocyte before and 2.35 ± 0.77 after maturation, $p<0.05$). Despite this, some animals exhibited significant variation after maturation (indicated by * in Table 1). Differences appeared in ATP content between cows (ID superscripts) and higher ATP content tended to be associated with higher developmental rates. Oocyte quality (same animal) did not affect ATP content either before or after maturation except for one animal in which Q3 had significantly smaller values than Q1 and 2 oocytes (2.90 ± 0.39; 4.07 ± 0.99; 4.00 ± 0.73 respectively).

Table 1: ATP contents and blastocyst production

ID	Maturation Status	N	ATP Content Mean ± SD	Mean Blast. Prod. Rate [2]
Cow E [bc]	Before	30	3.00 ± 0.60*	50.4 [a]
	After	23	2.38 ± 0.69	
Cow A [b]	Before	42	3.07 ± 0.58	34.3 [ab]
	After	22	2.84 ± 0.38	
Cow B [c]	Before	40	2.73 ± 0.74	31.7 [bc]
	After	33	2.54 ± 0.31	
Cow C [d]	Before	33	2.12 ± 0.50*	26.4 [bcd]
	After	27	1.83 ± 0.28	
Cow F [a]	Before	31	3.12 ± 0.31*	19.3 [cd]
	After	24	3.81 ± 0.95	
Cow D [d]	Before	28	2.15 ± 0.48	12.4 [d]
	After	30	2.12 ± 0.34	
Control [d]	Before	53	1.46 ± 0.52	-
	After	86	1.79 ± 0.54	

Values with * differ at the 0.05 level (ATP content for the same animal)
Cows with the same superscript are statistically not different at the 0.05 level

These results do not support the presence of a clear-cut relationship between oocyte ATP content and blastocyst rate. Neither does the evolution of ATP content after maturation (increase/decrease) seem clear-cut. Another interesting finding is the significantly lower ATP content of slaughterhouse control oocytes compared to OPU individual oocytes.

Further investigations to compare differences among oocytes from these animals from different oocyte developmental abilities (metabolism, first embryonic cleavage, mitochondrial activity) will contribute to the elucidation of the factors responsible for the maternal influence on oocyte developmental competence.

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Notes

IDENTIFICATION AND QUANTIFICATION OF DIFFERENTIALLY EXPRESSED TRANSCRIPTS IN *IN VITRO* PRODUCED PREIMPLANTATION STAGE CATTLE EMBRYOS

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Understanding the physiological timetable of gene expression during *in vitro* development of bovine embryos affords a tool for the further optimization of the system to produce high quality embryos from genetically superior animals for embryo transfer practices in animal production. The main objective of this study was to identify differentially expressed transcripts and to quantify their expression pattern in preimplantation stage embryos. For this, total RNA, isolated from pools of 15, 35, 94 and 188 blastocysts, morula, 16-cell and 8-cell embryos respectively, was reverse transcribed and subjected to Differential Display PCR (DD-PCR) using 26 upstream primers designed by Bauer *et al*., Nucleic Acids Res 1993;21:4272-4280. RT-PCR screening and quantification of identified clones was performed by fluorescence monitored real time PCR. mRNA, isolated from pooled matured oocytes (MO), 2-cell, 3-cell, 4-cell, 8-cell, 16-cell, morula (Mor), blastocyts (Bl), day 21 (D21) and day 25 (D25) whole embryos, were utilised for real time PCR screening and quantification using SYBR® PCR master mix in an ABI prism 7000 SDS instrument (Applied biosystems).

Applying DD-PCR a total of 305, 205, 162 and 239 cDNA bands were conserved in 8-cell, 16-cell, morula and blastocyst stages, respectively. Of the potential 40 differentially expressed cDNA bands, sixteen transcripts were cloned and sequenced. Of these, clone 1C14 (337 bp long) was found differentially expressed at the blastocyst stage and shares sequence homology with bovine 187461 MARC 4BOV cDNA (Acc. No. BE685292) which is derived from library made of pooled RNA from bovine tissues (Smith *et al*., Genome Res 2001; 11(4) 626-30). A pair of primers were designed to amplify and quantify 133 bp long product throughout preimplantation stage embryos. The real time PCR results were normalised with ß-actin gene. The relative expression level of clone 1C14 (Y-axis) to blastocyst stage is indicated in the figure below.

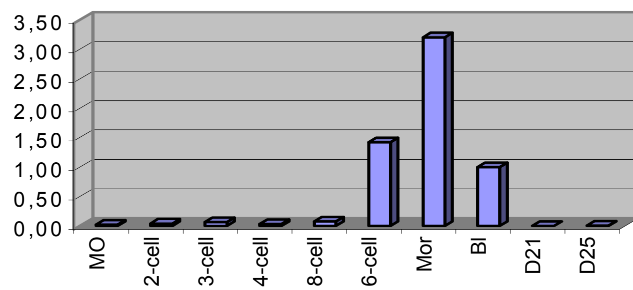

Figure. Relative expression of clone 1C14 in preimplantation stage embryos

Morula and 16-cell stage embryos revealed a 3 and 1.5 fold expression respectively relative to the blastocyst stage. Absence of expression up to the 8-cell stage indicates that clone 1C14 is from embryonic genome activation. Quantification of this clone at single embryo level in *in vitro* vs. *in vivo* derived embryos of different qualities may supplement our results.

Notes

EFFECTS OF FOLLICULAR CELLS ON *IN VITRO* MATURATION OF HORSE OOCYTES

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In mammalian oocytes meiosis is arrested in the dictyate stage of the first meiotic division. When immature cumulus oocyte complexes (COCs) are removed from their follicle, resumption of meiosis is induced spontaneously, suggesting that follicular components actively suppress maturation in the follicle-enclosed oocyte (Pincus and Enzmann, J.Exp.Med. 1935; 62:665-675). Unfortunately, limited information is available on the factors that maintain the oocyte in meiotic arrest and regulate the resumption of meiosis in the horse. To this end, this study investigated the role of the follicular cells in the maintenance of meiotic arrest (germinal vesicle-GV stage) in horse oocytes. Cumulus oocyte complexes (n = 435) were isolated from non-preovulatory follicles (10-20 mm diameter) of abattoir ovaries and cultured for 38 h with parts of the follicular wall in TCM199 supplemented with 10 µl penicillin/streptomycin (both Gibco Life Technologies, Breda, NL) without gonadotrophic hormones. Coculture conditions (Table 1) were such that the COCs were 1) attached to the follicular wall, 2) separated from the follicular wall but cultured in its vicinity, 3) attached to membrane granulosa (COCGs), 4) as COCGs and cocultured with theca cell layers, and 5) isolated COCs. COCGs were also cultured for 38 h in theca-cell conditioned medium.

When the oocytes were cocultured maintaining their attachments to the follicular wall, 79% remained in the GV stage. However, when oocytes were cultured in the vicinity of follicular wall, meiosis was resumed and similar proportion of oocytes underwent germinal vesicle breakdown (GVBD; 79%) as compare to cultures of isolated COCs (85%). Similarly, when COCs were cultured attached to a piece of membrane granulosa, the majority of the oocytes underwent GVBD (82%). However, when COCGs were cultured in the presence of theca cell or in theca-cell conditioned medium a significantly higher proportion of oocytes remained in GV stage (64% and 52% respectively; $P<0.05$) as compared to isolated COCs (15%) or COCGs (18%). This may indicate that the suppressive effect of the theca cells is exerted via the secretion of a meiosis-inhibiting factor. However, this suppressive effect was significantly less effective than that for the oocytes cultured attached to their follicular wall (GVBD stage oocytes: 21% versus 48%, respectively; $P<0.05$). These results support the role of the follicular wall in the maintenance of the horse oocyte in meiotic arrest. In particular, theca cells play an important role in this meiotic arrest mechanism, most likely exerted via a secreted inhibitory factor, since contact between these cells and the COC is not strictly necessary.

Table 1. The number (and percentages) of oocytes at various stages of nuclear maturation after IVM with different follicular components.[a]

Nuclear stage	COCs	COCs attached to follicular wall	COCs +Follicular wall pieces	COCGs	COCGs+ theca cells	COCGs+ theca cells conditioned medium
GV	19 (15)	41 (79)	11 (21)	9 (18)	59 (64)	32 (52)
GVBD	106 (85[b])	11 (21[c])	41 (79[b])	42 (82[b])	34 (36[d])	30 (48[d])
Nm oocytes	125	52	52	51	93	62

[a] GV, Germinal vesicle stage; GVBD, Germinal vesicle breakdown stage
[b-d] Values with different superscripts are significantly different ($P< 0.05$)

Notes

HAND-MADE SOMATIC CELL NUCLEAR TRANSFER IN CATTLE: RECENT IMPROVEMENTS

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Preservation of the zona pellucida during nuclear transfer requires the use of micromanipulators. However, zona-free embryos can also be cultured *in vitro* in modified dishes without any decrease in the blastocyst rates (Vajta *et al*., Mol. Reprod. Dev. 55:256-264, 2000). A technique for somatic cell nuclear transfer performed on zona-free matured oocytes and consequently excluding the use of micromanipulators has been established recently (Vajta *et al*., Cloning 3:89-95, 2001).

In our present work, efforts have been focused to increase efficiency regarding both blastocyst rates and duration of manual work required. Modifications have included omission of oocyte selection based on the presence of polar bodies; group fusion of cytoplasts and somatic cells; extension of the time of DMAP incubation at activation and improved embryo culture conditions.

In 14 consecutive experiments with either 1 week old granulosa cells or fetal fibroblasts as nuclear donors, an average rate of 51% (43 to 64%) blastocyst per reconstructed embryo was achieved. The total manual work per produced blastocyst was approx. 6 min. The developmental rate and morphological quality of blastocysts (evaluated by stereomicroscope and inverted microscope) did not differ from zona-intact in vitro produced embryos. Differential staining of 20 average quality blastocysts produced with this method resulted in 216 ± 52 total cell count, and 52 ± 10 cells in the inner cell mass. The average inner cell mass / trophectodermal cell ratio was 35%.

As the result of the improved steps, *in vitro* efficiency regarding the blastocyst per oocyte, blastocyst per working hour rates were among the highest compared to other techniques described so far for somatic cell nuclear transfer. Morphological examination of the produced blastocysts did not reveal any alteration. Our data suggest that the new, improved hand-made somatic cell nuclear transfer procedure may have considerable perspectives in bovine cloning.

Notes

RESTORATION OF OVARIAN CYCLICITY AS MONITORED BY TRANSRECTAL ULTRASONOGRAPHY FOLLOWING OVUM PICK-UP BEFORE AND AFTER THE LH SURGE IN COWS TREATED FOR SUPEROVULATION

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In cattle breeding programs, superovulation (SO) with a timed LH surge allows collection of high quality oocytes for IVP by Ovum Pick-Up (OPU) at specific stages. Donor cows, however, like after regular SO with embryo flushing, have to get pregnant soon after SO with OPU. It can be expected that this procedure disturbs the normal processes of ovulation and luteinization, in particular when OPU is carried out before the LH surge. Therefore, we investigated development of ovaries after OPU at different times during SO with a controlled LH surge, and in addition the effect of treatment with a PRID/PG on restoration of ovarian cyclicity.

Synchronized, cyclic animals (HF cows and heifers, n = 20) were treated for SO using a standard procedure with oFSH, PG and norgestomet ear implant. In each animal, oocytes were collected from all preovulatory follicles (>8 mm) by OPU carried out twice that is 2 h before and 22 h after a GnRH-controlled LH surge on the left and right ovary, respectively. In the majority (75%) of the animals also all small follicles (4-8 mm) were punctured to improve monitoring afterwards. After the 2nd OPU, 5 animals were administered a PRID (Sanofi Santé BV, Maassluis, The Netherlands) for 9 d and 15 mg PG (Prosolvin, Intervet International BV, Boxmeer, The Netherlands) on day 7 after OPU. Morphological development of the ovaries was monitored daily by transrectal ultrasonography (Aloka type SSD-500, 7.5 MHz linear array probe, Tokyo, Japan). Degree of luteinization was classified as 0 (none), 0.5 (bad; occasional luteinization of follicular walls), 2 (poor; 1-2 follicles filled with luteal tissue), 6 (average; regular luteinization and/or presence of a CL) and 9 (much; massive luteinization). Likewise, abundance of follicular structures in different size classes was scored from 0 (none), 1 (1-3 follicular structures), 2 (4-6), 3 (7-9) and 4 (>9). Blood samples were taken daily to measure the progesterone concentration.

On average 12 + 12 (left+right) preovulatory follicles were punctured during SO. Follicles punctured before LH (Fig. 1a) in general remained more often visible as follicular structures >15 mm than when punctured after LH, until their regression started after 8 d after OPU. Luteinization occurred almost exclusively on the right ovary for follicles punctured after LH (Fig. 1b); the left ovary in general did not exceed the score 2, poor. Time of regression of luteinization was quite variable per animal and always preceded return to oestrus (Fig. 1c), in 13 of 15 animals at 25 d (range 9-40). PRID/PG treatment resulted in a significant reduction and synchronization of this interval, in 5 of 5 animals 12 d (range 11-13).

In conclusion: when follicles are punctured before LH luteinization is prevented probably due to termination of local blood flow, but when punctured after LH luteinization has already been initiated. The resulting luteal tissue eventually regresses and is sensitive to PG at an early stage similar to a normal CL.

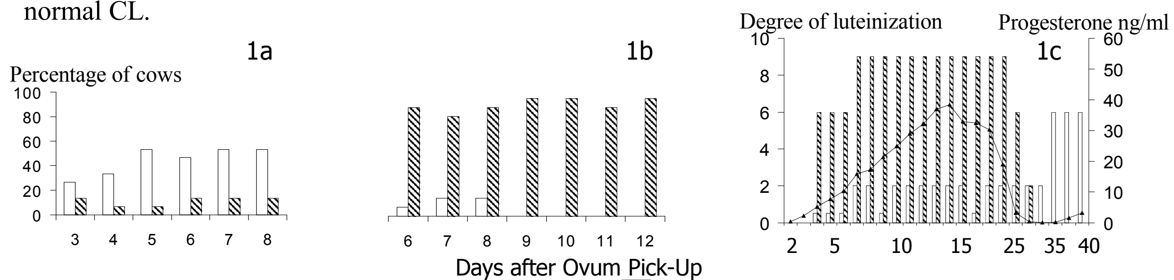

Figure 1 *Development of ovaries after OPU before (left ☐) and 22 h after the LH surge (right ▩) in cows (n = 15) treated for superovulation. Proportion of cows with higher degree on left or right ovary of 1a: follicular structures >15 mm, 1b: luteinization; 1c: representative cow returning to oestrus.*

Notes

EFFECT OF DIFFERENT DMSO-SUCROSE-SOLUTIONS ON *IN VITRO* DEVELOPMENT OF 8-CELL RABBIT EMBRYOS

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Morula and early blastocyst embryos have been preferably used in cryopreservation programs due to their tolerance to osmotic changes and chemical toxicity which take place in the cryopreservation process, which permits the establishment of embryo banks and the diffusion of animals with high genetic value. In addition, the development of successful cryopreservation programs of oocytes and early embryonic stages (pronuclear to 8-16 cells) could support biotechnological activity (nuclear transfer and transgenic production). Nevertheless, early embryonic stages are more sensitive to cryopreservation than morula or early blastocyst embryos, and in very few works their *in vivo* viability has been studied. The aim of this preliminary study was to evaluate the tolerance of 8-cell rabbit embryos exposed to several DMSO-solutions. Embryos were recovered from the oviducts of donor does which were slaughtered 48 hours after insemination; five replicates of the experiment were done. Eight-cell embryos scored as morphologically normal were exposed to DMSO-sucrose-solutions during 45 min at 20ºC (table 1). After that, they were washed twice in M199 and incubated in 0.5 ml of M199+20%FCS at 37.5ºC, 5%CO2 in air and 96% humidity for 72 hours. Embryo development was evaluated after 48h and 72h of culture as the percentage of hatching blastocyst. A chi-squared test with Yate's correction was used to analyse the effect of the solutions on the *in vitro* embryo viability.

Rabbit embryos in the eight-cell stage exposed to DMSO-sucrose solutions showed a survival rate similar to that observed for control group (92%) when the level of DMSO was 2M without sucrose or with 0.5 M sucrose. The principal effect of prolonged exposition to high levels DMSO or sucrose was the arrest of development to 8-cell stage; only 8 embryos over the total subjected to DMSO solutions reached the morula stage and did not develop to blastocyst stage. These preliminary results demonstrated a high tolerance of 8-cell embryos to 2M DMSO solutions without or with 0.5M sucrose, which allows to design further cryopreservation assays using only favourable DMSO solutions.

Table1. Viability of 8-cell embryos after prolonged exposition to different DMSO-sucrose solutions.

	Control	2M DMSO	2M DMSO 0.5M Sucrose	2M DMSO 1 M sucrose	3.5M DMSO	3.5M DMSO-0.5M sucrose	3.5M DMSO-1M sucrose
Nº 8 cell	63	50	49	44	42	42	36
Morula (%)	60 (95.2)	46 (92.0)	40 (81.6)	22 (50.0)	14 (33.3)	18 (42.9)	10 (27.8)
Hatching blastocyst (%)	58 (92.0)^{a}	46 (92.0)^{a}	39 (79.6)^{a}	22 (50.0)^{b}	12(28.6)^{b}	14 (33.3)^{b}	9 (25.0)^{b}

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

Notes

EXPRESSION OF CYCLIN B1 AND CDK1 MESSENGERS DURING *IN VIVO* MATURATION AND *IN VITRO* CULTURE OF CATTLE OOCYTES

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Although some morphological aspects of final oocyte differentiation are known, underlying molecular mechanisms remain mysterious. The oocyte acquires developmental competence during late folliculogenesis. This acquisition may be linked to the storage of RNAs and proteins involved in maturation, fertilisation or early development. We followed by RT-PCR the quantities of cyclin B1 and cdk1 mRNAs, coding for the two subunits of the meiosis promoting factor (MPF), key regulator of oocyte meiotic resumption, during *in vivo* and *in vitro* maturation of bovine oocytes and during in vitro culture of oocytes maintained under meiotic inhibition by roscovitine, a purine inhibiting the MPF kinase activity.

In vivo oocytes were collected by OPU (ovum pick up), 40 h (immature) or 60 h (mature) after prostaglandin treatment of FSH stimulated heifers. For the *in vitro* treatments, oocytes were collected by aspiration of growing follicles on slaughterhouse ovaries. Only oocytes surrounded by more than three layers of compact cumulus cells were selected and transferred for 24 h in maturation (TCM 199 +/- 10 ng/ml EGF) or inhibition medium (with 25 µM of roscovitine). For the semi-quantitative RT-PCR*,* total RNA was extracted (Sigma, Tripure isolation reagent) with a carrier (glycogen) and a reporter gene (0.1 pg of rabbit globin mRNA per oocyte). The RNA was reverse transcribed using oligo d (T) primers. The globin cDNA was co-amplified with cdk1 or cyclin B1 using specific primers. PCR products were analysed on agarose gels.

In all cases, the relative representation of cyclin B1 messenger was higher than cdk1. During *in vivo* oocyte maturation, cdk1 and cyclin B1 expression were increased (fig 1.A). On the contrary, these expressions decreased during *in vitro* maturation (fig 1.B).

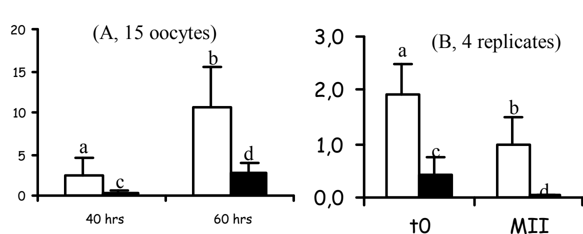

Fig 1 : Relative expression (ratio to globin) of cyclin B1 (white) and cdk1 (black) during oocyte maturation A) in vivo and B) in vitro. a.b and c.d: p<0.05.

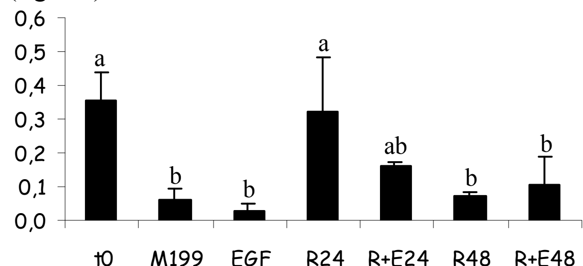

Fig 2 **.** *Effects of culture conditions on cdk1/globin ratio. a,b :p<0.05. (3 replicates).*

In the presence of roscovitine, the quantity of cdk1 mRNA was maintained. This quantity was decreased after a second culture without roscovitine (R 48 and R+E 48, fig 2). The quantity of cyclin B1 mRNA was also maintained during meiotic inhibition (not shown).

In conclusion, during the final maturation of bovine oocyte *in vivo*, there is an accumulation of cdk1 and cyclin B1 mRNA. This accumulation is not reproduced during *in vitro* maturation. This might be due to inappropriate maturation conditions or timing. Therefore, cdk1 and cyclin B1 messengers may represent suitable candidates of markers reflecting the developmental competence of bovine oocytes. Such markers could be used to improve oocyte culture conditions.

Notes

KINASE ACTIVITIES IN THE BOVINE OOCYTE AFTER ENUCLEATION AND FUSION WITH A SOMATIC CELL

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Embyo cloning by nuclear transfer involves the fusion of a somatic donor cell with an enucleated recipient oocyte. The efficiency of this procedure depends on nucleo-cytoplasmic interaction occurring in the newly reconstituted embryo and the evolution of the recipient cytoplasm in the cell cycle may be of major importance. Oocytes at the metaphase II stage are commonly used as recipient cytoplasts in nuclear transfer experiments in the bovine species. The procedure involves enucleation, fusion of the cytoplast with a somatic diploid cell and activation of the oocyte. These manipulations lead to an apparent suitable environment for the reconstitution of nuclear transfer embryos with a functional nucleus, but the biochemical characteristics of such cytoplasts are not really known although they may be determinant in the process of nuclear remodelling. In previous experiments, we evidenced that only 15 to 20% of the transferred nuclei in such oocytes underwent premature chromosome condensation (PCC). Our aim in the present study was to determine the activities of three key regulators of the cell cycle, MPF, MAPK, and Cdc25C, in bovine oocytes processed for their use as recipient cytoplast in nuclear transfer.

Bovine cumulus-oocytes complexes were recovered from slaughterhouse derived ovaries and matured *in vitro* for 22h. Matured oocytes were denuded and enucleated within 1h to produce recipient cytoplasts. The cytoplasts were submitted to two electric pulses of 2.0 kV/cm for 30 µsec which are our usual parameters for fusion and activation in the nuclear transfer experiments. Two groups of oocytes were used as control, the first one was at the germinal vesicle stage (GV), and the second was at the end of the maturation period (MII). The experimental group (EF) was analysed immediately after enucleation and electric stimulation. In each group, MPF and MAPkinase activities were estimated in individual oocyte by measuring the phosphorylation of exogenous histone H1 and by Western blotting respectively. The activation of Cdc25C was determined by analysis of its phosphorylation state by Western blotting.

In the GV group, we observed the nonphosphorylated forms of MAPkinase and Cdc25C, and a low H1 kinase activity; characteristic of interphase. In MII group, MPF activity was high, and MAPkinase and Cdc25C exhibited their phosphorylated form, attesting their activation. In comparison, in the oocytes of the EF group, MAPK and Cdc25C activities (as shown by their elevated phosphorylation state) remained as high as in the MII group, while MPF activity decreased to the basal level in 50% of the oocytes. These data suggest that 50% of the oocytes that have been handled in the same manner as those used in nuclear transfer are no more in a metaphase-II stage but have already progressed toward the interphase stage. However, MAPK and Cdc25C behaviour indicates that their activities still correspond to those of the metaphase-II oocytes. We conclude that such recipient cytoplasts prepared in our conditions may be in an interphase-like stage, between metaphase and interphase, at the time when a foreign nucleus is introduced. This situation may explain our observations that the remodelling of nuclei upon transfer into the cytoplast seems to be not systematically associated with a PCC and a nuclear envelope breakdown.

Notes

SEMEN QUALITY EVALUATIONS OF NELORE AND SIMMENTAL BULLS, KEPT UNDER TROPICAL CONDITIONS, USING THE HYPO-OSMOTIC SWELLING TEST AND A HEMI-ZONA ADHERENCE TEST

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Semen characteristics of Nelore and Simmental bulls, kept in a tropical environment (Mato Grosso do Sul, Brasil), were assessed by means of a hypo-osmotic swelling test and a hemi-zona adherence test. Semen samples of Nelore (n = 6) and Simmental (n = 6) bulls were obtained by electro-ejaculation, because of the low libido of Nelore bulls under field conditions, and evaluated for volume, color, mass movement, and motility. Fresh semen samples were used to test sperm membrane integrity by means of an hypo-osmotic swelling test. A 10 µl sample was added to 1 ml of a hypo-osmotic (100 mOsm/l) and to 1 ml of an iso-osmotic (control, 300 mOsm/l) solution and kept at 37°C (30 min), after which cells were fixed with formaldehyde. Cells presenting the typical swollen tail were considered intact and therefor positive for the test. Subsequently, semen samples were frozen following a standard sperm freezing protocol with glycerol. To conduct the hemi-zona adherence test, oocytes were aspirated from slaughterhouse ovaries, denuded by repeated aspiration with a micro-pipet and stored at 5°C in physiological saline. They were cut in half by a microblade and the hemi-zonas were washed with saline to remove the cytoplasm and then transferred to saline droplets covered with mineral oil (24 hrs). Semen was thawed, washed in Tyrode's solution, centrifuged for 10 minutes to remove the cryoprotectant and placed in the incubator (5% CO_2, 38°C) for one hour. The supernatant was removed and sperm concentration was adjusted to 5×10^5 mobile spermatozoa per ml. Hemi-zonas were then placed in groups of 3 in fertilization medium droplets. Each droplet was inseminated with 100 µL of semen while control droplets were inseminated with the sperm of a bull with proven fertility. After 4 hours of incubation, the hemi-zonas were evaluated by means of a phase interference inverted microscope and the number of spermatozoa adhered to the exterior side of the hemi-zonas was counted. The data were analyzed using SAS for Windows. The results are summarized below.

Table 1. Average percentage of spermatozoa surviving the osmotic shock and the average percentage of spermatozoa bound to the hemi-zonas for Nelore and Simmental bulls.

Breed of bulls	Average percentage of spermatozoa surviving the osmotic shock (intact membrane) (Mean ± SD)		Average percentage* of spermatozoa bound to the hemi-zona (Mean ± SD)
	Fresh semen samples	Frozen-thawed semen	
Nelore (n = 6)	58.8 ± 14.0	7.5 ± 10.2	29.1 ± 22.9
Simmental (n = 6)	60.9 ± 24.4	6.5 ± 6.2	22.4 ± 10.6

*(number of bound sperm cells of the trial bull/ number of bound sperm cells of the control bull) x 100

No statistical differences could be found between both breeds concerning the resistance towards an osmotic shock and the binding capacity of the spermatozoa. Freezing clearly impairs sperm membrane integrity, as indicated by the decrease of the number of intact spermatozoa after the hypo-osmotic test on frozen-thawed sperm for both bull breeds.

This leads to the preliminary conclusion that the reproductive performance of a moderate climate bull breed (Simmental) is not affected by the presence in tropical conditions. More bulls need to be tested for pregnancy rates to confirm this conclusion.

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