17ème COLLOQUE SCIENTIFIQUE

17th SCIENTIFIC MEETING

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Professor Josef FULKA

Special Celebration

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Lyon 07th and 08th September 2001

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C O N T E N T S

Professor Josef FULKA

A.E.T.E. Pioneer Award 2001

Professor Josef FULKA
Pioneer of Physiology of Animal Reproduction

Josef Fulka was born in the North Bohemia. In this very beautiful sub-mountain area, there are good conditions for farming, especially for cattle breeding. His family farm had a long tradition and he has had a great respect to daily work of animal breeders and farmers.

He graduated university studies oriented to animal production at the Agricultural University in Prague in 1951. In that uneasy period for our country and mainly for our agriculture, Josef Fulka decided for a research career at the Institute of Animal Production in Uhrineves, near Prague. It was the only place at that time where the research oriented to animal production took a place in Czechoslovakia. It is hardly believable that he obtained PhD degree in 1955. His supervisor, Professor Josef Smerha deeply influenced his scientific attitude and so he followed him without any hesitation when a new Institute in Libechov (about 40km from Prague) was established. We wrote an institute with a capital letter I, but at this time it was a large farm. Only the enormous enthusiasm allowed him to build step-by-step laboratory facilities.

An imperative for efficient farming at this time was eradication of infectious congenital diseases and only a new strategy of artificial insemination could fulfill this task. Josef Fulka gave the full young energy in rapid spreading of AI among farmers, in optimalization of sperm diluents. So the first research program in Libechov was oriented to bull semen and it was tightly connected with daily problems of farmers. Also the next logical step, deep freezing of bull semen, was tested in his research group and it was spread from the Institute to all insemination stations in Czechoslovakia. It is really symbolic that Chris Polge and Josef Fulka are good, extremely good friends. Their discussions, of course with full glasses of beer, influenced positively research orientation in Libechov.

His “male interest” continued also during his stay at the Institute of Reproduction, Veterinary and Agricultural University in Copenhagen. All original papers devoted to epididymal physiology, done in cooperation with H. H. Koefoed-Johnsen, were frequently cited. This high level of publication quality is the main credo through the whole Josef Fulka’s research career and this strict criterion helped to build the research group with international reputation.

The key decision came in 1967 when Antonin Pavlok entered in the team and Josef Fulka has oriented his PhD thesis to mammalian embryology. The subsequent line of excellent papers dealing with fertilization and in vitro fertilization in the mouse and rabbit and later also in the pig, sheep and cattle confirmed that this orientation was correct. Josef Fulka has had always in mind a connection of research activity with efficient agriculture and so it is not surprising that estrus synchronization with natural prostaglandin F2alfa started just in 1976 and he gave the substantial impetus for synthesis and production of Estrophan in Czechoslovakia. The efficient estrous synchronization was not only substantial help for large cooperative farms but it helped to develop embryo transfer technology first in the surgical and later in the non-surgical version. His research team experience helped substantially to introduced this method in Czechoslovakia and in all other East European countries.

Embryo transfer was for several years a leading subject for his group of Physiology of reproduction but he has stimulated all coworkers in numerous discussions, always connected with his necessary cigarette, to go in more basic aspects of oocyte maturation, fertilization and embryo development. It has been a great pleasure for him to modulate in this way also his son Josef. From that reason, we can find in all renowned journals in the field of developmental biology papers with co-authorship of father and son.

It is not possible to overlook his creative role for the Czech science after 1989. He gave all his experience of an active scientist in formation of the Grant Agency of the Czech Republic. It is no doubts that he imprinted in rules of the Agency an international peer reviewing as a main principle. He worked for several years in this body which substantially improved funding of competitive research teams in our country. His recent activity in the Council of Research Centers has also the attributes to bring the Czech biological research to an international level.

Josef Fulka has been working for many years as a vice-director of "his" Institute of Animal Physiology and Genetics. His wisdom and prudence help us to solve any complicated situation in our life of scientists. Due to his up-date literature knowledge all young colleagues come permanently to discuss and ask him for an advice. We hope to have a share in his life optimism and cleverness also in future years.

Jan Motlik
Jiri Kanka

CONTRIBUTION TO BETTER UNDERSTANDING OF MAMMALIAN EMBRYO GENERATION

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The Institute of Animal Physiology and Genetics in Libechov was established in 1955 as a small unit with the aim to help to solve daily problems of rapidly spreading artificial insemination in cattle and sheep. Gradually, as the time passed its activity has been slowly modified and the attention was paid to the introduction of new techniques that were used abroad in the reproduction of farm animals. This resulted in the stabilization of breeding programs and rapid to application of deep freezing technologies for the storage of bull semen. Already in year 1968 nearly exclusively all bull ejaculates were frozen and this strategy significantly increased the production of milk and meat. Our research of that period culminated in studies oriented almost exclusively on male population and the exploitation of male germ cells in breeding systems. But the new information mainly from England and France and partly also from Russia indicated that in future more interest will be paid to the role of females in reproduction and the exploitation of female germ cells for the improvement of genetic quality of herds. At that time it has been made key decision for the fate of our laboratory – to accept this field of research for future. It should be confess that the initial intentions were not so clear, but step by step, particularly under the influence of published results, personal contacts and with respects to the laboratory equipment as the area of our further studies we chose mammalian egg, fertilization and early embryonic development. At the beginning in all our studies we used laboratory animals – mice and rabbit as the supply of a suitable biological material to learn how to manipulate these particular biological objects.

During this initial period were achieved few observations that were published in international journals where articles underwent critical peer reviewed international evaluation. Among others, the attention certainly deserves those studies addressed to the stability of DNA during sperm storage *in vitro*. It was demonstrated by autoradiography together with Danish coworkers that even after a long time storage no loss of DNA from sperm heads occurred (Koefoed-Johnsen *et al*., 1968). Despite this, after insemination with stored semen, evaluation of flushed embryos and retransfer to oviducts revealed drop of sperm fertilizing ability and the significant increase of embryonic mortality in relation to the sperm age (Koefoed-Johansen *et al*., 1971). Remarkable results about fertilization of mouse oocytes published Pavlok (1967, 1968). He obtained, for the first time, high fertilization rate and the development to blastocysts when the fertilization was carried out in explanted oviducts instead of a free medium. In later experiments the role of cumulus cells in fertilization in the mouse in vitro was defined and contributed to better understanding of sperm – egg interactions (Pavlok and McLaren, 1972). During next few years we published several additional papers, some in the collaboration with French colleagues, dealing with sperm penetration in laboratory and farm animal oocytes under various experimental conditions, which elucidated specific demands for in vitro fertilization of different mammalian species.

This theoretical background and experiments with laboratory animals gave us the experience, which was extremely important for embryo transfer in farm animals. The transposition of previous knowledge to the technique that would be suitable for this new experimental field appeared to be very helpful. However, it should be stressed that we were limited with all problems associated with the application of bovine embryo transfer to farm level. Some of them, as surgical flushing and transfer were eliminated step by step, while some others as predicable success of superovulation outlast to now, irrespective to extensive studies focused to the regulation of follicular growth. Our contribution to the general improvement of transfer technology was rather low. On the other hand, as helpful might be appreciated our teaching activity and demonstration of basic laboratory techniques to members of teams operating in field

conditions. In the context with bovine embryo transfer deserve the attention the results describing the quality of heat in heifers and cows after synchronization of estrus with the analogue of PGF_{2x} – cloprostenol. In these experiments we found the relation between selected clinical parameters and the conception rate after insemination that can serve as an indicator for the selection of potential recipients (Fulka *et al*., 1978).

Seeking for alternative sources of competent oocytes.

Low efficiency of superovulation stimulated the effort for finding of ways leading to the production of oocytes, which would after fertilization develop to embryos suitable for transfer. From original observations of Pincus and Enzman (1935) it was know that the oocytes removed from antral rabbit follicles and placed to suitable environment matured to metaphase II, i.e. to the stage identical with the ovulated oocytes. Many experiments carried out later repeatedly confirmed that the fertilization and subsequent development were heavily compromised, irrespective to their normal morphological appearance. After couple of years, Thibault and Gerard (1970 and 1973) identified the deficiency as absence of unknown cytoplasmic factor in the cytoplasm of in culture matured oocytes. The absence was manifested by the arrested development of male pronuclei after sperm penetration also in many other mammalian species, including cattle at least in the half *in vitro* matured oocytes (Fulka Jr. *et al.*, 1982).

Since that time in various laboratories performed many experiments with the aim to define requirements for the physiological maturation and the results demonstrated to importance of follicular cells which form with the oocyte functional unit not only during growth phase but also at the resumption of meiosis and during GVBD. Therefore, it became obvious the necessity to create for the maturation such environment that would simulate the situation in antral follicle. Particular initial period after resumption of meiosis was found to be critical. In the pig, Motlík and Fulka (1974) reported that the oocytes aspirated for maturation from preovulatory follicles at GV stage possess after fertilization much higher frequency of developmental anomalies than counterparts removed from follicles at diakinesis. The oocytes cultured from M I stage were comparable with ovulated control. Similar conclusion made Moor and Trounson (1977) after maturation of ovine oocytes in intact follicles and obtained after fertilization blastocysts that developed after transfer to term. Beneficial effect of rabbit follicular cells hormonally pretreated before addition to culture systems enhanced the developmental competence, too (Motlík and Fulka, 1981). Taking together all previous observations suggested by Staigmiller and Moor (1984) to enrich culture medium with follicular cells. Although the cell are not able to supply the oocytes with all natural signals in proper level as in preovulatory follicle they help to improve the quality of cytoplasm and after fertilization to increase also the production of health embryos particularly in sheep and cattle.

For cultivation, oocytes (pig, cattle, sheep) are typically isolated from follicles of 3-5 mm in diameter and morphological criteria do not allow us to evaluate the actual physiological state of oocyte-cumulus complexes. The collected oocyte population thus exerts a very high heterogeneity. When examining the time course of germinal vesicle breakdown (GVBD) in fully grown porcine oocytes from prepubertal gilts we found that during 24 hr of culture most of them already underwent nuclear envelope breakdown (NEBD) and were in late diakinesis (LD) or metaphase I (MI) stage, whilst remaining oocytes still posses remnants of nuclear membrane and visible karyoplasm. This means that within usually used 40 hr of culture *in vitro* some oocytes mature more quickly and some of them are rather slow. Significantly lower heterogenity was observed in oocytes from hormonally stimulated gilts (Motlík and Fulka, 1976). Similar variability in the frequency of identical morphological stages was observed also during GVBD in bovine oocytes (Motlík *et al*., 1978). It is well documented that bovine oocytes maturing more rapidly have greater developmental potential when fertilized *in vitro* and thereafter cultured up to the blastocyst stage. Although the meiotic progression is an important part of maturation process it is not the only and sufficient criterion for the completion of full maturation. Cytoplasmic maturation represents parallel complexes of events providing the oocyte with new proteins and certain specialized classes of mRNA, which determine its full developmental competence. Present cultivation techniques used for the production of farm animal embryos provide an adequate environment only for nuclear maturation and allow almost to all oocytes to reach M II stage (with slight differences depending on species). Due to the lack of objective indicators for the assessment of cytoplasmic maturation and to the heterogenity of oocytes used, the results after

fertilization are less consistent. Irrespective of the relatively high fertilization rate, only 25-30% of oocytes used develop to the blastocyst stage that is suitable for transfer. Moreover, not all of them survive the freezing and thawing procedures (Galli and Lazzari, 1996).

To produce embryos more efficiently, the attention was paid also to the reduction of the heterogenity of oocytes. As it is evident from results published by Moor and Trounson (1977), oocytes isolated from smaller ovine follicles (2 mm) possess lower maturation competence than those isolated from larger follicles (3-5 mm). Also the development to blastocysts was strongly reduced after fertilization of oocytes derived from small follicles. The evident correlation between the ability to resume and to complete meiosis in pig oocytes was demonstrated also by Motlík *et al*. (1984). Oocytes from small antral follicles underwent GVBD in the lower rate and arrested meiotic maturation in M I comparing to oocytes isolated from large antral follicles. Full competence to undergo completely at least nuclear maturation was observed consistently only in oocytes isolated from follicles of different diameter, matured and fertilized *in vitro* and thereafter cultured up to the blastocyst stage (Pavlok *et al*., 1992). Much lower penetration rate was observed in the group of oocytes from follicles <2 mm comparing to oocytes derived from follicles in 2-4 mm and 4-8 mm. Moreover, subsequent development of embryos derived from the first follicular class was heavily compromised and only exceptionally embryos cleaved to 5-8 cells. In contrast, oocytes from the other follicular classes reached the blastocysts stage in 21% and 28% cases. These results were confirmed later by Lonergan *et al*. (1994) and Fair *et al*. (1995).

Detailed studies indicated that oocytes from small and larger follicles differ in several aspects. The oocytes from small bovine antral follicles show very intensive synthesis of RNA in the nucleoplasm and nucleoli. This synthesis gradually decreases with oocyte growth and stops in full sized oocytes (3 mm). Decreased intensity of RNA synthesis is accompanied with remarkable changes in nucleolar morphology. As oocyte attains the competence to mature the fibrillo-granular material becomes more compacted and forms dense fibrillar areas with a large central vacuole (Crozet *et al*., 1986). Sequential changes in nuclear morphology during bovine oocyte growth were described in details by Hyttel *et al*. (1997). These authors found a clear correlation between RNA transcription and the ability of oocytes to mature. These transcripts are required for synthesis of new proteins, including cell cycle regulating kinases and phosphatases, which are necessary for both, the resumption and completion of meiosis and for normal development after fertilization.

The events characteristic for oocyte maturation were, at the very beginning, monitored according to well defined changes during GVBD, progression of maturation and the time of formation of M II with the first polar body. The activity responsible for the induction of initiation of maturation was first described in 1971 by Masui and Markert in amphibians and was named as Maturation Promoting Factor (MPF). That the same activity is responsible for the initiation of mammalian oocyte maturation was first showed by Hanna Balakier (1978) with the help of induced cell to cell fusion. This approach help us, during early days, to characterize MPF activity not only in the mouse but also in farm animals oocytes. The activity of MPF in fully grown mammalian oocytes showed basically the same characteristics as in amphibian and starfish oocytes. Surprisingly, very interesting results were observed when growing oocytes were used for fusion. Our experiments showed the ability of growing oocytes fused to fully grown oocytes to prevent the initiation of GVBDs in giant cells (Fulka Jr. *et al*., 1985). Some further research was addressed to effect of MPF dilution on nuclear membrane dissolution and chromosome condensation (Fulka Jr. *et al*., 1986) and the autocatalytic amplification of MPF under various experimental protocols (Fulka Jr. *et al*.,1987). Moreover, these detailed studies resulted in the discovery that porcine and cattle oocytes need, contrary to mouse oocytes, newly synthesized proteins for the initiation of GVBD (Fulka Jr. *et al*., 1986).

It should be stressed that during these early days the presence and activity of MPF was assessed only by microscopical evaluation of morphological changes manifested on nuclear level. Gradual introduction of biochemical methods allowed later on to identify MPF as complex formed by cyclin B and $p34^{cdc2}$, a regulatory and a catalytic subunit of cdc2 kinase. But even now, after many years of an intensive research, not everything is clear and the cell cycle regulation is still intensively studied. These results contribute significantly to our better understanding of mammalian oocyte maturation and the reprogramming of nuclei in cloning experiments. In addition, some findings help us to modify our IVM techniques.

Two – step culture of mammalian oocytes.

Due to the fact that oocytes originate from different follicular categories individual cells of population are not equally prepared for fertilization and subsequent cleavage. Whilst the culture period of 24 h seems to be sufficient for the completion of nuclear maturation of bovine and ovine oocytes, the cytoplasmic maturation does not reach the level which is optimal for further development after fertilization. These considerations led us to suggestion that the artificially reversible arrest of nuclear maturation can provide the oocyte with a sufficient time necessary for the generation of vital molecules required for further development. To do this, different culture systems using follicular components or certain cell cycle inhibitors were tested with the aim to improve the quality of cultured oocytes.

Experiments with rodent oocytes demonstrated that GVBD can be reversibly blocked by several inhibitors, mainly with those which increased or sustain high intracellular levels of cAMP. Unfortunately, these inhibitors are not suitable for farm animals oocytes. On the other hand, the addition of cycloheximide or 6-dimethylaminopurine to the culture media prevents GVBD effectively (Lonergan *et al.*, 1997). However, these drugs decrease the developmental potential of inhibited oocytes after fertilization (Avery and Greve, 1997).

It may well be that more promising candidates for two step culture systems will be found in a new generation of newly discovered specific cell cycle inhibitors. Recently, Mermillod *et al.* (2000) used the inhibitor called roscovitin for the arrest of nuclear maturation in bovine oocytes. They found the high reversibility after cultivation for 24 hr in the concentration 25 μM. At the end of this time interval 83% oocytes remained in GV stage and H1 kinase activity was maintained at a basal level. After washing and further cultivation for additional 24 hr almost 90% of oocytes completed meiosis to MII. The cleavage rate of experimental and control oocytes was almost similar – the development to the blastocyst stage was 36% and 41% in treated and control group, respectively and with a comparable number of cells in both types of embryos.

Another inhibitor studied – butyrolactone I (BL I) blocks GVBD very effectively in bovine oocytes during the incubation of 24 hr or 48 hr (Kubelka *et al.*, 2000; Motlík *et al*. 2000). Full reversibility was associated with the normal fertilization capacity and low incidence of polyspermy. The analyses of several kinases that are typical for cell cycle progression indicate a high specificity of BL I and thus its suitability for two step maturation technology. In addition BL I may be convenient for the improvement of a meiotic and developmental competence of those oocytes which are isolated from small follicular categories. At least, new results published by Pavlok *et al.* (2000) show that oocytes isolated from follicles of 1-2 mm which mature to M II only exceptionally, became more competent when incubated in the presence of BL I for 48 hr. During the subsequent culture for 24 hr in BL I free medium, more than 80% mature to M II, whilst in a control group only 30% reached this stage. The original intensive incorporation of ^{3}H-uridine to nuclear and nucleolar structures gradually decreases throughout the oocyte preincubation in BL I and reached the level typical for oocytes from larger follicular classes. The drop of transcription coincides with the transformation of nucleoli, i.e. fibrillo-granular components are substituted by more compact fibrillar structures. The experiments addressed to the assessment of developmental competence of those oocytes are in progress at present. In any case these results clearly demonstrate the ability of specific inhibitors to influence, at least partially, those processes that may be responsible for the generation of fully functional oocytes.

The limited information did not allow us to evaluate the two step culture systems advantages for sure. However, it can be expected that the near future will give us some new more specific inhibitors for the regulation of cell cycle having the potential impact for the culture systems of mammalian oocytes. At present it is only known with certainty, that these new inhibitors are able to prolong the time period of RNA and protein synthesis in oocytes isolated from small antral follicles and this may contribute to the acquisition of better maturation competence *in vitro*. In meiotically competent oocytes the preincubation time has no obvious harmful effect on fertilization and further development to blastocysts stage. If this method is able to reduce heterogeneity of cultured population and to support the equipment of oocytes with vitally important molecules resulting in higher efficiency of *in vitro* produced embryos waits for clarification. However, only numerous transfer experiments may offer a final objective answer.

This short review would like to demonstrate some fragments of research activity in our laboratory from the begining until present days. In fact the programs were broader and covered also other aspects of sperm-egg interaction including activation of embryonic genome in mammals. Quite few papers about these topics were published in close collaboration with Dr. Václav Kopecn_, who introduced for detailed studies such methods as autoradiography and

electronmicroscopy. His valuable results are not included in this short review, they would deserve special article. From originally very limited staff, the number of coworkers gradually increased and new modern methods enabling to study selected processes on molecular level are employed now. We believe that this approach will allow us to contribute more effectively to the development of new biotechnologies with significant impact for animal breeding in future.

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National Statistical Data of Bovine Embryo Transfer Activity in Europe (2000).

TABLE : 1 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: AUSTRIA

A.E.T.E 2001

Data collected by
Dr. Fischerleitner Franz

Total number of approved E.T. teams in the country	19
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	218	B / A=	10.69
	Embryos collected	**B**	2331	C / A=	6.57
	Embryos transferable	**C**	1433	C / B=	61%
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**	3		
Total i*n vitro* embryos		**F**	3	=(D+E)	
Total number of transferable embryos		**G**	1436	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	399		
In vivo	Frozen	**I**	511		
In vitro	Fresh	**J**	2		
In vitro	Frozen	**K**	16		
Total embryos transferred		**L**	928	H+I+J+K=	
Number of frozen stored embryos		**M**	904		
% of *in vitro* embryos transferred		**N**	1.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	57%	(I+K) / L=	%

Number of E.T. calves born (2000)

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 2000

COUNTRY: **BELGIUM**

A.E.T.E 2001

Data collected by
Dr. Beckers Jean-François

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1791	B / A=	6.19
	Embryos collected	**B**	11088	C / A=	4.51
	Embryos transferable	**C**	8074	C / B=	73%
In vitro *(OPU)*	Nb of oocyte donors		277		
	Nb of OPU sessions		504		
	Nb of transferable embryos	**D**	1492		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	23		
Total *in vitro* embryos		**F**	1515	=(D+E)	
Total number of transferable embryos		**G**	9589	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3040		
In vivo	Frozen	**I**	4439		
In vitro	Fresh	**J**	734		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	8213	H+I+J+K=	
Number of frozen stored embryos		**M**	5468		
% of *in vitro* embryos transferred		**N**	8.9%	(J+K) / L=	
% of frozen embryos transferred		**O**	54%	(I+K) / L=	%

Number of E.T. calves born (2000)

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

* data not available

TABLE :3 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: CZECH REPUBLIC

A.E.T.E 2001

Data collected by
Dr. Petelikova Jirina

Total number of approved E.T. teams in the country	5
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	1027	B / A=	8.51
	Embryos collected	**B**	8744	C / A=	4.80
	Embryos transferable	**C**	4930	C / B=	56%
In vitro *(OPU)*	Nb of oocyte donors		9		
	Nb of OPU sessions		40		
	Nb of transferable embryos	**D**	23		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	318		
Total i*n vitro* embryos		**F**	341	=(D+E)	
Total number of transferable embryos		**G**	5271	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	2618		
In vivo	Frozen	**I**	2061		
In vitro	Fresh	**J**	147		
In vitro	Frozen	**K**	102		
Total embryos transferred		**L**	4928	H+I+J+K=	
Number of frozen stored embryos		**M**	2399		
% of *in vitro* embryos transferred		**N**	5.1%	(J+K) / L=	
% of frozen embryos transferred		**O**	44%	(I+K) / L=	%

Number of E.T. calves born (2000)

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 2000

COUNTRY: DENMARK

A.E.T.E 2001

Data collected by
Dr. Callesen Henrik

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	741	B / A=	9.22
	Embryos collected	**B**	6837	C / A=	6.30
	Embryos transferable	**C**	4671	C / B=	68%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	*	=(D+E)	
Total number of transferable embryos		**G**	4671	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1404		
In vivo	Frozen	**I**	2164		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3568	H+I+J+K=	
Number of frozen stored embryos		**M**	3185		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	61%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	1455
Number of calves born from *in vitro* embryos	
Total	

* data not available

TABLE : 5 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: FRANCE

A.E.T.E 2001

Data collected by
Dr. Guérin Bernard

Total number of approved E.T. teams in the country	29
Number of teams providing data	25

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	6716	B / A=	9.31
	Embryos collected	**B**	62538	C / A=	5.40
	Embryos transferable	**C**	36263	C / B=	58%
In vitro *(OPU)*	Nb of oocyte donors		163		
	Nb of OPU sessions		230		
	Nb of transferable embryos	**D**	1095		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	5621		
Total *in vitro* embryos		**F**	6716	=(D+E)	
Total number of transferable embryos		**G**	42979	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	17836		
In vivo	Frozen	**I**	15647		
In vitro	Fresh	**J**	555		
In vitro	Frozen	**K**	24		
Total embryos transferred		**L**	34062	H+I+J+K=	
Number of frozen stored embryos		**M**	12347		
% of *in vitro* embryos transferred		**N**	1.7%	(J+K) / L=	
% of frozen embryos transferred		**O**	46 %	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	4053
Number of calves born from *in vitro* embryos	99
Total	4152

TABLE : 6 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: GERMANY

A.E.T.E 2001

Data collected by
Dr. Clauss Karin

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	3942	B / A=	12..28
	Embryos collected	**B**	48417	C / A=	7.51
	Embryos transferable	**C**	29629	C / B=	61%
In vitro	Nb of oocyte donors		*		
(OPU)	Nb of OPU sessions		2748		
	Nb of transferable embryos	**D**	3697		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	3697	=(D+E)	
Total number of transferable embryos		**G**	33326	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	10484		
In vivo	Frozen	**I**	11375		
In vitro	Fresh	**J**	2203		
In vitro	Frozen	**K**			
Total embryos transferred		**L**	24062	H+I+J+K=	
Number of frozen stored embryos		**M**	19991		
% of *in vitro* embryos transferred		**N**	9.2%	(J+K) / L=	
% of frozen embryos transferred		**O**	47%	(I+K) / L=	%

Number of E.T. calves born (2000)

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 2000

COUNTRY: GREECE

A.E.T.E 2001

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	**A**	8	B / A=	6.75
	Embryos collected	**B**	54	C / A=	5.25
	Embryos transferable	**C**	42	C / B=	78%
In vitro (*OPU*)	Nb of oocyte donors		6		
	Nb of OPU sessions		24		
	Nb of transferable embryos	**D**	21		
In Vitro (*Slaughtered donors*)	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	21	=(D+E)	
Total number of transferable embryos		**G**	63	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	42		
In vivo	Frozen	**I**	0		
In vitro	Fresh	**J**	8		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	50	H+I+J+K=	
Number of frozen stored embryos		**M**	13		
% of *in vitro* embryos transferred		**N**	16%	(J+K) / L=	
% of frozen embryos transferred		**O**	0%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	27
Number of calves born from *in vitro* embryos	2
Total	29

TABLE : 8 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: HUNGARY

A.E.T.E 2001

Data collected by
Dr. Solti Laszlo

Total number of approved E.T. teams in the country	11
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	406	B / A=	9.67
	Embryos collected	**B**	3928	C / A=	4.83
	Embryos transferable	**C**	1963	C / B=	50%
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**	248		
Total i*n vitro* embryos		**F**	248	=(D+E)	
Total number of transferable embryos		**G**	2211	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	212		
In vivo	Frozen	**I**	217		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	429	H+I+J+K=	
Number of frozen stored embryos		**M**	1621		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	51 %	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	270
Number of calves born from *in vitro* embryos	*
Total	*

* data not available

TABLE : 9 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: IRELAND

A.E.T.E 2001

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**	866	B / A=	7.84
	Embryos collected	**B**	6788	C / A=	4.08
	Embryos transferable	**C**	3532	C / B=	52%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**	1560		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	62		
Total *in vitro* embryos		**F**	1622	=(D+E)	
Total number of transferable embryos		**G**	5154	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1736		
In vivo	Frozen	**I**	1536		
In vitro	Fresh	**J**	914		
In vitro	Frozen	**K**	47		
Total embryos transferred		**L**	4233	H+I+J+K=	
Number of frozen stored embryos		**M**	1212		
% of *in vitro* embryos transferred		**N**	22.7%	(J+K) / L=	
% of frozen embryos transferred		**O**	37%	(I+K) / L=	%

Number of E.T. calves born (2000)

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

* data not available

TABLE : 10 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: ITALY

A.E.T.E 2001

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**	1221	B / A=	11.18
	Embryos collected	**B**	13650	C / A=	5.89
	Embryos transferable	**C**	7196	C / B=	53%
In vitro	Nb of oocyte donors		174		
(OPU)	Nb of OPU sessions		916		
	Nb of transferable embryos	**D**	1954		
In Vitro	Nb of transferable embryos	**E**	6092		
(Slaughtered donors)					
Total i*n vitro* embryos		**F**	8046	=(D+E)	
Total number of transferable embryos		**G**	15242	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3676		
In vivo	Frozen	**I**	3427		
In vitro	Fresh	**J**	100		
In vitro	Frozen	**K**	5270		
Total embryos transferred		**L**	12475	H+I+J+K=	
Number of frozen stored embryos		**M**	7714		
% of *in vitro* embryos transferred		**N**	43%	(J+K) / L=	
% of frozen embryos transferred		**O**	70%	(I+K) / L=	%

Number of E.T. calves born (2000)

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

* data not available

TABLE : 11 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: NETHERLANDS *A.E.T.E 2001*

Data collected by
Dr. de Ruigh Lisette

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	4600	B / A=	8.20
	Embryos collected	**B**	37673	C / A=	4.58
	Embryos transferable	**C**	21073	C / B=	56%
In vitro	Nb of oocyte donors		349		
(OPU)	Nb of OPU sessions		3425		
	Nb of transferable embryos	**D**	3880		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	74		
Total i*n vitro* embryos		**F**	3954	=(D+E)	
Total number of transferable embryos		**G**	25027	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	3991		
In vivo	Frozen	**I**	13101		
In vitro	Fresh	**J**	1687		
In vitro	Frozen	**K**	1837		
Total embryos transferred		**L**	20616	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**	17.1%	(J+K) / L=	
% of frozen embryos transferred		**O**	72%	(I+K) / L=	%

Number of E.T. calves born (2000)

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 2000

COUNTRY: NORWAY

A.E.T.E 2001

Data collected by
Dr. Refsdal Arne Ola

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	52	B / A=	10.48
	Embryos collected	**B**	545	C / A=	4.98
	Embryos transferable	**C**	259	C / B=	48%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	*	=(D+E)	
Total number of transferable embryos		**G**	259	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	33		
In vivo	Frozen	**I**	228		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	261	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**		(J+K) / L=	
% of frozen embryos transferred		**O**	87%	(I+K) / L=	%

Number of E.T. calves born (2000)

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 2000

COUNTRY: PORTUGAL

A.E.T.E 2001

Data collected by
Dr. N. Chagas E Silva

Total number of approved E.T. teams in the country	7
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	30	B / A=	4.86
	Embryos collected	**B**	146	C / A=	3.97
	Embryos transferable	**C**	119	C / B=	82%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	119	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	41		
In vivo	Frozen	**I**	109		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	150	H+I+J+K=	
Number of frozen stored embryos		**M**	77		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	73%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	57
Number of calves born from *in vitro* embryos	
Total	57

TABLE : 14 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: ROMANIA

A.E.T.E 2001

Data collected by
Dr. Ilinca Nicolae

Total number of approved E.T. teams in the country	4
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	18	B / A=	12.55
	Embryos collected	**B**	226	C / A=	7.50
	Embryos transferable	**C**	135	C / B=	60%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	135	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	15		
In vivo	Frozen	**I**	187		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	202	H+I+J+K=	
Number of frozen stored embryos		**M**	11		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	93%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	72
Number of calves born from *in vitro* embryos	0
Total	72

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: RUSSIA

A.E.T.E 2001

Data collected by
Dr. Erokhin Anatoly S.

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

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	23	B / A=	4.0
	Embryos collected	**B**	92	C / A=	3.35
	Embryos transferable	**C**	77	C / B=	84%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	77	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	0		
In vivo	Frozen	**I**	66		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	66	H+I+J+K=	
Number of frozen stored embryos		**M**	11		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	100%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	28
Number of calves born from *in vitro* embryos	0
Total	28

* data not available

TABLE : 16 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: SPAIN

A.E.T.E 2001
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**	459	B / A=	8.60
	Embryos collected	**B**	3948	C / A=	4.15
	Embryos transferable	**C**	1904	C / B=	48%
In vitro *(OPU)*	Nb of oocyte donors		21		
	Nb of OPU sessions		201		
	Nb of transferable embryos	**D**	219		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	219	=(D+E)	
Total number of transferable embryos		**G**	2123	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	391		
In vivo	Frozen	**I**	928		
In vitro	Fresh	**J**	27		
In vitro	Frozen	**K**	46		
Total embryos transferred		**L**	1392	H+I+J+K=	
Number of frozen stored embryos		**M**	1582		
% of *in vitro* embryos transferred		**N**	5.2%	(J+K) / L=	
% of frozen embryos transferred		**O**	70%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	276
Number of calves born from *in vitro* embryos	2
Total	278

* data not available

TABLE : 17 EMBRYO TRANSFER ACTIVITY IN 2000

COUNTRY: SWEDEN

A.E.T.E 2001

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=	7.60
	Embryos collected	**B**	2232	C / A=	4.32
	Embryos transferable	**C**	1270	C / B=	57%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	*	=(D+E)	
Total number of transferable embryos		**G**	1270	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	679		
In vivo	Frozen	**I**	1004		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	1683	H+I+J+K=	
Number of frozen stored embryos		**M**	*		
% of *in vitro* embryos transferred		**N**	0%	(J+K) / L=	
% of frozen embryos transferred		**O**	60%	(I+K) / L=	%

Number of E.T. calves born (2000)

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 2000

COUNTRY: SWITZERLAND *A.E.T.E 2001*

Data collected by Dr. Rainer Saner

Total number of approved E.T. teams in the country	4
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	311	B / A=	11.44
	Embryos collected	**B**	3558	C / A=	7.85
	Embryos transferable	**C**	2440	C / B=	69%
In vitro	Nb of oocyte donors		36		
(OPU)	Nb of OPU sessions		355		
	Nb of transferable embryos	**D**	138		
In Vitro	Nb of transferable embryos	**E**			
(Slaughtered donors)					
Total *in vitro* embryos		**F**	138	=(D+E)	
Total number of transferable embryos		**G**	2578	=(C+F)	
EMBRYO TRANSFER					
In vivo	Fresh	**H**	672		
In vivo	Frozen	**I**	1695		
In vitro	Fresh	**J**	0		
In vitro	Frozen	**K**	84		
Total embryos transferred		**L**	2451	H+I+J+K=	
Number of frozen stored embryos		**M**	1886		
% of *in vitro* embryos transferred		**N**	3.4%	(J+K) / L=	
% of frozen embryos transferred		**O**	73%	(I+K) / L=	%

Number of E.T. calves born (2000)

Number of calves born from superovulated embryos	1380
Number of calves born from *in vitro* embryos	21
Total	1401

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2000

I. EMBRYO PRODUCTION

(Data collected from 18 countries)

***In vivo* produced embryos (superovulation)** - number of flushed donors - number of transferable embryos - mean number per flushed donor	**22 723** **125 005** **X = 5.50**
***In vitro* produced embryos:** From OPU - number of oocyte donors - number of OPU sessions - number of blastocysts produced From slaughterhouse collected ovaries - number of blastocysts produced Total *in vitro*	 **1 035** **8 443** **14 079** **12 441** **26520**
Total number of transferable embryos	**151 525***

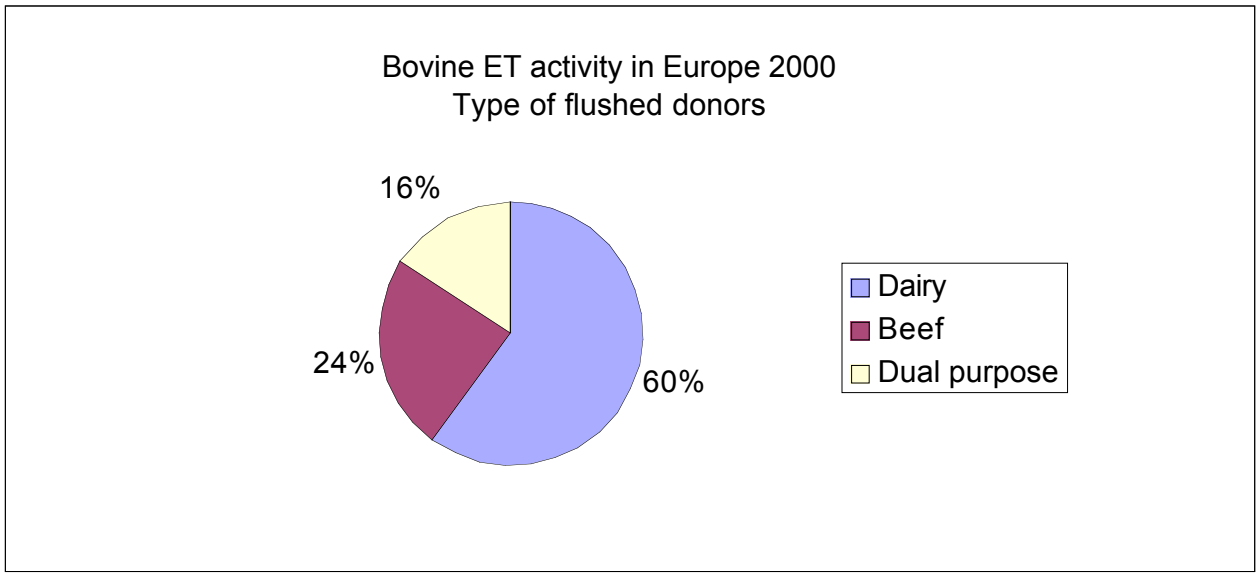
* This number is underestimated as the data from 2 countries were not available and not included

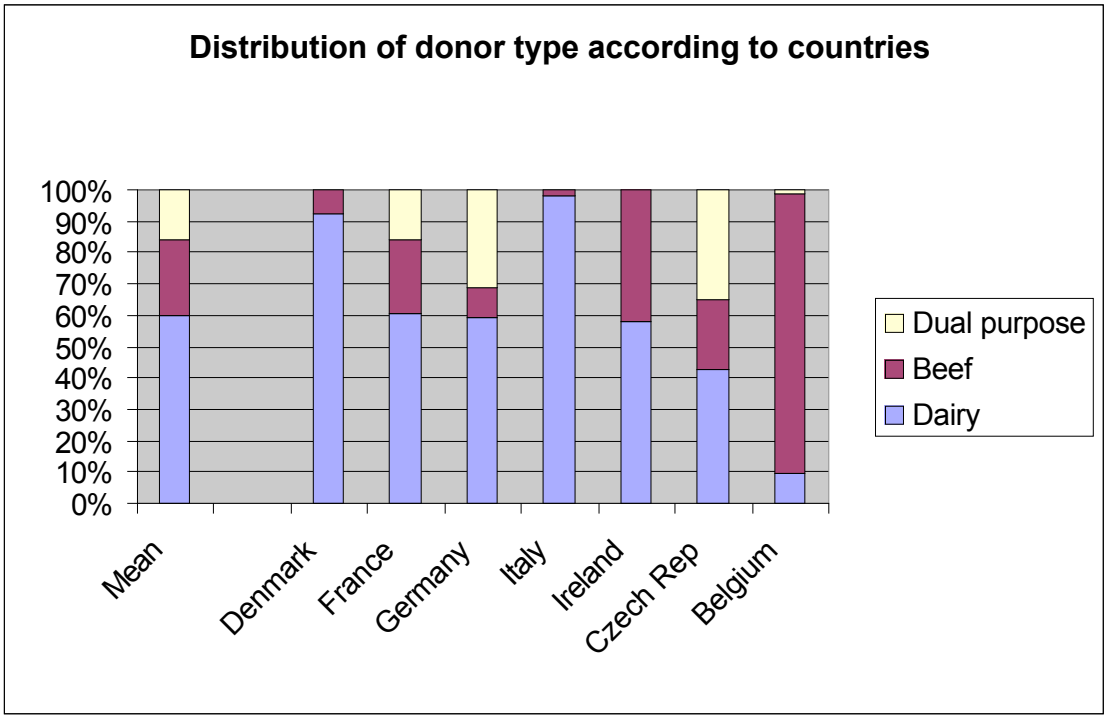
(Y.HEYMAN, AETE Lyon 2001)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2000

II. DISTRIBUTION OF BREEDS





OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2000

III. EMBRYO TRANSFERS

(Data collected from 18 countries)

	Number of recipients transferred
From *In* vivo produced embryos -	**105 964**
From *In vitro* produced embryos -	**13 803**
Total number of embryo transfers	**119 769***
Proportion of IVF embryos transferred	**11.52%**
Proportion of frozen embryos transferred	**55%**
Total number of embryos stored in LN2	**58 541**

* This number is underestimated as the data from 2 countries were not available and not included

(Y.HEYMAN, AETE Lyon 2001)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES*
EUROPE year 2000

Species	Embryo Production	Embryo Transfers	Countries
Sheep	**847**	**440**	Czech rep Greece Hungary Norway Portugal Romania
Swine	**816**	**619**	Czech Rep Romania
Goat	**243**	**145**	Czech Rep France Norway Portugal Romania
Horse	**563**	**226**	Czech Rep France Norway Portugal

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

(Y. HEYMAN, AETE Lyon, 2001)

INVITED LECTURES

RISKS OF TRANSMISSIBLE DISEASES IN RELATION TO EMBRYO TRANSFER

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Introduction

Initially limited to national or international trade with live animals or semen, the exchanges of genes are now widely associated with embryo transfer. This biotechnology which is mainly used to improve faster the genetic potential is not a way of transmission of different infectious diseases. Indeed, some epidemiological studies of numerous infectious diseases [i .e. Maedi-Visna or more recently infectious bovine rhinotracheitis (IBR), foot-and-mouth disease (FMD), porcine reproductive and respiratory syndrome (PRRS)] clearly highlighted the close relationship between the importation of infected animals in a free country and the resulting spread of the disease. Therefore, to avoid this kind of disease transmission, the perfect knowledge and the assessment of the sanitary risks associated with embryo production and embryo transfer are of highest interest for scientists and veterinary authorities. The aim of this article is to evaluate these risks by using the Hazard Analysis Criticial Control Points (HACCP) method that is commonly used in the agroalimentary industry. That means that the risk of disease transmission must be evaluated at each step of embryo production, storage and transfer. Then, we should be able to focus our attention on the critical points, to evaluate or define new procedures that would enable to deal with the potential risks and either to answer specific questions like this one "when we are face to a sanitary problem (bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD), tuberculosis, brucellosis…) in a herd of high genetic merit, can we collect oocytes or embryos?" In other words, can the ET be a way to save the genetic of a herd ?

In vivo and *in vitro* produced embryos may be contaminated during the different steps of production and transfer. The pathogenic agent may potentially originate from the donor male (semen) or the donor female (oocytes, embryos). It can be present in various environments, such as serum or media used for the handling of oocytes and embryos, *in vitro* maturation of oocytes, *in vitro* fertilization and culture. Pathogens may also be added during the whole process of embryo production, from collection to transfer, including storage conditions. Finally it can also transmitted by the recipient during the pregnancy.

Thus we will investigate the risks of disease transmission at each stage of the embryo production and evaluate the prevention measures. In order to obtain a good assessment of the sanitary aspects associated with embryos transfer, we should have and use both experimental and field data.

Sanitary risks associated with the semen

A lot of pathogen organisms are present in the genital glands or testis, in lymphatic system, in blood system (during a bacteriemie or a viremie) or in urinary system. Therefore, all of these microorganisms may be easily transmitted in semen by the way of seminal liquid, spermatozoid, or with urine contact. This is confirmed by the fact that nearly all of the pathogen microorganisms listed in the OIE have been isolated from semen.

When a pathogen agent is not detected in semen, we should wonder if the test we use to detect it is enough sensitive. For instance, there was no evidence of semen excretion of *Mycobacterium paratuberculosis* during several years, because of the dilution of semen in the extender and the low concentration of this pathogen in semen [35;36]. The semen excretion could be confirmed with more sensitive tests such as polymerase chain reaction or enhanced culture systems (Figure 1).

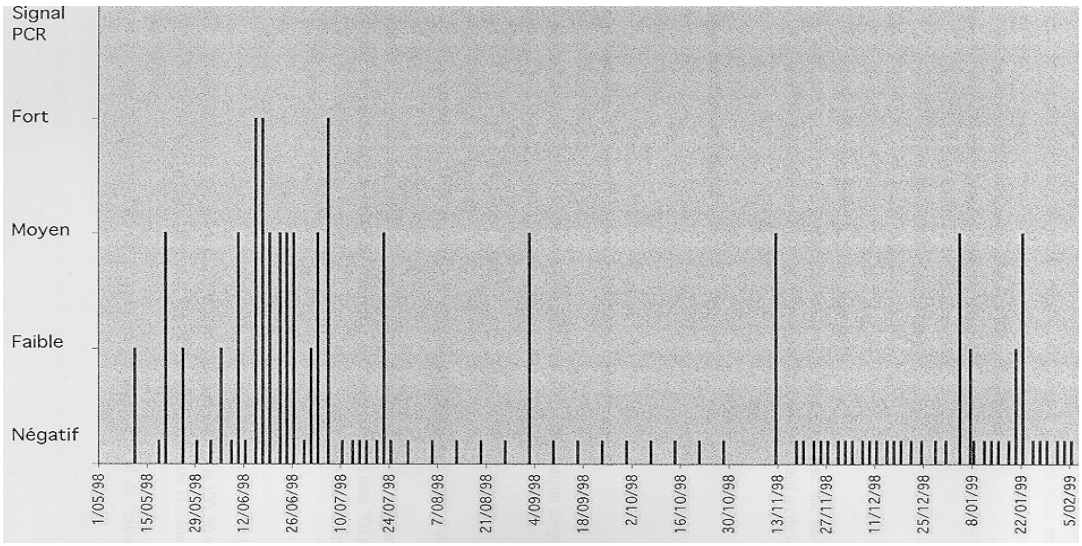

Figure 1 : Detection of DNA of *M. paratuberculosis* in semen by PCR test for a bull during nine months (Le Tallec, unpublished)

These microorganisms (virus or bacteria) can be present in semen either in seminal plasma and/or associated with spermatozoa [59]. This contamination can play a role during fertilization and can also be transmitted to the embryo or to the recipient. This may be illustrated by two different examples :

Bovine Viral Diarrhoea Virus (BVDV)

The experiments with BVDV realised in our laboratory have shown that the presence of virus in semen dramatically affects *in vitro* embryonic development which was dramatically reduced to 2,1 to 4% [1;30]. Moreover, all normal or degenerated embryos were contaminated after the 10 recommended washes. This negative effect of BVDV on *in vitro* development rates may depend on the virus strain and on its cytopathopathic effect [13]. For *in vivo* produced embryos, the effects of a BVDV contamination after insemination with infected semen is not well documented.

Mycoplasma spp.

Bielansky *et al*. (2000) performed in-vitro fertilization with semen experimentally infected with *Mycoplasma bovis* or *Mycoplama bovigenitalium* and showed that those pathogens can be transmitted through the IVF system and therefore infect embryos. Furthermore, their experiments showed that the supplementation of the media used for *in vitro* culture with standard antibiotics and the washing procedure of the embryos as recommended by IETS were not effective in rendering IVF embryos free from *M. bovis* and *M.bovigenitalium [4]*.

In fact, the different studies testing contamination of *in vitro* or *in vivo* produced embryos show that the risk of transmission of pathogen microorganisms when using infected semen is potentially very high.

Prevention measures

In order to avoid contamination of semen and to control related sanitary risks, different measures have to be taken. The first one and the most important one is the epidemiological rule specifically applied in AI centres : to be sure to collect semen free from pathogens, it is necessary to entry and keep a bull free from pathogens in a pathogen free centre where all animals are free from the disease and regularly controlled. If this rule can't be achieve and if a bull with high genetic merit has to be used for ET, the only way to lower the risk of contamination (without eliminating it) is to examine each ejaculate by testing a couple of straws with a sensitive test.

Besides these major pathogens for which males are strictly controlled in AI centres, semen can be contaminated by other species, such as ubiquitous pathogens. This contamination may be limited by hygienic measures which are applied especially on housing, collection and processing of semen. The respect of these sanitary procedures may be verified by the sanitary rule linked to the

ability to produce semen of high microbiological quality (<500 CFU/ml). Addition of antibiotics to the extenders are also efficient to limit the number of bacteria present in extended semen.

Sanitary risks associated with the donor female

In vivo produced embryos

Oocytes and embryos are surrounded by the zona-pellucida (ZP), which protects the embryos and plays a role during all the phases of embryonic development, from maturation through fertilization until early embryonic development. In general, the ZP of mammalian species is composed of three glycoproteins, which are assembled in a complex three-dimensional structure. Changes in the structure of the glycoproteins occur during fertilization and also during the passage of the embryo through the oviduct. As a consequence, pathogens can no longer bind to and/or penetrate the intact ZP after fertilization, which becomes an effective barrier against the contamination by most of the pathogens investigated. Therefore, the main parameter is related to the integrity of the ZP and it is generally accepted that the only way of transmission of pathogens to recipients is via adhesion to the ZP [50]. Nevertheless, it must be taken into account that adhesion characteristics are depending on pathogens as well as species.

In cattle, foot and mouth virus [38;39;45], blue-tongue virus [23;53], brucellosis [3;25;47;51], tuberculosis [11] and enzootic bovine leukosis [26] do not seem to represent any risk of transmission by ET. On the contrary, the risks associated with the bovine herpes virus-1 and non conventional agents such as prions are more questionable.

For Bovine herpes virus-1, the virus ability to attach the zona pellucida was highlighted for the first time after *in vitro* studies [46]. Indeed, BHV-1 was isolated in follicular fluid, follicular cells, oocytes and embryos after experimental infection of donor cows [31]. In that context, the related risk could be considered as high. However, field data on embryos exported in France over many years indicated that none of the recipients of embryos seroconverted even though some of the donors from which embryos had been collected were positive for antibodies [52]. Moreover, it has been shown that all viral particles are eliminated after the IETS washing procedures with trypsin [48]. As a consequence, if no transmission of pathogens to recipients is observed after transfer, the risk associated with transfer of embryos from infected donors can be considered to be negligible. These results have finally led to the following conclusion : **Ruminant ZP intact embryos do not appear to be a vehicle for the transmission of IBR.**

What about prions?

For scrapie, conflicting results have been obtained. The results of two studies are presented in table 1, indicating that transmission can occur after transfer of embryos collected from experimentally infected donor to free recipients, this in relation with the washing procedure of the embryos. Further studies are needed to investigate the effects of washing embryos and to be sure that ET could be used to control natural scrapie in sheep.

Table 1: Data on the risk of scrapie transmission via ET

Authors	Number of ewes	Time after infection	Clinical disease after collection	Number of washes	Number of positive lambs
Foster, 1992 [29]	6	6 mo	1-3 mo	0	6/20 *
Foote, 1993 [27]	74	3-21 mo	6-35 mo	3	0/67

*with Sip and PrP genotyped experimentally-infected sheep

Bovine Spongiform Encephalopathy (BSE)

Data on the risk of BSE transmission is being investigated in the UK, where 1000 embryos have been collected from confirmed BSE cows and some have been transferred into 347 heifers imported from New Zealand in 1991-1992. The results that have been partly published show that neither the embryos nor the flush fluids contained detectable BSE infectivity, assessed by injections in BSE-susceptible strains of mice [60;61]. In 1997, there was no evidence of BSE in the recipients and in their embryo transfer offspring, but the absence of transmission of BSE via ET has to be confirmed by their complementary results.

In goats, there is no evidence of transmission of the BSE agent to the offsprings via ET: Foster *et al*. (1999) have observed that *in vivo* embryos collected from donors which were experimentally infected with BSE and transferred into BSE-free recipients did not develop TSE-like-disease [28].

Although some exception could occur especially during fertilization, it is generally accepted that the only way of transmission is via adhesion of pathogens to the zona pellucida. Some problems have been observed with pathogens which express a high binding potential with ZP, such as *Heamophilus somnus* [54], ureaplasmas [24], mycoplasmas [9] or *Escherichia coli* [41]. Indeed, the 10 washes alone were ineffective for removal pathogens and even embryos could remained infected although addition of antibiotics to the washing media [41;43;54].

Nevertheless, washing of the embryos may been considered as the best method to eliminate different kinds of pathogenic or ubiquitous agents (slightly attached to the zona pellucida or present in the flushing fluids) and the fundamental rule expressed by the IETS a few years ago can be generally applied: If the ZP of the embryos is intact and if the embryos are processed according to the IETS recommendations, then ruminant and swine ZP-intact embryos **do not appear to be a vehicle for the transmission of most infectious agents.**

Prevention measures

The sanitary procedures associated with in-vivo production of embryos have been described in the manual of the IETS. Their recommendations about the selection of the donor female, the sanitary handling of embryos, the washing procedure of embryos and the general hygiene practices (laboratory sanitation, culture medium, sterilisation of material) should assure ET teams of the risk of disease transmission via embryo transfer [50].

In vitro produced embryos

The results obtained for *in vivo* produced embryos cannot be transposable to *in vitro* produced embryos. It is well recognise that IVF-generated embryos differ morphologically and physiologically from embryos fertilized *in vivo*. For example, IVF embryos are less compacted and may have a reduced number of cells. There may also be differences in the structure of the zona pellucida. These differences are also noticeable in terms of viability, freezability or speed of development that are significantly reduced for IVF embryos due to differences in ZP structure, interactions pathogen-ZP seem to occur in different conditions than those observed with *in vivo* produced embryos.

Risks of contamination of the oocytes

The origin of the oocytes is of considerable importance in the sanitary risks associated with production of *in vitro* embryos. The oocytes are collected either by Ovum Pick Up (OPU) in live donor females or by punction of slaughterhouse ovaries. In case of OPU, oocytes are collected from ovaries of identified donor females with a well defined health status and oocytes can easily be treated separately if necessary. On the contrary, ovaries from slaughterhouse are randomly collected, so that the donor females may present a potential risk of clinical or subclinical disease. Thereafter, ovaries are pooled for transportation to the IVF laboratory and consequently the pool of oocytes is undergoing IVM, IVF and IVC protocols.

The structural aspects of the bovine zona pellucida of IVF embryos and in vitro matured (IVM) oocytes have been recently studied by several authors with scanning electron microscopy and fluorescent microspheres. Vanroose *et al*. (2000) concluded that the intact bovine ZP of IVM oocytes and IVF embryos is constructed in such a way that BVDV and BHV-1 should not be able to traverse the ZP and reach the embryonic cells [58]. However, Bielanski and Surujballi (1998) have observed *Leptospira hardjo* in the pores, matrix and channels of ZP and in the embryonic cells, indicating the ability of these pathogenic agents to attach the ZP or penetrate into the embryos [19].

At collection, the oocytes used for IVF are surrounded by cumulus cells. As a consequence, contamination can directly become from the oocytes or be linked to the surrounding cells. The intrafollicular oocyte can incur either genomic or cellular contamination. Genomic contamination has never been highlighted [33] and intracellular contamination has been only reported for *porcine parvovirus* [2], *Campylobacter fetus* [16] or *Leptospira hardjo* [18;19]. Indeed, the role

of the surrounding cells is of great importance in the contamination process during IVF. Pathogens, such as BVDV or BHV-1 can contaminate follicular fluid, granulosa and cumulus cells and even cells from the ovarian stroma [22]. Thereafter, these pathogens can bind to the ZP and penetrate into the oocyte during fertilization. Most studies have reported lower fertilization and development rates [5;6;31;32;57], which may depend on the infectious dose [57] and the strains of the pathogens [56]. Nevertheless, the development of embryos is possible and could lead to disease transmission via ET.

Risks of disease transmission via contaminated IVF embryos

As well as for *in vivo* produced embryos, the risks of disease transmission via ET is conditioned by the possibility to remove the pathogens from the ZP before transfer. The washing procedure recommended by the IETS has been tested by many authors after experimental exposure of the oocytes to different pathogens, such as *bluetongue virus* [34], BHV-1 [5;15;31], BVDV [12;20;21;49;55], FMDV [37] and bacteria such as like *Actinomyces pyogenes bovis, E. coli* and *Streptococcus agalactiae* [17;42]. All those studies have shown that the washing procedure was ineffective for removing viruses from IVF embryos, even if a reduction of the infection rate has been observed after a standard trypsin treatment for BHV-1 [15] and BVDV [55]. Similarly, bacteria were not removed effectively by washing alone, but antibiotics had to be added to the *in vitro* culture media for removing or killing them.

The conclusions are different from the *in vivo* embryos. It is important to notice that all studies reported the effects of an artificial exposure to the pathogen, with high infectious doses and exposure time. Guérin *et al*. (1990) have observed that IVF embryos remained infected after washing above a threshold dose of viral exposure [31]. Moreover, conflicting results were obtained with oocytes originating from infected donors. Some studies confirmed the insufficient effects of washing on IVF embryos infected with BHV-1 [6], BVDV [62], whereas no evidence of pathogen after washing was observed in case of BVDV [7]. The discrepancies between these results raise doubts on the transposition of the *in vitro* contamination to natural infected animals and further studies are needed to clear the risks of disease transmission through transfer of in vitro embryos produced from natural contaminated donor females.

Prevention measures

Until it is not established that the oocytes that are infected are non-viable and that there is an effective disinfection method for IVF embryos, the only way to prevent the potential transmission of pathogens through embryo transfer is to control the health status of donor cows. As a consequence, the herd of origin of the donor female should be free of signs of contagious disease and should comply with the sanitary standards of the country. The donor female should be free of tuberculosis, leukosis and brucellosis, as well as BVD and IBR, in view of the potential for introduction of these viruses into the IVF system [40]. However, this measure is very difficult to apply in the case of slaughterhouse ovaries, because their sanitary status is generally no guarantied. Consequently, each IVF laboratory should process separately oocytes and embryos from slaughterhouse and from OPU.

For OPU oocytes, extended recommended procedures would be suitable : 1/ serological control of donor cows. 2/ control of biological products. 3/ quality control for Embryo Production Teams. 4/ Use of high microbiological quality semen. If the oocytes are collected from slaughterhouse ovaries, the risks are higher but quality controls on biological products and semen and on procedures with a quality control of Embryo Production Teams should be performed to guarantee such embryos. To be efficient control procedures must be precisely defined for each pathogen (or group of pathogens).

So far, washing embryos has been considered as the best way to eliminate the pathogenic agents from the embryos. In the case of IVF embryos, there is some evidence that the procedure may be insufficient to remove completely the pathogens from the ZP. However, the experimental conditions have generally tested severe contamination protocols and the washing procedure recommended by the IETS remains a good way to decrease or eliminate some pathogens, especially when associated with the following additional disinfection methods: 1/ trypsin treatment (0.25% for 90 s) for a more effective removal of pathogens such as BHV-1, without negative effects on the development rate of embryos [15], 2/ treatment with biochemicals and biological substances like hematoporphyrin derivative active on BHV-1 and

BVDV [8;10], 3/ antibiotic treatment for the removal of bacteria like *Actinomyces pyogenes bovis*, E. coli and *Streptococcus agalactiae* [42].

Sanitary risks associated with the environmental conditions

Numerous contaminations may result from serum or media used for the culture or the handling of embryos. Contaminations can also be added during the manipulation of embryos (collection of the oocytes or embryos, washing, culture or transfer)

Risks of environmental contamination

Somatic cells used for co-culture

For somatic cells, the preliminary results demonstrate that these tissues are sometimes contaminated by various pathogens. Therefore, additional risks may result in the use of co-culture cells obtained from ovaries or oviducts collected in slaughterhouse with an unknown health status. Oviductal cells from slaughterhouse material can be contaminated with BVDV or BHV-1 [14], so that the co-culture system may induce a contamination to initially healthy oocytes or embryos.

As a prevention measure, the preparation in a near future of fully synthetic media appears to be the most reliable and efficient measure to comply with sanitary security. When systems of co-culture are used, the best way to control sanitary risks is to use established cell lines, which can be tested before use for the presence of pathogens.

Fetal Calf Serum and products from animal origin

Various media containing products of animal origin are used for the collection, handling, washing, cryopreservation, in vitro culture of embryos and represent also a non negligible sanitary risk. Most particularly, fetal calf serum can be contaminated with viruses, such as BVDV [33;44]. Irradiation of commercial sera may limit those risks, but this procedure is not always applied. In the case of IVF systems, all biological products should be tested for the presence of BVDV and BHV-1 ans sera should be inactivated and tested again before use [40]. To conclude, application of the IETS recommendations reduces the sanitary risks related to products from animal origin, but the best way to avoid risks of contamination would be to replace sera by substitutes.

Bacterial contaminations

The presence of different bacteria strains usually considered as ubiquitous pathogens has been noted on some occasions (in particular, *Staphyloccus spp*., *Streptococcus spp*., *Pseudomonas spp*., *Bacillus spp*). These bacteria species are common contaminants of bovine semen or female genital tract (including vaginal mucosa and oviductal cells), but they are also present in the environment or even sometimes in the antibiotic powder usually added to the culture media. These bacteria may invade the IVF system including the oviductal cells used for co-culture of embryos, which leads to reduced fertilization rates and is usually followed by degeneration and high rates of embryonic death (Marquant-Le Guienne unpublished observations).

The hygienic conditions associated with the manipulation is also of great interest. Related to that particular point, a sanitary control of ET teams working on the field has been developped in France since 1986. This quality control is based on total bacterial count and identification of various pathogens including BVDV and BHV-1 from flushing and washing media and embryo samples. The preventive measures and special procedures defined in the IETS manual by Nibart *et al*. (1998) should be applied to *in vitro* production units to lower the sanitary risks associated with in vitro production [40].

Conclusion

It is well established that *in vivo* produced embryos are associated with low risk if handling is performed according to recommended IETS procedures. The risks associated with IVF embryos are more difficult to manage. On the one hand, the particular ability of pathogens to attach the zona pellucida of IVF embryos must be taken into account and extended recommended controls for donor cows should be systematically performed before OPU. On the other hand, sanitary controls are necessary on all the biological products used in media or culture system. In any case, it is important to use semen with a high microbiological quality. It remains some incertitude as regards

efficient disinfection methods for IVF embryos and prions. Especially, field studies are still necessary to confirm experimental studies.

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FACTORS INFLUENCING OOCYTE AND EMBRYO QUALITY IN CATTLE

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Introduction

While conditions of culture during the various steps of *in vitro* embryo production (IVP) can undoubtedly affect developmental rates, the relatively low level of efficiency achieved, manifested by the frequent failure of up to 60% of immature oocytes to reach the blastocyst stage, is almost certainly related to the intrinsic quality of the oocyte at the beginning of maturation.

In addition to the proportion of oocytes developing to the blastocyst stage, the quality of these embryos is important. Despite extensive research in terms of increasing the yield of blastocysts from immature oocytes, the quality of *in vitro*-produced embryos, in terms of survival following cryopreservation, has continually lagged behind that achieved with *in vivo*-derived embryos.

The aim of this paper is to briefly review the literature in relation to the factors controlling both blastocyst yield and blastocyst quality in cattle as well as describing some recent findings from our own laboratory.

Definitions of oocyte and embryo quality

It is important to distinguish between the terms "oocyte quality" and "embryo quality". The ultimate test of the quality of an oocyte is its ability to be fertilized and develop to the blastocyst stage, to establish a pregnancy and ultimately to produce a live calf. Similarly, the best measure of blastocyst quality is the ability to establish a pregnancy and produce a calf. Unfortunately, in most instances we do not have the luxury of transferring every blastocyst produced from IVP. Therefore, we have to settle for using parameters such as cleavage and blastocyst production as the best measures of oocyte quality. With regard to embryo quality, as pointed out by Bavister [7], the selection of the "best" blastocysts from a pool for transfer may not be indicative of the quality of that entire population. From a laboratory point of view, one useful measure of quality is the ability of the embryo to survive cryopreservation, as essentially all embryos from a given treatment can be tested.

Parameters used to assess quality - Importance of the kinetics of early cleavage divisions

We have previously demonstrated a clear relationship between the time of first cleavage post-insemination *in vitro* and developmental competence, with those oocytes cleaving earliest after IVF being more likely to reach the blastocyst stage than their later-cleaving counterparts [18, 49]. This phenomenon is common to many species [8, 54, 69, 71, 78, 84]. In addition we have demonstrated that this timing of first cleavage is related to the polyadenylation status of several developmentally important gene transcripts [12]. Subsequently, we demonstrated differences in gene expression in the early embryo that are reflective of differences in developmental competence between early- and late-cleaving zygotes [47]. The factors that control the time of first cleavage are unclear. Although culture conditions can influence the kinetics of early development [42, 62], it is likely that the main factors controlling this parameter are intrinsic to the oocyte [12, 47, 49], the sperm [15, 21, 83] or both. Indeed, in mice, a gene controlling the rate of preimplantation cleavage division and subsequent embryo survival (*Ped*: preimplantation embryo development) has been identified [84].

Differences between *in vivo*- and *in vitro*-produced embryos

Differences between *in vivo*- and *in vitro*-derived embryos have been reviewed by several authors [53, 87]. These differences include darker cytoplasm, lower density [64], more lipids, specifically, more triglycerides and less lipids from other classes [1], swollen blastomeres [81], a more fragile zona pellucida [19], differences in intercellular communication [10], and a higher incidence of chromosomal abnormalities [72, 82]. All of these factors may contribute to the higher sensitivity to cryoinjury exhibited by IVP embryos.

However, the causes behind these differences are unknown. It is unclear which parts of the process of embryo production are important in determining such parameters as blastocyst yield and blastocyst quality.

Oocyte maturation *in vivo* vs *in vitro*

There is evidence in the literature to suggest that oocytes matured *in vivo* are more developmentally competent than those matured *in vitro*. The oocyte undergoes significant modulations in the dominant follicle that play a key role in the acquisition of developmental competence. A number of ultrastructural and molecular changes occurring during oocyte development have been linked to developmental competence [5, 31]. Also, *in vitro* maturation has been associated with certain abnormalities in the oocyte [32-34].

Assey *et al*. [4] reported that bovine oocytes aspirated from dominant follicles before the LH surge display alterations in their nuclear and cytoplasmic morphology, which, according to the authors, are a prerequisite for the acquisition of full developmental competence. This would indicate that not only final oocyte maturation (i.e., the processes occurring between LH surge and ovulation) is significant, but also the period preceding the LH surge may be important for the establishment of developmental competence.

We carried out an experiment to evaluate the importance of the events surrounding oocyte maturation [68]. Four groups of oocytes were used: (1) immature oocytes from 2-6 mm follicles from slaughterhouse ovaries, n = 389, (2) immature oocytes from >6 mm follicles from slaughterhouse ovaries, n = 99, (3) immature oocytes recovered *in vivo* by ovum pick up (OPU) just before the LH surge, n = 102, and (4) *in vivo* matured oocytes recovered by OPU, n = 134. Following recovery, Groups 1-3 were submitted to IVM for 24 h, while Group 4 oocytes were immediately inseminated. The experimental design was such that oocytes from all 4 groups were inseminated at the same time.

There was no difference in oocyte cleavage rate following IVF; however, significantly more blastocysts developed from oocytes matured *in vivo* (58.2%) than those recovered just before the LH surge (39.2%) or those from 2-6 mm follicles (38.9%). Oocytes from large follicles (>6 mm) resulted in an intermediate blastocyst yield (46.5%). In terms of blastocyst quality (Figure 1), survival following vitrification was relatively low in all groups ranging from <40% at 24 h post warming to <20% in all groups by 72 h post-warming.

These results clearly demonstrate that oocytes matured *in vivo* are more competent than those matured *in vitro*. This in agreement with several previous studies [11, 26, 45, 52, 80]. The data also support the notion that oocytes derived from large follicles are more competent than those derived from small follicles following IVP [50, 61]. In addition, the data demonstrate that blastocyst quality is unrelated to source of oocyte.

Differences have been reported between *in vivo*- and *in vitro*-matured oocytes which may explain the observed differences in developmental competence. Cumulus expansion is usually more extensive following maturation *in vivo* [75]. In addition, there is a high degree of homogeneity amongst oocytes matured *in vivo* at the ultrastructural level; this contrasts with the ultrastructural heterogeneity exhibited by *in vitro* matured oocytes, even when a uniform population of the latter is selected prior to *in vitro* maturation [17].

Oocyte fertilization *in vivo* vs *in vitro*

There are few reports in the literature comparing fertilization *in vivo* versus *in vitro*. In an experiment designed to assess the importance of fertilization *in vitro* or *in vivo* on subsequent blastocyst development and quality, *in vivo* matured oocytes were either (1) recovered by OPU just prior to ovulation and fertilized *in vitro* (n = 134) or (2) fertilized *in vivo* by artificial insemination and the resulting presumptive zygotes surgically recovered on Day 1 (n = 69). Both groups were then cultured *in vitro* in parallel. As a control, a group of oocytes (n = 194) recovered from 2-6 mm follicles from the ovaries of slaughtered heifers were put through IVM/IVF/IVC at the same time.

There was no difference in cleavage rate when *in vivo* matured oocytes were fertilized *in vivo* (92.8%) or *in vitro* (87.3%). *In vivo* fertilized oocytes had a significantly higher blastocyst yield ($P<0.01$) than both *in vitro* fertilized groups (73.9% vs 58.2% and 39.2%, for *in vivo* fertilized, *in vivo* matured/*in vitro* fertilized and *in vitro* matured/*in vitro* fertilized oocytes, respectively). In addition, *in vivo* matured/*in vitro* fertilized oocytes yielded significantly more blastocysts ($P<0.001$) than *in vitro* matured oocytes. In terms of blastocyst quality, survival and hatching rate following vitrification was not different between the groups, with survival ranging from <40% at 24 h to <20% at 72 h post warming, suggesting that site of fertilization is not a determining factor of blastocyst quality.

The results of this experiment in which a higher proportion of *in vivo* matured oocytes developed to blastocysts following fertilization *in vivo* compared with fertilization *in vitro* suggests that the events around the time of fertilization might be important in determining the developmental competence of the oocyte. However, whether or not fertilization *in vivo* per se was solely responsible for the observed increase in blastocyst yield is questionable. It should be noted that the *in vivo* fertilized oocytes were ovulated oocytes; this is in contrast to the *in vivo* matured/*in vitro* fertilized group, in which oocytes were recovered from preovulatory follicles just prior to the expected time of ovulation. In unstimulated cattle, ovulation occurs approximately 24 h after the LH peak, while following superovulation ovulations occur from 24 – 33 h after the peak [13]. This would suggest that a proportion of the oocytes removed from preovulatory follicles may not have completed maturation and this may have contributed to a lower blastocyst yield.

To address this question we attempted the fertilization of *in vitro* matured bovine oocytes in the sheep oviduct using GIFT, involving the transfer of matured oocytes and sperm to the oviduct simultaneously, or the transfer of matured oocytes to the oviduct of a ewe previously inseminated with bovine sperm (results not shown). Irrespective of the method used only a very low proportion of oocytes were fertilized and none developed to blastocysts. Other authors have similarly attempted the *in vivo* fertilization of *in vitro* matured bovine oocytes in the inseminated rabbit [30, 73, 79], sheep [73] and cow oviduct [58, 60, 79] with limited success, although Newcomb *et al*. [60] did report the birth of twin calves following one such attempt. It would seem that such an approach is fraught with technical difficulties which only cloud the issue.

Embryo culture *in vivo* vs *in vitro*

In vivo, the oviduct is the site of fertilization and early embryo development. The oviductal environment can support embryonic growth up to the blastocyst stage across a wide range of species following trans-species transfer. Ligated rabbit oviducts have been used extensively for the development of embryos from many species including sheep [6, 43], cattle [9, 20, 74], pigs [63], and horses [3]. Sheep oviducts have been shown to support the growth of cow [22, 23, 25, 27] and pig [65] embryos. The mouse oviduct has also been used to support the development of zygotes from the cow [40, 70] and hamster [56].

We carried out two experiments to assess the impact of the culture *in vitro* or *in vivo* on the yield and quality of blastocysts. In the first, presumptive zygotes produced by IVM/IVF were cultured either *in vitro* in synthetic oviduct fluid (n = 463), or *in vivo* in the ewe oviduct (n = 775). The cleavage rate of *in vitro* cultured zygotes was 82.5%; a figure for the cleavage rate of *in vivo* cultured zygotes was not obtainable due to the degeneration of non-developing embryos in the oviduct. However, there was no difference in the proportion of oocytes developing to the blastocyst stage between the two culture systems (34.1 vs 34.5%). In contrast, there was a marked

difference in the quality of the blastocysts produced in the two culture systems; following vitrification and warming, significantly more blastocysts ($P<0.001$) from the ewe oviduct survived at all time points and hatched than their *in vitro* counterparts (88.0 vs 5.6% survival, respectively at 72 h). This would suggest that the culture system is critical in determining blastocyst quality.

The observation that blastocyst yield from IVM/IVF oocytes was unaffected irrespective of whether culture took place *in vitro* or *in vivo* in the ewe oviduct is consistent with a previous report from our group [22]. Jimenez *et al*. [35] observed that culture in the ligated sheep oviduct resulted in similar blastocyst yields to culture *in vitro* (26 vs 27%). However, if ligation was not carried out and the embryos were allowed to go into the uterus, development was significantly reduced [35]. This observation is diffcult to reconcile with the results of Rexroad and Powell [67] and Talbot *et al*. [76] who demonstrated that the uterus of the ewe supports normal development of bovine blastocysts after transfer at Day 7 and recovery at Day 14, demonstrating that bovine embryos can undergo continued development in the reproductive tract of ewes when transferred either as 4-cell embryos or as expanded or hatched blastocysts.

In the second experiment, *in vivo* matured/*in vivo* fertilized zygotes were either surgically recovered on Day 1 and cultured *in vitro* in synthetic oviduct fluid (n = 69), or remained *in vivo* and were non-surgically recovered on Day 7 (n = 70). As a control, a group of zygotes (n = 388) produced by IVM/IVF were cultured *in vitro* in parallel. The cleavage rate and blastocyst yield of *in vivo* produced zygotes was unaffected by the site of culture (*in vitro* vs cow oviduct). In addition, both *in vivo* groups resulted in significantly higher cleavage ($P<0.05$) and blastocyst yield ($P<0.001$) at all time points than the *in vitro* control. In terms of blastocyst quality, as in the first experiment, it was clear that culture system had a dramatic effect on survival following vitrification; *in vitro* culture, irrespective of origin of the zygote, resulted in significantly lower survival and hatching ($P<0.001$) than culture in vivo (69.6% vs 0% and 1.8% survival at 72 h, for *in vivo* matured/fertilized/cultured, *in vivo* matured/fertilized/*in vitro* culture and *in vitro* matured/fertilized/cultured embryos, respectively).

While culture of IVM/IVF zygotes in the ewe oviduct did not affect blastocyst yield, the oviduct environment of the intermediate recipient clearly improved the overall quality of IVM/IVF blastocysts, as measured by survival following cryopreservation. Similarly, the *in vitro* culture of zygotes derived from oocytes of high developmental potential (*in vivo* matured/fertilized) is sufficient to result in blastocysts of low cryotolerance, similar to those resulting from IVM/IVF/IVC. Consistent with these and previous results from our group [22], Pugh *et al*. [66] observed that culture of bovine embryos in the sheep oviduct improved the frozen but not the fresh embryo survival following transfer. Tervit *et al*. [77] found that, while culture in the oviduct did not affect the proportion of sheep embryos judged to be of freezeable quality, the percentage of embryos surviving post thaw was higher for IVM/IVF embryos after culture in the oviduct than culture in SOF. Holm *et al*. [29] demonstrated that IVC of IVM/IVF derived ovine zygotes reduced embryo viability by 15 to 25% compared with *in vivo* culture. Galli and Lazzari [25] reported that there were no differences between culture in the ewe oviduct or culture *in vitro* in terms of blastocyst formation at Day 8. However, in agreement with our results, they observed major differences in quality with embryos cultured in the ewe oviduct and those collected from superovulated donors being superior in terms of sensitivity to freezing/thawing.

The combined results of these two culture experiments provides further evidence that the intrinsic quality of the oocyte determines the blastocyst yield, where *in vitro* matured/fertilized oocytes resulted in a blastocyst yield of approximately 35% irrespective of whether they were cultured *in vitro* or *in vivo*, while >70% of *in vivo* matured/fertilized oocytes reached that stage following culture either *in vitro* or *in vivo*. These results clearly demonstrate that the proportion of oocytes developing to the blastocyst stage is not determined by the culture system, but rather by the origin of the oocytes. In addition, it is clear that oocytes from a common source will result in similar blastocyst development even when culture takes place in different environments.

What controls the development of a blastocyst of high quality?

Differences have been described at the ultrastructural level which may in part explain the variation in cryotolerance observed amongst groups of embryos. In a recent study from our group [24] it was demonstrated that blastocysts derived from the *in vivo* culture in the ewe oviduct of IVM/IVF zygotes displayed only minor morphological differences from those produced completely *in vivo* by superovulation. In contrast, *in vitro* produced blastocysts exhibited a range

of characteristics associated with reduced cryotolerance such as vacuoles in the trophoblast cells, a sparse population of microvilli, a greatly reduced network of intercellular connections and a large increase in lipid content. Similar observations have been reported by other authors [2, 16].

Differences in gene expression exist between *in vitro*- and *in vivo*-derived embryos which may explain the differences in quality observed. Wrenzycki *et al*. [86] demonstrated that expression of the connexin 43 gene at the blastocyst stage differs between bovine embryos produced *in vitro* and those produced *in vivo*. This gene is involved in the formation of a protein that gives rise to gap junctions between cells. Poor gap junction formation is associated with poor cell compaction and is a common occurrence in IVP embryos. Also, accelerated development *in vitro* due to serum addition [14, 51] may affect gene regulation and transcription, resulting in well-documented developmental abnormalities such as foetal oversize in the bovine [88].

It has also been demonstrated that the gene expression in the developing embryo can be influenced by the culture environment *in vitro* [36, 44, 59, 85]. Knijn *et al*. [38] compared gene expression in blastocysts derived from *in vivo*- or *in vitro* matured bovine oocytes. No differences were observed in relative abundance for four genes studied, suggesting that maturation is not the major step in the IVP process affecting expression of these genes in the embryo. This would be consistent with the findings of Wrenzycki *et al*. [85] and those of the present study, reinforcing the point that the culture system is the major determinant of blastocyst quality, irrespective of the origin of the oocyte.

The culture environment can also have a significant effect on embryo metabolism which may have implications for embryo quality. Embryos generated in a completely defined medium have lower rates of glycolysis than those in serum [39]. Khurana and Niemann [37] examined energy metabolism in *in vivo*- and *in vitro*-derived bovine embryos. In general, the pattern was similar; however, IVP embryos exhibited a 2-fold higher rate of anaerobic glycolysis and produced more lactate. Culture for 72 h of *in vivo*-produced blastocysts resulted in lactate production similar to that of *in vitro* produced blastocysts.

Conclusion

In conclusion, events before ovulation determine the ultimate fate of the oocyte but events occurring between the zygote and blastocyst stages determine the blastocyst quality. As pointed out by Hendriksen *et al.* [28], the observation that oocytes with identical developmental conditions up to the LH surge (ie initiation of meiotic resumption) differ in their ability to reach the blastocyst stage depending on whether they undergo maturation *in vivo* or *in vitro* highlights the fact that current *in vitro* maturation methods can still be improved. One route towards improving embryo yield may be the prematuration of the oocyte prior to maturation. While some recent results are encouraging in that oocytes can be reversibly maintained at the germinal vesicle stage without affecting subsequent blastocyst yield [41, 46, 48, 55, 57], it has not yet been demonstrated that such an approach can improve the developmental ability of an oocyte. In addition, there is little if any evidence to demonstrate that such blocked oocytes can result in the birth of normal offspring. A more feasible approach in the short term at least will probably be to modify the conditions of *in vitro* culture to ensure that those blastocysts that do develop are of the highest possible quality.

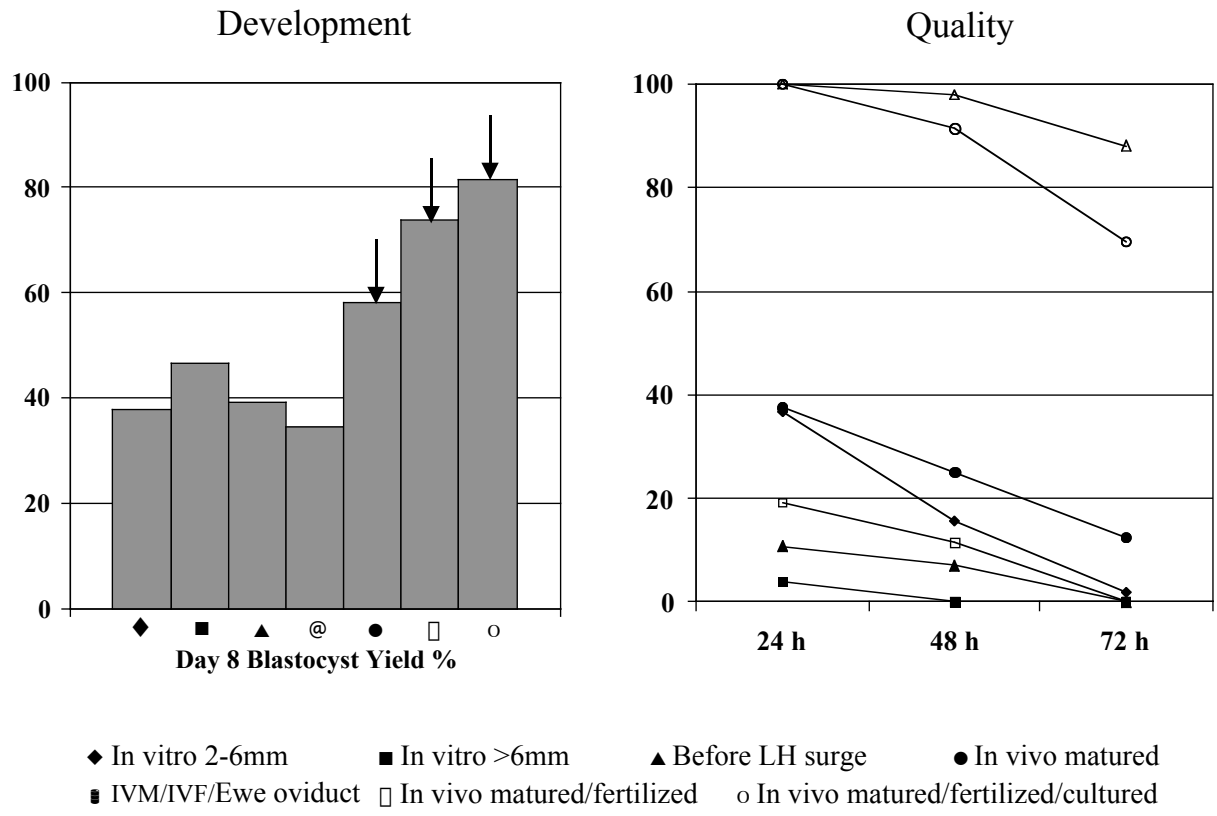

Figure 1. Summary graph of all the data from the three experiments relating to oocyte quality measured in terms of blastocyst development, and blastocyst quality measured in terms of survival following vitrification. Blastocysts were derived from oocytes (1) from slaughterhouse ovaries from 2-6 mm or (2) >6 mm follicles, (3) recovered by ovum pick up just prior to the expected time of the LH surge, or (4) following maturation *in vivo*, (5) matured and fertilized *in vitro* and cultured in the ewe oviduct, (6) matured/fertilized *in vivo* and cultured *in vitro*, or (7) matured/fertilized/cultured *in vivo*. Arrows indicate that the further along the developmental axis the oocyte/embryo is removed from the *in vivo* environment the greater the blastocyst development. In addition, embryo culture *in vivo*, irrespective of the origin of the oocyte, results in blastocysts of superior quality to culture *in vitro*.

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RENDEZ-VOUS IN THE OVIDUCT:
Implications for superovulation and embryo transfer

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1. Introduction

The meeting between the male and the female gamete, ultimately leading to fertilization, involves a number of finely tuned processes that in principle are liable to be adversely affected by the morphological and endocrine changes induced by the superovulatory treatment. In the following review, aspects of male and female events leading to fertilization will be discussed with special emphasis of aspects related to embryo transfer in cattle. It is by no means intended to comprise a complete review of the processes leading to fertilization, but merely highlighting the steps where the events may be adversely affected in the superovulated animal.

2. Preparation

2.1 The oocyte

Following the preovulatory LH-surge the oocyte and its surrounding cumulus cells, designated the cumulus oocyte complex (COC), undergoes a number of sequential changes which are essential for the oocyte to obtain fertilizing capability. The oocyte maturation has been studied in great detail in cattle by Kruip *et al.* (1983), Callesen *et al*. (1986) and Hyttel *et al*. (1986, 1989). The changes include disruption of the junctions between the cumulus cell projections and the oocyte as well as breakdown of the envelope of the oocyte nucleus (ONBD) within approximately 12 h after the LH-peak (maximum value of LH in plasma during the LH-surge). Around 15 h after the LH-peak, the metaphase of the first meiotic division (MI) and rearrangement of mitochondria and vesicles are seen. Around 19 h after the LH-peak the first polar body is abstricted and the second metaphase (MII) appears. Around 22 h after the LH-peak, the cortical granules migrate from the centre of the ooplasma to solitary positions just under the oolemma, the Golgi compartment decreases and the smooth endoplasmic reticulum transforms. The COC leaves the follicle around 24 h in unstimulated and from 24-33 h after the LH-peak in superovulated cattle.

It has however become increasingly clear that the normal process of oocyte maturation, both the nuclear and the cytoplasmic events described above, may be perturbed in animals treated with gonadotrophins. This includes premature maturation of the oocytes induced by the LH-contamination of the exogenous gonadotrophins (Callesen *et al*., 1986; Callesen, 1995; Goff *et al*., 1986) and/or deviant peripheral endocrine profiles (premature or absent LH-surge; high progesterone concentrations at the time of heat) leading to deviant follicular steroidogenesis and abnormal oocyte maturation ultimately resulting in decreased capacity for the oocyte to undergo normal fertilization and embryonic development (Callesen *et al*., 1986; Hyttel *et al*., 1986). Thus, a proportion of oocytes from superovulated animals will have an inherent abnormal structure that renders them inferior for subsequent normal embryonic development. The deviant follicular steroidogenic pattern, which includes abnormalities in the ratio between estradiol-17 and progesterone, may influence not only oocyte maturation but also the transport of oocyte and spermatozoa in the female genitalia, an aspect that will be addressed in a later section.

Besides the oocyte maturation, the cumulus investmen t undergoes certain essential changes including expansion and mucification. These events are important at least for the separation of the COC from the follicular wall and thus for assuring the ovulation process per se (Hunter, 1988).

Furthermore, the COC becomes extremely sticky (Greve *et al*. 1984) and one may speculate whether this feature is important for the subsequent pick up by the ostium of the tuba uterina. Certainly, the mucification of *in vivo* produced cattle COC´s is much more pronounced than in their *in vitro* produced counterparts (Greve, personal observation). Besides endocrine (steroids, prostaglandins) factors, autonomic nervous regulation may be involved in the process of ovulation by inducing contraction of the follicular wall prior to the collapse of the preovulatory follicle (O`Shea and Phillips, 1974). Other studies have however shown that denervation of the ovary had no adverse effect on the ovulatory process so the effect of the nervous system is still not clear (Wylie *et al*., 1985). The duration of the ovulatory process is estimated to be approximately 6 min in the mare (Townson and Ginther, 1987), 1.8 h in pigs (Soede *et al*. 1992) and 4-12 h in the superovulated cow (Callesen *et al*., 1986; Callesen, 1995). Needless to say that the variation in the time of ovulation hardly can affect the development stages of cattle embryos recovered on day 7 (Callesen *et al*., 1995).

The approximate time interval from the occurrence of the pre-ovulatory LH-peak to ovulation(s) varies between animal species. For cattle it is between 24-33 h (Callesen *et al*., 1986; Hunter, 1988), pig 40-42 h (Hunter, 1988) and for horses around 36 h (Grøndahl *et al*. 1993). This time interval is obviously important for planning the proper breeding or insemination time both in cases of spontaneous occurrence of the LH-surge and in cases where ovulation is induced by exogenous hormones.

At the time of ovulation the ciliated epithelium of the fimbriated infundibulum sweeps the surface of the ovary to pick up the COC´s (Flechon and Hunter, 1980). Whether the cumulus cells are essential for this process is still under discussion (Hunter, 1988) but observations from our laboratory have shown that at least in cattle, the oocytes are void of cumulus cells soon after ovulation. Following the entry into the ostium, the oocyte is transported within minutes to the site of fertilization that is known to be the ampullar-isthmic region of the oviduct (Hunter, 1988). This transport is facilitated by beat of the cilia and smooth muscle contractions, both of which are very active around the time of ovulation. During this passage and until the time of fertilization, the oocyte is exposed to an environment which is substantially different from the follicle and this is likely to induce certain metabolic and structural changes (Hunter, 1988). The transport to the ampullar-isthmic region is regulated by estradiol-17 and progesterone, and abnormalities in the production of these hormones - or rather their ratio which may be seen in the superovulated animal (e.g. Callesen *et al,* 1986, Goff *et al.,* 1986) - can certainly interfere with the normal oocyte transport to the site and thus the process of fertilization and the subsequent embryonic development.

A proportion of the oocytes reaching the site of fertilization in the ampullar-isthmic region is ready to be fertilized and it is important that the Rendez-Vous between the oocyte and the spermatozoa takes place within a certain time interval. As reviewed by Hunter (1988) the viable life span of the oocytes in the oviduct is 10-12 h in cattle and sheep, 8-12 h in the pig and about 8-10 h in horses. For the bitch it may be as long as 24-48 h (Bysted, 2001). Furthermore the age of the oocyte is inversely related to the estimated fertilization rate (Hunter and Greve, 1997). One could however argue that not all oocytes or eggs have the same viability, as a certain proportion will not be fertilized even when exposed to spermatozoa capable of fertilizing an oocyte or undergo either embryonic or foetal death following fertilization. Extensive use of the in vitro fertilization technique has clearly substantiated that not all oocytes of even similar appearance have the same developmental potentials. It is still unknown, however, which factor or factors contribute to the inherent quality, but the so-called "prematuration" of the oocytes seems to be important (Hyttel *et al*., 1997).

2.2. The spermatozoa

Following ejaculation where semen is deposited either in the anterior vagina (e.g. cattle, sheep) or in the cervix and/or uterus (pig and horse), the spermatozoa commence a long journey towards the site of fertilization. There are numerous reviews and articles on sperm transport in the female genital tract (e.g. Hunter *et al*. 1980, 1982; Hunter, 1988; Hawk, 1987) and the main objective with the transport is obviously to establish a population of fertilizable or viable spermatozoa near the oocyte prior to the time of ovulation. This requires establishment of a functional sperm reservoir and in the following this aspect shall be devoted most attention.

After deposition in the vagina, a large proportion of the spermatozoa gain access to the cervical canal by their own motility and quite a large number migrates into the cervical crypts where they may remain motile for as long as 3.5 days (Hunter and Nichol, 1983; Hunter, 1988; Scott, 2000). These spermatozoa form some kind of a reservoir but it is questionable whether they are able to be released from the crypts and contribute to the fertilizing pool of spermatozoa as they are intimately bound to the crypt cells by the nostril part of their head (Hunter, personal communication 1998) and backwards swimming movements will be required to let these spermatozoa return to the cervical canal.

It is most likely that the spermatozoa which pass through the mucus in the cervical canal are those that will establish the reservoir in the oviduct. The sperm transport through the uterus probably occurs as a combination of sperm motility and myometrial contractions (Hunter, 1988). In cattle the uterine tone is greatly increased during estrous whereas this is not the case in for example the mare, so different mechanisms may exist. The period in the uterus is probably important for removal of certain components from the ejaculate and may thus contribute to the capacitation process (Hunter, 1988). It is worth noting that a population of living spermatozoa may be present in the uterus of the bitch for as long as 11 days (Doak *et al*., 1967) whereas as previously mentioned this period in cattle and pigs is probably in the magnitude of hours.

The first spermatozoa reach the oviduct within minutes, but most studies have clearly shown that it takes from 6-8 h to establish a functional spermatozoa reservoir in the oviduct (Hunter *et al*., 1980; Hunter and Wilmut, 1983; Hunter, 1988). This reservoir is established in the utero-tubal junction (UTJ) and the lower part of isthmus of the oviduct (Hunter, 1998; Suarez, 1998; Scott, 2000). This is true for sheep (Hunter *et al*., 1982), pigs (Hunter, 1984) and cattle (Hunter and Wilmut, 1983) and the spermatozoa remain in this region until close to the time of ovulation. During their storage in the lower part of the isthmus and UTJ the spermatozoa bind intimately to the epithelium as it can be visualized by electronmicroscopy (Lefebvre *et al*., 1997). The binding of at least un-capacitated bull spermatozoa to the oviduct epithelium seems to involve some kind of recognition of specific carbohydrates, lectins, on the sperm surface and oligo-saccharides on the epithelial surface (Lefebvre *et al*., 1997; Suarez, 2000). The very narrow lumen of the isthmic part may in fact facilitate this binding by reducing the speed of the movement of the individual spermatozoa (Suarez, 2000). During the course of capacitation the previously mentioned lectins may be lost on the surface enabling the sperm to leave the epithelium and move towards the site of fertilization (Suarez, 2000). It is obviously important that the spermatozoa remain uncapacitated in order for them not to become hyperactivated and move towards the oocyte too early. In horses it has been shown that maintenance of a low intracellular Ca^{2++} concentration is important in this context (Dobrinski *et al*., 1997) but other microenvironmental factors may also be important (Hunter, 1998; Hunter *et al*., 1998). The maintenance of a lower Ca^{2++} concentration is apparently achieved by the close contact between the sperm head and the epithelial surface (Dobrinski *et al*., 1997).

The fertilizing ability of the spermatozoa is obtained after some period of contact to the epithelium and it is anticipated that the final part of the capacitation takes place during this period (Scott, 2000). A completed capacitation is a prerequisite for leaving the oviduct epithelium (Lefebvre and Suarez 1996), for penetration of the COC and for undergoing acrosome reaction (for review, see Hunter, 1988). One of the very important functional changes which occurs in the spermatozoa is the so-called hyperactivation, where the sperm movements change from slow progressive to vigorous flagellar movements (Suarez *et al*., 1991; Nichol *et al*., 1997; review: Scott, 2000; personal observation 1987). It is beyond any question that the local oviduct-ovarian endocrine environment has a decisive influence on the release and thus the final transport of the spermatozoa from the isthmic reservoir to the site of fertilization in the ampulla (Hunter, 1988; Hunter *et al*., 1998, 1999). It was thought that the follicular fluid could enter the oviduct and thus directly affect the flux of spermatozoa but there is now convincing evidence that the effect of the follicular fluid progesterone is indirect by inducing changes in the gradients of the Ca^{2++} which will trigger the release of the spermatozoa**.** Thus, the local endocrine environment probably acts through the calcium route. It goes without saying that disturbances in the follicular production of steroid hormones (e.g. progesterone and 17-estradiol) as seen in association with superovulation (e.g. Callesen *et al*. 1986, 1987) might have an adverse effect not only on the oocyte maturation as mentioned previously but also on the sperm transport by either enhancing or delaying the release and transport of the spermatozoa towards the site of fertilization. This may ultimately

lead to formation of inferior (aged) zygotes or lack of fertilization, both of which are more frequently observed in the superovulated than unstimulated animal.

3. The meeting

The changes in the follicular fluid steroids (mainly progesterone and estrogens) which occur towards the impending ovulation is the starting signal for the spermatozoa to make the final journey towards the oocyte which is located in the distal part of the ampulla where the meeting, the process of fertilization, will take place. It is quite appropriate that fertilization has been denominated a process as it involves several well-defined steps. The timing of sperm penetration and pronucleus formation has been examined in pigs by means of light microscopical observations (Hunter, 1972; Baker and Polge, 1976) and detailed ultrastructural studies of the in vivo fertilization process have been presented by Crozet (1984) and Hyttel *et al.,* (1988a, 1989). Valuable information on this process has also been obtained by studies of the *in vitro* fertilization process (Hyttel *et al*. 1988b, c). The cumulus cells are shed very soon after ovulation (<10 h, Crozet, 1984), so the spermatozoa can make direct contact to the zona pellucida where the acrosome reaction occurs (Talbot, 1985; Hunter, 1988; Töpfer-Petersen, *et al*., 1988; Hyttel *et al*., 1989). The acrosome reaction is a vesiculation that originates from fusion of the plasma membrane and the outer acrosomal membrane and results in formation of vesicles. In the scanning electron microscope this reaction appears as a fenestration of the membrane coats of the sperm head. Upon penetration of the zona pellucida, the spermatozoa's equatorial segment first touches and is then gradually internalised into the ooplasma. At the same time the release of the cortical granules takes place and this process will prevent entrance of more spermatozoa into the ooplasma.

The subsequent nuclear changes will lead to the formation of the paternal and maternal pronuclei and following an apposition period, these structures will fuse at around 19-20 h following insemination in cattle and immediately thereafter the first cleavage division takes place (Hyttel *et al.* 1988a). For details on the similar processes in pigs, see Hunter (1972). The fertilization process does not always occur in this normal fashion. One of the conditions particularly seen in pigs, but also in cattle, is polyspermic penetration (polyspermi). At least in cattle this condition can be directly related to deviant oocyte maturation involving abnormal distribution and release of the cortical granules as a consequence of treatment with exogenous gonadotrophins (Hyttel *et al*. 1989). Oocytes matured in vitro experience this condition even more frequently (Hyttel *et al.* 1988c). The biochemistry of fertilization will not be discussed in this paper, but one substance that allegedly facilitates or promotes the events leading to fertilization is the oviductal glycoproteins (Verhage *et al.* 1998). They may increase capacitation and the subsequent fertilization rates in cattle (King *et al*. 1994) and if purified and synthetically produced addition to semen might be used to improve embryonic developmental rates *in vitro*. Other studies have shown that certain proteins exist in the seminal plasma of bulls with high fertility (26 kDa and 55 kDa proteins, Killian *et al*. 1993).

The number of spermatozoa in the zona pellucida, denominated accessory or supernummary spermatozoa (AS), varies with species. In pigs it reaches an average number of 106 at 6 h after insemination (Hunter, 1972). In cattle the number of AS depends on a number of factors as reviewed by Saacke *et al.* (1994, 1998a, b and 2000), namely breeding method (more AS following natural service), timing of breeding (more AS following 24 h versus 12 h and 0 h breeding after first mount), embryos recovered from single versus multiple ovulating animals (more AS in single ovulating eggs) and finally the embryo quality (more AS in excellent/good embryos versus degenerate/unfertilized (Saacke *et al*. 2000)). In ewes it has also been observed that superovulation will inhibit sperm transport (Hawk *et al*. 1987) leading to fewer spermatozoa in all parts of the female genital tract and a lower number of AS in the zona pellucida. Again, natural breeding gave a significantly higher number of AS than artificial breeding (single ovulating 127 versus 23 or 17 per embryo)

The impairment of sperm transport and reduction in fertilization rates in the superovulated animal is a commonly accepted condition and it may be caused by the adverse endocrine environment induced by the peripheral and the follicular endocrine deviations, which by and large is a significant increase in the estrogen levels (Greve *et al*., 1989). This may affect not only sperm storage, release and transport but it may also create microenvironment which is incompatible not only with gamete transport but with proper fertilization and early development

which is dependant on a very delicate balance in the oviductal secretions of small peptides (Hunter, 1994; Hansen, 2000). The reduction in the number of AS may be explained by the subtle changes in the oviductal motility induced by the superovulatory treatment. It is however more difficult to give an explanation for the direct relationship between embryo quality and AS. Why are there more AS in good embryos? Because the oocytes were of higher quality, and as the oocytes compete for more fertilizing spermatozoa, then the better wins more? It is evident though that the number of AS in embryos of good quality should be around 10-20 per embryo. It is finally interesting to note that abnormal spermatozoa do not gain access the zona and thus do not constitute part of the AS population (Saacke *et al*., 1998b).

4. Deep uterine insemination

With the emergence of sex selected semen it will be necessary to use lower doses of semen (Seidel *et al*., 1997) because the sorting process is still rather slow. Deep intrauterine insemination may thus be an alternative to the standard cervical or uterine body deposition (Hunter and Greve, 1998). The advantage of this method would be an increase of a fertilizing population of spermatozoa close to the functional isthmic sperm reservoir resulting in a rise in non-return rates by elevating the subsequent fertilization rate. Even when not using sexed semen this aspect would be economically important. However, potential risks might be perforation of the uterine wall if the inseminator is not properly trained as well as an increase in polyspermic fertilization.

In a recent experiment conducted in our laboratory (Dahlgaard, 2001), we tried to use deep intrauterine insemination with reduced sperm concentration in both single ovulating and superovulated animals. The results were disappointing, as we did not obtain a satisfactory number of embryos in spite of a reasonable ovulation rate. Could this be due to an inflammatory reaction induced in the endometrium by the semen or the extender leading to impaired and even complete lack of embryonic development, maybe even total destruction?

5. Final comments: superovulation

From this review it has hopefully become clear that the meeting between and the final fusion of the gametes in the Fallopian tube (uterine tube or the oviduct) is a long and meticulously well tuned process which may be perturbed in several ways as a result of a superovulatory treatment. The abnormalities induced will include deviant oocyte maturation as well as abnormal peripheral and follicular endocrine events. Deviant oocyte maturation will eventually lead to formation of a gamete with reduced developmental potentials and a hostile endocrine environment. This will predispose for impaired storage and transport of the spermatozoa in all parts of the female genital tract yielding fewer or no available sperm in the vicinity of the oocyte at the time when fertilization should have taken place. The abnormal activity of the oviduct induced by the adverse endocrine conditions may also have the consequence that the transport of the zygotes and embryos are hastened leading to premature entry into the uterus. This asynchrony may well be incompatible with normal subsequent embryonic development.

Thus, there are ample risks for the whole process to run of the track, but despite all odds: superovulation and embryo recovery is a successful technique in most European countries as evidenced by the AETE statistics.

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SHORT COMMUNICATIONS

SUCCESSFUL BOVINE SOMATIC CELL NUCLEAR TRANSFER BY A SIMPLE ZONA-FREE MANIPULATION TECHNIQUE

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Contemporary nuclear transfer (NT) techniques require skilled personnel and extended periods of micromanipulation. Here we present details of the development and application of a bovine NT technique for somatic cells that is simpler and faster than traditional methods. This comprises the bisection of zona-free oocytes and the reconstruction of embryos comprising two half cytoplasts and a somatic cell using phytohaemagglutinin (PHA), followed by culture in microwells (termed WOWs; Vajta *et al.*, 2000, Mol. Reprod. Dev., 55, 256-264) to prevent embryonic disaggregation. The system was initially developed, as described here, by a) selecting the optimal primary activation agent that induced the lowest lysis rate but highest blastocyst yield of parthenogenetically activated oocytes, b) evaluating the quantity and quality of zona-free blastocysts cultured in WOWs from the zygotic stage, and c) establishing any potential embryotoxic effects of PHA on zona-free zygotes.

The *in vitro* production system comprised maturation of cumulus-oocyte complexes in TCM-199, fertilisation in TALP medium and culture in modified SOFaaci. The agents used for primary parthenogenetic activation of oocytes comprised either 10 μM calcium ionophore (1.5–5 min), 5 μM ionomycin (1.5–5 min) or electropulse (0.6-1.2 kV/cm, 20 μs) followed by 2 mM DMAP (4 h) as the secondary activation agent. Following evaluation of these protocols, the method selected for NT embryo activation comprised 0.63 μM calcium ionophore (5 min) followed by 2 mM DMAP (4 h). NT cytoplasts were prepared by removing the zona of MII oocytes with pronase, bisecting them with a micro-knife and discarding the half containing the metaphase plate (visualised with Hoechst stain). Each half cytoplast was adhered to a granulosa cell using PHA (observed under a stereo microscope) and each couplet was fused to another half cytoplast by electropulse (1.2 kV/cm, 30 μs). All zona-free embryos were cultured in WOWs.

The initial data indicated that of calcium ionophore A23187, ionomycin and electropulse treatments as primary activation agents, the two former were equally efficient even with reduced exposure times. WOW-culture of zona-free vs. zona-intact zygotes were not different in either blastocyst yield (44.6 ± 2.4 vs. 51.8 ± 13.5% [mean ± SEM]) or quality (126.3 ± 48.4 vs. 119.9 ± 32.6 total cells), and exposure of zygotes to PHA did not reduce blastocyst yields compared to vehicle control (40.8 ± 11.6 vs. 47.1 ± 20.8% of cultured zygotes). Subsequent application of the optimised technique for NT using 9 different granulosa cell primary cultures (cultured in 0.5% serum for 5-12 days) gave 88.8 ± 1.9% fusion and 37.6 ± 3.9% (11 replicates) [range; 16.4-58.1%] blastocysts per successfully fused and surviving reconstructed embryo (after activation), and 33.6 ± 3.7% blastocysts per attempted reconstructed embryo. Mean Day 7 total blastocyst cell numbers from 5 clone families was 128.1±15.3. Ongoing pregnancy rates of recipients each receiving 2 NT blastocysts are 8/13 recipients pregnant at Day 30 after transfer. These results suggest that the zona-free NT technique generates blastocysts of equivalent quantity and quality compared to conventional micromanipulation methods, requires less technical expertise, is less time consuming and can consequently increase the daily output of reconstructed embryos.

Notes

PLOIDY OF BOVINE NUCLEAR TRANSFER BLASTOCYSTS RECONSTRUCTED USING GRANULOSA CELLS

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Numerical chromosome abnormalities in nuclear transfer (NT) embryos could play a role in the high rates of preimplantation and foetal losses observed in embryonic NT embryos, a phenomenon which is exaggerated in NT embryos reconstructed using somatic cells. We have previously reported (Booth *et al.*, 2000, Cloning, 2, 63-8) in embryonic NT embryos, that when chromosome errors are present, such NT embryos are more severely affected than *in vitro* produced (IVP) embryos. In this report, we extend our observations to somatic NT embryos.

Bovine NT embryos were reconstructed using the zona-free manipulation technique (Booth *et al.*, 2001; see accompanying abstract). Briefly, cytoplasts were made from zona-free oocytes by bisection. Two half oocytes and one granulosa cell (serum starved primary cultures) were fused together and activated by Ca ionophore followed by DMAP before culture in SOFaacis for 7 days in microwells. Nuclei were extracted from all grades of Day 7 blastocysts and fluorescent in situ hybridisation was performed using chromosome 6- and 7-specific probes (Viuff *et al.*, 1999, Biol Reprod, 60, 1273-8). The results are presented in Table 1.

Table 1: Chromosome abnormalities in all blastocysts from 5 somatic nuclear transfer clones.

Granulosa cell line No.	No. NT embryos	% nuclei examined/ embryo	% chromosome complement					% total ploidy error
			Diploid	Triploid	Tetraploid	≥Pentaploid	P*	
1	14	83.1 ± 4.4	87.2 ± 5.9	3.0 ± 1.1	9.4 ± 4.8	0.4 ± 0.2		12.8 ± 5.9
2	24	73.0 ± 3.3	73.9 ± 8.2	3.6 ± 1.7	21.5 ± 7.32	1.0 ± 0.4	a	26.1 ± 8.2
3	9	76.3 ± 4.3	96.3 ± 1.3	0.7 ± 0.4	2.9 ± 1.2	0.1 ± 0.1	b	3.7 ± 1.3
4	17	74.0 ± 3.9	90.7 ± 4.8	2.8 ± 1.3	6.3 ± 3.7	0.2 ± 0.2		9.3 ± 4.8
5	48	71.1 ± 2.5	86.2 ± 3.8	3.0 ± 1.2	9.9 ± 2.6	0.9 ± 0.4		13.8 ± 3.8
Total	112	75.5 ± 2.1	86.9 ± 3.7	2.6 ± 0.5	10.0 ± 3.1	0.5 ± 0.2		13.1 ± 3.7

* Comparison of ploidy distributions by chi-square. a vs b = $P<0.01$

On average, over 85% of nuclei in somatic clones were diploid with tetraploid cells comprising the major ploidy abnormality (see Table 1). An effect ($P<0.01$) of granulosa cell line on ploidy distribution was observed between lines 2 & 3. Blastocysts of line 2 possessed a high proportion of tetraploid nuclei and indeed 4 blastocysts (all possessing cell numbers of <100) were 100% polyploid. Blastocyst yield was not correlated to % total ploidy error, while an inverse relationship ($P<0.01$) between blastocyst total cell number and % chromosome abnormality was observed (data not shown). Categorisation of the blastocysts into 3 quality grades (good; good + medium; good + medium + poor) and comparison of the distribution of ploidies when classified into 0, 0.1-5.0, 5.1-10.0, 10.1-15.0, 15.1-20.0, 20.1-25.0, 25.1-50.0 and >50% errors within embryos, indicated that only inclusion of poor grade embryos caused the distributions to be different ($P<0.05$) to that of good quality IVP embryos as recorded by Viuff *et al.* (1999, Biol Reprod, 60, 1273-8).

These results suggest that (a) the line of somatic cells could be a potential contributor to ploidy errors in somatic NT embryos but deserves further clarification, (b) 100% polyploid blastocysts can exist, (c) good and medium quality NT embryos possess no greater ploidy errors than IVP embryos, (d) foetal losses of somatic NT embryos are probably unrelated to ploidy errors.

Notes

SIMPLIFICATION OF PORCINE SOMATIC NUCLEAR TRANSFER BY APPLICATION OF THE ZONA-FREE MANIPULATION TECHNIQUE

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Successful somatic pig nuclear transfer (NT) has recently become a reality (PPL Therapeutics; The Times newspaper, March 15, 2000) but is still extremely inefficient as a result of poor *in vitro* maturation and culture systems as well as other undefined factors that hinder *in vitro* development. The latter could be more efficiently and rapidly identified by simplification of the traditional NT technique which is currently both intensive and time-consuming. We have developed a zona-free manipulation technique for bovine somatic NT (Booth *et al.*, 2001; see accompanying abstract) which we have now applied to pig somatic NT as described here.

Post-pubertal pig cumulus-oocyte complexes were matured for 40h in TCM-199. Cumulus and zona pellucidae were removed sequentially in hyaluronidase and pronase. Cytoplasts were made by bisecting oocytes in cytochalasin and Hoechst 33342 with a microknife using a micromanipulator. Halves containing the metaphase plate were discarded. One cytoplast was adhered to one porcine granulosa cell (primary cultures; cultured in 0.5% serum for 2-5 days prior to use) in 300 μg/ml phytohaemaglutinin. Each cytoplast-granulosa cell complex was then aligned next to another cytoplast and all were simultaneously fused together (0.8-1.0kV/cm, 30 μs) in 0.3M mannose containing 0.1 mM MgSO4. Successfully reconstructed NT embryos were then activated with Ca ionophore (0.63-1.3 μM, 5 min) followed by DMAP (2mM, 4 h) and then cultured in microwells (WOWs) in NCSU-23 containing 4 mg/ml FAF-BSA for 7 days. The experiment was repeated 5 times.

Of 279 attempted reconstructed NT embryos, 85.0 ± 2.8% (mean ± SEM) successfully fused and survived activation and 78.5 ± 2.7% embryos cleaved. The blastocyst rate (per successfully fused and surviving embryo) was 4.8 ± 2.3% (11/236; range 0-12.8%) The blastocyst rate of control oocytes (parthenogenetically activated and zona-free) propagated under the same conditions was 17.1 ± 2.9% (34/207 embryos; 2 replicates). 54.4 ± 2.3% (53/96 embryos; 3 replicates) of developmentally halted embryos (that could still be evaluated on Day 7) possessed anucleate blastomeres, the latter representing 53.5 ± 6.6% of the blastomeres in such embryos.

The blastocyst rate described here is equivalent to other published results (Tao *et al.*, 1999, Cloning, 1, 55-62). The low blastocyst yield appears to be independent of activation efficiency, and is likely caused by insufficient nuclear remodelling, reprogramming, imprinting or other effects. The data also indicate that fragmentation is a considerable problem which could conceivably contribute to halted development in a high proportion of embryos.

We conclude that the zona-free maniplulation technique can be successfully applied to pig somatic NT. Although such zona-free early cleavage stage embryos cannot be transferred to recipients at present, this technique permits simplification of the NT technique for application in basic research until pig blastocyst non-surgical transfer becomes a realistic option. Understanding the basic requirements for the cytoplasmic and reprogramming events that occur during early cleavage development deserve attention in porcine somatic NT to permit wider application of this technology.

Notes

EMBRYO SURVIVAL AFTER TRANSFER OF *IN VITRO* AND *IN VIVO* PRODUCED GOAT EMBRYOS.

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Normal offsprings were obtained after transfer of *in vitro* produced goat embryos (Crozet *et al.*, 1993, Therio., 39, 206). However, to our knowledge, no body has compared the embryo survival rate of *in vivo* and *in vitro* produced embryos after transfer in this specie.

The aim of this study was to compare the pregnancy rate and the embryo survival after transfer at day 7 of *in vitro* and *in vivo* produced goat embryos.

Synchronization of estrus was performed in recipients and donors (*in vivo* embryo production) Alpine/Saanen dairy goats by intramuscular injection of 50 µg of cloprostenol (Estrumate) on day 9 of 11-d progestagen treatment (Chronogest-Intervet, Angers, France). For *in vivo* embryo production, donor goats were superovulated with pFSH (16 Armour Units, Mérial, Belgium) and inseminated the day of oestrus. The embryos were collected surgically on day 7 after oestrus. For *in vitro* embryo production, oocyte-cumulus complexes were collected from slaughtered ovaries, matured, fertilized and zygotes were cultured in SOF-BSA in presence of serum to the blastocyst stage as previously described (Cognie *et al.*, 1995, AETE, 146). At day 7 post insemination, compacted morulae, blastocysts, expanded and hatched blastocysts were transferred surgically to recipients (2 embryos per recipient). Pregnancy rate was diagnosed by progesterone assay on Day 21, confirmed by ultrasound on Day 41 and at term.

Table 1 : Pregnancy rate and embryo survival of *in vitro* and *in vivo* produced goat embryos

Embryo production method	Recipients n (embryos)	Pregnancy rate % (n) day 21	Pregnancy rate % (n) day 41	Kidding rate % (n)	Embryo survival % (n)
In vitro	18 (36)	83 (15)	61[a] (11)	61[a] (11)	47[a] (17)
In vivo	19 (38)	89 (17)	89[b] (17)	89[b] (17)	71[b] (27)

[a, b] Values differ significantly ($P < 0.05$ chi-square test).

Gestation length and birth weight of kids were similar in the two groups. Moreover, there was no significant difference between development stages of transferred embryos on embryo survival.

The kidding rate and the embryo survival were significantly lower after transfer of the *in vitro* produced embryos than after transfer of the *in vivo* produced embryos ($P<0.05$) and resulted from pregnancy failure between Days 21 and 41. However, the embryo survival of the *in vitro* produced embryos was as good as embryo survival of *in vivo* frozen /thawed embryos (Baril *et al.*, 7th Int. Conf. Goats, 2000, 1030 : 47.3%).

Our conditions of goat embryo *in vitro* production allow, after transfer into recipient, an acceptable embryo survival rate, with totally normal offsprings.

Notes

EMBRYO PRODUCTION IN SANTA INES SHEEP AFTER pFSH TREATMENT: PRELIMINARY RESULTS

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Sheep explored in the northeast of Brazil represent an important economical source for the farmers, being explored mainly for the meat and skin production. The Santa Inês breed presents a notable capacity to produce meat, besides supplying a skin of excellent quality. The embryo transfer promotes a faster diffusion of the genetic material of superior animals with safety and at a smaller cost compared to acquisition of pure animals. The aim of this study was to evaluate the quanti-qualitative production of embryos in Santa Inês ewes after superovulatory standard treatment with pFSH. The estrus of 15 adult Santa Inês ewes was synchronized by insertion of intravaginal sponges containing 60 mg MAP (Promone-E, Rhodia Mérieux Ltda.) for 14 days. Sixty hours before sponge removal ewes begin to receive a superovulation treatment with 200 UI pFSH (Pluset, Serono S.p.A.) administered every 12 h in 6 decreasing doses. The ewes were naturally mated by two Santa Inês rams using hand mating from onset of estrus during 2 days at 12 h interval. The embryos were recovered surgically 6-7 days after estrus and were classified according to their stage of development and quality. Estrus was detected in all treated ewes. The mean interval sponge removal to estrus onset and the mean estrus length was 36.0 ± 11.1 h and 43.2 ± 14.2 h, respectively. Eleven ewes showed a superovulation response (73.3%) with a mean ovulation rate of 9.9 ± 3.6. The recovery rate was 79.8% (7.9 ± 3.7 structures per ewe). A total of 87 structures was collected and the average number of embryos recovered per ewe was 4.2 ± 3.4. After evaluation, it was observed 52.9% of embryos (46/87) and 47.1% (41/87) of oocytes. From the recovered embryos 32.6; 34.8; 13.0 and 19.6% were of I, II, III and IV/degenerating grade, respectively. Although the Santa Inês ewes had showed a good ovulate rate, fertilization failure could be significantly reduced using uterine AI as shown in others breeds.

Notes

COMPARISON BETWEEN TWO EMBRYO TRANSFER METHODS OF VITRIFIED SHEEP BLASTOCYSTS

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Ovine blastocysts were produced by uterine insemination of superovulated adult animals. Dulbecco's PBS supplemented with 20% FBS was used as the basic cryopreservation solution. Seventy embryos (Group I) were exposed to the vitrification solution in three steps as follows: 10% glycerol (G) for 5 min, 10% G + 20% ethylene glycol (EG) for 5 min. Then they were loaded into 25% G + 25% EG (Yang *et al.*, 1992) of Open-pulled-straw (OPS) and plunged within 30 sec into LN_2. Other 15 embryos (Group 2) were exposed to a different vitrification solution in two steps as follow: 10% EG + 10% DMSO for 3 min. Then they were loaded into 20% EG + 20% DMSO + 0.3 M Sucrose (Vajta *et al.*, 1998) of OPS and plunged within 30 sec into LN_2. Warming was done placing the OPS into a Falcon tube with 8 ml of 0.5 M Sucrose solution at 37°C for 3 min. Then 42 embryos from Group I were transferred into TCM199 supplemented with 20% FBS for 24 h of culture at 39°C with 5% CO_2 in humidified air in incubator. Only the re-expanded blastocysts (32 out of 42) were transferred, in pairs, to synchronised recipient ewes with a tomcat catheter connected to an insulin syringe: indirect transfer group (group IT). The other 43 embryos vitrified (28 from Group I and 15 from Group 2) were warmed as described above. During the 3 minutes of exposition into the Falcon tube they were retrieved from the sucrose solution and were transferred directly (group DT) into synchronised recipient ewes inserting the tomcat catheter into the OPS. Out of 42 blastocysts of the IT group 24 lambs were born (57%) and out of 43 embryos of the DT group 26 lambs were born. 17/28 (60%) of the Group 1 and 9/15 (60%) of the Group 2 respectively. In conclusion these results show that the OPS direct transfer technique can be used with good efficiency in sheep whatever the cryopreservation method used.

Notes

EFFECT OF ADMINISTRATION OF FSH TO PREPUBERTAL HEIFERS ON FOLLICULAR DEVELOPMENTAL KINETICS

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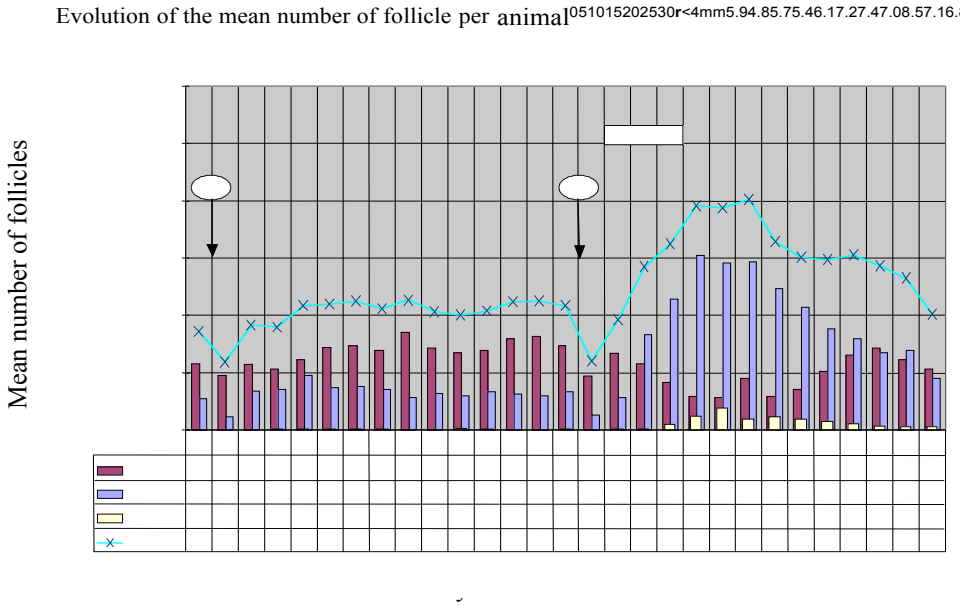
The aim of the experiment was to determine the follicular developmental kinetics before, during and after FSH stimulation in prepubertal calves.

Ovarian follicles of 14 prepubertal calves (aged 7 months) were counted and measured daily for 28 days (Hitachi EUB 405 ultrasound, 7.5 MHZ EUP-F331 probe). Ovaries were maintained manually through the rectum and the probe was introduced through the vagina. On D1and D14, all visible follicles were removed from the ovaries by OPU. On D16-17-18, a total amount of 22 U FSH (0% LH) was administered (morning and evening) in decreasing doses. Blood was sampled daily for detection of 17-beta-Estradiol and Progesterone. For analysis, follicular diameters were divided in 3 classes (small [<4 mm]; medium [4-10 mm] and large [>10 mm]). Results are presented in figure 1:



Follicular development : - before FSH, the follicular population consisted of a majority of small , a minority of medium and few large follicles. After FSH, there was a 43% increase in follicle numbers with a majority of medium, a minority of small and some large follicles for about 8 days following the last FSH injection..

Total numbers started to decline 3 days after the last FSH injection.

Hormone levels (data not shown) : -3 of 14 animals showed an increase (>2 ng/ml) in progesterone levels a few days after FSH, probably due to CL following ovulation;

- none of the animals showed signs of estradiol production, before, during or after FSH. This was surprising and is currently under investigation.

In this study, FSH administration was found to increase the number and diameter of follicles in prepubertal calves, and influenced progesterone but not estradiol production.

Notes

A COMBINATION OF TWO SERUM REPLACEMENTS DURING THE *IN VITRO* CULTURE OF BOVINE EMBRYOS

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The aim of this study was to evaluate the effect of combining two serum replacements (CPSR3®, SIGMA –CP- and ULTROSER® -US-, LIFE Technologies) added to the culture medium at different concentrations during two periods of *in vitro* bovine embryo development.

Presumptive zygotes produced as using standard techniques in our laboratory (Duque *et al*, 2000; AETE Meeting, pp 140) were vortexed and cultured up to Day 5 in SOF (Holm *et al.*, Theriogenology 52: 683-700) to which serum replacements were added as follows:

1. Group 6CCC: 6% of CPSR3® (42h post-FIV)
2. Group 2UUC: 2% of ULTROSER® (42h post-FIV)
3. Group 6UUC: 6% of ULTROSER® (42h post-FIV)
4. Group OOC: no serum replacement

Day 5 morulae and early blastocysts were placed in SOF + 6% CPSR3® and allowed to develop up to Day 8. Culture conditions were 39ºC, 5% CO_2 in air and high humidity. Results are shown in Table 1. Data were analysed by Duncan's test and expressed as a mean ± SEM.

Table 1. Development rates of Day 5-morulae and early blastocysts produced in SOF supplemented with 6% CP (6CCC), 2% US (2UUC), 6% US (6UUC) or without supplement (OOC). Culture from Day 5 up to Day 8 was performed in SOF + 6% CP.

	Treatment			% M+BJ (M)	Blastocysts		
Group	D 2-5	D 5-8	N	D5	D7	D8	Expansion
6CCC	6%CP	6%CP	146	35.7 ± 3.7(51)	71.7 ± 13.0^{a}	71.7 ± 13.0	34.9 ± 10.6^{a}
2UUC	2%US	6%CP	134	39.1 ± 4.1(52)	51.5 ± 11.3^{b}	51.5 ± 11.3	22.3 ± 8.2^{b}
6UUC	6%US	6%CP	132	35.4 ± 2.1(46)	34.0 ± 9^{c}	43.2 ± 10.6	15.4 ± 5.2^{c}
OOC	CP(-)US(-)	6%CP	126	35.9 ± 3.7(46)	56.2 ± 14.1^{b}	59.5 ± 14.0	29.9 ± 9.6^{b}

N: oocyte number; % M+BJ: morulae and early blastocysts rate at Day 5. Blastocyst rates are a proportion of Day 5-morulae and early blastocysts (M). Different superscripts within columns differ significantly ($p<0.05$). Number of replicates: 3.

The use of 6% of CPSR3® throughout the whole culture period produced blastocyst and expansion rates higher than the other treatments. Day 5 morulae and early blastocysts produced in SOF + 2% US (2UUC) or without serum replacement (OOC) developed at similar rates once in SOF + 6% CP. Day 5 embryos produced in SOF + 6% US (6UUC) had the lowest developmental ability.

These results show that concentration and period of development influence the effect of CPSR3® and ULTROSER® when used in combination as serum replacements during the *in vitro* culture of bovine embryos.

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Notes

COMPARISON OF FOLLICULAR DEVELOPMENT AND OOCYTE MATURATION IN MANGALICA AND LANDRACE GILTS[#]

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Mangalica is a native Hungarian pig breed. This race was the most typical swine in Hungary until the early 1950s. Then it was replaced by modern breeds because its characteristics were unsuitable for breeders and consumers (low fecundity, long growing period, fatty meat). At the end of 1970s the population reached a critical level. Nowadays there is increasing public interest for preservation of native breeds. The number of Mangalica stock is rising slowly. This breed has some excellent properties, which support the preservation of Mangalica, like delicious taste of meat, the plasticity of adaptation for extensive environment and maternal instinct. However low fecundity of the breed hampers the propagation. Therefore, preovulatory follicular development and oocyte maturation of Mangalica and of Landrace gilts were investigated.

Altogether 18 puberal Blond and Swallow Belly Mangalica (10-12 month of age, body weight of 100-110 kg) and 19 Landrace gilts (8,5-9 month of age, body weight of 120-125 kg) were synchronized by per os administration of altrenogest (Regumate, Hoechst Roussel), 24 h after follicular growth was stimulated by 1000 IU PMSG (Folligon, Intervet) and the ovulation was induced by 750 IU hCG (Choriogonin, Richter Gedeon) 80 h after PMSG.

Endoscopic oocyte recovery (ovum pick up) was carried out 34 h after hCG. The morphology of cumulus-oocytes-complexes (COCs) was determined under stereomicroscope and was classified as compact, expanded or denuded. Thereafter COCs were prepared for evaluation of nuclear configuration. They were divided in three groups as 1) immature - germinal vesicle (GV), with diplotene chromatin; 2) meiosis resumed - GV breakdown, diakynesis, Metaphase I to Anaphase I; or 3) mature - Telophase I or Metaphase II (T I /M II).

Data were analysed by Student t-test and Chi-square, respectively and $p<0.05$ was considered to be statistically significant.

The average number (± SD) of preovulatory follicles was less in Mangalica 6.8 ± 1.4 than in Landrace 19.6 ± 6.6 ($p<0.05$). Differences were observed in COCs morphology and nuclear status of oocytes between breeds. There was a higher percent of oocytes with compact cumulus in Mangalica compared to Landrace (31 vs. 16%) and less had expanded cumulus (62 vs. 78%, $p<0.05$). The rate of oocytes with mature chromatin configuration (T I /M II) was higher (27 vs. 62%, $p<0.05$) in Landrace.

These results indicate that both diminished follicular development and delayed oocyte maturation may be connected with low fecundity in Mangalica.

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Notes

TRANSRECTAL ULTRASONOGRAPHY IN SUPEROVULATED EWES

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A non invasive technique to evaluate ovarian response in superovulated ewes is particularly useful in embryo transfer programs. The objective of this study was to evaluate the relationship between ovarian follicle number determined by transrectal ultrasonography at time of oestrus and CL number determined by laparotomy at time of embryo recovery 6-7 day after estrus (day 0 of Santa Inês ewes. A real-time B-Mode array ultrasound unit (Concept LC/ Dynamic Imaging, England) with a 7,5MHz linear rectal probe was used to evaluate the number of ovarian follicles. The estrus was synchronized in 15 ewes using intravaginal sponges impregnated with 60 mg of medroxyprogesterone acetate (Promone-E, Rhodia-Mérieux Veterinary), for 14 day, and 200 UI of porcine follicle stimulating hormone (Pluset, Serono) for superovulation, divided in six injections, administered every 12 hours in 6 decreasing doses during the last three days of progesterone treatment. Ovarian follicles were evaluated using transrectal ultrasonography once a day, during 3 days after onset of estrus. The estrus was observed twice a day. Visualization of ovary in connection with the beginning of estrus was 71% at 0 hour, 87.5% at 12 hours, 100% at 24 hours, 50% at 36 hours, 71% at 48 hours and 37% at 60 hours. The relationship between preovulatory follicles at transrectal ultrasonography and corpus luteum number at laparotomy was better at 12 hours after the onset of estrus $r=0.87$ ($P<0.05$). Transrectal ultrasonography is an appropriate method to predict corpus luteum response before embryo recovery in superovulated ewe.

Notes

BOVINE BLASTOCYST APOPTOSIS INDUCED BY OXIDATIVE STRESS: PROTECTIVE EFFECT OF ß-MERCAPTOETHANOL AND TROLOX®

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In previous studies, we have shown that addition of pro-oxidants {AAPH; 2.2' azobis (2-amidinopropane) dihydrochloride, an exogenous radical initiator, or BSO; buthionine sulfoximine, an inhibitor of gluthatione synthesis} in the culture medium from Day 5 pi (1) increased the apoptotic index in Day 7-blastocysts mainly in the ICM region (Feugang *et al.*, 2000) and (2) induced blastocyst degeneration at Day 8 pi. The addition of ß-mercapthoethanol (ß-ME, a precursor of gluthatione synthesis) or Trolox® (a water soluble analog of vitamin E) partly prevented blastocyst degeneration (Feugang *et al.*, AETE proceedings, 2000; p 144). The present study aimed to evaluate the protective effect of these antioxidants on apoptosis in Day 7-blastocysts in the presence or absence of pro-oxidants.

After IVM/IVF, bovine oocytes were cultured in SOF medium containing 5% FCS, at 39°C under 5% O_2, 5% CO_2 and 90% N_2. Morulae were recovered (Day 5 pi) and distributed into groups of 5 embryos, in 20-µl drops of fresh culture medium. Three conditions were tested: 1) medium alone ; 2) 0.01mM of AAPH and 3) 0.4 mM of BSO. ß-ME was added from Day 5 pi and during exposure to pro-oxidants (from Day 6 to Day 7 pi), and Trolox® was added simultaneously with the two pro-oxidants (from Day 5 to Day 7 pi). The blastocysts were recorded and fixed at Day 7 for evaluation of apoptosis by the TUNEL technique (Boerhinger, Mannheim).

The blastocyst rate at Day 7 pi was not affected by the treatments, and varied from 68% to 80%. AAPH and BSO induced A significant increase in the percentage of TUNEL positive nuclei in blastocysts (Table). In the presence of antioxidants, this increase was prevented. Moreover, ß-ME but not Trolox® decreased the rate of apoptotic nuclei in the control group.

Table: Effect of ß-mercaptoethanol (ß-ME) or Trolox® on apoptosis in bovine blastocysts at Day 7 pi.

Groups		ß-ME (0.1 mM)			Trolox (0.4 mM)		
		N	% of TUNEL+ Nuclei (± sem)	Mean cell number (±sem)	N	% of TUNEL+ Nuclei (± sem)	Mean cell number (±sem)
Control	-	32	3.7 ± 0.4 [a]	112 ± 6 [a]	32	2.8 ± 0.7 [a]	115 ± 8 [a]
	+	30	2.8 ± 0.4 [b]	107 ± 6 [a]	31	3.4 ± 0.7 [a]	101 ± 7 [a]
AAPH (0.01 mM)	-	27	5.6 ± 0.5 [c]	116 ± 6 [a]	31	5.5 ± 0.7 [b]	106 ± 5 [a]
	+	31	2.6 ± 0.5 [b]	116 ± 6 [a]	28	3.5 ± 0.7 [a]	106 ± 5 [a]
BSO (0.4 mM)	-	31	6.3 ± 0.5 [c]	109 ± 6 [a]	27	5.8 ± 0.7 [b]	114 ± 6 [a]
	+	31	2.7 ± 0.6 [b]	126 ± 7 [a]	31	2.8 ± 0.6 [a]	112 ± 5 [a]

[a,b,c] Values with different superscripts in the same column are significantly different ($P<0.05$ – ANOVA 2). Total of 8 replicates.

These results show that (1) Trolox® and ß-ME can prevent apoptosis induced by oxidative stress generated by different ways in IVP bovine blastocysts and (2) ß-ME could reduce apoptosis in bovine blastocysts. This could explain the increased hatching rates and cell number observed one day later (Feugang *et al.*, AETE proceedings, 2000; p 144).

Notes

PMSG PRIMING DURING THE CYCLE PRECEDING SUPEROVULATION IMPROVES EMBRYO QUALITY IN DAIRY HEIFERS

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To improve embryo yield after superovulation, different priming treatments based on an hormonal injection during the oestrus cycle of the superovulatory treatment have been tested with contrasting results. It was hypothesised here that the stimulation of follicular growth should occur earlier. The effects of a PMSG priming at the end of the oestrus cycle preceding superovulation on embryo production results were investigated.

A total of 75 cycling Holstein heifers, 12 to 18 months of age, superovulated for the first time, were included from December 2000 to May 2001 by two ET teams of Brittany (France). Oestrus was synchronized with a norgestomet implant (CRESTAR, Intervet, Fr) left for 9 days and a prostaglandin (PG) injection 2 days before implant removal. At implant withdrawal, ET technicians injected blindly 300 IU of PMSG (FOLLIGON, Intervet, Fr ; PMSG group, n = 34) or isotonic saline (CONTROL group, n = 41). A norgestomet implant was inserted between D7 and D10 after oestrus and left for 5 days. The superovulatory treatment started 2 days later (total dose of 400 µg of pFSH, 8 injections in decreasing doses 12 h apart). A PG injection was administered at the time of the 5th FSH injection. Heifers were inseminated 12 and 24 h after estrus detection. Embryos were collected non surgically on D7 after AI. The influence of treatment on mean numbers of total (TOT), transferable (TRA), Grade 1 (GR1), degenerated (DG) embryos and unfertilized oocytes (UFO) was tested using a mixed analysis of variance (MIXED procedure of SAS) adjusted on ET team, TOT embryos and paternal origin of donor as a random effect.

The distribution of heifers between treatments was similar for ET team, bull of AI and age. Number of collections with no embryo and no TRA embryo were 4 (9%) and 6 (17%) respectively in the PMSG group and 2 (5%) and 4 (10%) in the CONTROL group ($p>0.05$). PMSG priming enhanced the quality of embryos as shown in Table 1. Mean numbers of GR 1 were significantly improved in the PMSG group ($p = 0.02$) and the rate of collections with 5 UFO or more was decreased in the PMSG group (PMSG: 9% vs CONTROL: 27%; $p<0.05$). The viability of embryos increased with age of donor at collection on account of lower numbers of UFO ($p<0.05$). The quality of embryos was also influenced by the ET team, whereas paternal origin of donor heifers influenced TOT embryos, ranging from 1 to 26 between origins.

Table 1. Embryo yield in PMSG and control heifers (Ls means ± sem; a vs b: p=0.02)

	N	TOT	TRA	GR 1	DG	UFO
PMSG	34	8.18 ± 1.77	7.82 ± 0.59	5.08 ± 0.55^{a}	0.91 ± 0.25	1.46 ± 0.43
Control	41	8.82 ± 1.71	6.77 ± 0.59	3.35 ± 0.50^{b}	1.35 ± 0.23	2.29 ± 0.40

To conclude, the use of a PMSG priming during the cycle before superovulation may be a good way to improve the quality of embryos in heifers. Further experiments are planned to optimise the dose of PMSG and to investigate the effects of such a priming on dairy cows.

Notes

IN VITRO SURVIVAL OF VITRIFIED GOAT EMBRYOS : COMPARISON OF TWO VITRIFICATION METHODS

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Vitrification is an effective freezing method for goat embryos : Day 7 *in vivo* produced embryos are able to support the cryoperservation by vitrification with an embryo survival rate of 40% after transfer (Traldi *et al.*, 2000). Recently, vitrification with OPS method of goat expanded blastocyst (Day 7, *in vivo* produced) allowed 60% of birth *vs* 42% with conventional slow freezing (El-Gayar *et al.*, 2001). However, few data compared the survival of early (morula) and late (blastocysts) embryos.

The aim of this study was to compare 2 methods of vitrification with in *vivo* goat embryos at different stages of development (compacted morulae, early and expanded blastocysts) in relation to the hatching rate after *in vitro* culture.

Alpine and Saanen dairy goats were superovulated with pFSH (16 Armour Units) and inseminated. The embryos were collected on day 7 or day 8 after oestrus and were OPS – vitrified as described by Vajta *et al.* (1998) or classically vitrified as described by Mermillod *et al.* (1997). After thawing, OPS – vitrified embryos were cultured in 100 µl drops of SOF + 20%FCS during one hour and then in 100 µl drops of SOF + 10% FCS, whereas classically vitrified embryos were cultured directly in 100 µl drops of SOF + 10% FCS. All embryos were cultured during 96 hours. Data were analysed by a general linear model taking into account the trial (Venables & Ripley, 1994).

Table 1: Hatched blastocysts (%) obtained after *in vitro* culture of *in vivo* produced and vitrified caprine embryos

	Hatched blastocysts (%)	
	OPS method	Classical vitrification
Compacted morulae and early blastocysts	19 / 32 (59.3%)	10 / 31 (32.2%)
Expanded blastocysts	62 / 80 (77.5%)	64 / 81 (79%)

The *in vitro* embryo survival rate after thawing was significantly different between stages of development: expanded and hatched blastocysts survive better than compacted morulae and early blastocysts ($P<0.001$). The vitrification method had no effect on the hatching rate after *in vitro* culture ($P=0.36$). However according to embryo stage of development, the hatching rate tended to be better for the compacted morulae and the early blastocysts after OPS than after classical vitrification ($P=0.07$).

It is well known that in ruminants early stage embryos do not survive very well after freezing. However, the OPS-method could increase the survival rate of morula stage embryos.

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Notes

GLUCOSE INDUCES APOPTOSIS IN IVP BOVINE BLASTOCYSTS

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Adverse effects of high concentrations of glucose on the embryonic development of bovine embryos produced *in vitro* have been reported. However, the mechanism by which high levels of glucose exert a detrimental effect on embryo development *in vitro* has not been elucidated. The purpose of this study was to investigate if in bovine embryos, as early as the blastocyst stage, glucose may modulate the expression of two genes: BAX (Bos taurus apoptosis regulator box-α) an apoptosis regulatory gene, and the mitochondrial (Mn) SOD (Bos taurus supoeroxide dismutase) implicated in the formation of H_2O_2.

Bovine oocytes recovered from slaughterhouse ovaries were put through IVM and IVF. One-cell embryos were cultured *in vitro* in SOF+10%FCS under two different conditions: (a) with glucose (11,2 mM), and (b) control without glucose. Total mRNA was isolated from two pools (15/pool) of blastocysts and semi-quantitative reverse transcription was performed using specific reverse primers to Bax or SOD plus β-actin. The expression of β-actin was used as a reference value to quantify Bax and SOD in the semi-quantitative PCR. Triplicate reactions from each sample were displayed. Expression of Bax mRNA was 2.5-fold higher and the expression of SOD mRNA was 2-fold higher in embryos cultured in the presence of glucose than in absence, indicating that hyperglucemic conditions *in vitro* modulate the expression of Bax, and that glucose may generate reactive oxygen free-radicals not only by inhibiting hypoxanthine phosphoribosyl tranferase (HPRT) activity but also by increasing mRNA encoding for mitochondrial (Mn) SOD.

Apoptosis is a normal process in the mammalian preimplantation blastocyst that protects the embryo by the elimination of abnormal cells. Here we demonstrate that expression of Bax, a death-promoting member of the Bcl-2 family of proteins, is increased at the blastocyst stage in the presence of high concentrations of glucose. These findings emphasize the importance of tight glycemic control at the earliest stages after conception and it provides evidence of how a metabolite such as glucose, the presence of which *in vivo* is determined by different environmental factors, could be related to embryo mortality.

Notes

THE ROLE OF THE PRE-IMPLANTATION EMBRYO IN THE VERTICAL TRANSMISSION OF NATURAL SCRAPIE INFECTION IN SHEEP

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The objective of this study is to determine the role of the pre-implantation stage embryo in the vertical transmission of natural scrapie. Scrapie is one of a group of diseases called transmissible spongiform encephalopathies (TSE's) which also include Bovine Spongiform Encephalopathy (BSE) and Creutzfeldt-Jacob Disease (CJD). Scrapie is a widespread, insidious and untreatable disease of farm animals which culminates in brain degeneration. Despite the fact that scrapie has been recognised for two centuries, the way in which it spreads from mother to offspring is not well understood. This lack of knowledge hinders the development of effective control programmes for the eradication of scrapie.

Once the mode of vertical transmission of natural scrapie infection is determined, the results can be used :

a) to provide advice and a model to allow the possible reduction in levels of TSE infection in the national flock
b) to allow informed decisions to be made by import/export regulatory authorities on the relative risks of importing disease in embryos from infected flocks/countries
c) to potentially increase agricultural exports

The epidemiology of scrapie has been the subject of extensive research for several decades and, whilst it has been shown that maternal transmission from ewe to lamb does occur, the mechanism by which the lamb becomes infected remains unknown. It is possible that infection may be transovarian, it may occur after fertilisation (before implantation or transplacentally), or it may be that lambs are infected at parturition or in early neonatal life. Identification of the exact method of transmission is required for the design of sensible control strategies. This project is designed to determine whether or not the pre-implantation embryo can act as a carrier of infection and whether it can be treated to remove infection.

Frozen embryos which were collected from naturally scrapie-infected sheep in the UK and embryos which were collected in the UK from non-infected sheep of New Zealand (NZ) origin have been transferred to isolated recipient sheep, known to be free of scrapie (New Zealand origin), in the UK. The embryo collection procedure involved orthograde flushing of the donor's uterine horns following surgical exteriorisation of the uterus. Embryos were held in ovum culture media supplemented with NZ lamb serum (ICP, NZ). Each donor's embryos were passed through 5 washes of culture medium, 2 washes of Trypsin and a further five washes of culture medium before being packaged into straws for freezing.

The scrapie-exclusion facility was established and populated with the New Zealand-imported Suffolk sheep in the autumn of 1998. In total 124 NZ Suffolk sheep (119 mixed age ewes, 3 entire rams and 2 vasectomised rams) were introduced. 10 genotyped (prion protein genotyped homozygous for glutamine, i.e. QQ at codon 171) NZ ewes were superovulated and flushed to produce control embryos for freezing in the spring of 1999. In December 1999, 47 control embryos were transferred into 22 NZ recipient ewes (genotyped QQ and RQ, i.e. the latter being heterozygous arginine-glutamine at codon 171) and 17 pregnancies were established. QQ is the prion protein (PrP) genotype which confers the greatest susceptibility to natural scrapie infection in Suffolk sheep; RQ confers moderate susceptibility. In December 1999, 95 embryos from naturally infected ewes were transferred into 50 NZ recipient ewes (genotyped QQ and RQ) and 57 pregnancies were established. The scrapie strain from the naturally-infected flock affects Suffolks of the QQ and RQ genotypes and the incubation period in the field varies from 19 to 64 months with the majority of clinical scrapie cases between 23 and 36 months.

The ewes carrying control embryos (the control group) were separated from those ewes into which naturally infected embryos had been transferred (the experimental group). However, the 2 groups were fed the same food and graze pasture with the same history within the quarantined sheep unit. A small number of ewes (QQ and RQ genotypes) were run with the control and experimental recipient ewes to act as sentinels for scrapie infection.

Lambings commenced 20th March 2000 and finished 29th March 2000. 15 live lambs (6 male, 9 female) were obtained from the 17 pregnancies in the control group and 52 live lambs (29 male and 23 female) were obtained from the 57 pregnancies in the experimental group. Post mortem examinations were carried out on the 7 dead lambs and formalin-fixed and fresh tissues, frozen at –70^{o} Celsius, were collected and stored for future examination (as is the case for all animals which are euthanased or die). Additionally, frozen and formalin-fixed placentae were collected from 12 of the 14 control ewe lambings and 36 of the 38 experimental ewe lambings. All 67 lambs were confirmed homozygous QQ at codon 171.

All sheep receive routine veterinary care including vaccination against pasteurellosis, footrot and clostridial infection. All sheep are grazing the established paddocks except for a group of 15 thinner experimental sheep which were housed for 3 months during winter 2001. All sheep are monitored for signs of scrapie (clinically, post mortem, histopathology and PrP analysis). So far, 7 'adult' sheep have been euthanased and were negative for scrapie on clinical, post mortem, histopathology and PrP immunohistochemistry.

Future landmarks in the project are : November 2002 – embryo recipient ewes of QQ genotype to be slaughtered and undergo investigation for scrapie. April 2003 – sheep that were experimental and control embryos and embryo donor ewes to be slaughtered and undergo investigation for scrapie. November 2003 – embryo recipients of RQ genotype and all sentinel ewes to be slaughtered and undergo investigation for scrapie.

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Notes

GENETIC EFFECTS ON BREEDING PERFORMANCES AND EMBRYO PRODUCTION RESULTS AFTER SUPEROVULATION IN RABBIT SPECIES

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For most of domestic species, embryo production results after a superovulation treatment are extremely variable between and within breeds or strains. This variability can be explained mainly by environmental factors but genetic factors should be also taken into account, even if it is very difficult to evidence direct effects. The aim of this study was to evaluate the influence of genetic effect on breeding performances and on embryo production results after superovulation treatment in rabbit species.

Two divergent lines have been selected on the uterine efficiency and the foetal viability from the INRA 1029 synthetic rabbit strain. But after 4 generations, the two lines only diverged on ovulation rate: line 1 with low ovulation rate and line 2 with high ovulation rate. Breeding performances (527 observations on line 1 and 474 on line 2) were scored by endoscopy at Day 11 after ovulation. On the other hand, part of these females (60 in line 1 and 56 in line 2) was superovulated with a pFSH treatment to produce embryos for cryobanking. Embryos were recovered at compacted morula stage (Day 3) and corpora lutea (CL) on ovaries were counted. Only excellent and good quality embryos (668 in line 1 and 808 in line 2) were frozen. After thawing, embryos (193 in line 1 and 217 in line 2) were transferred into recipients, and the number of pups was scored at birth. The effect of genotype on the number of corpora lutea, implanted embryo and frozen embryo per doe and on the prenatal and embryo survival rate per doe was estimated by analysis of variance (table 1).

Table 1: Effect of genotype on breeding performances and embryo production after superovulation

Genotype	Breeding performances			Embryo production results after superovulation		
	Corpora lutea per doe	Implanted embryos per doe	Prenatal survival rate[(*)]	Corpora lutea per doe	Frozen embryos per doe	Embryo survival rate[(**)]
Line 1-(low)	10.8 [(a)]± 0.2	8.2 [(a)]± 0.2	67.1% [(ns)]	28.0 [(a)]±1.5	11.1 [(a)]± 1.1	60.7% [(ns)]
Line 2-(high)	13.0 [(b)]± 0.2	10.1 [(b)] ±0.2	65.6% [(ns)]	33.3 [(b)]± 1.6	14.4 [(b)]± 1.2	61.5% [(ns)]

Means ± SEM for corpora lutea and embryos per doe

[(*)] Prenatal survival rate = number of pups born / number of corpora lutea

[(**)] Embryo survival rate = number of pups born / number of embryos transferred into recipients

Embryo production estimated by the number of CL and embryos implanted at day 11 was significantly higher ($p<0.01$) for line 2 in comparison with line 1, but there was no significant difference ($p>0.05$) for embryo viability (prenatal survival rate). These observations were also confirmed for embryo production after superovulation treatment (number of CL and frozen embryo at day 3 significantly higher for line 2; $p<0.01$) and for embryo viability after transfer of frozen embryos (no significant differences on embryo survival rate; $p>0.05$).

In conclusion, we have shown that genetic effects on ovulation rate evidenced by divergent selection influence embryo production results after a superovulation treatment.

Notes

THE BIOCHEMICAL CHANGES IN THE CONTENT OF GLYCOPROTEINS IN THE BOVINE ZONA PELLUCIDA WITH EXPERIMENTALLY INDUCED HARDENING

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Hardening of the zona pellucida (HZP) can be observed *in vivo* as a consequence of senescence of ovulated oocytes, caused by the oviductal environment. It is also possible to induce HZP *in vitro* by holding IVM oocytes into oviduct or oviductal fluid. In our earlier experiments we have shown that HZP significantly reduced developmental competence of bovine IVM/IVF oocytes (K_tska *et al.*, 1999). Noguchi *et al.* (1997) analyzed the bovine zona pelucida (ZP) by SDS-PAGE after enzymatic deglycosylation and reported the ZP consisted of 3 major glycoproteins under non-reducing condition. ZP from ovarian egg emerged as 3 bands with molecular mass of 78 kDa, 64 kDa and 21 kDa. However, under reducing condition ZP could also be separated into 4 glycoproteins, named ZP1-ZP4. It has supposed that ZP2 and ZP4 were cleavage products of the parent protein ZP1. Therefore, correlation of the specific cleavage with HZP remains to be determined.

The aim of the present study was to investigate how differentiated degree of the HZP affects the morphology and biochemical changes in the content of glycoproteins.

Bovine cumulus-oocytes complexes, recovered from slaughterhouse ovaries, were matured *in vitro* in TCM 199 + 20% ECS for 24h at 39^0C. To induce HZP, mature oocytes freed of cumulus cells, were placed into bovine oviductal fluid (OF), aspirated from oviducts of slaughterhouse heifers, for 20, 30 and 40 min. at 39^0C. After incubation the oocytes were used for evaluation of the ZP digestibility (in 0.1% pronase) and ZP thickness was measured by microscopic micrometer. Empty ZP, isolated mechanically from IVM oocytes, and then incubated in a similar way as mature intact oocytes, were used for evaluation ZP digestibility. For analysis of the ZP glycoprotein fractions by SDS-PAGE electrophoresis immature, control *in vitro* matured and HZP oocytes incubated into OF, were used.

The dissolution time of the ZP in 0.1% pronase solution increased in proportion to the incubation time of IVM oocytes in OF. It amounted to 36.0, 69.0 and 129.0 min after 20, 30 and 40 min. of the exposition to OF, respectively. For the control oocytes only 2.5 min were required for dissolution of the ZP. The dissolution time of empty ZP was much higher in comparison to the results presented above, and after 20, 30 and 40 min of incubation it resulted 69.0, 96.5 and 129.0 min. For the control empty ZP of IVM oocytes only 4.1 min were required for digestion. The ZP thickness of HZP oocytes increased in proportion to the incubation time of the IVM oocytes into OF. It amounted 19.05; 21.15 and 23.2 μm after 20, 30 and 40 min of the exposition to OF, and for the control oocytes-16.05 μm.

On the SDS-PAGE gel we have observed many bands between 45.0-21.5 kDa in the lane belongs to the HZP oocytes (n = 467) compared to immature (n = 400) and IVM oocytes (n = 423). There have been additional glycoproteins in ZP derived from HZP oocytes in contrast to ZP in immature or IVM oocytes. We suppose that these additional proteins may cause HZP. Glycoprotein contents in OF have been proceeded.

In conclusion, oviductal factor, responsible for HZP also influences the specific changes in the content of the zona pellucida components.

Notes

COMPARISON OF EXPRESSION OF 6 DEVELOPMENTALLY IMPORTANT GENE TRANSCRIPTS IN DAY 7 AND DAY 8 BOVINE BLASTOCYSTS

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Expression of developmentally important gene transcripts can be used as a quality parameter of preimplantation embryos. It is known that faster developing blastocysts are of higher quality than slower developing blastocysts based on cell number, end-stage development and pregnancy rate after transfer. Therefore, we investigated whether embryos reaching the blastocyst stage at day 7 p.i. differ in expression of 6 developmentally important gene transcripts from blastocysts formed at day 8 p.i..

Three groups of blastocysts were collected from 1) immature oocytes from 3- to 8-mm follicles after IVM, IVF and IVC (SOF medium was used), 2) prematured oocytes from preovulatory-sized, FSH-stimulated follicles after IVM, IVF and IVC, 3) oocytes as in (2) but after *in vivo* maturation, IVF and IVC. In groups 1, 2, and 3 blastocysts and expanded blastocysts were collected at day 7 p.i. and early blastocysts and morulae were cultured for one additional day. On day 8 newly developed blastocysts and expanded blastocysts were collected. In these 3 groups similar numbers of embryos were analyzed and all groups showed the same differences between day 7 and day 8 embryos. Therefore these groups were combined (*in vitro* group) (Fig. 1). Furthermore a control group of blastocysts was collected from FSH-stimulated cows after fertilization and development completely *in vivo*. The blastocysts in this *in vivo* group were collected by flushing of the uterus at day 7 after AI (*in vivo* group).

For the analysis, a semi-quantitative RT-PCR was performed using rabbit globin mRNA as an external standard. Poly(A)RNA was prepared using a Dynabead oligo-dT mRNA purification kit (Dynal A.S., Oslo, Norway). PCR products were separated on 2% agarose gels and were stained with ethidium bromide. The gene transcripts were quantified by digital imaging and the relative abundances were calculated.

This study provided convincing data that blastocysts developed at day 7 p.i. differ in expression of several gene transcripts from blastocysts reaching that stage at day 8 p.i. It would therefore be tempting to suggest that these differences in expression could be related to the quality of the blastocysts. The mRNA levels of the day 7 blastocysts were, however, not unlike those of the *in vivo* embryos. For statistical analysis more replicates are needed.

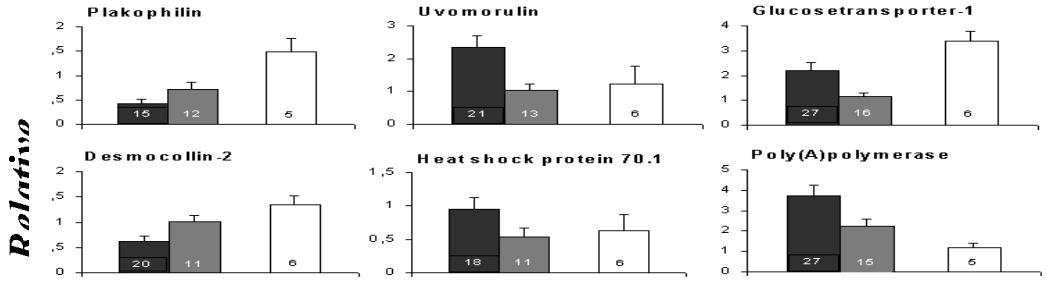

Fig. 1. Relative abundance of six gene transcripts (values shown as mean ± SEM) in *in vitro* – day 7 blastocysts (black bars), *in vitro* - day 8 blastocysts (dark grey bars) and *in vivo* – day 7 blastocysts (open bars). Numbers of embryos are indicated in the bars.

Notes

BOVINE EMBRYO TRANSFER TECHNIQUE EVALUATION

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Pregnancy rate of embryos frozen in the same conditions varies from 30% to 70% (Moroz T.A., Malinovsky A.M 1998), which requires an objective evaluation of both absolute and relative parameters of biological object viability, taking into consideration the recipient's physiological status. The aim of our work was development of an evaluation method for mammalian embryo transfer technique, using relative parameters of the biological object viability.

The subjects of the research were bovine embryos at the developmental stages from late morula to expanded blastocyst . Embryo freezing was performed using a device whose principle of operation is based on passive cooling of a cooling unit inside the X-34B Dewar the vessel neck (Ostashko F.I., Bezugly N.D. *et al.* 1991). Introduction of a quantitative parameter of embryo viability (95% is excellent quality, 85% is good quality, 70% is satisfactory quality, and 30% is poor quality) allows a quantitative assessment of the investigatel embryo pool (Gorbunov L.V., Bugrov A.D. *et al.* 2000).

To prevent the effect of variation in quality of biological objects on the assessment of embryo cryopreservation and transfer techniques, a relative parameter – survivability – was used. Survivability for the two techniques was determined as a ratio of thawed embryo viability to that of fresh ones and ratio of embryo pregnancy rate to viability, respectively. Embryo pregnancy rate was determined as a ratio of pregnancies to the number of transferred embryos. Whereas the conventional parameter of the embryos cryopreservation technique – survival rate – varies from 92.59 ± 46% for excellent embryos to 76.85 ± 2.39% (n = 331) for good quality, the relative parameter – survivability – is nearly the same for both groups 95.70 ± 1.13% and 94.48 ± 1.16%, respectively. Consistency of survivability values of frozen/thawed embryos is based on their viability. The same distribution pattern can be observed in the analysis of techniques applied for transfer of both fresh and thawed embryos of various quality. Pregnancy rates for native and thawed embryos are the same and different only among embryos of different quality: 53.20 ± 3.20% and 51.17 ± 1.16% for excellent embryos, 40.20 ± 3.10% and 37.80 ± 3.70% for good embryos, and 21.60 ± 5.90% and 19.10 ± 6.20% for satisfactory embryos, respectively. Survivabilities of transferred thawed excellent, good and satisfactory embryos are very close and 56.2 ± 3.8%, 47.12 ± 4.2%, and 45.04 ± 6.7%, respectively. Consistency of relative survivability values for transferred embryos of different qualities, in contrast to discrepancy of absolute survival rates, shows the effect of variance in biological objects on the technique evaluation results. The significant difference in survivability of thawed (95.11 ± 1.12%) and transferred (52.59 ± 3.28%) embryos of excellent and good quality demonstrates a relatively low level of embryos transfer technique efficiency on the one hand, and low resistance of transferred embryos on the other. The objective parameter of a transfer technique for bovine both native and thawed embryos of excellent, good and satisfactory quality is their survivability, which is 50.40 ± 3.16% (n = 1204) and 48.92 ± 2.91% (n = 886), respectively.

Notes

MILK PRODUCTION AND ENERGY METABOLISM AFFECT EMBRYO PRODUCTION IN MONTBELIARDE DAIRY CATTLE IN FRANCE.

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The relationship between milk production, nutrition and embryo production was investigated in 97 Montbeliarde dairy cows from 82 herds in Franche-Comté, France.

The cows were superovulated with a total amount of 320 μg (heifers, n = 19) and 500 μg (cows, n = 78) of FSH (Stimufol®, Merial, France) which was given as 8 injections of decreasing amounts over 4 days. An injection of cloprostenol (Estrumate®, Schering-Plough-veterinaire, France) was given together with the 5th FSH injection. Cows were inseminated 12 and 24 hours after standing estrus (Day 0). Embryos were collected on Day 7 in 92 cows. Five cows did not respond to treatment. Blood samples were collected on the day of the 1stFSH injection, on Day 0 and Day 7. Plasma cholesterol and triglyceride concentrations were measured. Composition of diet, body condition score (BCS) and body weight (BW) were recorded at the beginning of treatment. Milk production, milk protein and fat content were measured during the month preceding treatment. Cows were allocated to one of two classes according to Embryo/Unfertilized Ova (EUO) production (High, EUO ≥ 9, n = 46 vs Low, EUO < 9, n = 46) and to Transferable Embryo (TE) production (Embryos evaluated has 1 to 3 on the IETS scale, High, TE ≥ 5, n = 46, vs Low, TE < 5, n = 46). Individual characteristics and the percentage of requirements covered by the diet were compared between high and low producer cows by T test.

Cows were flushed 182 ± 213 days after calving and produced 8.4 ± 4.8 embryos/unfertilized ovas and 5.1±4.1 transferable embryos. High EUO produced more milk (34.6 ± 5.8 vs 30.9 ± 7.0 kg/day, $p<0.05$) with lower protein concentration (30.4 ± 2.0 vs 32.1 ± 3.4 g/kg, $p<0.05$) than low EUO. They had lower plasma cholesterol and higher plasma triglyceride concentrations than low EUO before stimulation, on Day 0 and on Day 7. High TE produced more milk (34.6 ± 6.5 vs 31.0 ± 6.2 kg/day, $p<0.05$) and tended to have higher plasma triglyceride concentrations than low TE before stimulation, on Day 0 and Day 7. BCS (3.1 ± 0.5), BW (674 ± 88 kg) and percentage of energy, protein, phosphorus and calcium requirements covered by the diet did not influence embryo production.

Table 1 : Plasma triglyceride and cholesterol concentrations compared between high vs. low EUO and TE

		Before supersitmulation	Day 0	Day 7
Triglyceride	High/low EUO	0.71±0.15 vs 0.62±0.18**	0.72±0.18 vs 0.65±0.17*	0.71±0.19 vs 0.64±0.19*
(g/l)	High/low TE	0.70±0.17 vs 0.64±0.17*	0.72±0.18 vs 0.66±0.16*	0.71±0.18 vs 0.64±0.19*
Cholesterol (g/l)	High/low EUO	1.76±0.59 vs 2.05±0.59**	1.72±0.59 vs 1.93±0.59*	1.76±0.60 vs 1.98±0.57*

*$p<0.10$ **$p<0.05$

As shown in previous reviews, there was a negative effect of high plasma cholesterol concentrations on production. Contrary to other studies, the cows which produced the least milk were those which produced the least EUO and TE. This, associated with the high triglyceride concentrations seems to indicate that there is a deficient in energy metabolism in the low EUO and TE. Plasma concentrations of non-esterified fatty acid, β hydroxy-butyrate, leptin, progesterone and estradiol are being analysed and should give more information about the energy status of the superovulated cows.

Notes

NUCLEAR AND MICROTUBULE BEHAVIOUR IN BOVINE SOMATIC NUCLEAR TRANSFER EMBRYOS.

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In bovine cloning, *in vitro* matured oocytes (MII) are generally used as recipient cytoplasts and different activation procedures have been used with success. In our bovine cloning protocol, i.e. simultaneous fusion and activation, 30 % of the reconstructed embryos reached the blastocyst stage (Vignon *et al.*, Theriogenology 2000; 53:245). It is generally assumed that the competence of IVM bovine oocytes in reprogramming of somatic cell nuclei is related to a high MPF activity which leads to premature donor chromatin condensation (PCC). The objective of this study was to examine the kinetics of cytoplasmic activation by analysing the interphasic microtubule distribution and the nuclear modifications in recipient oocytes and reconstructed embryos during our procedure of somatic nuclear transfer.

Bovine cumulus-oocytes complexes were recovered from slaughterhouse ovaries and matured *in vitro* for 23 h. Matured oocytes were denuded and enucleated within 1 hour to produce cytoplasts. For both groups, cytoplasts and reconstructed embryos, electric activation/fusion (two electric pulses of 2.0 kv/cm for 30 μs) was performed at 24 hours after onset of maturation.

MII oocytes used were divided into five groups: group A (n = 47), cytoplasts pulsed and incubated for 1h in 10 μg/ml cycloheximide; group B (n = 34), cytoplasts pulsed and incubated for 2h in cycloheximide and group C (n = 42), control MII oocytes. In some oocytes of groups A and B, only a cytoplasmic vesicle and the first polar body were removed instead of the metaphase plate. In group D (n = 34), quiescent skin fibroblast donor cells were fused with non-enucleated oocytes and in group E (n = 33), they were fused with cytoplasts. Reconstructed embryos were incubated for 1 h in cycloheximide. Cytoplasts and reconstructed embryos were fixed in 2.5% paraformaldehyde, incubated with anti-tubulin mouse monoclonal antibody and then, incubated with FITC-conjugated anti-mouse IgG. Nuclei were stained with 10 μg/ml propidium iodide. Cytoplasts and embryos were finally mounted onto slides and observed using a confocal laser scanning microscope.

In the group A, 13% of cytoplasts presented thin microtubules localized in the cortex with a nuclear progression to anaphase II and 87% showed no interphasic microtubules. In the group B, microtubules were present in 60% of cytoplasts and the oocytes reached the post-telophase stage (activation). In control group C, interphasic microtubules were not detected and all oocytes were still at MII stage. In groups D and E, 65% and 15% of foreign nuclei underwent condensation (PCC) respectively, ($P<0.05$).

Our results show that after nuclear transfer, using MII cytoplasts as recipients (group E), only a limited proportion (15%) of the transferred somatic nuclei are remodeled by PCC when observed at 1h post-fusion. This proportion of PCC was significantly higher (65%) when somatic cells were fused to non-enucleated oocytes. This suggests that removing of the maternal metaphase plate induces the loss of factors involved in the chromatin condensation. Together, these observations indicate that a prolonged chromatin condensation may not be essential to obtain a high rate of *in vitro* development of nuclear transfer embryos to the blastocyst stage.

Notes

THE ROLE OF CUMULUS INTEGRITY AND CULTURE CONDITIONS IN MATURATION AND *IN VITRO* DEVELOPMENT OF BOVINE OOCYTES

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The purpose of this study was to investigate the role of cumulus cells and culture conditions on maturation, fertilization and *in vitro* and *in vitro* development of bovine oocytes obtained from undefined stage follicles. In total 2,288 oocytes were used. Four categories of COCs were examined: control intact COC+ and COC+/-, COC-/+ and denuded COC-, which were prepared from selected COCs+ by mechanical removal of 1/3, 2/3 and entire cumulus, respectively. Oocytes maturel in M199 medium supplemented with gonadotrophins, ECS and with or without EGF and developed to blastocyst in Menezo B2 medium supplemented with ECS. Part of oocytes was used for kinase H1 assay which was used as a criteria of oocyte maturation quality and others were subjected to IVF.

The levels of activity of H1 kinase after 22h of maturation did not differ significantly among groups irrespective of the presence or absence of EGF in culture medium. This finding suggests that the presence of cumulus cells during maturation is not necessary for nuclear maturation of bovine oocytes *in vitro*. On the other hand, partial or entire removal of cumulus cells before maturation resulted in a significant decrease in the number of blastocysts (23.2% COC+; 18.2% COC+/-; 9.2% COC-/+ and 0.0% COC-, $p<0.05$). The negative of partial removal of cumulus cells was reversed in EGF supplemented coditions in COC groups (21.2 COC+; 22.0 COC+/-; 4.4% COC-/+ and 1.2% COC-).

The results suggest that the presence of cumulus cells during maturation is important for acquisition of developmental competence of oocytes *in vitro* and that EGF may improve maturation and developmental potential in COC with partially deficient cumulus.

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Notes

A DIET SUPPLEMENTATION WITH NUCLEOR TRANSPLAN IMPROVES EMBRYO QUALITY FOLLOWING SUPEROVULATION IN THE MONTBELIARDE BREED

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Nutrition is an important variation factor influencing embryo production results. Embryo recovery is mainly performed in high producing dairy cows, which need to be fed with high levels of energy. Nucleor Transplan (Chemoforma SA) is a diet supplement containing nucleotides precursors and barm, which aims to optimise the processes of digestion and nutriment resorption. This trial aimed to investigate the effects of a supplementation of donor cows with Nucleor Transplan (Chemoforma SA) on embryo production after superovulation in the Montbeliarde breed.

A total of 50 donor cows were included between November 1999 and October 2000 and were supplemented with 75 g per day from 4 weeks before superovulation to embryo recovery. The diet supplement was conditioned in sacks blindly numbered (1 to 50) and containing 2.6 kg of Nucleor Transplan (n = 25) or a placebo diet (n = 25). Each donor cow was superovulated with a total dose of 500 µg of pFSH (8 injections i.m. during 4 days; decreasing doses; STIMUFOL ND, Merial, Fr) together with a prostaglandin injection at time of the 5thFSH injection. AI were performed 12 and 24 hrs after standing oestrus. Embryos were collected on Day 7 and their viability estimated according to IETS morphological criteria. Individual parameters such as date and rank of calving, milk production level, fat and protein content of milk were also recorded.

The effects of the supplementation on embryo production results were assessed by ANOVA (SAS, GLM procedure). Results are presented as means ± s.e.m.

The rank of calving, the level of milk production and milk composition were comparable between both diet groups. The donor cows supplemented with Nucleor Transplan produced greater numbers of transferable and grade 1 embryos than the control donor cows (Table 1; $p<0.05$). Fat content of milk seemed to influence the quality of embryos, with a significant decrease of the number of viable embryos for fat contents lower than 34‰ and higher than 39‰ (<34‰ (n = 12) : 3.3 ± 0.9 ; 34-39‰ (n = 22) : 7.0 ± 1.0 ; ≥39‰ (n = 11) : 3.9 ± 1.4 ; $p<0.05$).

Table 1 : Effect of a supplementation with Nucleor Transplan during 4 weeks before superovulation on embryo production results

	Total	Transferable	Grade 1	Degenerated	Unfertilised oocytes
Nucleor Transplan (n=23*)	10.0 ± 0.9	6.7 ± 1.0[a]	3.4 ± 0.8[a]	1.5 ± 0.5	1.7 ± 0.7
Placebo (n=25)	8.4 ± 0.9	4.4 ± 0.7[b]	1.7 ± 0.5[b]	2.3 ± 0.4	2.0

a vs b : $P<0.05$; * one cow which deviated from the protocol was excluded, one cow has not been collected

To conclude, this trial shows that a supplementation with Nucleor Transplan may improve embryo quality in Montbeliarde donor cows. This positive effect should be further

investigated to explain the mechanisms which are involved and adjust the plane of supplementation.

Notes

SIMMENTAL SPERM SEPARATION PROTOCOLS, EVALUATION FOR IVF

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Simmental purebreed cattle is grown for meat and milk purpose in the farms of Croatia. Eighty percent of the herd is under selection programmes and subjected to A.I.. The recent adoption of *in vitro* technologies with aim to improve our selection possibilities give the opportunity to test our sires for the *in vitro* fertilization. In the first phase of this program we tested three protocols for sperm separation and clean-up for *in vitro* fertilization.

The sperm is routinely frozen in the Center for Reproduction and Animals Breeding of Croatia in a TRIS- Citrate-Egg yolk-Glycerol diluent, in 0.5 ml straws. Only straws with more than 50% post-thaw motility were stored for A.I. For this evaluation one sire was selected. The non return rate of this bull is 66.6 % over 2.910 inseminations. Four straws were thawed for each of five repetitions and the motility, concentration, membrane integrity and acrosome status were evaluated before and after sperm separation. Straws were thawed at 39°C for 1 minute, and the concentration was determined in a Thoma chamber. Motility was evaluated subjectively by a trained evaluator under phase-contrast microcospe. The sperm membrane integrity was evaluated using the HOS Test. The status of the acrosome was evaluated using the Trypan blus-Giemsa stain and the total number of spermatozoa with intact acrosome was counted under 400X magnification. The sperm separation and clean up for IVF were performed with three different protocoles. a) Diluation and Washing of the thawed sperm in Tyrode medium supplemented with Pyruvate and BSA and without Calcium (Medium without Calcium: M-Ca), two washings with 10 and 5 ml of medium respectively, centrifugated at 200 x g for 5min, the pellet was resuspended in medium for fertilization (mIVF: Tyrode supplemented with BSA, Penicillamine, Epinefrine, Heparine, Pyruvate and Caffeine). b) Swim up protocol: in M-Ca (1 ml, one hour at 39°C), the upper fraction of 1 ml was collected and washed twice with 3 ml of M-Ca and resuspended with mIVF. c) Discontinuous Percoll gradient centrifugation (45-90 % Percoll in M-Ca), centrifugated at 700 x g for 15 min, washed in M-Ca, and resuspended in mIVF. The final concentration of the sperm suspension was adjusted to $1x10^6$ spermatozoa/ml in the mIVF with oocytes. An average of 40 *in vitro* matured oocytes were used for each of five repetitions. The oocytes were fertilized with the separated frozen/thawed bull semen. Cleaved embryos were cultured *in vitro* in SOFaaBSA medium till the Day 10. On the 2nd day after IVF we registered the number of cleaved embryos, the total number of morulas (M) and blastocysts (B1) by day 7 and finally the number of hatched blastocysts (hB1) by day 10. The statistical comparison between protocols was done by ANOVA (GraphPad, Instat, V2.00) using the arcsin transformation of the percent values.

The initial parameters of the thawed sperm were: 105 x 10^6 (± 27) spermatozoa/ml and 70 % (± 7.1) progressive motility, 51% (± 19.8) membrane integrity and 12.5% (± 2.12) of spermatozoa with intact acrosome.

Table 1. Comparison of three sperm separation protocols: final sperm quantity and quality and IVP results.

Separation Protocol	Motility % (± S.D.)	Concentration x10^6 sp./ml (± S.D.)	Membrane Integrity % (± S.D.)	Intact Acrosome % (± S.D.)	Cleavage Day 2 % (± S.D.)	M+ B1 Day 7 % (± S.D.)	hB1 Day 10 % (± S.D.)
Washing	62 (7.6)	35.4 (10.4)[a]	60.5 (0.7)	48 (2.8)	76.6 (12.2)	22.4 (8.2)	11.8 (6.5)
Swim up	65 (10.0)	11.0 (2.7)[b]	55 (4.2)	14 (5.7)	73.6 (16.6)	24.8 (17.9)	11.7 (10.4)
Percoll	69 (7.4)	70.0 (16.5)[c]	55.5 (12)	22.5 (14.9)	67.5 (13.8)	20.9 (8.8)	10.8 (7.6)

Values in the same column with different superscripts differ significantly (P<0.05).

Although the studied parameters showed differences in the final concentrations and slight differences in the percent of intact acrosome recovered after sperm separation protocoles, the sperm used for *in vitro* fertilizations showed the same results in terms of Cleavage, percent of Morulae and Blastocysts on Day 7 and hatched Blastocyts on Day 10 for all three protocols.

The final concentration and quality parameters of the separated sperm not seem to be related with the results of IVP, at least in the range obtained in our experiments. So, we cloncluded that all three protocols gave suitable sperm for IVF and can be used for *in vitro* production under our conditions.

Notes

DE-NOVO PROTEIN SYNTHESIS AND PHOSPHORYLATION BY *IN VIVO* AND *IN VITRO* PRODUCED CATTLE EMBRYOS AROUND COMPACTION

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The widespread exploitation of embryo based biotechnology in cattle is currently hampered by the reduced viability of *in vitro* produced (IVP) embryos. A major factor affecting the viability of IVP embryos is their failure, in a high proportion of cases, to undergo compaction in the manner of *in vivo* embryos and this failure to compact ultimately leads to developmental problems and compromise viability. The timing of compaction at around days 5 to 6 also coincides with the first period of embryonic loss *in vivo.* The objective of this study was to compare the rate of *de novo* protein synthesis and phosphorylation around compaction by *in vivo* and IVP embryos.

In vivo (n=137) and IVP (n=242) embryos from the 8-16 cell (pre-compaction) to the expanded blastocyst stage (post compaction) were cultured in KSOM under oil and gassed with 5%$C0_2$, 5%0_2 and 90%N_2. ^{35}S methionine (500µCi.ml-^{1}) or ^{32}P orthophosphate (1mCi.ml^{-1}) were added to measure protein synthesis and phosphorylation respectively. After 4 hr in culture the embryos were washed and lysed in 1% SDS buffer at 60°C. Radiolabel incorporation into trichloroacetic acid (TCA) insoluble material was determined by counting an aliquot of lysate on TCA impregnated filters. Data from six replicates were analysed by ANOVA using PROC GLM (SAS) using a model containing the effect of embryo source and developmental stage and their interaction. A probability value of $P<0.05$ was considered significant. Results are presented as arithmetic means ± S.E.M.

De novo protein synthesis increased in both *in vivo* and IVP embryos from the 8-16 cell stage to the blastocyst stage (Fig.1). The incorporation of ^{35}S methionine was higher ($P<0.05$) in IVP embryos compared to *in vivo* embryos at the 8-16 cell, compacting and compacted stages but was not different at the blastocyst stage. Incorporation of ^{32}P orthophosphate via ATP by *in vivo* or IVP embryos did not differ at the 8-16 cell or at compacting stages (Fig. 2). Following compaction incorporation of ^{32}P by *in vivo* and IVP embryos increased significantly ($P<0.05$) and remained so at the blastocyst stage. In contrast to ^{35}S methionine, ^{32}P incorporation did not differ between *in vivo* and IVP embryos at any developmental stage. Preliminary analysis by SDS-PAGE indicates increased synthesis by IVP embryos of a protein with an apparent molecular mass of 60kDa possibly a heat shock protein. The appearance of two newly phosphorylated proteins at compaction may be the first biochemical indicators of compaction.

These data indicate that significant changes in the rate and pattern of protein synthesis, and phosphorylation occur in cattle embryos around the timing of compaction. Differences in these activities also occur between *in vivo* and IVP embryos, and may be due to sub optimal culture conditions associated with the IVP system used in this study. Compaction in cattle embryos is, nevertheless, accompanied by synthetic changes that may need to be successfully completed in order to ensure continued embryo survival.

Acknowledgements. This work was supported by the EC 4th Framework Programme, Contract No. CT-98-0032

Notes

NEW DEVELOPMENTS IN A RAZA DE LIDIA EMBRYO TRANSFER PROGRAMME. 1/ USING F.S.H.

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After nearly 10 years of embryo transfer practice in the Spanish fighting cows Raza de Lidia we still could not obtain more than 1.6 embryos per flush. To increase the number of progeny per female flushed we tried to use a different treatment.
First we decided to use F.S.H. as the hormone for superovulation rather than P.M.S.G. and Neutra-P.M.S.G. (ND)

The major problem in this breed is the handling of animals because they are wild. Apart from feeding time they never see humans. The use of F.S.H. hormone for superovulation in domestic cattle is very successful but the F.S.H. must be injected twice daily over a period of three or four days. This practice is impossible in this breed. We ran such a programme with 8 cows 6 years ago and the results were very bad This is the reason we used the P.M.S.G.

N°cows treated	N°cows collected	N°embryos	N° good embryos	N° E / flush
12	8	14	9	1.1

In 2000 we tried to flush some cows using three injections of F.S.H. (day 1 morning, day 2 morning, day 3 morning with Prostavet (ND) and removal of CID'R (ND). This programme was acceptable for the animals, because the animals are caughted only once more than with the P.M.S.G. programme. Results obtained are sumarized below.

N° cows treated	N° cows collected	N° embryos	N° good embryos	N° E / flush
5	3	5	0	0

In January 2001, we ran another experiment with F.S.H. but this time to avoid handling of the animals, we used an osmotic pump introduced under the skin in the neck of the cow, with the following programme

Day 1: ovaries checking + put in CID'R (ND) without oestrogen
Day 8: put in pump with 35 mg of F.S.H.
Day 10: removal of the CID'R (ND)+ 2 ml prostavet (ND)
Day 12: removal of the pump + artificial insemination twice with fresh semen morning and evening
Day 13: artificial insemination in the morning

N° cows treated	N° cows collected	N° embryos	N° good embryos	N° E / flush
8	4	5	0	0

Our results using F.S.H. were very bad, but it was only one experiment with one group of cows and we must do some more experiments. However the animals' owner prefers to use the old protocol with P.M.S.G. We must also find a way of preparing the F.S.H. more easily because 80µl is a very small quantity to dilute the F.S.H.

Notes

NEW DEVELOPMENTS IN A RAZA DE LIDIA EMBRYO TRANSFER PROGRAMME. 2/ EMBRYO SPLITTING.

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As we explained in our previous paper about E.T. on Raza de Lidia (A.E.T.E. 2000), the number of embryos produced per cow is very low, undoubtedly caused by the stress of the animals during the treatement. So to improve the results of our E.T. programme we decided to split the embryos into two parts to obtain twins, to increase number of calves.

A first experiment was made in the Victorino Martin's ganaderia. This is one of the most famous in Spain and has been so for almost 25 years.The manager of the herd Dr. Victorino Martin is also a veterinary surgeon, so it was easier to run the programme here.

The aim of this experiment was to produce twins and also to implant each twin in female of a different breed to see if there is any difference between the behaviour of the identical twins carried by a domestic recipient or a Raza de Lidia recipient.

After a first visit to prepare the programme, a group of 10 donors was selected. A group of 12 recipients of the Raza de Lidia breed, and a group of 10 heifers of the Retinta breed (suckling spanish breed) was also synchronised. We used our previous protocol with CID'R, P.M.S.G. and neutra-P.M.S.G.

The embryos were flushed at day 7, then washed in holding medium 10 times before placing in the final solution. They were held with a micropipette and split in two identical parts. Each half was transfered in a recipient Raza de Lidia or Retinta.

Donors prepared	Donors flushed	Embryos recovered	Usable embryos	Embryos split	Half embryo transfered	Entire embryo transfered	PD+ with half	PD+ with entire	Calves born
10	8	22	5	2	4	3	0	0	0

These results show firstly that we had a problem with fertilisation of the embryos caused by the quality of semen used (only 5 good embryos). Secondly the recipients Retinta did not come into heat properly (only 2 transferable) and also not enough care was given to recipients after transfer.

We did a second experiment in May 2000 in France in Occitania, the farm where we usually run an E.T. programme. Preparation of donors and recipients was the same but we transfered the 2 half embryos into the same recipient (Aubrac breed).

Donors prepared	Donors flushed	Embryos recovered	Usable embryos	Embryos split	Entire embryo transfered	PD+ with two half	PD+ with entire embryo	Calves born	Paire of identical twin born
11	9	17	15	9	3	3	1	6	2

Unfortunately the pregnancy rate was not as good (33%): usually we reach 55% with fresh embryos. May be that the very high temperature on the day of the transfer caused the decrease.. However we are very pleased to have obtained two couples of identical female twins and proved that splitting embryos in Raza de Lidia is possible. That was the first stage of our project which is to obtain identical twins out of recipients from different breeds (Aubrac and Raza de Lidia) to investigate that the behaviour of the animals comes from genetics and not from conditions of breeding.

Notes

QUALITY OF *IN VIVO*- AND *IN VITRO*-PRODUCED OVINE EMBRYOS ASSESSED AFTER VITRIFICATION AND EMBRYO TRANSFER

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It is well known that *in vitro* produced embryos have an increased sensitivity to cryopreservation than their *in vivo* counterparts. The aim of this study was to examine the quality of ovine embryos produced *in vivo* following superovulation and embryo recovery or *in vitro* following maturation, fertilization and culture of ovarian oocytes.

For the production of blastocysts *in vivo*, donor ewes were (n = 30) synchronized using an 13 day intravaginal progestagen pessary (FGA, Chrono-gest, Intervet). They received 1250 IU eCG 2 days before pessary withdrawal. Ewes were mated 2 days after pessary withdrawal and embryos were recovered surgically 6 days later. Recipients (n = 95) were synchronized as above and received 500 IU eCG at the time of pessary removal.

Blastocysts were produced *in vitro* using standard techniques (Byrne *et al.* 2000, Anim Reprod Sci, 62: 265-275). Blastocysts produced by both methods were transferred to recipients (2 embryos per recipient) either fresh or following open pulled straw vitrification/warming. The results are shown in the Table.

Table 1. Pregnancy rate following transfer of *in vitro*- or *in vivo*-produced ovine embryos either fresh or following vitrification.

Embryo source		No. transfers	Pregnant (%)	Singles (%)	Twins (%)	Embryo Survival (%)
IVP	Fresh	35	19 (54.3)[a]	15 (79.0)	4 (21.0)	23 (32.8)[a]
	OPS	40	2 (5.0)[b]	2 (100.0)	0	2 (2.5)[b]
In Vivo	Fresh	10	9 (90.0)	3 (33.3)	6 (66.7)	15 (75.0)
	OPS	10	5 (50.0)	3 (60.0)	2 (40.0)	7 (35.0)

$P<0.05$

These results demonstrate that IVP embryos are inferior in quality to their *in vivo*-produced counterparts as measured in terms of their ability to establish a pregnancy following transfer. This difference is further attenuated following exposure to a stress such as cryopreservation. It is clear that conditions of *in vitro* culture must be improved in order to ensure the production of embryos of acceptable quality. In addition, the results highlight a gap between the viability of *in vivo* produced embryos transferred fresh or following vitrification suggesting that optimization of cryopreservation techniques is needed to reduce chilling injury.

Notes

STUDY OF THE FERTILIZING CAPACITY OF FROZEN BOAR SEMEN FOR AN HYPOTHETIC USE IN *IN VITRO* PRODUCTION EMBRYO SYSTEMS

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Polispermy still remains as one of the major problems found in *in vitro* production of pigs embryos (IVPe) (Long *et al*, Theriogenology 1999, 51:1375). The number of spermatozoa per penetrated oocyte is related to the fertilizing capacity of a semen sample (Gadea *et al.*, Anim Reprod Sci 1998, 54: 95). Since frozen boar semen show a reduced fertilizing capacity, normally due to its poor quality (Hofmo & Almlid, 2nd Conference on Boar Semen Preservation, 1991), beneficial consequences to this technology could be achieved. However, this suboptimal quality could also lead to reduced penetration rates and would counteract the hypothetic beneficial effect on polispermy. Therefore, we evaluated the suitability of boar semen frozen with an improved technology for an hypothetic use in an IVPe system.

Semen was frozen and thawed as described by Bwanga *et al.* (Acta vet scand 1991, 32:431) and evaluated for motility (MOT) and the percentage of live sperm cells with normal acrosomes (lvNAR). Cumulus-oocyte complexes were matured in TCM-199 supplemented with 10 ng/ml EGF, 400 ng/ml FSH and 10% follicular fluid for 44-48 hours. 25 denuded oocytes were then cocultured for 24 hours in 500 µl of tris buffered medium (TBM, Abeydeera and Day, Theriogenology 1997, 48: 537) with 50 x 10^3 frozen-thawed (FT) sperm cells (spz). As a first objective, we set out to evaluate the fertilizing potential of different FT ejaculates. Freshly collected semen samples, to whom no decrease in fertilizing abilities was supposed, were used as controls and to study the response in penetrating capacity with increased number of spz (x10^3: 25, 50, 250) in the fertilizing medium. The percentage of penetrated matured ova (PEN), the percentage of polyspermic penetrated ova (PS) and the number of swollen sperm heads and/or male pronucleus (mPN) were taken as measures of fertilizing capacity. Results are shown in Tables 1 and 2.

Table 1. Quality and fertilizing capacity values of frozen semen.

	SEMEN QUALITY		*PENETRATION RESULTS*		
	MOT (%)	*lvNAR*	*PEN*	*PS*	*mPN*
Frozen-A	*18.33 ± 2.89a*	*54a*	*42.86a*	*22.22*	*2.80 ± 1.55*
Frozen-B	*45.00 ± 5.00b*	*50ab*	*29.17b*	*17.86*	*2.60 ± 0.89*
Frozen-C	*25.00 ± 5.00a*	*45b*	*29.81ab*	*19.35*	*3.00 ± 1.09*
Frozen-D	*26.67 ± 2.89a*	*29c*	*11.49c*	*17.31*	*2.22 ± 0.44*
Frozen-E	*21.67 ± 2.89a*	*13d*	*15.04c*	*11.76*	*2.50 ± 0.71*

Within a column values with different letters differ significantly. (MOT: a, b: P<0.05, One-way ANOVA; lvNAR: a, b, c, d: P<0.05, Fisher's exact test; PEN: a, b, c: P<0.05, Fisher's exact test). Three replicates per sample. Fifty oocytes per replicate in each sample.

Table 2. Fertilizing capacity values of fresh semen.

	PEN			*PS*			*mPN*		
	25000	*50000*	*250000*	*25000*	*50000*	*250000*	*25000*	*50000*	*250000*
Fresh-1	*75a*	*94.44ab*	*100b*	*100*	*100*	*100*	*5±2.70a*	*6.29±2.20a*	*17.68±5.11b*
Fresh-2	*93.6*	*98.03*	*100*	*97.73*	*98.04*	*100*	*6.95±2.71a*	*11.21±3.23b*	*18.11±4.88c*
Fresh-3	*100*	*97.37*	*100*	*100*	*100*	*100*	*8.34±2.75a*	*12.46±4.21b*	*33.52±10.99c*

Within a row values with different letters differ significantly. (PEN: a,b: P<0.05, Fisher's exact test; mPN: a,b,c: P<0.05, One-way ANOVA). One replicate per sample. Fifty oocytes per replicate in each sample.

FT semen samples showed a highly reduced fertilizing ability, as it is borne out from values for fresh semen, whose penetration capacity also increases with greater number of spz. The maintenance of this ability is possible with frozen semen (Abeydeera & Day, Biol Reprod 1997, 57: 729), but preliminary experiences undertaken with sample E did not show this tendency. From results of samples D and E one may infer that a certain impairment between survival to freezing and capacity to support the fertilization process exists. Moreover sample A showed different penetration rates with regard to B, both having resisted the freezing process (lvNAR values) in a similar manner. Interestingly, the PS data of this good quality FT semen samples is lower than that normally reported in the literature, maybe reflecting a high loss of fertilizing capacity, but penetration rates would only make sample A suitable for a profitable IVPe. It is known that the evolution of the capacitation status (Wang *et al*, J Reprod Fertil 1995, 104: 305) and the study of sperm chromatin alterations of frozen semen (Royere *et al*, Int J Androl 1991, 14: 328) can be related to fertility. From the above results it is clear that deeper analysis for an accurate prediction of fertility are needed, since good survival rates of samples B and C do not explain their penetration results.

Notes

ABILITY OF THREE DIFFERENT ANTISERA TO RECOGNIZE PREGNANCY-ASSOCIATED GLYCOPROTEINS IN HEIFERS DURING THE FIRST FIFTY DAYS OF GESTATION

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Pregnancy-associated glycoproteins (PAGs) are expressed in the superficial layer of the ruminant placenta according to a temporal and spatial expression pattern and probably the PAG 67 is not the first molecule expressed in the trophoblast (Green *et al.*, Biol. Repr., 2000, 62, 1624-1631). Some of them are secreted into the maternal blood circulation providing a useful tool to follow up an ongoing pregnancy. This study was undertaken to determine the ability of three different antisera to recognize PAGs in blood samples collected frequently from three heifers through the first 50 days of pregnancy.

In the three RIA systems, a pure 67 kDa PAG preparation was used as tracer (labelled by iodine-125 isotope using the chloramin T method) and as standard. In RIA 1, the antiserum was produced against a pure preparation of 67 kDa PAG (PAG I_{67}) purified according to Zoli *et al.* (Biol. Repr., 1991, 45, 1-10). In RIA 2 and 3, the antisera were obtained by immunizing rabbits against two preparations of PAG purified from caprine cotyledons according to Garbayo *et al.* (Biol. Repr., 1998, 58, 109-115). The PAG profiles for the 3 heifers are shown in Fig. 1, Fig. 2 and Fig. 3, respectively.

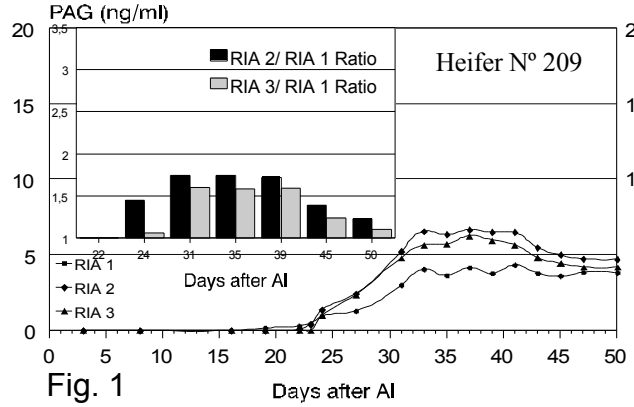

Fig. 1

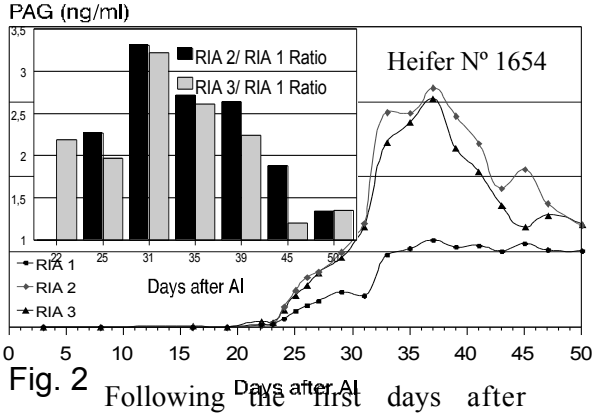

Fig. 2

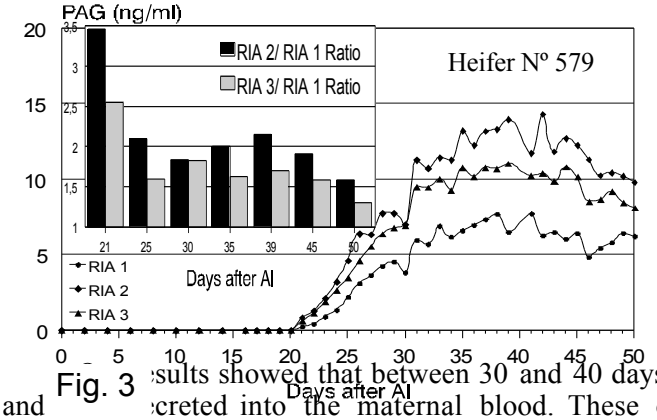

Fig. 3

Following the first days after fertilization PAG levels were undetectable in all the three RIA. PAG levels started to increase intensively from day 20-23 after AI. Between day 30 and 40, RIA 2 and RIA 3 systems showed higher concentrations when compared to those of RIA 1, the RIA 2/RIA 1 and the RIA 3/RIA 1 ratios were higher than 1.5 (Fig. 1-3).

Results showed that between 30 and 40 days after AI, PAGs better recognized by RIA 2 and [illegible] secreted into the maternal blood. These observations support the hypothesis on the early expression of PAG molecules different from PAG I_{67}.

Notes

BOVINE OOCYTES BLOCKED FOR 24 H IN GV STAGE BY A COMBINATION OF BUTYROLACTONE I AND ROSCOVITINE MAINTAIN A NORMAL DEVELOPMENTAL CAPACITY

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Butyrolactone I (BL-I) and Roscovitine (ROS), two specific and potent inhibitors of M-phase Promoting Factor (MPF) kinase activity, were used to block germinal vesicle breakdown (GVBD) of cattle oocytes. A concentration 6.25 μM BL-I and 12.5 μM ROS blocked over 93.3 ± 2.5% of oocytes at the germinal vesicle (GV) stage during a 24 h culture period. Following a second 24 h culture step in maturation medium (IVM) almost all (91.5 ± 3.0%) inhibited oocytes resumed meiosis and reached the metaphase II (MII) stage.

The MII kinetics was different for inhibited and control oocytes. 50% MII was reached at 13-14 h in BL-I + ROS treated oocytes, compared to 18 h in control oocytes. Therefore, control oocytes were fertilised (IVF) after 22 h IVM and inhibited oocytes after 16 h or 22 h IVM. After IVF, the percentage of grade 1 freezable embryos on day 7 (D + 7) as well as the percentage of blastocyst formation on D + 8 in the group of BL-I + ROS treated oocytes fertilised after 16 h IVM were higher ($p<0.05$) compared with the other experimental group fertilised after 22 h IVM but not different in comparison with the control.

Table 1: Development of controls and oocytes inhibited with BL-I + ROS

	n° of oocytes	% Cleavage	% Total Blastocysts (D + 7)	% Freezable Blastocysts (D + 7)	% Total Blastocysts (D + 8)
CTR-IVM	360	72.4 ± 4.8	25.7 ± 8.1	14.8 ± 4.4[a]	43.0 ± 7.8[a]
BL-I + ROS (IVF 16 h post-IVM)	280	73.2 ± 0.7	26.2 ± 9.3	12.1 ± 3.1[a]	42.4 ± 11.2[a]
BL-I + ROS (IVF 22 h post-IVM)	294	68.5 ± 4.4	16.2 ± 4.5	6.9 ± 4.5[b]	28.1 ± 7.5[b]

Student's t-test was used for statistical analysis.
Different superscripts within columns indicate significant differences ($p<0.05$) .

Survival to freezing and thawing of grade 1 embryos frozen on D + 7 was employed as viability criteria and was similar in all groups: there was no significant difference in viability and hatching rate between control and treated oocytes during 48 h of culture post thawing.

Thus, the presence of BL-I + ROS in the prematuration medium of bovine oocytes determines a reversible meiotic block, without compromising their subsequent developmental competence.

Notes

IN VITRO MATURATION OF BUFFALO OOCYTES VIS-À-VIS ESTRUS SERUM SUPPLEMENTATION OF CULTURE MEDIA

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Using a prospective comparative design, *in vitro* buffalo oocyte maturation, vis-à-vis culture treatments, with three basal culture media – Ham's F-10, Ham's F-12 and TCM-199; supplemented with 20 per cent estrus sera – buffalo (BES) and cow (CES) were evaluated. A total of 1020 cumulus oocyte complexes (COC) were aspirated from 902 buffalo ovaries, procured from a slaughterhouse. *In vitro* maturation (IVM) was carried out at 38.5°C in 5% CO_2 and 95% RH up to 30 hours. Maximum maturation rate of 55.6, 74.2 and 66.3 per cent were obtained in TCM-199 with BES, CES and BES+CES supplementation respectively at 28h of IVM. For Ham's F-10 and Ham's F-12 media the corresponding IVM rate were 48.7, 69.6 and 59.0, and 52.2, 70.9 and 61.3 per cent, respectively.

Table: *In vitro* maturation (%) of Buffalo oocytes: effect of estrus serum

Medium	Serum	COC	Hours after start of IVM				CD between IVM time
		(N)	22	24	28	30	
Ham's F-10	BES	111	22.1 ± 1.9	40.9 ± 3.3	48.7 ± 0.6	48.7 ± 0.6	5.5
	CES	115	35.4 ± 2.4	64.8 ± 1.5	69.5 ± 0.9	69.5 ± 0.9	4.7
	BES+CES	107	20.9 ± 3.1	53.9 ± 1.5	59.0 ± 1.0	59.0 ± 1.0	4.8
Ham's F-12	BES	109	20.2 ± 1.4	51.4 ± 1.6	52.2 ± 1.3	52.2 ± 1.3	4.1
	CES	114	49.0 ± 1.2	70.0 ± 1.1	70.9 ± 1.4	70.9 ± 1.4	4.9
	BES+CES	108	20.4 ± 3.6	58.2 ± 0.7	61.3 ± 1.1	61.3 ± 1.1	6.0
TCM-199	BES	117	30.0 ± 0.9	54.0 ± 0.9	55.6 ± 0.8	55.6 ± 0.8	3.6
	CES	117	41.9 ± 1.1	71.1 ± 0.7	74.2 ± 0.8	74.2 ± 0.8	3.4
	BES+CES	122	25.0 ± 1.7	60.7 ± 0.8	66.3 ± 1.0	66.3 ± 1.0	4.5

Data from 8 replications are pooled; values presented as Mean ± SEM; CD significant at $P<0.05$

IVM rates for TCM-199 were significantly higher ($P<0.05$) than for Ham's F-10 and Ham's F-12 media. Ham's F-12 resulted in higher IVM rates than Ham's F-10 but the differences were non-significant. The use of CES in culture media for IVM of buffalo oocytes was investigated for the first time and yielded better IVM rate than BES supplementation. TCM-199 as culture medium was superior to Ham's F-10 and Ham's F-12 for IVM of buffalo oocytes.

Notes

EFFECT OF MODIFICATION OF SYNTHETIC OVIDUCT FLUID (SOF) COMPOSITION ON YIELD AND QUALITY OF BOVINE BLASTOCYSTS

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The aim of this study was to investigate the effect of modification of the standard SOF recipe used in our laboratory on the yield and quality of blastocysts produced from bovine oocytes following maturation, fertilization and culture *in vitro*.

Twenty hours following IVF, zygotes were denuded and randomly allocated to 1 of 3 groups for IVC. They were cultured in (1) SOFAA containing 3 mg/ml BSA with 10% FCS added on Day 2 of culture, (2) SOFAA containing 3 mg/ml BSA without FCS, (3) SOFAA containing 16 mg/ml BSA without FCS. Cleavage rate was assessed at 48 h post insemination and blastocyst yield on Days 6 to 9. A representative number of Day 7 blastocysts were vitrified and warmed using the OPS technique. *In vivo* produced blastocysts were vitrified at the same time as a control.

Table 1. Development of bovine IVM/IVF zygotes in synthetic oviduct fluid

SOF	N	Cleaved %	Blastocyst Yield %			
			Day 6	Day 7	Day 8	Day 9
1	330	86.1	20.0[a]	32.1	41.2	42.1
2	391	84.1	4.6[b]	28.4	37.1	39.1
3	372	83.3	11.6[c]	27.7	35.8	36.6

P<0.05

Table 2. Survival of bovine blastocysts following vitrification/warming

SOF	N	Survival %			Hatched %
		24 h	48 h	72 h	
1	53	34.0[a]	32.1[a]	22.6[a]	9.4[a]
2	48	72.9[b]	64.6[b]	54.2[b]	31.3[b]
3	49	42.9[a]	36.7[a]	24.5[a]	10.2[a]
Vivo	23	100.0[c]	91.3[c]	69.6[b]	43.5[b]

P<0.05

These results demonstrate that the addition of FCS to SOF significantly increases the speed of embryo development, with more blastocysts appearing by Day 6. However, the overall blastocyst yield is unaffected. Increasing the concentration of BSA also increased the speed of development, though to a lesser extent than FCS. Blastocyst quality, as measured by survival following vitrification, was significantly reduced in the presence of FCS or an increased concentration of BSA.

Notes

DYE EXCLUSION TECHNIQUE TO ASSESS THE VIABILITY OF PRE-IMPLANTATION BOVINE EMBRYO BASED ON A MURINE MODEL

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Dye exclusion assay was evaluated for assessing viability of pre-implantation murine and bovine embryos along with morphological examination, rolling, sucrose-induced shrinkage and *in vitro* culture. Different concentrations of the dyes were also investigated to determine the optima. A total of 460 murine and 79 bovine embryos, on morphological evaluation revealed 71.52 and 69.62 per cent viable, respectively. Dye exclusion assay of murine embryos using Trypan Blue (0.05%), Eosin-Y (0.12mM), Rose Bengal Red '0.5mM) and Eosin-B (0.12mM) followed by developmental competence indicated 82.35, 81.25, 78.57 and 50.0 per cent viable, respectively. The corresponding values in case of bovine embryos were 80.0, 87.0, 75.0 and 61.5 per cent. The differences in viability estimates due to dyes were statistically significant ($P<0.05$). Sucrose induced shrinkage of murine bovine embryos indicated 71.06 and 65.45 per cent viable, respectively. Corresponding value by *in vitro* culture were 73.62 and 70.9 per cent. The differences between morphological assay and other methods were significant ($P<0.05$) but the differences between dye exclusion assay and developmental competence of embryos in culture medium were not statistically significant. Eosin-Y and Rose Bengal Red dyes were used for the first time.

Table Dye exclusion and developmental competence of morphologically viable bovine embryos

Dye/ concentration	No.	Morphology Viable		Dye exclusion Viable		Developmental competence after dye exclusion Viable	
		No	%	No	%	No	%
Eosin Y							
0.12mM	15	11	73	8	72.7	7	87.5
Eosin b							
0.12mM	24	18	75	13	72.2	8	61.5
Trypan Blue							
0.05%	20	15	75	10	66.6	8	80.0
Rose Bengal Red							
0.5mM	17	11	64.7	8	72.7	6	75.0
	76	55	72.36a	39	70.09b	29	79.38b

Figures with different superscripts vary significantly ($P<00.05$)

Notes

THE ASCORBIC ACID CONTENT OSCILLATIONS IN COW AND HEIFER OVARIAN, UTERINE AND ADRENAL TISSUES

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The ascorbic acid, accordingly the literary data, plays a key role in promotion of collagen synthesis, hormone production, and has an ability to protect cells from free radicals. These facts may explain its reproductive actions. Ascorbate accumulates in both ovary and testis. Both gonads exhibit cycles of tissue remodeling and of peptide and steroid secretion that can be assumed to be ascorbate-dependent. Ascorbic acid may also prevent gametes from damage by free radicals during production and fertilization. The supply of ascorbic acid to the ovary might be a limiting factor in the ability of the preovulatory follicle to grow in response to gonadotropin stimulation. It is concluded that ascorbic acid is a key compound in gonadal physiology

The present study had on its purpose the investigation of ascorbic acid concentration in homogenates of cow and heifer endometrium, ovarian stroma, corpus luteum, adrenal gland and in the fluid of the large ovarian follicles under conditions of sexual cycle, pregnancy and functional pathology of the ovary.

The results of the study are provided in the table.

Concentration of ascorbic acid (mkg / %) in some of the cow and heifer tissues during the sexual cycle, pregnancy and functional pathology of the ovary. M+m, n = 4

Cycle stage, ovary state	Endometrium	Ovarian stroma	Corpus luteum	Follicular fluid	Adrenal gland
Cows					
Follicular stage	26.7 ± 4.7	38.2 ± 5.8		10.3 ± 2.1	82.7 ± 19.4
Ovulation	27.0 ± 8.6	41.7 ±12.3		11.3 ± 3.3	92.3 ± 28.1
Luteal stage	27.3 ± 3.2	41.1 ± 7.2	110.7 ± 18.6	12.6 ± 2.2	96.5 ± 31.4
Pregnancy before D45	32.4 ± 6.4	50.6 ± 16.7	131.8 ± 24.3	18.3 ± 3.7	172.1 ± 42.0
Pregnancy after D45	24.3 ± 4.1	46.5 ± 15.0	125.3 ± 18.7	17.8 ± 5.2	160.8 ± 34.3
Luteal cyst	25.5 ± 6.7	48.4 ± 14.9	127.6 ± 39.2		108.2 ± 32.7
Follicular cyst	28.9 ± 8.2	40.7 ± 12.8			143.4 ± 43.0
Persistent corpus luteum	29.3 ± 7.6	32.5 ± 10.4	52.4 ± 15.1	15.4 ± 4.7	96.1 ± 27.9
Ovarian hypofunction	18.9 ± 6.1	32.0 ± 8.0			82.2 ± 22.3
Heifers					
Follicular stage	22.3 ± 5.3	37.5 ± 4.8		12.8 ± 4.1	81.0 ± 22.7
Ovulation	23.8 ± 4.9	40.2 ± 12.5		14.7 ± 3.8	104.6 ± 29.1
Luteal stage	24.2 ± 7.3	43.3 ± 9.7	100.4 ± 23.0	16.2 ± 3.5	120.3 ± 17.8
Ovarian hypofunction	16.1 ± 4.4	26.8 ± 8.2			62.8 ± 12.4

These data suggest that ascorbic acid concentration in all investigated tissues is under direct dependence of ovarian steroidogenic activity, and especially—the luteal function competence. This competence is highest on the beginning of the pregnancy and during the luteal stage of sexual cycle, when corpus luteum plays the role of the main source of progesterone in the body.

The highest ascorbic acid concentrations are in the tissues of adrenal gland and corpus luteum where active steroidogenesis takes place.

All these results correspond with our previous data that tissue respiration processes, namely oxidative phosphorilation and respiration enzymes activities, in cow and heifer ovarian, uterine and adrenal tissues mitochondria are higher in animals with actively functioning corpus luteum or placenta.

Notes

MATERNAL INFLUENCE IN BOVINE OOCYTE AND *IN-VITRO* EMBRYO PRODUCTION

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The oocyte is an essential element in embryo development. Oocyte developmental competency is influenced by the maturation conditions and the oocyte capacitation, which is the preparation of the oocyte during folliculogenesis and later phases of follicular growth. Although the paternal effect in bovine embryo development has been well documented, there is still a lack of information regarding the maternal influence in *in vitro* embryo production. The objective of this study was to evaluate the maternal influence in oocyte capacitation and blastocyst formation using repeated ovum pick-up (OPU) and *in vitro* fertilization (IVF) on the same donors with varied genetic origin.

Six Holstein cows of similar age (3-4 years) raised in the same farm, with the same nutrition and without any common relative for at least two generations were selected. They were estrous synchronized (CRESTAR®-INTERVET) and OPU (twice weekly) started on day 4 after induced estrus for an average of 9 weeks on each of the 4 experimental sessions. The recovered oocytes (from experimental sessions one to three) were matured for subsequent IVF. The sperm from three bulls with high *in vitro* fertility (one bull for each session) was used. Oocyte and blastocyst production data for each cow were recorded and statistically analyzed using SPSS.

A total of 1946 oocytes were recovered in 73 "OPU days", with a mean of 4.64 ± 0.29 oocytes/cow/OPU and ranging from 0 to 22. The mean oocyte production in each experiment sessions (1 to 4) was $4.53^{a} \pm 0.45$, $5.89^{a} \pm 0.63$, $3.54^{b} \pm 0.51$, and $3.62^{a} \pm 0.61$ respectively (values with different superscript differ significantly at the 0.05 level). This statistical difference can be attributed to the fact that one cow performed poorly after a temporary health problem. Despite this variance, when cows were ranked using multiple stepdown range tests (MSRT) and compared for oocytes production, the order (statistical ranking) was preserved in each session.

Blastocyst production was analyzed as a pool as well as individually for each of the experimental session. The mean pooled (sessions 1 to 3) blastocyst rate was 28.7 ± 3%. Mean individual blastocyst rate production ranged from 52.4 ± 12% to 11.7 ± 5%. Session blastocyst rate differed significantly with mean rates of $35.8^{a} \pm 5.8\%$, $19.7^{b} \pm 4.9$ % and $31.1^{a} \pm 7.8$ % (values with different superscript differ significantly at the 0.05 level) for session 1, 2 and 3, respectively. This difference can be explained by the paternal effect (three genetically different males) and/or by the fact that for the experimental session 2 FIV was made on a newly established on farm laboratory. Again, despite this variation, when cows were ranked for blastocyst rate (MSRT) the rank order was highly preserved (in all three sessions) despite the use of semen from different bulls. Results also revealed a negative correlation ($\rho = -0.07$) between oocyte production and blastocyst rate.

The data supports the hypothesis of maternal (genetics) influence on *in vitro* embryo production. Further studies are necessary to identify the reasons behind oocyte developmental differences: timing, gene expression, metabolism, etc…. Identification of the factor(s) may enable a fertility laboratory test to be performed in animals before entering an *in vitro* embryo production programme.

Notes

SEMEN BATCH EFFECT ON *IN VITRO* BOVINE EMBRYO YIELD

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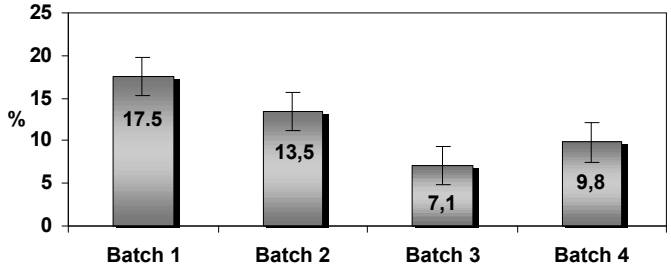
Data from a commercial laboratory producing *in vitro* bovine embryos were analysed with the aim of finding out whether the semen batch significantly affected embryo yield or not.

Routine work consisted in the collection of oocytes from ovaries of slaughtered heifers of various breeds, by means of slicing the ovarian cortex. Cumulus-oocyte complexes (COCs) were matured in TCM-199 medium supplemented with 1 mg/ml BSA, 1μg/ml estradiol, 0.5 μg/ml p-FSH and 0.03 I.U. hCG (Eckert *et al.*, 1995, Theriogenology, 1211-1225). Semen came from a commercial distributor and it met standard commercial requirements; four batches, from a single Blue-Belgian bull, were included in the analysis.

Fertilization took place in Fert-talp medium supplemented with 6 mg/ml BSA and 10 μg/ml sodium heparin, after selection of motile spermatozoa through a swim-up procedure; sperm concentration was adjusted at $1.0x10^6$ sperm/ml. After 20 hours of co-incubation, presumptive zygotes were cultured in TCM-199 medium supplemented with 3 mg/ml BSA. Maturation, fertilization and culture were carried out in 100 μl droplets, under paraffin oil, at 38,5 °C and in a humidified atmosphere with 5% CO_2. Cleavage rate and number of freezable embryos (morphological evaluation) were recorded on D-7 of culture. Data were analysed with a one-way ANOVA considering the effect of semen batch. The minimun number of replicates for a batch was 2 and the maximun was 6, for an overall number of 1,740 oocytes.

Embryo yield, expressed as the percentage of morulae and blastocysts over the total number of oocytes (Fig. 1) varied from 7.1% to 17.5% . The effect of semen batch on embryo yield approached but did not reach a statistical significance (P = 0.07). Cleavage rate and percentage of morulae and blastocysts over cleaved oocytes, were not affected by the batch of semen either.

Figure 1. Percentage of morulae and blastocysts (on the total number of oocytes) for each batch of semen.



One of the factors which is generally recognized as responsible for the large variability of *in vitro* bovine embryo production is the different bull fertility. Previous researches already investigated the variability among ejaculates or semen batches of single bulls, but the conclusions are not univocal. Otoi *et al.* (1993, Theriogenology, 713-718) and Astiz Blanco *et al.* (1999, XV AETE Meeting, 118) did not find any significant variation among different lots of semen while Zhang *et al.* (1997, Theriogenology, 221-231) showed a great variability among batches.

We conclude that under our working conditions, embryo yield was not affected by the different batch of semen.

Notes

MORPHOLOGICAL CARACTERISATION OF MORULAE DERIVED FROM PREPUBERTAL EWES OOCYTES

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The objective of this experiment was to determine the capacity of the prepubertal ewes' oocytes to undergo "*in vitro*" maturation and fertilization and the early development of embryos in culture. Prepubertal ewes' oocytes were obtained by ovariectomies from 9 females (8-10 month) with induced oestrous and superovulated in 12 days with intravaginal progestagen pessary and PMSG (FGA, chronogest, Intervet).The procedure used for "*in vitro*" maturation and fertilization were similar to the method of INRA–PRC Nouzilly (Poulain N., Cognie Y., 2000). Ovaries collection was made by ovariectomied of superovulated prepubertal ewes. The ovaries were kept for 10-20 min in sterile PBS at 35°C. Cumulus oocyte complexes were aspirated from 1-6 mm follicles. Oocytes with cumulus enclosed were maturated for 24 hours in M 199 with 10% follicular liquid and FSH (100 ng/ml) at 38.5° in an atmosphere of 5% CO_2 in air at maximum humidity. Freshly collected ram semen was capacitated in DMH medium supplemented with 20% ewes oestrous serum and then used for fertilization of denuded oocytes. After 24 h, (2 replicates) IVF oocytes were cultured in SOF medium for 6 days. The results of this experiment are presented in Tables 1 and 2.

Table 1 Oocyte collection from superovulated ovaries of prepubertal ewes

Experiment	Female ewes	Recuperated oocytes (n) from follicles (mm)			Total
	(n)	1-2	3-4	5-6	Oocytes (n)
1. (10.10.2000)	4	32	18	12	62
2. (18.11.2000)	5	40	21	11	72

Table 2 *In vitro* fertilization and development of prepubertal ovine oocytes

Experiment	Selected Oocytes	IVM		IVF		Cleavage		M		Bl	
	(n)	n	%	n	%	N	%	n	%	n	%
1	59	48	81.5	36	75.0	23	63.8	15	65.	3	20.
2	61	43	70.9	33	76.4	25	75.5	17	68.0	6	35.9

M-morulae; Bl-blastocyst;

The experiment shows that prepubertal sheep oocytes obtained are capable to producing morulae and blastocysts by totally "*in vitro*"procedure. The difference in efficiency of embryos development are discussed in relation with follicular diameter.

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