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Original article

Survival and viability of fresh and frozen-thawed *in vitro* bovine blastocysts

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Summary — Three experiments were undertaken to investigate the effect of some factors on survival and viability of bovine blastocysts produced *in vitro* in serum-free oviduct-conditioned media. *In vitro* survival to freezing was higher for blastocysts appearing on days 6 and 7 than for day 8 blastocysts (67 and 50% hatching rate *vs* 12%). Transfer success of day 8 blastocysts to recipient heifers was 35% (9/26) calves born *vs* 19% (4/21) for cows. Embryo mortality occurred mainly during the peri-implantation period (between days 21 and 45) for cows compared with heifers. Calving rate after direct transfer of frozen blastocysts without dilution was 24% (4/17) compared with 31% (5/16) for control unfrozen blastocysts.

bovine / *in vitro* / embryo / freezing / transfer

Résumé — Survie et viabilité de blastocystes bovins frais et congelés produits *in vitro*. *Des blastocytes bovins obtenus* in vitro *dans un milieu conditionné par des cellules d'oviducte en l'absence de sérum ont été congelés dès leur formation depuis le jour 6 jusqu'au jour 8. Les taux d'éclosion* in vitro *ont été respectivement de 67%, 50% et 12% pour les blastocystes des jours 6, 7 et 8. Le taux de vêlage après transfert de blastocystes de J8 à des génisses receveuses est de 35% (9/26) comparé à 19% (4/21) lorsque les receveuses sont des vaches. Chez ces dernières, la mortalité embryonnaire se produit essentiellement pendant la période péri-implantatoire. Le taux de vêlage après transfert direct sans dilution de blastocystes congelés est de 24% (4/17) et celui après transfert de blastocystes frais de 31% (5/16).*

Bovin* / in vitro / *embryon / congélation / transfert

INTRODUCTION

The first calf produced from *in vitro* fertilization of an *in vivo* matured oocyte was a male born in 1981 (Brackett *et al,* 1982). Over the past 10 years, major advances have been made in controlling the *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of cow oocytes and eggs and several groups have reported the occurrence of pregnancies and the birth of calves following IVM and IVF (see review by Greve and Madison, 1991; Mermillod *et al,* 1992a). Information on the viability of IVM/IVF/IVC embryos is obviously critical to the future use of these *in vitro* techniques in animal breeding. For example, the so-called MOET (multiple ovulation and embryo transfer) plan aims at establishing nucleus breeding herds, and also at widening the application of embryo transfer technology on the farm (Gordon and Lu, 1990; Gordon, 1991; Van Soom *et al,* 1994).

Reports on the cryopreservation of *in vitro*-produced embryos are more recent (see Leibo, 1992; Leibo and Loskutoff, 1993) and survival after thawing is quite variable. Here again, full documentation of the birth and viability of animals produced from cryopreserved *in vitro* embryos is scarce (Goto *et al,* 1989; Tachikawa *et al,* 1993; Zhang *et al,* 1993). Many factors other than those inherent to the *in vitro* technique itself affect the viability of *in vitro*-produced embryos.

The objectives of this study were: 1) to test *in vitro* survival after freezing of embryos reaching the blastocyst stage on days 6–8 of culture, in serum-free, conditioned medium; 2) to evaluate the viability of bovine blastocysts produced *in vitro* in a serum-free conditioned medium after transfer to recipient heifers or cows; and 3) to compare the developmental competence of control unfrozen blastocysts with that of frozen-thawed blastocysts, both groups of which were produced *in vitro* in serum-free medium, by transfer into recipient heifers.

MATERIALS AND METHODS

In vitro *production of embryos*

Ovaries from adult cows were collected from a local abattoir and transported to the laboratory in a 9‰ saline solution at 20–25°C. The contents of small follicles (2–6 mm) were aspirated and oocytes surrounded by several layers of compact cumulus cells were selected. Intact cumulus oocyte complexes were matured in tissue culture medium 199 supplemented with 10% heat-treated fetal calf serum and hormones pFSH, p LH and oestradiol (100 oocytes in 500 µl maturation medium). After 24 h at 39°C under 5% CO_2 in a humidified air atmosphere, cumulus-expanded oocytes were fertilized in 4-well tissue culture plates by 10^6 Percoll-treated frozen-thawed spermatozoa in 250 µl/well of heparin (10 µg/ml) supplemented tyrode albumin lactate pyruvate (TALP medium). Oocytes and sperm were co-incubated for 18 h. Presumed zygotes were vortexed to remove cumulus cells and cultured for 8 d (210 h post-insemination) in droplets (50 embryos/50 µl) of serum-free oviduct conditioned 199 medium under mineral oil (Mermillod *et al,* 1992b and c). Conditioned medium was prepared by seeding oviduct cells in 25 cm^2 flasks in M 199 + 10% fetal calf serum. When the cells reached confluence (after 6 d) the oviduct monolayer was washed thoroughly with pure M 199 and then this same medium was incubated with the cells 3 times 48 h and the 3 fractions were pooled, aliquoted and stored at –80°C.

Use of embryos

Embryos were produced both in France at the INRA Physiology of Reproduction Station in Nouzilly and in Belgium at the University of Louvain in Louvain-La-Neuve. Three experiments were undertaken.

Experiment 1

This experiment tested the *in vitro* survival after freezing of embryos reaching the blastocyst stage on days 6, 7 or 8 of culture and was performed in Belgium. All expanding blastocysts appearing on days 6, 7 or 8 of culture (162, 186 and 210 h post

insemination, respectively) were picked up. Embryos with a good morphological aspect were exposed to the freezing medium for 10 min at room temperature (20–25°C): glycerol (1.4 M) and sucrose (0.25 M) in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS). Groups of 5 embryos were loaded in 0.25 ml French straws (IMV, France) and frozen-thawed according to Massip *et al* (1993a). After thawing (15 s in a 20°C water bath), the contents of each straw was expelled into a petri dish and mixed by shaking rapidly; after 10 min at room temperature, embryos were washed twice in PBS for 10 min. They were then cultured on feeder-layer of mouse inactivated fetal fibroblasts (as described for ES cell culture in Rudnicki and McBurney, 1987) in 4-well plates (NUNC) with 500 µl of 199 medium supplemented with 10% FCS. Survival (reexpansion) was scored after 24 and 48 h and hatching rate after 72 h. As a control, fresh day 7 *in vitro* produced blastocysts were cocultured in the same conditions.

Experiment 2

This experiment involved the evaluation of the viability of blastocysts transferred to recipient cows or heifers with normal cyclicity. This experiment was performed in France. Grade 1 or 2 day 8 blastocysts were non-surgically transferred individually to day 7 Holstein recipient heifers (21 grade 1 and 5 grade 2 embryos) or cows more than 3 years old which had calved at least twice (16 grade 1 and 5 grade 2 embryos). The proportions of grade 1 and 2 embryos was similar between heifers (5/26 = 19% grade 2) and cows (5/21 = 24% grade 2). The recipients belonged to the experimental herd of the station and were synchronised with a Norgestomet ear implant. Pregnancy was diagnosed by progesterone assay on day 21, echography on day 35 and rectal palpation on day 120 and follow up between these dates by daily examination of returns in heat.

Experiment 3

This experiment was a comparison of the viability of *in vitro* produced blastocysts transferred to recipient heifers either without freezing or after freezing and thawing. For this purpose, embryos were obtained and transferred in Louvain-La-Neuve. A control group of 16 unfrozen blastocysts were transferred individually soon after their first appearance in culture (days 6–8) to 16 recipient heifers during their natural cycle. A group of 17 blastocysts was frozen when they first appeared in culture from day 6–8 and transferred directly after thawing (without dilution) to 17 synchronous or –1 d asynchronous recipient heifers during their natural cycle. Pregnancy was diagnosed by progesterone determination on day 21, PAG (pregnancy associated glycoprotein assay) from day 35 to 63 post-oestrus (Zoli *et al*, 1992) and rectal palpation on day 90. Birth and normality of calves were scored for each transfer protocol.

Statistics

The data in tables I–III were compared using chi-square analysis. The data in figure 1 were compared using the SAS procedure lifetest (Cox and Oakes, 1984).

RESULTS

In vitro *survival of blastocysts frozen-thawed on days 6, 7 or 8 of culture*

The results of survival for blastocysts frozen on day 6, 7 or 8 are given in table I. They

Table I. *In vitro* survival and hatching of bovine *in vitro*-produced blastocysts frozen at day 6, 7 and 8 of culture. *

Day of freezing	n	*24 h survival (%)*	*48 h survival (%)*	*72 h hatching (%)* [d]
D6	37	73[a]	62[a]	67[a]
D7	38	79[a]	76[a]	50[a]
D8	47	34[b]	32[b]	12[b]
Unfrozen	27	100[c]	100[c]	60[a]

* These results were presented as a poster at the *8th Meeting of the European Embryo Transfer Association* (Mermillod *et al*, 1992d); [abc] values within the same column with different superscripts are significantly different (chi-square, $P < 0.05$); [d] hatching rate was calculated from 24 h surviving embryos.

Table II. Pregnancies after transfer of day 8 *in vitro*-produced blastocysts to day 7 synchronous heifers or cows.

Recipient	*No of transferred embryos, 1 embryo/recipient,* n	*Pregnancies*			*Calves born,* n
		D21 n *(%)* [a]	*D35* n *(%)* [a]	*D120* n *(%)* [a]	
Heifers	26	17 (65)	15 (58)	9 (35)	9 (6 M,3 F)
Cows	21	13 (62)	9 (43)	4 (19)	4 (4 M)
Total	47	30 (64)	24 (51)	13 (28)	13 (10 M, 3 F)

[a] All the percentages are with respect to the number of transferred embryos.

Table III. Pregnancies following transfer of unfrozen or frozen-thawed *in vitro*-produced bovine blastocysts. *

Embryos	*No of transferred embryos,* n	*Pregnancies*			*Calves born,* n *(%)*
		D21 n *(%)* [a]	*D35* n *(%)* [a]	*D90* n [a]	
Unfrozen	16	5 (31)	5 (31)	5 (31)	5 (31) (4 M,1 F)[b]
Frozen-thawed	17[c]	7 (41)	5 (29)	4 (24)	4 (24) (3 M,1 F)

* These results were presented as a poster at the *9th Meeting of the European Embryo Transfer Association* (Massip *et al,* 1993b); [a] all the percentages are with respect to the number or transferred embryos; [b] 1 calf died 2 d after birth (cardiac anomaly); [c] at the time of revision of the manuscript, 2 more day 6 blastocysts (17 + 2) have been transferred successfully after 1 year of storage in LN_2 increasing the pregnancy rate by day 35 to 37%.

indicate that the survival of the embryos that developed into blastocysts on day 6 or 7 is significantly higher than for day 8 blastocysts (73 and 79% *vs* 34% after 24 h; 62 and 76% *vs* 32% after 48 h) but lower than controls (100%) ($p < 0.05$). On the other hand, the hatching rates of frozen day 6 and 7 blastocysts were similar to that of non-frozen controls. Frozen day 8 blastocysts had a poor hatching rate.

Viability of transferred day 8 in vitro-*produced blastocysts to heifers or cows*

The pregnancy rate did not differ significantly between heifers and cows either on day 21 (65 *vs* 62%) or day 120 (35 *vs* 19%) (χ^2 test, table II). In heifers, 8 embryonic deaths occurred between days 21 and 120 (47%), 4 between 21 and 45 d, 3 between 46 and 100 d, and 1 at 108 d. In cows, 9

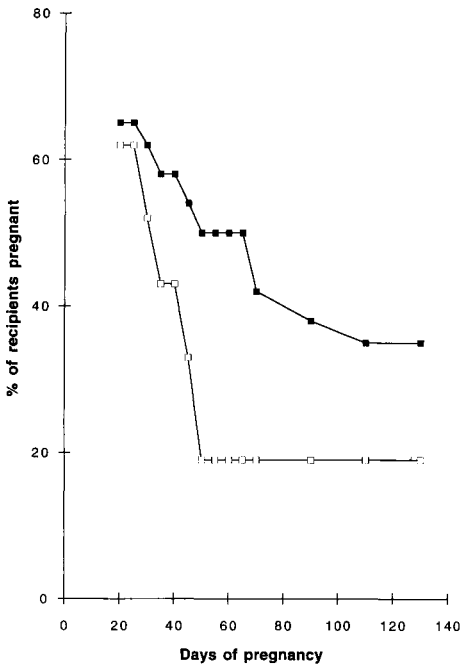

Fig 1. Pregnancy course after transfer of day 8 *in vitro*-produced blastocysts to day 7 synchronous recipient heifers (■, $n = 26$) or cows (□, $n = 21$).

embryos died, all of them between days 21 and 49. Thirteen calves were born (10 m, 3 f) weighing from 43 to 65 kg but 4 died (2 dystocic calvings, 1 cardiac malformation and 1 harelip). The average pregnancy length was 280 d (259–301). Figure 1 shows the loss of pregnancies with time. A marked drop was observed during the periimplantation period (days 25–40) in cows.

Viability of frozen blastocysts transferred directly after thawing

Of 16 control blastocysts (9 day 8, 6 day 7 and 1 day 6) transferred without freezing soon after culture, 2 day 8 and 3 day 7 (= 31%) developed to term and gave birth to 5 normal calves (4 m, 1 f) weighing 40–60 kg (table III). The mean gestation length was 290 d (277–295).

Seven pregnancies were initiated after direct transfer of 17 frozen-thawed blastocysts (1 day 8, 9 day 7 and 7 day 6) and 4 (24%) were maintained to term (2/9 day 7 and 2/7 day 6) giving 4 normal calves (3 m + 1 f) between 40 and 60 kg after a mean gestation period of 290 d (276–310). Two more pregnancies from day 6 blastocysts have been confirmed recently, increasing the pregnancy rate to 37% (7/19) at day 35.

These low numbers did not allow statistical comparison of calving rates after transfer of unfrozen and frozen blastocysts. Embryonic mortality occurred only in the frozen group between day 21 and 35 for 2 embryos and after day 35 for 1 embryo but a high proportion of transferred embryos did not maintain pregnancy until day 21 (69 and 59% respectively).

DISCUSSION

In these experiments, the culture solution was tissue culture medium TCM 199 conditioned by oviduct cells in the absence of serum.

The results in table I show that blastocysts appearing on days 6 and 7 of culture had a higher post-freezing survival rate than those appearing on day 8. As shown by the data in table III, only those embryos frozen as blastocysts on day 6 and day 7 developed to term after transfer. Similar observations were reported by Del Campo *et al* (1993) and Massip *et al* (1993b). A time-lapse analysis of embryo development in our conditioned media (Grisart *et al*, 1994) showed a link between early cleavage kinetics and further development. The results from cytogenetic studies by Plante and King (1992) show that as the interval between insemination and cleavage increases, the ability to develop to the hatched blastocyst

stage decreases. This failure in development may be related to the higher incidence of abnormalities in the 'late' groups (King, 1991).

A significant difference could not be demonstrated in the pregnancy rates in heifers or cows after transfer of day 8 blastocysts either at days 21 or 120 (table II). However in cows, the 9 embryo deaths occurred between days 27 and 49 of gestation, whereas, in heifers, only 4 of the 7 embryo deaths were noted during this interval, suggesting a problem in uterine environment at the time of implantation (Wathes, 1992).

The pregnancy rate of 35% in heifers is comparable to that reported by Reichenbach *et al* (1992) after unilateral transfer of 129 *in vitro* late morulae and blastocysts to −1 day asynchronous recipient heifers (38%). In their study, 43.4% of transferred embryos failed to initiate pregnancy and the embryonic mortality rate between days 21 and 250 was 32.9%. In our case, the respective values in heifers were 35 and 47%. In the practice of embryo transfer, embryos are generally transferred into heifers because the results are known to be more consistent.

As shown by the results in table III, the calving rate with unfrozen embryos was 31%. In this case, 69% (11/16) of transferred embryos failed to maintain pregnancy until day 21 but there was no embryonic mortality after day 21. After transfer of frozen embryos, 59% of recipients did not maintain pregnancy until day 21. The reason for such poor results compared with those from *Experiment 2* is unknown. In *Experiment 3,* which was carried out in Belgium, recipients were provided by a salesman without knowledge of their gynaecological background and they were used several times because of their small number. On the contrary at the INRA Station in Nouzilly (France), the recipients were selected from their own experimental herd, synchronised with ear implants and used only once or twice *(Experiment 2).*

Calving rate after direct transfer of frozen embryos was 24% (4/17). Seven pregnancies were initiated, although 2 mortalities occurred between days 21 and 35 and 1 between day 35 and term. Two additional pregnancies resulting from the transfer of 2 day 6 blastocysts have recently been confirmed (one at 3 months, the other at 35 d). A recent publication by Tachikawa *et al* (1993) reported the birth of 5 live calves from 20 *in vitro* blastocysts vitrified and transferred to recipients and 5 calves from 22 fresh embryos.

Reichenbach *et al* (1991) have frozen day 6 *in vitro* late morulae and early blastocysts. After thawing, the investigators cultured them for 8 to 10 h and selected only the viable ones for transfer. Of 248 frozen and thawed embryos, 65 (26%) were transferred, giving a pregnancy rate on day 35 of 43% (28/65) and representing 11% of total frozen embryos. In a study by Kajihara *et al* (1992), IVF-derived embryos that developed into blastocysts after 168 to 192 h post-fertilization (day 0 = day of fertilization) (corresponding to 186 and 210 h in our case, day 0 = first day of culture) were frozen by a classical method in 10% glycerol with 0.2 M sucrose solution. After thawing, they were transferred to recipient heifers ($n = 408$) or cows ($n = 458$) on days 6 to 9 of the oestrous cycle. Total pregnancy rate (between 50 and 90 d) was 40.5% for heifers and 35.2% for cows. Transfer on day 7.5 of the oestrous cycle in heifers gave the best pregnancy rate (48.2%).

These 2 reports, as well as that of Reichenbach *et al* (1992) on transfer without freezing, may be considered as better reflecting the real situation with *in vitro*-produced embryos as they were established on a large scale. Although our data are somewhat limited, we have reported the final calving rate (tables II and III). A similar study by Van Soom *et al* (1994) gives a calv-

ing rate of 30.6% after 62 unilateral transfers of unfrozen embryos and 7.4% with frozen-thawed embryos. In the same report, of 35 calves born, 25 were male, which means a sex ratio of 71.4%. We have observed the same tendency. Of 22 calves born in our investigations, 17 were male (77%) confirming the results of Avery *et al* (1992). They found that at 7 d post-insemination the most advanced group of *in vitro*-produced bovine embryos, consisted predominantly of male embryos as was also reported by Xu *et al* (1992) and Yadar *et al* (1993).

In conclusion, survival to freezing of blastocysts produced *in vitro* in serum-free conditioned medium was higher for day 6 and 7 blastocysts than for day 8. The synchrony between embryo and recipient seems to be dependent on embryo quality, although –1 d asynchrony would be preferable. Finally, from these results it appears that the viability of *in vitro*-derived embryos is still lower than for *in vivo* embryos. Morphological, cellular and physiological differences have been described (Iwasaki *et al,* 1990; Van Soom and De Kruif, 1992; Leibo and Loskutoff, 1993; Pollard and Leibo, 1993, 1994) which affect the viability and sensitivity to cooling and freezing. For example, *in vitro* embryos have a more buoyant density (< 1.2) than *in vivo* ones (> 1.3). The sensitivity of the zona pellucida to pronase digestion is greater in embryos produced *in vitro* than *in vivo* (Pollard and Leibo, 1993). This could explain the discrepancy between the hatching rate *in vitro* and the implantation rate. *In vitro* embryos are able to hatch *in vitro* but unable to implant *in vivo* perhaps due to deficient production of luteotrophic factors (Hernandez-Ledezma *et al,* 1993). In this case, co-transfer of these embryos with trophoblastic vesicles might improve the transfer success (Heyman *et al,* 1987). In any case, the quality of *in vitro*-derived embryos and consequently their survival and viability after transfer or cryopreservation depend on the improvement of the *in vitro* methodology. Finally, the rate of early cleavage and freezing resistance could represent reliable criteria for assessment of the viability of such embryos (Greve *et al,* 1993).

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REFERENCES

Avery B, Jorgense CB, Madison V, Greve T (1992) Morphological development and sex of bovine *in vitro* fertilized embryos. *Mol Reprod Dev* 32, 265-270

Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA (1982) Normal development following *in vitro* fertilization in the cow. *Biol Reprod* 27, 147-158

Cox DR, Oakes D (1984) *Analysis of survival data.* Chapman and Hall, London, UK

Del Campo MR, Donoso MK, Palasz AT, Garcia A, Mapletoft RJ (1993) The effect of days in co-culture on survival of deep frozen bovine IVF blastocysts. *Theriogenology* 39, 208

Gordon I (1991) Potential application of cattle *in vitro* fertilization in commercial practice and research. *IETS Embryo Transfer Newsl* 9, 4-9

Gordon I, Lu KH (1990) Production of embryos *in vitro* and its impact on livestock production. *Theriogenology* 33, 77-87

Goto K, Kajihara Y, Kosaka S *et al* (1989) Normality of calves obtained from the transfer of blastocysts produced by totally *in vitro* techniques. *Aust J Anim Sci* 2, 591-593

Greve T, Madison V (1991) *In vitro* fertilization in cattle: a review. *Reprod Nutr Dev* 31, 147-157

Greve T, Avery B, Callesen H (1993) Viability of *in vivo*- and *in vitro*-produced bovine embryos. *Reprod Dom Anim* 28, 164-169

Grisart B, Massip A, Dessy F (1994) Cinematographic analysis of bovine embryo development in serum-

free oviduct-conditioned medium. *J Reprod Fertil* 101, 257-264

Hernandez-Ledezma JJ, Mathialagan N, Vallanueva C, Sikes JD, Roberts RM (1993) Expression of bovine trophoblast interverons by *in vitro*-derived blastocysts is correlated with their morphological quality and stage of development. *Mol Reprod Dev* 36, 1-7

Heyman Y, Chesné P, Chupin D, Ménézo Y (1987) Improvement of survival rate of frozen cattle blastocysts after transfer with trophoblastic vesicles. *Theriogenology* 27, 477-484

Iwasaki S, Yoshiba N, Ushijima H, Watanabe S, Nakahara T (1990) Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *J Reprod Fertil* 90, 279-284

Kajihara Y, Kometani N, Shitanaka Y *et al* (1992) Pregnancy rates and births after the direct transfers of frozen-thawed bovine IVF embryos. *Theriogenology* 37, 233

King WA (1991) Cytogenetics of bovine embryos produced from oocytes matured and fertilized *in vitro*: an update. *Proc 7th N Amer Colloq on Domestic Animals, Cytogenet Gene Mapping,* 15-19

Leibo SP (1992) Techniques for preservation of mammalian germ plasma. *Anim Biotech* 3, 139-153

Leibo SP, Loskutoff NM (1993) Cryobiology of *in vitro*-derived bovine embryos. *Theriogenology* 39, 81-94

Massip A, Mermillod P, Wils C, Dessy F (1993a) Effect of dilution procedure and culture conditions after thawing on survival of frozen bovine blastocysts produced *in vitro*. *J Reprod Fertil* 97, 65-69

Massip A, Van Langendonckt A, Mermillod P, Wils C, Grisart B, Dessy F (1993b) Viability of bovine blastocysts produced by totally *in vitro* technique in serum-free oviduct conditioned media and transferred immediately or after cryopreservation. *Proc 9th Eur Embryo Transfer Meeting,* Fondation Mérieux, Lyon, September 10-11, 240

Mermillod P, Massip A, Dessy F (1992a) *In vitro* production of cattle embryos: a review and Belgian results. *Int J Dev Biol* 36, 185-195

Mermillod P, Wils C, Massip A, Dessy F (1992b) Collection of oocytes and blastocysts production *in vitro* from individual, slaughtered cows. *J Reprod Fertil* 96, 717-723

Mermillod P, Mourmeaux JL, Wils C, Massip A, Dessy F (1992c) Protein-free oviduct conditioned medium supports complete bovine embryo development. *Vet Rec* 130, 13

Mermillod P, Wils C, Massip A, Dessy F (1992d) *In vitro* survival of frozen-thawed *in vitro*-produced cattle blastocysts. *Proc 8th Eur Embryo Transfer Meeting,* Fondation Mérieux, Lyon, September 11-12, 194

Plante L, King A (1992) Effect of time to first cleavage on hatching rate of bovine embryos *in vitro*. *Theriogenology* 37, 274

Pollard JW, Leibo SP (1993) Comparative cryobiology of *in vitro*- and *in vivo*-derived bovine embryos. *Theriogenology* 39, 287

Pollard JW, Leibo SP (1994) Chilling sensitivity of mammalian embryos. *Theriogenology* 41, 101-106

Reichenbach HD, Leibrich J, Berg U, Brem G (1991) Pregnancy results following transfer of frozen-thawed *in vitro*-produced bovine embryos to recipients. *Proc 7th Eur Embryo Transfer Meeting,* Cambridge, UK, September 14-15, 198

Reichenbach HD, Leibrich J, Berg U, Brem G (1992) Pregnancy rates and births following unilateral or bilateral transfer of *in vitro*-produced bovine embryos. *J Reprod Fertil* 95, 363-370

Rudnicki MA, McBurney MW (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. *In: Teratocarcinomas and embryonic stem cells. A Practical approach* (EJ Robertson, ed) IRL Press, Oxford, UK, 19-49

Tachikawa S, Otoi T, Kondo S, Machida T, Kasai M (1993) Successful vitrification of bovine blastocysts derived by *in vitro* maturation and fertilization. *Mol Reprod Dev* 34, 266-271

Van Soom A, De Kruif A (1992) A comparative study of *in vivo*- and *in vitro*-derived bovine embryos. *Proc 12th Int Congress Anim Reprod,* The Hague, The Netherlands, August 23-27, 3, 1363-1365

Van Soom A, Mitjen P, Van Vlaenderen I, Van Den Branden J, Mahmoudzadeh AR, De Kruif A (1994) Birth of double-muscled Belgian blue calves after transfer of *in vitro*-produced embryos into dairy cattle. *Theriogenology* 41, 855-867

Wathes DC (1992) Embryonic mortality and the uterine environment. *J Endocrinol* 134, 321-325

Xu KP, Yadav BR, King WA, Betteridge KJ (1992) Sex-related differences in developmental rates of bovine embryos produced and cultured *in vitro*. *Mol Reprod Dev* 31, 249-252

Yadar Br, King WA, Betteridge KJ (1993) Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated *in vitro*. *Mol Reprod Dev* 4, 434-439

Zhang L, Barry DM, Denniston RS, Bunch TD, Godke RA (1993) Birth of live calves after transfer of frozen-thawed bovine embryos fertilized *in vitro*. *Vet Rec* 132, 247-249

Zoli AP, Guilbault LA, Delahaut P, Benites-Ortis W, Beckers JF (1992) Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application in pregnancy diagnosis. *Biol Reprod* 46, 83-93