Factors Affecting the Survival of Frozen Thawed Bovine *In Vitro* Produced Blastocysts[a]

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ABSTRACT : Factors Affecting the Survival of Frozen Thawed Bovine *In Vitro* Produced Blastocysts. The effect of some factors on the post-thaw survival of a total of 240 *in vitro* produced bovine blastocysts was investigated using logistic regression analysis. The explanatory variables tested were: type of culture medium before freezing (TCM 199 supplemented with BSA, BSAITS (BSA+insulin+transferrin+selenium), ECS (estrous cow serum) with or without BOEC (bovine oviductal epithelial cells), age of the blastocyst (Day 7, Day 8+9), morphological appearance before freezing (distinct=Q1 or indistinct=Q2 inner cell mass) and type of cryoprotectant (glycerol, 1.0 M or ethylene glycol, 1.6 M). The survival after thawing based on the post-thaw quality and the development after co-culture with BOEC for 24 and 48 hours. Day 7 blastocysts had an almost three times better chance of survival than Day 8+9 blastocysts. Q1, Day 8+9 blastocysts had higher odds to survive after 48 hours in culture than Q2 blastocysts ($p<0.05$). Blastocysts produced in BSAITS medium had the best chances of survival; however, the odds were not always significant. Blastocysts frozen in glycerol had a better post-thaw quality rating than those frozen in ethylene glycol; however, the difference in post-thaw development at culture was not significant. The relationship between post-thaw quality and post-thaw development at culture was significant ($p<0.05$). The developmental stage and/or age of the embryo and culture medium where development up to blastocyst takes place affect the post-thaw survival of the bovine embryos. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 1 : 7-12*)

Key Words : Bovine, Embryo Age, Insulin-Transferrin-Selenium, Glycerol, Ethylene Glycol, Post-Thaw Quality Score

INTRODUCTION

Since the birth of the first calf following transfer of a frozen-thawed embryo 27 years ago (Wilmut and Rowson, 1973), significant progress has been made in the cryopreservation of bovine embryos. Today, frozen-thawed, *in vivo* developed embryos transferred to recipients under well-controlled conditions lead to pregnancy rates comparable to those for fresh embryos (Hasler, 1992; Leibo and Loskutoff, 1993).

In vitro maturation and fertilisation of bovine oocytes and subsequent *in vitro* culture to the late morula or blastocyst stage (in the text hereafter referred to as IVMFC-embryos) is today an alternative to *in vivo* production of embryos by superovulation. Embryos developed from *in vitro* fertilised oocytes are comparable to those collected from superovulated cattle with respect to the pregnancy rate (Gordon, 1989).

The first calf after transfer of a frozen-thawed IVMFC-embryo was born already in 1987 (Fukuda et al., 1990) and there are many births of calves reported from several laboratories since then. However, frozen-thawed IVMFC-embryos often result in lower pregnancy rates than do the *in vivo* produced embryos (Leibo and Loskutoff, 1993; Greve et al., 1993).

IVMFC-embryos exhibit morphological alterations that might influence the freezing resistance (Greve et al., 1992; Shamsuddin et al., 1992; Shamsuddin and Rodriguez-Martinez, 1994b). There are also differences in physical properties such as density and resistence of the zona pellucida to pronase digestion (Leibo and Loskutoff, 1993). Factors related to culture conditions such as culture atmosphere, addition of cells to the medium, hormonal supplements and addition of growth factors influence not only embryo development but also survival after freezing and thawing (Fukui et al., 1991; Massip et al., 1993; Shamsuddin et al., 1994). Furthermore, the quality and developmental stage of the embryo are also of importance for the viability after freezing and thawing (Pavasuthipaisit et al., 1993; Han et al., 1994). Also the type of cryprotectant and other factors related to the freezing and thawing procedure can affect post-thaw survival (Suzuki et al., 1993).

The objective of the present study was to retrospectively evaluate effects of embryo culture

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methods, culture period and type of cryoprotectant on survival *in vitro* after thawing of bovine IVMFC-blastocysts produced in our laboratory.

MATERIALS AND METHODS

In vitro maturation and fertilisation

Oocytes were obtained by aspirating follicles (<7 mm) from ovaries collected at slaughter of the cow. The oocytes were cultured in tissue culture medium 199 (TCM-199; Biochrom, Berlin) supplemented with 10% oestrus cow serum (ECS) according to a procedure described by Shamsuddin and Larsson (1993). After 24 h of culture, the oocytes were inseminated with spermatozoa selected by a modified swim-up through a hyaluronic acid preparation (Shamsuddin and Rodriguez-Martinez, 1994a). After 20-24 h of sperm- oocyte co-incubation in fert-TALP, the cumulus cells surrounding the oocytes were removed by repeated pipetting. The zygotes were pooled and allocated to 4 different embryo culture treatments with at least 2 treatments running in parallel.

Embryo culture

Ten-to-twenty zygotes were cultured in 50-μl droplets of medium. The 4 culture media were based on TCM 199 supplemented with, 1) bovine serum albumin (BSA), 2) BSA and insulin, transferrin and selenium (BSAITS), 3) ECE alone or 4) ECS with bovine oviduct epithelial cells (BOEC). The bovine oviduct epithelial cells were prepared according to the procedure described by Shamsuddin et al. (1993). Briefly, oviducts were collected at slaughter, washed 5 times in low bicarbonate TALP (Parrish et al., 1988) and cultured in TCM 199 supplemented with 10% ECS. After 24-48 h of culture, the cells were washed twice with TCM 199 with ECS by repeated centrifugation. The cell pellet was resuspended with the same medium at a ratio of 1:400. Droplets of the cell suspension were prepared and incubated overnight. Twenty-four hours after the initiation of embryo co-culture, 50 μl of TCM 199 with ECS were added to the droplets. The procedure for preparation of the BSA, BSAITS and ECS media has been described in detail by Shamsuddin et al. (1994). The BSA medium was prepared by adding 10 mg BSA (Sigma, St. Louis, MO, USA) per ml. To obtain the BSAITS medium, 5 μg/ml insulin (Sigma, St. Louis, MO, USA), 5 μg/ml transferrin (Sigma, St. Louis, MO, USA) and 5 ng/ml sodium selenite (Sigma, St. Louis, MO, USA) was added to the BSA-medium. The ECS medium was prepared by supplementing TCM 199 with 10% ECS (National Veterinary Institute, Uppsala, Sweden). Embryos were evaluated on Day 2 (day of insemination=Day 0) for first cleavage and on Days 7, 8 and 9 for blastocyst formation. The experiment was done in 7 replicates.

Freezing and thawing procedures

Expanding blastocysts were selected and scored morphologically under a stereomicroscope (Wild M8, Germany) as follows: Q1=blastocyst with a distinct inner cell mass and a clear trophoblastic cell layer and Q2=blastocyst with a dispersed inner cell mass. The blastocysts were then washed in phosphate buffered saline (PBS) supplemented with foetal calf serum (FCS; 1%v/v, National Veterinary Institute, Uppsala, Sweden) and kanamycin (25 μg/ml, Sigma, St Louis, MO, USA). The embryos were allocated to freezing with either glycerol (1.0 M; The Royal Veterinary and Agricultural University, Copenhagen, Denmark) or ethylene glycol (1.6 M; E Merck, Darmstadt, Germany) as cryoprotectants in PBS supplemented with FCS and kanamycin. The embryos after equilibration for 10 minutes in freezing medium at room temperature were loaded into straws (1-6 embryos per straw). The straws were sealed and placed at +15℃ in a programmable freezing machine (Planar Biomed, R 206) and immediately cooled down to -7℃ (-3℃/min). After 5 min at -7℃, seeding was induced and 5 min later cooling at a rate of -0.3℃/min was initiated. At -37℃, the straws were plunged into liquid nitrogen. Straws frozen with glycerol were thawed in air and the glycerol was diluted in one step in 1.0 M sucrose (The Royal Veterinary and Agricultural University, Copenhagen, Denmark) in PBS supplemented with FCS and kanamycin. The straws frozen in ethylene glycol were thawed for 10 seconds in 30℃ water. The content in the straw was expelled into a dry petri dish and the blastocyst was transferred to PBS supplemented with FCS and kanamycin. All blastocysts were evaluated morphologically under a steromicroscope and scored into 1=morphology close to fresh expanded IVF-blastocyst, or collapsed blastocyst but with no other imperfections, 2=collapsed blastocyst with cells showing signs of degeneration (dark) and free cells in the perivitelline space or 3=degenerated with dispersed dark cells inside the zona pellucida.

Embryo culture after thawing

The blastocysts were washed twice in PBS, once in ECS medium and then co-cultured with BOEC in 50-μl droplets of ECS medium. The developmental stage of an embryo was evaluated and scored after 24 h and 48 h as 1=degenerated, collapsed, no signs of reexpansion, 2=blastocyst, 3=expanded blastocyst or 4=hatched blastocyst.

Statistical analysis

Each embryo was regarded as one unit of observation. Logistic regression analysis as applied in

PROC CAT MOD of SAS (SAS Institute Inc., 1985), was used to investigate the effect of exposure variables on embryo survival and development. Survival was based on embryo quality after thawing (PTQ; 1-3 scale) and development after 24 and 48 h *in vitro* culture (E24Q, E48Q), developmental stage 1 (no development) or developmental stage 2+3+4 (any development). Exposure variables were date of freezing (FRD) and of thawing (THD), quality before freezing (Q; 1-2 scale), age of embryo (Day 7 or 8+9), culture media before freezing (MEDIA: BSA, BSAITS, ECS or BOEC) and cryoprotectant (CRYO: eg or gl). The logistic regression models were developed by stepwise backward elimination of non-significant ($p>0.05$) for main effects and two-factor interactions. The final models were:

PTQ=FRD+THD+CRYO

E24Q=FRD+THD+DAY+MEDIA

E48Q=FRD+THD+Q+DAY+Q*DAY+MEDIA

The difference in proportion of embryos with different PTQ developed after 24 h and 48 h were tested by using a X^2-test. The strength of the association between different factors was expressed as odds ratio. Large odds ratio means that the odds for embryos exposed to a certain factor to develop are larger than for embryos not exposed to the factor.

RESULTS

A total of 240 IVMFC-blastocysts produced from 1234 presumptive zygotes were included in the analysis. The mean ± SEM percentages of blastocysts developed in BSA, BSAITS, ECS and BOEC were 7.7 ±1.4, 25.6±4.7, 20.3±3.6 and 23.9±3.6, respectively. Each embryo was exposed to several factors. The results for each factor are shown in table 1.

Twenty-one to 42% of the embryos scored grade 1 after thawing with respect to the factors studied. The percentage of survived embryos following 24-h *in vitro* culture ranged between 9 and 47%. Zero to 20% of the embryos hatched after 48-h *in vitro* culture.

A significant relationship ($p<0.01$) existed between the post-thaw morphology and the survival rates *in vitro* (table 2). The proportions of developed embryos decreased with decreasing PTQ.

As shown in table 3, there was a decreased chance for blastocysts to be PTQ 1 if ethylene glycol was used as cryoprotectant than if glycerol was used. No difference was seen between embryos scored as grades 2-3 post thawing regarding cryoprotectant.

Embryos frozen on Day 7 had an almost 3 times higher chance to survive freezing and thawing than for embryos frozen on Days 8-9 (table 4). Embryos cultured in BSAITS had the best chance of survival; however, the difference was not significant (tables 4 and 5). Day 7 blastocysts had a better chance to develop after 48 h culture than Day 8+9 blastocysts (table 1). For Q 2 blastocycts, the odds for Day 7 blastocysts were 9 times higher than for Day 8+9 embryos (table 5). The difference between Q 1 and Q 2 day 7 blastocysts in the odds to survive was not significant. However, day 8+9 blastocycts scored Q 1 had a higher ($p<0.05$) chance to survive than those scored Q 2 (table 5).

Table 1. Number and percentage of embryos morphologically scored after thawing as grade 1 and reexpanded and hatched after thawing and culture based on the quality score before freezing, age of embryo, culture media and cryoprotectant

FACTOR	No. of embryos	Scored grade 1		Re-expanded after 24 h		Hatched after 48 h	
	n	n	%	n	%	n	%
Embryo quality scope							
Quality 1	154	58	38	47	31	20	13
Quality 2	86	29	34	30	35	10	12
Age of embyro							
Day 7	136	51	38	51	38	22	16
Day 8+9	104	36	35	26	25	8	8
Culture media							
BSA	22	9	41	5	23	3	14
BSAITS	137	57	42	64	47	27	20
ECS	28	6	21	3	11	0	0
BOEC	53	15	28	5	9	0	0
Cryoprotectant							
Ethyleng glycol	101	30	30	36	36	19	19
Glycerol	139	57	41	41	29	11	8

[1] Details of the factors are explained in material and methods.

DISCUSSION

Embryos that had reached the blastocyst stage on day 7 and were frozen had an almost three times better chance to survive after thawing than embryos developed to blastocyst after culturing 8 or 9 days. This is in agreement with Greve et al. (1993), who found that the viability following cryopreservation was correlated with the rate of early embryonic development. Pollard and Leibo (1993) reported a significantly lower survival rate *in vitro* for frozen-thawed compacted morula compared with blastocysts (0% vs. 64%). Following transfer of IVMFC embryos, Day 8 embryos yielded higher pregnancy rates (36%) than Day 7 embryos (17%), however the developmental stage of these embryos was not reported (Suzuki et al., 1991). Altogether, our findings indicate a major influence of age and/or stage of development of IVMFC embryos on their freezing sensitivity.

It has earlier been shown that the choice of culture conditions has a marked effect on the development of fresh IVMFC embryos (Fukui et al., 1991). Also the culture conditions for IVMFC embryos influence their sensitivity to freezing (Leibo and Loskutoff, 1993; Shamsuddin et al., 1994; Leibo et al., 1996). In the present study, the blastocysts produced in the BSAITS medium showed a higher survival rate in general than the blastocysts produced by using other treatments. However, the odds were not always significant. The better survival rate of BSAITS medium-developed embryos might be due to their better morphology. Shamsuddin and Rodriguez-Martinez (1994b) compared the fine structure of bovine blastocysts developed in BSAITS medium or in BOEC. Five of 8 blastocysts developed in BSAITS fulfilled the criteria set for normal morphology of *in vivo* developed blastocysts but only 2 of 8 embryos co-cultured with BOEC reached those criteria, the authors stated. The BOEC cocultured-blastocysts showed more lipids in their cytoplasm than did BSAITS-cocultured ones (Shamsuddin and Rodriguez-Martinez, 1994b). The porcine embryos, which have high lipid concentration in their cytoplasm, are more sensitive to low temperature (Niwa and Funahashi, 1999).

In the present experiments, two cryoprotectants were compared with regard to the post-thaw survival of IVMFC embryos. The method using glycerol as cryoprotectant can be regarded as an effective method yielding high pregnancy rates for *in vivo* embryos (Niemann, 1991). Nevertheless, Voelkel and Hu (1992) recently reported promising results after using ethylene glycol as cryoprotectant. The high permeability of ethylene glycol (Szell et al., 1989) makes it possible to transfer embryos directly after thawing without removal from the straw, which is of significant practical interest. The vitrification procedure, which has been found simple in operation and less injurious to mouse embryos, may be another alternative for cryopreservation of bovine IVMFC embryos (Kasai, 1996). The present investigations revealed five times higher odds with respect to the post-thaw quality score Good for blastocysts frozen in glycerol than for those frozen in ethylene glycol. However, the difference between the cryoprotectants in development of frozen-thawed embryos at *in vitro* culture was not significant.

The post-thaw survival rates obtained in our laboratory were generally lower than those obtained from some other laboratories. Massip et al. (1993) reported *in vitro* survival rates of around 80% and

Table 2. Number of embryos (%) developed after 24 h and 48 h *in vitro* culture in relation to their post-thaw quality

Post-thaw quality	Development after 24 h	Development after 48 h
1	16 (47%)	15 (44%)
2	41 (37%)	40 (36%)
3	20 (21%)	18 (19%)

Table 3. Relationships expressed as odds ratios with 95% confidence intervals between post-thaw quality scores (PTQ) and cryoprotectant at logistic regression analyses

Cryoprotectant	Odds ratios	
	PTQ 1 vs. 3	PTQ 2 vs. 3
Ethylene glycol	0.2 (0.1-0.5)	0.8 (0.3-2.4)
Glycerol	1	1

[1] PTQ1=Morphology close to fresh good quality IVMFC-blastocysts.
PTQ2=Blastocysts with cells showing signs of degeneration and with free cells in the periviteline space.
PTQ3=Degenerated.

Table 4. Relationships, expressed as odds ratios with 95% confidence intervals, between embryonic development after 24 h *in vitro* culture (any development vs. no development) with respect to age of embryo and culture medium at logistic regression analyses

	Odds ratios
Age of embryo	
Day 7	2.9 (1.4-6.1)
Day 8+9	1
Culture medium	
BSA	1.8 (0.3-9.9)
BSAITS	3.9 (0.9-16.4)
ECS	1
BOEC	0.6 (0.1-2.8)

[1] Explained in material and methods.

hatching-rates of around 50% for IVMFC embryos frozen in glycerol; the author used basically the same method as in the present experiment. The use of ethylene glycol as cryoprotectant resulted in 90% and 50% survival and hatching, respectively (Suzuki et al., 1993). On the other hand, post-thaw *in vitro* survival rates in the range of 20-40% have been reported by others (Reichenbach et al., 1992; Rorie et al., 1990; Palasz et al., 1992). These indicate that undefined factors may significantly influence the survival rates after freezing and thawing. The system used for embryo development, such as types of medium, cell support, to mention a few, may be amongst the undefined factors.

The appearance of an IVMFC embryo under a light microscope differs in several aspects from that of *in vivo* embryos, making the morphological evaluation uncertain. IVMFC embryos are darker and have more vacuoles than *in vivo* embryos and the size of the inner cell mass varies considerably (Greve et al., 1992). In the present study, only embryos with a clear blastocoel and with a morphology largely similar to that of *in vivo* embryos described by Lindner and Wright (1983) were selected for freezing. Within this group of blastocysts, a further differentiation was made based on the appearance of the inner cell mass. The survival rates after 24 hours of culture were not affected by the morphological appearance of the inner cell mass before freezing. However, based on the development after 48 hours, day 8+9 blastocysts with a distinct inner cell mass had a significantly greater chance to survive than those with a more indistinct inner cell mass. This finding might reflect the higher sensitivity of the day 8+9 blastocysts to cryopreservation and indicates the need for a more critical morphological evaluation before freezing. To fully assess the developmental competence of blastocysts with so-called dispersed inner cell mass, they must be transferred to recipients to evaluate their ability to develop to viable offspring.

Table 5. Relationships of the quality of embryos before freezing and the media used for culturing up to the blastocyst stage with post-thaw development after 48 hours culture (any development vs. no development) at logistic regression analyses

		Odds ration (95% confidence intervals)
Quality before freezing	Age of embryo	
Quality 1	Day 7	6.7 (2.1-21.4)
	Day 8+9	4.7 (1.5-15.4)
Quality 2	Day 7	9.2 (2.7-30.7)
	Day 8+9	1
Culture media[1]		
BSA		1.8 (0.3-9.9)
BSAITS		3.9 (0.9-16.4)
ECS		1
BOEC		0.6 (0.1-2.8)

[1] Explained in material and methods.

The post-thaw embryo survival in our investigations based on both morphological evaluation under a steromicroscope and on the ability to reexpand and develop when cultured. In the morphological classification, post-thaw modified criteria for *in vivo* embryos were used (Lindner and Wright, 1983). Our analysis showed a significant relationship between embryo quality post-thaw and development during *in vitro* culture.

In conclusion, our data indicate that culture medium and developmental rate of the embryos before freezing affect the post-thaw survival of IVFMC embryos and that these embryos survive freezing with both glycerol and ethylene glycol as cryoprotectants. Morphological evaluation of IVMFC embryos post thaw was significantly related to their ability to develop in culture.

REFERENCES

Fukuda, Y., K. Ichikawa, K. Naito and Y. Toyoda. 1990. Birth of normal calves resulting from bovine oocytes matured, fertilised and cultured with cumulus cells *in vitro* up to the blastocyst stage. Biol. Reprod. 42:114-119.

Fukui, Y., L. T. Mcgowan, R. W. James, P. A. Pugh and H. R. Tervit. 1991. Factors affecting the *in-vitro* development to blastocysts of bovine oocytes matured and fertilised *in vitro*. J. Reprod. Fert. 92:125-131.

Gordon, I. 1989. Large-scale production of cattle embryos by *in vitro* culture methods. Ag. Biotech. News and Information. 1:345.

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

Greve, T., N. M. Loskutoff, B. C. Buckrell, C. R. Christian, S. P. Leibo and K. J. Betteridge. 1992. 5e'me colloque Franco-tchecoslovaque sur la reproduction des animaux domistiques. Jouy-en-Josas, France. p. 36.

Han, Y. M., H. Yamashina, N. Koyama, K. K. Lee and Y. Fukui. 1994. Effects of quality and developmental stage on the survival of IVF-derived bovine blastocysts cultured *in vitro* after freezing and thawing. Theriogenology. 42:645-654.

Hasler, J. F. 1992. Current status and potential of embryo transfer and reproductive technology in dairy cattle. J. Dairy Sci. 75:2857-2879.

Kasai, M. 1996. Simple and efficient methods for vitrification of mammalian embryos. Anim. Reprod. Sci. 41:67-75.

Leibo, S. P. and N. M. Loskutoff. 1993. Cryobiology of *in vitro*-derived bovine embryos. Theriogenology. 39:81-94.

Leibo, S. P., A. Martino, S. Kobayashi, J. W. Pollard. 1996. Stage-dependent sensitivity of oocytes and embryos to low temperature. Anim. Reprod. Sci. 42:45-53.

Lindner, G. M. and R. W. Wright Jr. 1983. Bovine embryo morphology and evaluation. Theriogenology. 20:407-416.

Massip, A., P. Mermillod, C. Wils and F. Dessey. 1993. Effects of dilution procedure and culture conditions after thawing on survival of frozen bovine blastocysts produced *in vitro*. J. Reprod. Fert. 97:65-69.

Niemann, H. 1991. Cryopreservation of ova and embryos from livestock: current status and research needs. Theriogenology. 35:109-124.

Niwa, K. and H. Funahashi. 1999. Recent development in embryo technology in pigs review. Asian-Aus. J. Anim. Sci. 12:966-975.

Palasz, A. T., L. Tan, M. R. Del Compo and R. J. Mapletoft. 1992. The effect of methods of thawing and glycerol removal on survival of frozen mouse and bovine embryos. Proc. 12th. Int. congr. on Anim. Reprod., The Hague, The Netherlands. 3:1472-1474.

Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Pavasuthiapaisit, K., C. Tocharus, C. Thonabulsomat and Y. Kitiyanant. 1993. The viability testing of frozen-thawed bovine embryos produced *in vitro*. Theriogenology. 39:280.

Pollard, J. W. and S. P. Leibo. 1993. Comparative cryobiology of *in vitro* and *in vivo* derived bovine embryos. Theriogenology. 39:287.

Reichenbach, H. D., J. Liebrich, U. Berg and G. Brem. 1992. Pregnancies following transfer of frozen-thawed *in vitro* matured, fertilised and cultured bovine embryos to recipients. Reprod. Dom. Anim. 27:59-60.

Rorie, R. W., K. P. Xu and K. J. Betteridge. 1990. Effects of culture on the post-thaw viability of cryopreserved *in vitro* fertilised bovine embryos. Theriogenology. 33:311.

SAS/STAT Guide for Personal Computers, SAS Institute Inc., Cary, NC. 1985.

Shamsuddin, M. and B. Larsson. 1993. *In vitro* development of bovine embryos after fertlization using semen from different donors. Reprod. Dom. Anim. 28:77-84.

Shamsuddin, M., B. Larsson, H. Gustafsson, S. Gustari, J. Bartolome and H. Rodriguez-Martinez. 1992. Comparative morphological evaluation of *in vivo* and *in vitro* produced bovine embryos. 12th. Int. Congr. on Anim. Reprod. 3:1333-1335.

Shamsuddin, M., B. Larsson, H. Gustafsson and H. Rodriguez-Martinez. 1993. *in vitro* development up to hatching of bovine *in vitro*-matured and fertilised oocytes with or without support from somatic cells. Theriogenology. 39:1067-1079.

Shamsuddin, M., B. Larsson, H. Gustafsson and H. Rodriguez-Martinez. 1994. A serum-free, cell-free culture system for development of bovine one-cell embryos up to blastocyst stage with improved viability. Theriogenology. 41:1033-1043.

Shamsuddin, M. and H. Rodriguez-Martinez. 1994a. A simple, non-traumatic swim-up method for the selection of spermatozoa for *in vitro* fertilisation in the bovine. Anim. Reprod. Sci. 36:61-75.

Shamsuddin, M. and H. Rodriguez-Martinez. 1994b. Fine structure of bovine blastocysts developed either in serum-free medium or in conventional co-culture with oviduct epithelial cells. J. Vet. Med. A. 41:307-316.

Suzuki, T., M. Yamoto, M. Oe, Y. Nishikata, K. Okamoto and T. Tsukihara. 1991. Effect of media on fertilisation and development rates of *in vitro* fertilised embryos, and of age and freezing of embryos on pregnancy rates. Theriogenology. 35:278.

Suzuki, T., M. Takagi, M. Yamamoto, A. Boediono, S. Saha, H. Sakakibara and M. Oe. 1993. Pregnancy rate and survival in culture of *in vitro* fertilised bovine embryos frozen in various cryoprotectants and thawed using a one-step system. Theriogenology. 40:651-659.

Szell, A., J. N. Shelton and K. Szell. 1989. Osmotic characteristics of sheep and cattle embryos. Cryobiology. 26:297-301.

Voelkel, S. A. and Y. X. Hu. 1992. Use of ethylene glycol as a cryoprotectant for bovine embryos allowing direct transfer of frozen-thawed embryos to recipient females. Theriogenology. 37:687-697.

Wilmut, I. and L. E. A. Rowson. 1973. Experiments on the low-temperature preservation of cow embryos. Vet. Rec. 92:686-690.