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Effects of different cryopreservation methods on post-thaw culture conditions of *in vitro* produced bovine embryos

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Summary

The aim of this work was to evaluate the effect of cryopreservation protocols on subsequent development of *in vitro* produced bovine embryos under different culture conditions. Expanded *in vitro* produced blastocysts ($n = 600$) harvested on days 7–9 were submitted to controlled freezing [slow freezing group: 10% ethylene glycol (EG) for 10 min and 1.2°C/min cryopreservation]; quick-freezing [rapid freezing group: 10% EG for 10 min, 20% EG + 20% glycerol (Gly) for 30 s]; or vitrification [vitrification group: 10% EG for 10 min, 25% EG + 25% Gly for 30 s] protocols. Control group embryos were not exposed to cryoprotectant or cryopreservation protocols and the hatching rate was evaluated on day 12 post-insemination. In order to evaluate development, frozen–thawed embryos were subjected to granulosa cell co-culture in TCM199 or SOFaa for 4 days. Data were analyzed by PROC MIXED model using SAS Systems for Windows®. Values were significant at $p < 0.05$. The hatching rate of the control group was 46.09%. In embryos cultured in TCM199, slow freezing and vitrification group hatching rates were 44.65 ± 5.94% and 9.43 ± 6.77%, respectively. In embryos cultured in SOFaa, slow freezing and vitrification groups showed hatching rates of 11.65 ± 3.37 and 8.67 ± 4.47%, respectively. In contrast, the rapid freezing group embryos did not hatch, regardless of culture medium. The slow freezing group showed higher hatching rates than other cryopreservation groups. Under such conditions, controlled freezing (1.2°C/min) can be an alternative to cryopreservation of *in vitro* produced bovine embryos.

Keywords: Bovine embryos, Cryopreservation, Embryo culture, *In vitro* production, Slow freezing

Introduction

A large number of bovine embryos can be *in vitro* produced at relatively low cost. This biotechnology has advanced remarkably in the past decades, especially after incorporation of the ovum pick-up technique (OPU) allowed commercial application of *in vitro* produced (IVP) bovine embryos (Pereira *et al.*, 2005). Up to now, the major obstacle associated with the extensive use of this technology is the lack of suitable methods to preserve IVP embryos (Mucci *et al.*, 2005). IVP embryos are more sensitive to cryopreservation than their *in vivo* counterparts (Thompson, 1997; Kaidi *et al.*, 2001; George *et al.*, 2008).

IVP embryos are held in culture medium for a longer period than *in vivo* embryos, with great effect on embryo quality, especially on timing of development, blastocyst quality, hatchability and total cell number. All these factors might contribute to the greater susceptibility of embryos to cryoinjury, and to the reduced pregnancy rates (Nedambale *et al.*, 2004; Lonergan *et al.*, 2006; Corcoran *et al.*, 2007). It is

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well known that preimplantation bovine embryos can adapt to an enormous variety of environments, even under adverse or inadequate culture conditions (Robert *et al.*, 2002). The poor cryotolerance of IVP embryos is probably due to ultrastructural and functional differences between *in vivo* and *in vitro* derived embryos (Leoni *et al.*, 2002). Cryotolerance of IVP embryos is lower than that obtained *in vivo*, and it can be affected by the embryo culture system (Campos-Chillon *et al.*, 2006).

It has been reported that freezing and thawing processes decrease embryo viability, which is attributed to physical and chemical damage induced during the cryopreservation process (Marquez-Alvarado *et al.*, 2004). Cryopreservation procedures are designed to avoid intracellular ice crystal formation based on the principle of dehydration. Cooling rates are optimized to remove water from the embryo, preventing ice crystal formation cryoinjury, while minimizing chemical toxicity/osmotic stress from exposure to high salt concentrations (Campos-Chillon *et al.*, 2006).

Currently, there are two approaches towards embryo cryopreservation (Silva & Metelo, 2005; Leibo, 2008). The first approach consists of a controlled, slow-freezing process that was first developed for mouse embryos, and later applied to bovine embryos produced *in vivo* (Silva & Metelo, 2005). Conventional cryopreservation requires removing water from cells by osmosis to prevent damaging ice crystal formation; this approach requires slow cooling rates and cryoprotectants (Barceló-Fimbres & Seidel, 2007) and is considered as an equilibrium freezing (Leibo, 2008). During the process, solutions contained in the straws turned opaque, a sign of crystallization and ice formation (Jin *et al.*, 2008). The second approach, called vitrification, involves high concentrations of the cryoprotectant and a very rapid freezing rate that prevents ice crystals formation (Marquez-Alvarado *et al.*, 2004; Campos-Chillon *et al.*, 2006). It requires high solution viscosity, rapid cooling rates, small volumes, and the use of high concentrations of cryoprotectant solutions to bring about a physical state similar to glass (Campos-Chillon *et al.*, 2006), which increases the risk of osmotic and toxic damage (Barceló-Fimbres & Seidel, 2007). This procedure is considered as a non-equilibrium freezing (Leibo, 2008). When cooled directly in liquid nitrogen (LN_2), vitrification solutions remained transparent (Jin *et al.*, 2008). Post-thaw embryo viability can be assessed by various methods; the most used is *in vitro* survival 24 or 48 h after of culture (Kaidi *et al.*, 1999). The aim of this work was to evaluate the effect of cryopreservation protocols on subsequent development of IVP embryos under different culture conditions.

Materials and methods

Chemicals and supplies

Unless otherwise were indicated, all chemicals were obtained from Sigma Chemicals. Tissue culture media (TCM), HEPES and sodium bicarbonate, and fetal calf serum (FCS) were obtained from Gibco.

In vitro production of bovine embryos

Slaughterhouse ovaries were transported to the laboratory at 30°C. Cumulus–oocyte complexes (COCs) from follicles 3–10 mm in diameter were aspirated with a sterile 21G needle attached to a disposable syringe and pooled in a 15 ml conical tube. The tubes were maintained in a water bath at 37°C for 10 min. After sedimentation, COCs were recovered and selected using a mouth pipette under a stereomicroscope. Oocytes with at least three layers of cumulus cell and homogenous cytoplasm were selected, washed three times in TCM199-HEPES and three times in maturation medium [TCM199 bicarbonate containing 10% serum, gentamicin (50 μg/ml), follicle stimulating hormone (FSH) (0.5 μg/ml), luteinizing hormone (LH) (50 μg/ml) and estradiol (1 μg/ml)]. Groups of 20–30 COCs were transferred to a 90-μl drop of maturation medium under mineral oil for 24 h at 39°C in an atmosphere of 5% (v/v) CO_2 in humidified air. For *in vitro* fertilization, COCs were washed three times in TCM199-HEPES and transferred to a 90-μl drop of fertilization medium [IVF-TALP: modified Tyrode stock solution supplemented with PHE (0.5 mM penicillamine, 0.25 mM hypotaurine and 25 μM epinephrine in 0.9% (w/v) NaCl), gentamicin (50 μg/ml) and 0.3% essentially fatty acid-free bovine serum albumin (BSA) under mineral oil]. Frozen–thawed spermatozoa from one bull were purified by discontinuous Percoll density gradient (45–90%). Oocytes were fertilized with $\sim 1 \times 10^6$ Percoll-purified spermatozoa/ml for 18 h at 39°C in an atmosphere of 5% (v/v) CO_2 in humidified air (day 0) (Tavares *et al.*, 2008).

Presumptive zygotes were partially denuded by gentle pipetting in TCM199-HEPES, washed once in culture medium (SOFaa). Groups of 20–30 presumptive zygotes were transferred to a 90-μl drop of culture medium in the same plate used for *in vitro* maturation (co-culture system) under mineral oil at 39°C in an atmosphere of 5% (v/v) CO_2 in humidified air. At day 3, co-culture drops were supplemented with another 90 μl culture medium and cleavage was assessed. Development rates were evaluated on days 7 to 9 and hatching rates were evaluated on day 12.

The co-culture system was established on the same plate used for *in vitro* maturation. *In vitro* maturation

drops were discarded and drop spots containing a monolayer of granulosa cells were washed once with culture medium. The washing medium was removed and a new culture medium drop of 90 μl was prepared. The same process was conducted to the co-culture plates used to evaluate development of frozen–thawed embryos using either TCM199 or SOFaa.

Embryo exposition (Experiment 1)

Excellent-quality expanded blastocysts selected on days 7 to 9 post-insemination were randomly distributed into three experimental groups: control group, EG10 and EG20/Gly20. As a control group, 72 expanded blastocysts were not exposed to the cryoprotectant. To EG10 group, 101 embryos were equilibrated in 10% ethylene glycol (EG) for 10 min. To EG20/Gli20 group, 100 embryos were equilibrated in 10% EG for 10 min, 20% EG + 20% glycerol (Gly) solution for 30 s. The cryoprotectant was removed in two steps of 3 min each. The first step was conducted in 0.5 ml phosphate-buffered saline (PBS) solution containing 0.3 M sucrose and 0.2% BSA and the second step was in 0.5 ml PBS solution containing 0.2% BSA. Embryos were re-placed in the same co-culture plates that embryos were produced to evaluated survival rates.

Cryopreservation and thawing (Experiments 2 and 3)

Excellent-quality expanded blastocysts selected on days 7 to 9 post-insemination were randomly distributed into four experimental groups: control group (embryos not exposed to the cryoprotectant or cryopreservation protocol), slow freezing group, rapid freezing group and vitrification group. In the slow freezing group, 199 expanded blastocysts were equilibrated in 10% EG and loaded into 0.25 ml plastic straws for 10 min. The straws were seeded into a methanol bath at −7°C for 5 min, cooled from −7 to −31°C (1.2°C/min) (PG20, HAACKE®, Germany) and plunged into LN_2 (Assumpção *et al.*, 2008). In the rapid freezing group 200 expanded blastocysts were equilibrated in 10% EG for 10 min, 20% EG + 20% Gly solution for 30 s and loaded into 0.25 ml plastic straws. The straws were placed in nitrogen vapor (0.8 cm over LN_2 at –170°C, Mello *et al.*, 2001) for 2 min and plunged into liquid nitrogen. In the vitrification group 201 expanded blastocysts were equilibrated in 10% EG for 10 min, 25% EG + 25% Gly solution for 30 s and loaded into 0.25 ml plastic straws. The straws were placed in nitrogen vapour (0.8 cm over liquid nitrogen at −170°C) (Mello *et al.*, 2001) for 2 min and plunged into LN_2. The straws were loaded in three columns separated by two air bubbles. The central column was cryoprotectant solution carrying six embryos and the other two columns were of PBS solution containing 0.3 M sucrose and 0.2% BSA. Embryos were maintained under LN_2 for at least 7 days. Thawing was accomplished by holding the frozen straw for 10 s in air and 10 s in a 25°C water bath. The cryoprotectant was removed in two steps of 3 min each. The first step was conducted in 0.5 ml PBS solution containing 0.3 M sucrose and 0.2% BSA and the second step was in 0.5 ml PBS solution containing 0.2% BSA. Embryos were randomly cultured in TCM199 (Experiment 2) or SOFaa (Experiment 3) and *in vitro* development was evaluated during 4 days up to the hatched blastocyst stage.

Table 1 Hatching rates of IVP embryos following granulosa cell co-culture in SOFaa after cryoprotectant exposition (Experiment 1)

Treatment group	Number of blastocysts	Mean ± SEM
Control group	72	58.94 ± 9.43
EG10 group	101	65.55 ± 9.43
EG20/Gly20 group	100	60.78 ± 9.43

Values with different superscripts (a, b) are significantly different ($p < 0.05$), PROC MIXED Test.

Statistical analyses

Data were presented as means and SEM and analyzed by ANOVA with PROC MIXED model of Software Statistical Analysis System for Windows SAS®. Dependent variables were the re-expansion and hatching rates and independent variables were treatment (cryopreservation methods), replicates and the drops. $p < 0.05$ was considered significant.

Results

Results are presented in Tables 1, 2 and 3. Two Experiments, 2 and 3, control embryos, not exposed to cryoprotectant or cryopreservation, had 46.09% hatching rate on day 12 post-insemination.

In Experiment 1, hatching rates were similar ($p > 0.05$) for all experimental groups (Control vs. EG10 vs. EG20/Gly20) (Table 1).

In a study of extracellular and intracellular ice formation during different cryopreservation protocols, Jin *et al.* (2008) observed the appearance of the solution in the straw visually, that is, whether it remained transparent (not crystallized) or turned opaque (crystallized) during cooling and warming. In our study, we adopted the same visual inspection and controlled freezing and quick-freezing solutions

Table 2 Re-expansion and hatching rates of IVP cryopreserved embryos following granulosa cell co-culture in TCM199 (Experiment 2)

		Mean ± SEM	
Cryopreservation group	Number of blastocysts	Re-expansion	Hatching
Slow freezing group	99	58.58 ± 6.94^{a}	44.65 ± 5.94^{a}
Rapid freezing group	103	1.61 ± 7.41^{b}	0 ± 6.46^{b}
Vitrification group	101	13.65 ± 7.76^{b}	9.43 ± 6.77^{b}

Values with different superscripts (a, b) are significantly different ($p < 0.05$), PROC MIXED Test.

Table 3 Re-expansion and hatching rates of IVP cryopreserved embryos following co-culture in SOFaa (Experiment 3)

		Mean ± SEM	
Cryopreservation group	Number of blastocysts	Re-expansion	Hatching
Slow freezing group	100	34.66 ± 5.39^{a}	11.65 ± 3.37^{a}
Rapid freezing group	97	4.32 ± 5.39^{b}	0.29 ± 3.37^{b}
Vitrification group	100	$19.62 \pm 6.95^{a,b}$	$8.67 \pm 4.47^{a,b}$

Values with different superscripts (a, b) are significantly different ($p < 0.05$), PROC MIXED Test.

turned opaque and the vitrification solution turned transparent.

In Experiment 2, re-expansion and hatching rates were higher ($p < 0.05$) for slow freezing group embryos than quick and vitrification groups when co-cultured in TCM199. There was no difference in re-expansion and hatching rates between rapid freezing and vitrification groups.

In Experiment 3, slow freezing group embryos showed high ($p < 0.05$) re-expansion and hatching rates than the rapid freezing group but similar to vitrification group while re-expansion and hatching rates of the quick and vitrification groups were similar.

Discussion

In previous study, Mucci *et al.* (2005) observed that immediately after thawing, embryos contracted showing a clear cytoplasmic outline of regular morphology. However, 30 min after culture embryos shrunk and acquired a dark appearance with cellular debris in the perivitelline space (Mucci *et al.*, 2005). In the present study, rapid freezing and vitrification group embryos showed the same morphological appearance and the lowest survival rates. Slow freezing group embryos had a shrunken appearance and an increase of perivitelline space. However, they reached a higher hatching rate 24 h following culture.

Culture conditions, as well as embryo quality, are relevant to cryopreservation of IVP embryos. The cryopreservation method plays a major role in this scenario. In addition to the choice of cryoprotectant and its method of use, the speed at which embryos are cooled and warmed are determinant of embryonic post-thaw survival (Fahning & Garcia, 1992). Hochi *et al.* (1996) to understand the effect of cooling and warming rates on survival of cryopreservation of embryos tested five different cooling rates. In that work, the slower rates (0.3°C/min and 0.6°C/min) showed higher survival rates than faster rates (1.2°C/min and 1.5°C/min). However, other groups showed that there was no difference in expanded blastocysts rates between two slow freezing protocols (cooling rates of 0.5°C or 1.2°C/min). Assumpção *et al.* (2008) postulated that reduction of cryopreservation procedure using a more rapid cooling rate (0.5°C × 1.2°C/min), without losing embryo viability after thawing has great importance on routine use of this technique. In agreement with Assumpção *et al.* (2008), in the current study a high cooling rate of 1.2°C/min was used. According to the present results the concentration of EG (10%) used was efficient to protect a great number of embryos from cryoinjury in a controlled freezing protocol (1.2°C/min).

Vajta *et al.* (1996) studied procedural elements such as the composition of the holding medium, temperature of incubation and development stage. The cryoprotectants chosen were 25% EG and 25% Gly in different holding medium and sucrose in the diluents thawing procedure. The group that used PBS and BSA showed a 28% hatching rate (Vajta *et al.*, 1996). In a previous study, Martinez *et al.* (2002), obtained rates between 44.8 and 67.8% when using vitrified day 7 blastocysts with 25% EG and 25% Gly and different

sucrose concentrations (0.0, 0.1, 0.3 and 0.5 M). Our work showed a 8.67 to 9.43% hatching rate with the same cryoprotectant concentration and the sucrose only to dehydratation procedure. This discrepancy in results could be due to different *in vitro* embryo production protocols.

The slow freezing method is a well established protocol with minor variations in large-scale trials. However, for vitrification there are many variations in protocols and most studies involved small numbers of embryos (Barceló-Fimbres & Seidel, 2007).

Tominaga *et al.* (2007) cryopreserved IVP bovine blastocysts after biopsy treatment by slow freezing in the presence of 0.7 M glycerol with or without sucrose. This work suggested that the presence of sucrose plays an important role in accelerating dehydration before and during slow cooling. Martinez *et al.* (2002) showed that only the use of a lesser sucrose concentration (0.1 and 0.3 M) had a positive effect, whereas a greater concentration (0.5 M) resulted in a lesser hatching rate. In our study, sucrose was added only to dehydration solutions after thawing. So, we suggested that dehydration was not sufficient and detrimental intracellular freezing occurred during the cryopreservation protocol. In addition, we suggested that the same problem occurred to rapid freezing and vitrification groups.

Direct comparisons between two cryopreservation methods (slow freezing vs. vitrification) led to different results, but it seems that vitrification procedures are mostly suitable for IVP embryos, especially when high cooling rates are applied (Mucci *et al.*, 2005). In a study that compared slow freezing versus vitrification it was shown that vitrification was less injurious to bovine embryos produced in sequential KSOM-SOF than was slow freezing (Nedambale *et al.*, 2004). Barceló-Fimbres & Seidel (2007) investigated the effects of hexoses, FCS, and phenazine ethosulfate (PES) during the culture of bovine embryos on blastocysts development and survival after cryopreservation by slow freezing and vitrification with similar survival rates to both cryopreservation protocols. However, in our study, slow freezing showed higher survival rates than rapid freezing or vitrification for bovine embryos produced in a granulosa cell co-culture system with SOFaa. It is possible that such discrepancy is due to different methods and protocols applied for the different work-groups. It is possible that, in the current work, the IVP system used resulted in embryos of poorer quality than other systems. As a result, embryos were more susceptible to rapid freezing and vitrification protocols. So, the slow freezing method was the best protocol for this system of production.

Embryo culture environment not only influences embryo development, but also exerts an effect on embryo survival following cryopreservation (Nedambale *et al.*, 2004). The ability of an embryo to survive and hatch after cryopreservation is a good indicator of its quality (George *et al.*, 2008). The use of blastocyst yield alone as a parameter for the evaluation of embryo culture systems remains common in several laboratories (Russel *et al.*, 2006). The ultimate test of embryo quality is the establishment of pregnancy after transfer to a recipient resulting in the birth of a live and healthy offspring. However, this procedure is time-consuming, expensive and often impractical. For these reasons is essential to develop reliable and practical test systems for quality evaluation of embryos (Russel *et al.*, 2006; Wrenzycki *et al.*, 2007) emphasizing several markers of developmental competency (Russel *et al.*, 2006).

In this work, embryos cryopreserved by the slow freezing method showed better survival rates when co-cultured in TCM199 in granulosa cells. This finding suggests that the culture conditions after cryopreservation can modify the survival of the embryos and makes difficult the choice of cryopreservation protocol. The true viability of cryopreserved embryos needs to be investigated by direct transfer into recipients because confirmation of pregnancy or birth of calves is the most rigorous criterion for assessment (Tominaga *et al.*, 2007). However, pregnancy rates cannot easily be compared (Van Wagtendonk-de Leeuw *et al.*, 1995). Our results showed that the same problem of comparisons of results of different work-groups happens with *in vitro* evaluation of embryo survival after cryopreservation.

Based on the data from this work, we suggest that, under our conditions, controlled freezing (1.2°C/min) can be an alternative approach for cryopreservation of IVP bovine embryos.

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References

Assumpção, M.E.A.O., Milazzotto, M.P., Simões, R., Nicacio, A.C., Mello, M.R.B. & Visintin, J.A. (2008). In vitro survival of *in vitro*-produced bovine embryos cryopreserved by slow freezing, fast freezing and vitrification. *Anim. Reprod.* **5**, 116–20.

Barceló-Fimbres, M. & Seidel Jr, G.E. (2007), Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during *in vitro* culture of bovine embryos on embryonic development after cryopreservation. *Mol. Reprod. Dev.* **74**, 1395–405.

Campos-Chillon, L.F., Wlaker, D.J., De La Torre-Sanchez, J. & Seidel Jr, G.E. (2006). In vitro assessment of a direct transfer

vitrification procedure for bovine embryos. *Theriogenology* **65**, 1200–14.

Corcoran, D., Rizos, D., Fair, T., Evans, A.C.O. & Lonergan, P. (2007). Temporal expression of transcripts related to embryo quality in bovine embryos cultured from the two-cell to blastocysts stage *in vitro* or *in vivo*. *Mol. Reprod. Dev.* **74**, 972–7.

Fahning, M.L. & Garcia, M.A. (1992). Status of cryopreservation of embryos from domestic animals. *Cryobiology* **29**, 1–18.

George, F., Daniaux, C., Genicot, G., Verhaeghe, B., Lambert, P. & Donnay, I. (2008). Set up of a serum-free culture system for bovine embryos: embryo development and quality before and after transient transfer. *Theriogenology* **69**, 612–23.

Hochi, S., Seemple, E. & Leibo, S.P. (1996). Effect of cooling and warming rates during cryopreservation on survival of *in vitro*-produced bovine embryos. *Theriogenology* **46**, 837–47.

Jin, B., Kusanagi, K., Ueda, M., Seki, S., Valdez Jr, D.M., Edashige, K. & Kasai, M. (2008). Formations of extracellular and intracellular ice during warming of vitrified mouse and its effect on embryo survival. *Cryobiology* **56**, 233–40.

Kaidi, S., Bernard, S., Lambert, P., Massip, A., Dessy, F. & Donnay, I. (2001). Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced *in vitro*. *Biol. Reprod*. **65**, 1127–34.

Kaidi, S., Van Langendockt, A., Massip, A., Dessy, F. & Donnay, I. (1999). Cellular alteration after dilution of cryoprotective solutions used for the vitrification of *in vitro*-produced bovine embryos. *Theriogenology* **52**, 515–25.

Leibo, S.P. (2008). Cryopreservation of oocytes and embryos: optimization by theoretical versus empirical analysis. *Theriogenology* **69**, 37–47.

Leoni, G., Bogliolo, L., Berlinguer, F., Rosati, I., Pintus, P.P., Ledda, S. & Naitana, S. (2002). Defined media for vitrification, warming, and rehydration: effects on post-thaw protein synthesis and viability of *in vitro* derived ovine embryos. *Cryobiology* **45**, 204–12.

Lonergan, P., Fair, T., Corcoran, D. & Evans, A.C.O. (2006). Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology* **26**, 137–52.

Marquez-Alvarado, Y.C., Gallina, C.S., Castilla, B., Leon, H. & Moreno-Mendoza, N. (2004). Evidence of damage in cryopreserved and fresh bovine embryos using the Tunnel technique. *Reprod. Dom. Anim.* **39**, 141–5.

Martinez, A.G., Valcárcel, A., De Las Heras, M.A., De Matos, D.G., Furnus, C. & Brogliatti, G. (2002). Vitrification of *in vitro* produced bovine embryos: *in vitro* and *in vivo* evaluations. *Anim. Reprod. Sci*. **73**, 11–21.

Mello, M.R.B., Queiroz, V.S., Lima, A.S., Tavares, L.M.T., Assumpção, M.E.O.A., Wheeler, M.B. & Visintin, J.A. (2001). Cryopreservation of mouse morulae through different methods: slow-freezing, vitrification and quick-freezing. *Braz. J. Vet. Res. Anim. Sci.* **38**, 160–4.

Mucci, N., Aller, J., Kaiser, G.G., Hozbor, F., Cabodevilla, J. & Alberio, R.H. (2005). Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology* **65**, 1551–62.

Nedambale, T.L., Dinnyés, A., Groen, W., Dobrinsky, J.R., Tian, X.C. & Yang, X. (2004). Comparison on *in vitro* fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. *Theriogenology* **62**, 437–49.

Pereira, D.C., Dode, M.A.N. & Rumpf, R. (2005). Evaluation of different culture systems on the *in vitro* production of bovine embryos. *Theriogenology* **63**, 1131–41.

Robert, C., McGraw, S., Massicotte, L., Pravetoni, M., Gandolfi, F. & Sirard, M.A. (2002). Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol. Reprod.* **67**, 1465–72.

Russell, D.F., Baqir, S., Bordignon, J. & Betts, D.H. (2006). The impact of oocyte maturation media on early bovine embryonic development. *Mol. Reprod. Dev.* **73**, 1255–70.

Silva, F.M. & Metelo, R. (2005). Relation between physical properties of the zona pellucida and viability of bovine embryos after slow-freezing and vitrification. *Reprod. Dom. Anim.* **40**, 205–9.

Tavares, L.M.T., Feitosa, W.B., Mello, M.R.B., Nicacio, A.C., Lima, A.S., Assumpção, M.E.A.O. & Visintin, J.A. (2008). Is the early reduction of fetal calf serum concentration in bovine *in vitro* embryo culture beneficial? *Anim. Reprod*. **5**, 34–8.

Thompson, J.G. (1997). Comparison between *in vitro*-derived and *in vitro*-produced pre-elongation embryos from domestic ruminants. *Reprod. Fertil. Dev.* **9**, 341–54.

Tominaga, K., Iwaki, F. & Hochi, S. (2007). Conventional freezing of *in vitro*-produced and biopsied bovine blastocysts in the presence of a low concentration of glycerol and sucrose. *J. Reprod. Dev.* **53**, 443–7.

Vajta, G., Holm, P., Greve, T. & Callesen, H. (1996). Factors affecting survival rates of *in vitro* produced bovine embryos after vitrification and direct in-straw rehydration. *Anim. Reprod. Sci.* **45**, 191–200.

Van Wagtendonk-De Leeuw, A.M., Den Daas, J.H.G., Kruip, T.H.A.M. & Rall, W.F. (1995). Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. *Cryobiology* **32**, 157–67.

Wrenzycki, C., Herrmann, D. & Niemann, H. (2007). Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* **68**, 77–83.