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Vitrification of *in vitro* produced Zebu embryos

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Abstract

Survival and pregnancy rates achieved after traditional, slow-cooling-rate cryopreservation of *in vitro* produced Zebu (*Bos indicus*) embryos are generally poor. Vitrification is considered an alternative to traditional methods for preservation of embryos. The aim of the present experiment was to evaluate the addition of sucrose to vitrification medium and the influence of embryonic diameter on survival rates of *in vitro* produced Zebu embryos within each treatment. Oocytes recovered by ovum pick-up (OPU) were matured for 24 hours, fertilized with frozen-thawed spermatozoa from a Nelore bull, and cultured *in vitro* in controlled conditions (5% CO_2, 5% O_2, 90% N_2, and saturated humidity). Two treatments were used to evaluate the effect of sucrose addition to the vitrification medium. All of the embryos were measured before vitrification to evaluate the influence of diameter on survival rate after warming. Day 7, excellent-quality blastocysts and expanded blastocysts were equilibrated for 40 seconds in either 25% ethylene glycol (EG) and 25% dimethylsulfoxide (DMSO; Treatment 1; n = 30) or 20% EG, 20% DMSO, and 0.5 M sucrose (Treatment 2; n = 34) and then loaded into open pulled straws (OPS) and immersed into liquid nitrogen. At warming, the open end of the OPS was immersed into 0.5 M sucrose solution, and embryos were subsequently rehydrated in decreasing concentrations of sucrose. Re-expansion and hatching rates were determined at 24 and 48 hours after warming and culture, respectively. There were no differences in the rates of re-expansion (30.0% vs. 44.1%) or hatching (13.3% vs. 23.5%) of embryos that had been cryopreserved using either of the two treatments. No differences were observed between diameters of viable embryos after vitrification and those that did not survive after treatments. Although more studies should be carried out to improve the viability of *in vitro* produced Zebu embryos after cryopreservation, at the present time, vitrification is probably the best method for cryopreservation of sensitive embryos.

Keywords: cryopreservation, sucrose, embryo, bovine, vitrification, Zebu.

Introduction

Cryopreservation of *in vitro* produced embryos derived from animals of high genetic merit has allowed genetically-superior embryos to be stored indefinitely (Dinnyés *et al*., 1999; Kaidi *et al*., 1999; Reichenbach, 2003). However, *in vitro* produced bovine embryos are sensitive to cooling and conventional freezing protocols (Massip *et al*., 1995). This fact is mainly due to the extreme sensitivity of these embryos to slow cooling (0.5°C per minute), principally at temperatures below -6°C (Mahmoudzadeh *et al*., 1994). This sensitivity may be due to the presence of large amounts of lipids in the cytoplasm of *in vitro* produced bovine embryos (Leibo and Loskutoff, 1993). Nevertheless, *in vitro* produced *Bos taurus* embryos have higher survival rates compared to *Bos indicus* embryos, when cryopreserved using a conventional freezing method. Vitrification, an alternative to conventional cryopreservation methods, is an ultra-fast method wherein a viscous, highly-concentrated cryoprotectant solution, submitted to sufficiently low temperatures (-120ºC), suffers direct passage of the liquid state to a vitrified and amorphous state (Rall and Fahy, 1985).

The addition of a sugar such as sucrose, which does not penetrate the cell membrane, to an ethylene-glycol-based vitrification solution promotes cellular dehydration. Besides, it may significantly reduce the amount of cryoprotectant required as well as the toxicity of ethylene glycol (EG) by decreasing its concentration. The non-permeating sucrose also acts as an osmotic buffer by reducing the osmotic shock associated with freezing (Liebermann *et al*., 2002).

Because the vitrification technique has shown better *in vitro* results for more sensitive embryos, the objectives of the present study were to evaluate the addition of sucrose to the vitrification medium and the influence of embryonic diameter on survival rates of *in vitro* produced Zebu embryos within each treatment.

Materials and Methods

All materials were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. The OPSs were generously supplied by Dr. Vajta and Dr. Mezzalira. Eleven Nelore cows, on the second or third parturition, were submitted to ovum pick-up (OPU) following a gynecological examination and body condition evaluation that, ranged from 3 to 3.5 for all donors. The animals were maintained on pasture and received

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mineral supplementation *ad libitum.* Ovum pick-up was performed every 15 days. The local mean temperature was 26ºC and air humidity 67%. Follicles with a diameter between 2 and 6 mm were aspirated, and the cumulus-oocytes complexes (COCs) were transported to laboratory in TCM-199 with HEPES at 37ºC within three hours after aspiration.

In vitro embryo production

For the *in vitro* development of bovine embryos, 4 replicates were conducted using the same procedure. Recovered COCs were selected (Leibfried and First, 1979), washed in TCM-199 with HEPES, and transferred within culture dishes to 100-µl drops of maturation medium consisting of TCM 199 with bicarbonate, 10% fetal calf serum (FCS; Nutricell - Campinas, SP), 22 µg/ml pyruvate, 50 µg/ml LH (Lutropin®; Vetrepharm, Canada Inc.), 0.5µg/ml FSH (Folltropin® –V; Vetrepharm, Canada Inc.), and 50 µg/ml penicillin G sodium and 50 µg/ml gentamycin. The drops containing 20 oocytes were covered with liquid mineral oil and cultured for 24 hours in a CO_2 incubator (5% CO_2 in humidified air) at 38.7°C. After maturation, oocytes were washed in TCM-199 with HEPES and transferred in groups of 10 to drops of 100 µl of fertilization medium-TALP (Parrish *et al*., 1995) supplemented with 50 µg/ml gentamycin and penicillin, 2.7 µg/ml penicillamine, 1 µg/ml hypotaurine and 0.3 µg/ml epinephrine, 5 µg/ml bovine serum albumin (BSA-code 8806), 22 µg/ml pyruvate, and 10 µg/ml heparin. For the *in vitro* fertilization (IVF) procedure, 0.5 ml straws of frozen semen from two Nelore bulls were thawed at 37°C for 30 seconds. The Percoll gradient technique was used to separate sperm (Palma, 1993). Sperm cell concentration was measured using a hemocytometer. Oocytes were fertilized with a final sperm concentration of $2x10^6$ spermatozoa/ml. Oocytes and spermatozoa were co-cultured in fertilization medium in an incubator at 38.7 °C in 5% CO_2 in air and saturated humidity for 20 hours. The day of fertilization was considered Day 0.

Following co-culture, oocytes and zygotes were submitted to partial or total mechanical removal of the cumulus cells using an automatic pipette. Subsequently, they were washed in TCM-199 with HEPES and transferred in groups of 10 to drops of 100 µl of synthetic oviduct fluid (SOF; Holm *et al*., 1999). Zygotes were incubated for 7 days at 38.7°C in 5% CO_2, 5% O_2, 90% N_2, and saturated humidity. A total of 4 replicates of *in vitro* embryo production were conducted. On Day 7 after fertilization, embryos were morphologically evaluated using a stereo microscope (200x). Blastocysts or expanded blastocysts classified as Quality 1, according to International Embryo Transfer Society (IETS; Robertson and Nelson, 1999), were selected for vitrification.

Vitrification

The vitrification experiment was replicated four times. The cryoprotectant agents, dimethylsulphoxide (DMSO), EG, and sucrose solution, were diluted in holding medium (HM) composed of TCM-199 and 20% FCS. Sucrose solution was prepared according to Vajta *et al*. (1997), and two vitrification treatments were tested: Treatment 1 (n = 30) - 50% HM + 25% EG + 25% DMSO (40 seconds at 39°C) and Treatment 2 (n = 34) - 50% HM + 20% EG + 20% DMSO + 0.5 M sucrose solution (40 seconds at 39°C).

Embryos were measured using a bright field microscope with an ocular micrometer (scale 1mm/100µm). After that, an adjustment factor obtained with objective micrometer was applied to the measurements recorded from the ocular micrometer (Ueno and Gonçalves, 1998). After measurement, the embryos were equally distributed between treatments according to their diameters. One to three embryos were loaded in an open pulled straw (OPS; Vajta *et al*., 1997), considering the bull used for fertilization, embryo diameter, and treatment. Each OPS contained embryos with a maximal 5 µm difference in diameter. All of the procedures were carried out at room temperature between 28 and 30°C, and the media were maintained at 39°C. Therefore, the temperature of vitrification solutions was between 34 and 36°C.

To reduce the toxicity of the cryoprotectants, embryos were exposed to vitrification solutions in four steps by increasing cryoprotectant concentrations: 1) HM (1 minute/39°C); 2) HM (5 minutes/39°C); 3) vitrification solution 1 (VS1) - 75% HM, 20% EG, 5% DMSO (1 minute/39°C); and 4) VS2-Treatment 1- 50% HM, 25% EG, 25% DMSO (40 seconds/39°C) or VS2-Treatment 2 - 50% HM, 20% EG, 20% DMSO, 10% sucrose 0.5 M (40 seconds/39°C). After the four-step process, embryos were loaded into OPSs and submerged into liquid nitrogen.

At warming, the open end of OPS was immersed into a 0.5 M sucrose solution, and the vitrification media became liquid in 2-3 seconds. Thereafter, embryos were rehydrated in sucrose solution in four steps (Vajta *et al*., 1998): 1) HM + 0.5 M sucrose solution (1 minute); 2) HM + 0.5 M sucrose solution (5 minutes); 3) HM + 0.3 M sucrose solution (5 minutes); and 4) HM (5 minutes). Step 1 was performed to remove the cryoprotectant solution.

After the embryos were cultured for 48 hours in an incubator at 38.7 °C in a saturated atmosphere of 5% CO2, 5% O2, and 90% N2, their survival was assessed. Re-expansion rate was evaluated at 24 hours and hatching rate at 48 hours post-culture. After measurement using a bright field microscope (200x), embryos that reached a diameter equal or greater than their diamete

prior to vitrification were considered re-expanded.

Statistical analysis

Data were analyzed using the SAS program (Statistical Analyses System). Rates of re-expansion and hatching following warming and culture as well as embryonic diameter were analyzed by the Chi-square test. A probability of $P < 0.05$ was considered significant.

Results

Embryo production, re-expansion, and hatching rates

From 406 recovered oocytes, a cleavage rate of 64.0% and an embryonic development rate of 32.7% on Day 7 were observed (Table 1). The embryonic re-expansion and hatching rates post-warming did not differ between Treatments 1 (Table 2).

Table 1. Embryonic production rates.

Mature oocytes (n)	Cleavage rate (Day 3)	Embryonic development rate (Day 7)
406	64.0% (259/406)	32.7% (132/406)

Table 2. Re-expansion and hatching rates of vitrified/thawed embryos.

Treatment	Number of blastocysts		
	Vitrified	Re-expanded (24 hours)	Hatched (48 hours)
1	30	9 (33.0%) [a]	4 (13.3%) [b]
2	34	15 (44.1%) [a]	8 (23.5%) [b]

[a,b]Values with different letters within each column are different ($P < 0.05$).

Embryo diameter, stage, and quality

Embryos were distributed in three categories according to their diameter: 160 to 175 µm (T1, n = 8; T2, n = 8), 180 to 200 µm (T1, n = 18; T2, n = 20), and > 205 µm (T1, n = 4; T2, n = 6). Neither diameter of vitrified embryos nor treatment influenced the survival rate after warming and culture for 24 hours.

Among the viable embryos, no significant difference in diameter was observed before and after vitrification in Treatment 1, while in Treatment 2, a significant increase in diameter after warming and culture for 24 hours was observed (Table 3).

All viable embryos were vitrified at the expanded blastocyst stage (14/64), while vitrified embryos at blastocyst stage did not survive (3/64). All vitrified embryos lost one or two points of classification after warming, except for two embryos in Treatment 1 that remained Quality 1 (excellent). One embryo from Treatment 1 had a fractured zona pellucida.

Table 3. Diameter of viable embryos before and after vitrification.

Treatment	n	Embryo diameter (µm)	
		Before vitrification	After warming and culture
1	9	212.2 ± 19.7 [aA]	208.8 ± 13.9 [bA]
2	15	201.0 ± 25.1 [aA]	214.0 ± 29.2 [bB]

[a,b]Values with different letters within each column are different ($P < 0.05$).
[A,B]Values with different letters within each row are different ($P < 0.05$).

Discussion

In the present experiment, the cleavage (64.0%) and embryonic development rates (32.7%) were similar to cleavage (60.7%) and development (36.5%) rates previously reported (Pugh *et al*., 2000) in an experiment that used the same culture medium. However, higher cleavage (92%) and embryonic development rates (37%; Han *et al*., 1994), as well as lower cleavage (44%) and blastocyst development (16%) rates, were also reported (Dinnyés *et al*., 1999). The difference in the results between previous studies and the present study was possibly associated with different oocyte sources. Most studies used oocytes recovered from ovaries collected from cows from slaughterhouses whereas oocytes used in the present study were obtained from OPU of Zebu cows. Besides, some factors like maturation medium, variations in fertilization procedure, and duration of each step of the process might result in different embryonic development rates.

While re-expansion and hatching rates of embryos vitrified with Treatment 1 of the present experiment were 33.0% and 13.3%, respectively, studies

that used the same vitrification solution reported higher re-expansion (83% to 97%) and hatching (49.6% to 72%) rates (Vajta *et al*., 1995; 1996; Saalfeld *et al*., 2002; Mezzalira *et al*., 2003). This could be explained by less exposure time (20 seconds) of the embryos to vitrification solution in reported studies compared to the exposure time used in the present experiment (40 seconds).

Likewise, embryo re-expansion and hatching rates in Treatment 2 were lower (44.1% and 23.5%, respectively) compared to studies that used a lower concentration of cryoprotectant (33% and 44%) in vitrification protocols (74% to 81% and 46% to 94%, respectively; Vajta *et al*., 1999; Lazar *et al*., 2000). Besides, exposure time of embryos to the vitrification solution was also lower (20 to 30 seconds) than that used in the present experiment (40 seconds). It is possible that lower cryoprotectant concentration associated with less exposure time of embryos to the vitrification solution could result in a decrease of vitrification medium toxicity (Rall and Fahy, 1985; Kasai *et al*., 1992; Varago, 2005). In contrast, when the same protocol of Treatment 2 was used, no re-expansion rate was reported (Siqueira-Pyles *et al*., 2003; 2004).

Time and temperature of embryo exposure to the vitrification solution are directly related to the toxicity of the permeant cryoprotectant. Toxicity depends on the concentration of cryoprotectant in the vitrification solution and the penetration rate of the cryoprotectant through the cells, which is influenced by temperature and exposure time of the embryo to the vitrification solution (Saha and Suzuki, 1997). Usually, in the vitrification protocols of *in vitro* produced *Bos taurus* embryos, the exposure time to vitrification solution does not exceed 30 seconds when medium temperature is at 23°C and 39°C and the concentration of cryoprotectant between 33% and 50% (Donnay *et al*., 1998; Vajta *et al*., 1998; Gutiérrez-Adán *et al*., 1999; Vajta *et al*., 1999; Lazar *et al*., 2000; Saalfeld *et al*., 2002). On the other hand, when the temperature of the vitrification solution was lowered (22°C to 4°C), the exposure time was increased from 45 seconds to 2 minutes (Mahmoudzadeh *et al*., 1995; Saha and Suzuki, 1997; Vajta *et al*., 1997; Oliveira *et al*., 1998). In the present experiment, temperature of the vitrification solution and exposure time of embryos were higher than those reported in other protocols, which may have been the cause of higher toxicity of vitrification solution.

As described with *Bos taurus* embryos, similar results were observed in the present experiment in which addition of 0.5 M sucrose to the vitrification medium did not improve embryonic survival rates (Mahmoudzadeh *et al*., 1995; Oliveira *et al*., 1998). Likewise, when another sugar (0.85 M galactose) was added to the vitrification medium, no significant increase in survival rate was reported (Donnay *et al*., 1998). During cryopreservation, a high sucrose concentration is required in order to promote dehydration and toxicity reduction of a permeant cryoprotectant when *in vitro* produced bovine embryos are exposed to the vitrification solution for a long time (40 seconds; Mahmoudzadeh *et al*., 1995). Therefore, it is possible that the sucrose concentration used in the present experiment was not enough to significantly increase the embryonic survival rate post-warming. Thus in the present experiment, the addition of sucrose to the vitrification medium probably did not reduce the toxicity of permeant cryprotectors to the embryos, which contradicts preview reports (Massip *et al*., 1987; Dobrinski *et al*., 1992; Liebermann *et al*., 2002; Guignot *et al*., 2006).

Although higher survival rates (100%) of expanded blastocysts larger than 150 µm and 180 µm have been reported by Dinnyés *et al*. (1999) and Donnay *et al*. (1998), respectively, embryonic diameter before vitrification did not influence embryo viability post warming regardless of treatment in the present experiment. The higher viability of *in vitro* produced bovine embryos of a larger diameter and in the developmental stage of an expanded blastocyst could be related to the acquisition of unknown properties that made them more resistant to the cryopreservation process (Carvalho *et al*., 1995; Hasler *et al*., 1997). Although it has not yet been proven for bovine embryos, these properties in swine embryos are described as modifications of lipid composition of the membrane during embryonic development, which reduces the embryo's sensitivity to cooling (Nagashima *et al*., 1992; Dobrinski, 1996). Nevertheless, in the present experiment, different methods for embryo production and vitrification were used and could have led to similar survival rates for embryos with different diameters.

Although survival rates were not influenced by embryo size and addition of sucrose, viable embryos in Treatment 2 had a significant increase in diameter after warming and culture for 24 hours. This embryo size increase could be due to a continued development, probably caused by the presence of sucrose in the vitrification medium. Moreover, the length of the *in vitro* culture period after warming may also play a role in determining the number of embryos that reach the re-expansion stage (Vajta *et al*., 1996). Therefore, an evaluation of re-expansion rate after culture for 48 hours could have resulted in higher re-expansion rates in both treatments.

When *in vitro* embryos were vitrified in 0.25 ml straws, rates of 20.4% to 27.0% of zona pellucida damage were reported (De Paz *et al*., 1994; Titterington *et al*., 1995). In the present experiment, one embryo from Treatment 1 (1.6%) had fracture of the zona pellucida. In closed straws, air bubbles shrink and expand rapidly in parallel with the changes in temperature, resulting in significant pressure changes and movements in the partially solidified solutions. Using the OPS method, no pressure changes occur around the solution, thus fracture damage is minimal

(Vajta *et al*., 1997). Probably, the use of the OPS method in the present experiment prevented fracture of the zona pellucida of *in vitro* produced Zebu embryos.

It has been reported that *in vitro* produced Zebu embryos contain a greater number of mitochondria and consequently mitochondrial membranes compared to *in vitro* produced *Bos taurus* embryos (Esper and Barbosa, 1991). Therefore, it can be suggested that a higher number of membranes could lead to a higher sensitivity of *in vitro* produced Zebu embryos to the cryopreservation process.

Although more studies should be carried out to improve the viability of *in vitro* produced Zebu embryos after cryopreservation, at the present time, vitrification is probably the best method for cryopreservation of sensitive embryos.

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References

Carvalho RV, Del Campo MR, Plante Y, Mapletoft R J. 1995. Effects of stage of development on sex ratio and survival after freezing of day 7 bovine IVF embryos. *Theriogenology,* 43:183. (abstract).

De Paz P, Sanchez AJ, Fernandez JG, Carbajo M, Dominguez JC, Chamorro CA, Anel L. 1994. Sheep embryo cryopreservation by vitrification and conventional freezing, *Theriogenology*, 42:327-338.

Dinnyés A, Carolan C, Lonergan P, Massip A, Mermillod P. 1999. Survival of frozen or vitrified bovine blastocysts produced "*in vitro*" in synthetic oviduct fluid. *Theriogenology*, 46:1425-1439.

Dobrinski JR, Stice SL, Phillips PE, Duby RT, Robl JM. 1992. Development of IVM-IVF bovine embryos following vitrification dilution treatments. *Theriogenology,* 37:202. (abstract).

Dobrinski JR. 1996. Cellular approach to cryopreservation of embryos. *Theriogenology,* 45:17-26.

Donnay I, **Auquier P**, **Kaidi S**, **Carolan C**, **Lonergan P**, **Mermillod P**, **Massip A**. 1998. Vitrification of *in vitro* produced bovine blastocysts: methodological studies and development capacity. *Theriogenology,* 52:93-104.

Esper CR, Barbosa IC. 1991. Ultraestrutura comparativa de embriões bovinos. *In*: Anais do 9º Congresso Brasileiro de Reprodução Animal, 1991, Belo Horizonte. Belo Horizonte, MG: CBRA. p.295. (abstract).

Guignot F, Bouttier A, Baril G, Salvetti P, Pignon P, Beckers JF, Touze JL, Cognie J, Traldi AS, Cognie Y, Mermillod P. 2006. Improved vitrification method allowing direct transfer of goat embryos. *Theriogenology,* 66:1004-1011.

Gutiérrez-Adán A, Granados J, De La Fuente J. 1999. Effect of post-thaw cell rehydration at 4°C on survival of frozen and vitrified IVP-derived bovine embryos. *Reprod Nutr Dev,* 39:517-520.

Han YM, Yamashina H, Koyama N, Lee KK, Fukui Y. 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 JF, Hurtgen PJ, Jin ZQ, Stokes JE. 1997. Survival of IVF-derived bovine embryos frozen in glycerol or ethylene glycol. *Theriogenology,* 48:563-579.

Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H. 1999. High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol or without serum-proteins. *Theriogenology*, 52:683-700.

Kaidi S, Van Langendonckt 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*, 52:515-525.

Kasai M, Nishimori M, Zhu SE, Sakurai T, Machida T. 1992. Survival of mouse morulae vitrified in an ethylene glycol-based solution after exposure to the solution at various temperatures. *Biol Reprod*, 47:1134-1139.

Lazar L, Spak J, David V. 2000. The vitrification of *in vitro* fertilized cow blastocysts by the open pulled straw method. *Theriogenology,* 54:571-578.

Leibfried L, First N L. 1979. Characterization of bovine follicular oocytes and their ability to mature "*in vitro*". *J Anim Sci*, 48:76-86.

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

Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. 2002. Potential importance of vitrification in reproductive medicine. *Biol Reprod*, 67:1671-1680.

Mahmoudzadeh AR, Van Soom A, Ysebaert MT, de Kruif A. 1994. Comparison of two-step vitrification versus controlled freezing on survival of *in vitro* produced cattle embryos. *Theriogenology,* 42:1389-1397.

Mahmoudzadeh AR, Van Soom A, Bols P, Ysebaert MT, de Kruif A. 1995. Optimization of simple vitrification procedure for bovine embryos produced *in vitro*: effect of developmental stage, two steps addition of cryoprotectant and sucrose dilution on embryonic survival. *J Reprod Fertil*, 103:33-39.

Massip A, Van der Zwalmen P, Ectors F. 1987. Recent progress in cryopreservation of cattle embryos. *Theriogenology,* 27:69-79.

Massip A, Mermillod P, Dinnyés A. 1995. Morphology and biochemistry of *in vitro* produced bovine embryos: implications for their cryopreservation. *Human Reprod*, 10:3004-3011.

Mezzalira A, Mezzalira JC, Barbieri DP, Lohn L,

Santos RM, Vieira AD, Damiani JG. 2003. Vitrificação de embriões bovinos produzidos *in vitro*: avaliação de dois protocolos. *Acta Sci Vet,* 31:480. (abstract).

Nagashima H, Yamakwa H, Niemann H. 1992. Freezability of porcine blastocysts at peri-hatching stages. *Theriogenology*, 37:839-850.

Oliveira ATD, Rodrigues JL, Niemann H. 1998. Vitrification of bovine IVMFC-derived embryos dehydrated with 3.6 M of ethylene glycol and sucrose. *Acta Sci Vet*, 26:335. (abstract).

Palma GA. 1993. Transferencia de los embriones. *In*: Palma GA, Brem G. *Transferencia de embriones y biotecnologia de la reproducción en la especie bovina*. Buenos Aires: Hemisferio Sur. pp.143-162.

Parrish JJ, Krogenaes A, Susko-Parrish JL. 1995. Effect of bovine sperm separation by either swim-up and percoll method on success of *in vitro* fertilization and early *embryonic* development. *Theriogenology,* 44:859-869.

Pugh PA, Tervit HR, Niemann H. Effects of vitrification medium composition on the survival of bovine *in vitro* produced embryos following in straw dilution *in vitro* and *in vivo* following transfer. *Anim Reprod Sci*, 2000; 58:9-22.

Rall WF, Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature*, 313:573-575.

Reichenbach HD. 2003. Transferência e descongelação de embriões bovinos: considerações práticas. *Acta Sci Vet,* 31:15-50.

Robertson I, Nelson R. 1999. Certificação e identificação de embriões. *In*: Stringfellow DA, Seidel SM (Eds.). Manual da Sociedade Internacional de Transferência de Embriões. 3. ed. *Savoy, IL:* IETS. pp.109-140.

Saalfeld MH, Durr GC, Pergoraro LM, Vetromila MA, Rhneingantz MG, Anghinoni LB, Pivato I, Vajta G. 2002. Vitrification of embryos from jersey cows by the OPS procedure. *Theriogenology,* 57:560. (abstract).

Saha S, Suzuki T. 1997. Vitrification of *in vitro* produced bovine embryos at different ages using one and three-step addition of cryoprotective additives. *Reprod Fertil Dev,* 9:741-746.

Siqueira-Pyles ESC, Fernandes CB, Peres KR, Landim-Alvarenga FC. 2003. Utilização de diferentes agentes crioprotetores na viabilidade de embriões bovinos submetidos à vitrificação em Open Pulled Straw. *Acta Sci Vet,* 31:598. (abstract).

Siqueira-Pyles ESC, Fernandes CB, Landim-Alvarenga FC. 2004. Utilização de etilenoglicol, dimetilsulfóxido e dimetilformamida na vitrificação de embriões bovinos em Open Pulled Straws. *Acta Sci Vet*, 32:162. (abstract).

Titterington JL, Robinson J, Killick SR, Hay DM. 1995. Synthetic and biological macromolecules: protection of mouse embryos during cryopreservation by vitrification. *Human Reprod*, 10:649-653.

Ueno H, Gonçalves PC. 1998. *Manual para diagnóstico de helmintoses dos ruminantes*. 2. ed. Brasília: Japan International Cooperation Agency.

Vajta G, Holm P, Greve T, Callesen H. 1995. Direct in-straw rehydration after thawing of vitrified *in vitro* produced embryos. *Vet Rec*, 137:672.

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.

Vajta G, Booth PJ, Holm P, Greve T, Callesen H. 1997. Successful vitrification of early stage bovine *in vitro* produced embryos with open pulled straw ("OPS") method. *Cryo-Lett*, 18:191-195.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. 1998. Open pulled straw ("OPS") vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev,* 51:53-58.

Vajta G, Murphy CN, Macháty Z, Prather RS, Greve T, Callesen H. 1999. In straw dilution of bovine blastocysts after vitrification with the open pulled straw method. *Vet Rec*, 144:180-181.

Varago FC. 2005. *Effect of sucrose addition to the vitrification medium of in vitro produced zebu embryos* [in Portuguese]. 46p. Belo Horizonte, MG: UFMG/Veterinary School. Dissertation.