Ciência Rural
ISSN: 0103-8478
cienciarural@mail.ufsm.br
Universidade Federal de Santa Maria
Brasil

Marques dos Santos, Rodrigo; Barreta, Marcos Henrique; Frajblat, Marcel; Córdova Cucco, Diego; Mezzalira, Joana Claudia; Bunn, Silvério; Buss Cruz, Fabiano; Diniz Vieira, Arnaldo; Mezzalira, Alceu
Vacuum-cooled liquid nitrogen increases the developmental ability of vitrified-warmed bovine oocytes
Ciência Rural, vol. 36, núm. 5, setembro-outubro, 2006, pp. 1501-1506
Universidade Federal de Santa Maria
Santa Maria, Brasil

ISSN 0103-8478

Vacuum-cooled liquid nitrogen increases the developmental ability of vitrified-warmed bovine oocytes

Nitrogênio super resfriado por vácuo melhora a capacidade de desenvolvimento de oócitos bovinos após vitrificação

Rodrigo Marques dos Santos[1] Marcos Henrique Barreta[1] Marcel Frajblat[2] Diego Córdova Cucco[1] Joana Claudia Mezzalira[1] Silvério Bunn[1] Fabiano Buss Cruz[1] Arnaldo Diniz Vieira[1] Alceu Mezzalira[1*]

ABSTRACT

The objective of this study was to determine the effects of vacuum-cooled liquid nitrogen on the development of vitrified immature (germinal vesicle stage; GV) and mature (metaphase II; MII) bovine oocytes after re-warming. Liquid nitrogen was exposed to either atmospheric pressure or to a vacuum (300mm Hg for 45sec); the latter decreased the temperature of the liquid nitrogen to -200°C. Partially denuded oocytes were vitrified either just after selection (GV) or after 22 hours of in vitro maturation (MII) in TCM 199 medium + 10% of estrous mare serum. For vitrification, oocytes were firstly exposed to an intermediate solution (10% EG + 10% DMSO) for 30sec, followed by the vitrification solution (20% EG + 20% DMSO + 0.5M sucrose) for 20sec. Groups of three or four oocytes were loaded into an open-pulled-straw and directly plunged into liquid nitrogen. Oocytes were subsequently re-warmed by exposure to air (25°C) for 4sec, followed by 5 min exposure to decreasing concentrations (0.3 and 0.15M) of sucrose. Fertilization (Day 0) was done with 2 x 10^6 spermatozoa mL^{-1} (selected by a swim-up procedure) and incubated for 18 to 22 hours. Presumptive zygotes were cultured at 39ºC in four-well dishes with SOFaaci medium, under 5% CO_2 and saturated humidity. Cleavage (Day 2) and blastocyst rates (Day 8) were 33.9 and 4.2%, respectively, for GV stage oocytes at atmospheric pressure, 41.2 and 8.8% for GV oocytes under vacuum, 43.5 and 6.7% for MII oocytes at atmospheric pressure, and 53.6 and 10.6% for MII oocytes under vacuum. In conclusion, vacuum-cooled liquid nitrogen improved developmental rates of vitrified-thawed bovine oocytes.

Key words: *vitrification; cryopreservation; oocytes; bovine; vacuum.*

RESUMO

O objetivo deste estudo foi determinar o efeito do nitrogênio liquido super resfriado por vácuo no desenvolvimento, após reaquecimento, de oócitos bovinos vitrificados imaturos ou maturados. O nitrogênio líquido foi mantido em atmosfera normal ou submetido ao vácuo (300mm Hg por 45s) este último reduzindo a temperatura do nitrogênio para -200°C. Oócitos parcialmente desnudos foram vitrificados logo após a seleção (estádio de vesícula germinativa; VG), ou após 22 horas de maturação (metáfase II; MII) em meio TCM 199 + 10% de soro de égua em estro. Para a vitrificação, os oócitos foram inicialmente expostos a uma solução intermediária (10% EG + 10% DMSO) por 30s e a seguir a uma solução de vitrificação (20% EG + 20% DMSO + 0,5M sacarose) por 20s. Grupos de 3 ou 4 oócitos foram envasados em palhetas estiradas e abertas e mergulhados no nitrogênio líquido. Os oócitos foram então reaquecidos por exposição ao ar (25°C) por 4s, seguido de exposição a concentrações decrescentes de sacarose (0,3 e 0,15M - 5 minutos cada). A fecundação (dia 0) foi realizada com 2 x 10^6 espermatozóides mL^{-1} (selecionados por "swim-up") e incubação por 18 a 22 horas. Os presumíveis zigotos foram cultivados a 39°C, em placas de quatro poços, com meio SOFaaci, com 5% de CO_2 e umidade saturada. As taxas de clivagem (Dia 2) e de blastocistos (Dia 8) obtidas foram de 33,9 e de 4,2%, respectivamente, para oócitos no estágio de VG / pressão normal, de 41,2 e 8,8% para oócitos VG / vácuo, 43,5 e 6,7% para oócitos MII / pressão normal e de de 53,6 e 10,6% para oócitos MII / vácuo. Conclui-se que o emprego de nitrogênio líquido super resfriado pelo vácuo melhora as taxas de desenvolvimento de oócitos bovinos após a vitrificação.

Palavras-chave: *vitrificação; criopreservação; oócitos; bovino; vácuo.*

INTRODUCTION

Cryopreservation facilitates storage of biological materials for prolonged intervals without loss

[1]Universidade do Estado de Santa Catarina, UDESC, 88520-000, Lages, SC, Brazil. *Autor para correspondência: E mail mezzalira@cav.udesc.br.
[2]Universidade do Vale do Itajaí, UNIVALI, Itajaí, SC, Brazil.

Received 07.25.05 Approved 04.12.06

of functional activity or genetic alteration. Cryopreservation is essential for widespread application of assisted reproductive technologies; in a recent survey more than 50% of transferred bovine embryos had been previously frozen (THIBIER, 2003). Although bovine oocytes are more difficult to freeze than late-cleavage stage embryos (PARKS & RUFFING, 1992; AMANN & PARKS, 1994), improvements in oocyte cryopreservation decrease the dependency on fresh oocytes and simplify management of genetic resources. Furthermore, oocyte cryopreservation would provide a steady source of materials and greater flexibility for the application of many technologies, e.g. somatic cell nucleus transfer, including intergeneric somatic cell transfer (ATABAY et al., 2004). Cryopreservation involves exposure to non-physiologic low temperatures, even before freezing occurs, and this induces changes in the membrane lipid organization (i.e. lipid phase transition) (ARAV et al., 1996; ZERON et al., 2002). Cell damage is most likely to occur between +15 and –90°C. Below 0°C, there is a risk of intracellular ice formation, which likely increases rapidly as the temperature continues to decline (RUFFTING et al., 1993). In addition to the protocols used, the meiotic stage of an oocyte also influences its ability to survive cryopreservation; the germinal vesicle (GV) stage was more sensitive than any other stages (PARKS & RUFFING, 1992; MARTINO et al., 1996a)

Studies demonstrated that vitrification at a higher cooling rate is better for oocyte cryopreservation (MARTINO et al., 1996b; ARAV & ZERON, 1997; ISACHENKO et al., 2001). To achieve the rapid cooling condition needed for vitrification, several methods use a small volume of vitrification solution, which is plunged directly into liquid nitrogen. Electron microscopy grids (MARTINO et al., 1996b), glass capillaries (MEZZALIRA et al., 1999), cryoloops (LANE et al., 1999) and open-pulled-straws (OPS) (VAJTA et al., 1997) have all been used to reduce the volume of vitrification solutions. Unfortunately, developmental rates obtained so far with cryopreserved bovine oocytes, especially those at the GV stage, are still very low. However, in the last years it was demonstrated (ARAV et al., 2000; ISACHENKO et al., 2001) that exposure of liquid nitrogen (N_2) into vacuum increased cooling speed and improved the viability of vitrified-warmed immature and mature oocytes. The objective of the present study was to determine the effect of vacuum-cooled liquid nitrogen on the post re-warming developmental ability of immature (GV) and mature (MII) vitrified bovine oocytes.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). For oocytes and embryos manipulations, four-well dishes (Nunc; Roskilde, Denmark) were used.

Recovery and selection of oocytes

Bovine ovaries were collected at an abbatoir and transported (for up to 4h post-collection) at 29 to 35ºC in phosphate buffered saline solution (PBS) with 65mg L^{-1} penicillin and 50mg L^{-1} streptomycin. Ovarian follicles of 2 to 8mm in diameter were aspirated with a 19-gauge needle connected to a vacuum pump (flow rate, 20mL min^{-1}). Cumulus enclosed oocytes were maintained in a 110mm Petri dish (Corning Ltd.; Corning, NY, USA) containing centrifuged follicular fluid (LEHMKUHL et al., 2002); those with compact cumulus cells layers and a homogeneous cytoplasm were selected for use.

Experimental groups

The selected oocytes were randomly allocated to one out of five groups. In Groups 1 (n=175) and 2 (n=172), GV-stage oocytes were vitrified in N_2 at atmospheric pressure and under vacuum, respectively. Similarly, in Groups 3 (n=170) and 4 (n=174), MII-stage oocytes were vitrified in N_2 at atmospheric pressure and under vacuum, respectively. Group 5 (Control) consisted of 282 oocytes that were matured, fertilized and cultured. The study was conducted in 10 replications, with 15 to 18 oocytes per replicate.

Vacuum production

An electric self-fabricated vacuum pump was connected by silicon hoses to a cylindrical acrylic container with 120mm diameter and 130mm length, covered with 12mm thickness Styrofoam and containing approximately 300mL N_2. The pump was operated for approximately 45sec, the container opened, and the open-pulled-straw (containing oocytes) was immediately plunged into the cooled N_2 In preliminary studies (unpublished), after 45sec the vacuum pump provided 300mmHg of vacuum and the liquid nitrogen temperature decreased to –200°C.

Vitrification and warming

Vitrification and warming procedures were adapted from VAJTA et al. (1998) with replacement of fetal calf serum (FCS) by estrous mare serum (EMS), and were performed on a 37°C heated plate in a room heated to 25°C. Oocytes were firstly exposed to a 10% ethylene glycol (EG) + 10% dimethyl sulfoxide (DMSO)

solution for 30sec, followed by exposure to a vitrification solution (20% EG + 20% DMSO + 0.5M sucrose) for 20 seconds. Both solutions were prepared in TCM 199 Earle salts medium (Gibco BRL, Grand Island, NY, USA), buffered with 6.5mg mL^{-1} Hepes, with the addition of 20% EMS. Groups of 3 or 4 oocytes were loaded in each OPS and directly plunged into liquid nitrogen in an ambient or vacuum atmosphere, according to the treatment. Oocytes were thawed by exposure to air (25°C) for 4sec, followed by 5min exposure to decreasing concentrations (0.3 and 0.15M) of sucrose solution in TCM 199 + 10% EMS, and then transferred to a sucrose-free medium.

In vitro maturation

Oocytes were matured in TCM 199 - Earle salts, with 26.2mM sodium bicarbonate, 25mM Hepes, 0.2mM sodium pyruvate, 0.01 IU FSH/mL (Folltropin; Bioniche, Belleville, ON, Canada) 0.5µg mL^{-1} of LH (Lutropin; Bioniche) and 10% EMS. Oocytes were cultured for 24h at 39°C in a 5% CO_2 incubator (Haeraeus Instruments, GmbH. Hanau, Germany) with saturated humidity. Prior to vitrification, hyaluronidase (0.5IU μL^{-1}) was used to partially denude matured oocytes. After re-warmine, immature vitrified oocytes were submitted to the same maturation process.

In vitro fertilization

Fertilization was performed with frozen-thawed semen from a single ***Bos taurus*** bull. Spermatozoa were selected by a swim-up technique and were present in a concentration of 2 x 10^6 spermatozoa mL^{-1}. Oocytes and semen were incubated for 18 to 22 hours in TALP-Fert medium supplemented with 30µg mL^{-1} heparin, 30µg mL^{-1} penicillamine, 15µM hypotaurine, and 1µM epinephrine.

In vitro culture

Cumulus cells were removed and presumptive zygotes transferred to four-well dishes with 400µL SOFaaci medium + 5% EMS (under mineral oil). After 24h of culture in 5% CO_2 at 39°C, the cleavage rate was determined and only cleaved structures were maintained in culture. Dishes were then placed in a foil bag with an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 and incubated for another 168 hours.

Embryo evaluation

The viability of in vitro-produced embryos (cleavage and blastocysts rates) was assessed at 48 and 192h after fertilization. Rates were converted to proportions, arc-sine transformed, and two-way ANOVA was used to determine the effects of vacuum, stage of development, and their interaction. Regular ANOVA was used to determine the effect of group, including Control. If there was a significant main effect or interaction, differences were evaluated with a Bonferroni test ($P<0.05$). All analyses were conducted with BioEstat software (AYRES et al., 2000).

RESULTS

As a 300mmHg vacuum pressure for 45 seconds in liquid nitrogen provides a decrease in temperature to -200°C, during OPS immersion in this cooled nitrogen it was observed a reduced boiling in comparison to normal pressure nitrogen.

Cleavage rates demonstrated a significant effect of vacuum (47.5% versus 38.7%) and maturational stage (48.6% maturated versus 37.6% immature), but there were no an interaction between these factors. As demonstrated in table 1, blastocyst rates demonstrated only a significant and positive effect of vacuum (9.7 versus 5.4%). On the other hand, when the condition of the liquid nitrogen (cooled or not) was not considered, there were no differences ($P>0.05$) on blastocysts rates of MII (8.9%) and GV (6.8%) oocytes.

To verify the differences among all experimental groups, data were submitted to a regular ANOVA. Control group (G5) proportionate better cleavage (80.3%) and blastocysts (30.8%) rates than all other groups ($P<0.05$). As shown in table 2, cleavage rates did not differ ($P>0.05$) among GV/normal pressure (33.9% - G1), GV/vacuum (41.2% - G2) and MII/normal pressure (43.5% - G3) vitrified oocytes. Better results were obtained with MII/vacuum (G4) which did not differ from G3 group, and both were lower than G5 Control group.

The lower blastocyst rate was observed in G1/normal pressure group (4.2%). Cooled liquid nitrogen provided an increase in blastocysts rates ($P<0.05$) of G2 (8.8%), which did not differ from G3 MII/normal pressure (6.7%) and G4 MII/vacuum (10.6%).

DISCUSSION

While the embryo freezing is a well-established practice, results from oocyte cryopreservation are still poor. This is determined by several factors like plasmatic membrane characteristics, high lipid content, presence of cortical granules, and the spindle system (CHEN et al., 2003) of oocytes. Even so, significant progress was obtained in the last decade, especially in cryopreservation of immature oocytes, with pregnancies and birth of healthy calves (VAJTA et al., 1998; VIEIRA et al., 2002).

Table 1 - Developmental rates following vitrification of bovine oocytes with vacuum or normal pressure liquid nitrogen.

Treatments	Vitrified n	Cleaved (%)	Blastocysts (%)
Vacuum nitrogen	346	47.5[a]	9.7[a]
Normal pressure nitrogen	345	38.7[a]	5.4[b]

[a,b]: different letters within a column by Bonferroni test ($P< 0.05$).

With GV vitrified oocytes in cooled nitrogen, the cleavage rate obtained (41.2%) is very close to the 50% observed by VAJTA et al. (1998) and 49.1% by MEZZALIRA et al. (2002) with vitrified MII oocytes, demonstrating a progress in the GV bovine oocytes cryopreservation. Our cleavage rates (33.9 to 53.6%) are satisfactory and indicate high survival of bovine oocytes following vitrification. Unfortunately blastocyst rates were very low (4.2 to 10.6%), suggesting that cleavage is not an accurate parameter to evaluate the viability of vitrified oocytes. Developmental capacity of cleaved oocytes following vitrification is lower than that of unvitrified control oocytes (FUKU et al., 1995; MEZZALIRA et al., 2002). It suggests the occurrence of injuries that may not avoid the cleavage, but reduces further embryo development (FUKU et al., 1995; HOCHI et al., 1998; MEZZALIRA et al., 2002).

In this study, when liquid nitrogen was submitted to a 300mmHg vacuum pressure for 45 seconds, its temperature decreased to -200°C, demonstrating that vacuum may be a simple alternative to increase the cooling rate during vitrification. A decrease in temperature (-210°C or -212°C) was also observed with liquid nitrogen slush (ARAV & ZERON, 1997) or with Vitmaster apparatus (ARAV et al., 2000; NOWSHARI & BREM, 2001). Although the temperature obtained in our study was not as lower as that obtained by ARAV et al. (2000) and NOWSHARI & BREM (2001), it provides a visible decrease in boiling activity during OPS immersion in liquid nitrogen. The reduced nitrogen boiling is believed to increase the heat transfer and improve developmental rates for both GV and MII vitrified oocytes.

The biological characteristics of maturated oocytes are related to its limited cryopreservation viability, and this is well-documented (OTOI et al., 1995; OTOI et al., 1997; SAUNDERS & PARKS, 1999; LE GAL & MASSIP, 1999; HYTTEL et al., 2000; SHAW et al., 2000). This problem could be solved with the cryopreservation of immature oocytes where the spindle system is not yet organized and the genetic material is protected inside germinative vesicle. Until now the cryopreservation of maturated oocytes has provided better results and as consequence the cryopreservation of immature oocytes was considered less viable (FUKU et al., 1992; YANG et al., 1998; LE GAL & MASSIP, 1999) and less investigated. Our study demonstrated that the use of vacuum in liquid nitrogen during vitrification provides similar blastocyst rates for maturated and immature oocytes (10.6 and 8.8%), respectively.

The vacuum cooled nitrogen provides better blastocyst rates in MII (10.6 versus 6.7%) and GV (8.8 versus 4.2%) vitrified oocytes. These data suggest that the high cooling speed resulted in less damage to vitrified oocytes, which agree with previous observation of ARAV et al. (2000).

The developmental rate of 8.8% obtained with GV oocytes was higher than the 6.1% obtained in a previous study (VIEIRA et al., 2002), and similar to that (8.0%) obtained by ABE et al. (2004), with nylon mesh. NOWSHARI & BREM (2001) demonstrated no effect with super cooled liquid nitrogen in pronuclear-stage mouse embryo cryopreservation. These observations disagree with our results, however the study was performed with different species (mouse), a

Table 2 - Developmental rates following bovine germinal vesicle (GV) and metaphase II (MII) oocytes vitrification with vacuum or normal pressure liquid nitrogen.

Group / Treatments		Vitrified oocytes N	Cleaved %	Blastocysts day 8 %
G1	GV/ normal pressure	175	33.9[a]	4.2[a]
G2	GV / vacuum	172	41.2[a]	8.8[bc]
G3	MII / normal pressure	170	43.5[ab]	6.7[ab]
G4	MII / vacuum	174	53.6[b]	10.6[c]
G5	Control	282	80.3[c]	30.8[d]

[a,b,c,d]: different letters within a column differ by Bonferroni test ($P< 0.05$).

different developmental stage (pronuclear-stage) and a different structure size that may proportionate a distinct situation during cryopreservation.

In conclusion, our results demonstrate that similar developmental rates following vitrification may be obtained with GV or MII bovine oocytes when vacuum cooled liquid nitrogen is used. Vacuum cooled liquid nitrogen provides higher developmental rates than normal liquid nitrogen following vitrification of GV and MII bovine oocytes. Finally, vacuum is a simple and low cost alternative to increase the cooling rate during vitrification.

ACKNOWLEDGEMENTS

We are grateful to Verdi and Fox Abattoirs for ovaries supplying and to the Brazilian Council of Research (CNPq) for financial support.

REFERENCES

ABE, Y. et al. *In vitro* maturation, fertilization and development of bovine immature oocytes cryopreserved by vitrification with stepwise exposure using a nylon mesh. **Reproduction, Fertility and Development**, v.16, p.1-2, 2004.

AMAN, R.R.; PARKS, J.E. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro matured bovine oocytes. **Biology of Reproduction**, v.50, p.103-110, 1994.

ARAV, A. et al. Phase transition temperature and chilling sensitivity of bovine oocytes. **Cryobiology**, v.33, p.589-99, 1996.

ARAV, A.; ZERON, Y. Vitrification of bovine oocytes using modified minimum drop size technique (MSD) is affected by the composition and concentration of the vitrification solution and by the cooling conditions. **Theriogenology,** v.47, p.341, 1997. (Abstract).

ARAV, A. et al. A new device method for vitrification increases the cooling rate and allows successful cryopreservation of bovine oocytes. **Theriogenology**, v.53, p.248, 2000. (Abstract).

ATABAY, E.C. et al. Vitrification of bovine oocytes and its application to intergeneric somatic cell transfer. **Theriogenology**, v.61, p.15-23, 2004.

AYRES, M. et al. **BioEstat 2.0 Aplicações estatísticas nas áreas das ciências biológicas e médicas**. Belém, PA: Sociedade Mamirauá, 2000. 72p.

CHEN, S.U. et al. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocytes freezing. **Molecular and Cellular Endocrinology**, v.202, p.101-107, 2003.

FUKU, E. et al. *In vitro* fertilization and development of frozen-thawed bovine oocytes. **Cryobiology**, v.29, p.485-492, 1992.

FUKU, E. et al. Ultrastructural changes in bovine oocytes cryopreserved by vitrification. **Cryobiology,** v.32, p.139-156, 1995.

HOCHI, S. et al. Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. **Theriogenology,** v.49, p.787-796, 1998.

HYTTEL, P. et al. Vitrification of bovine oocytes with the open pulled straw method: ultrastructural consequences. **Molecular Reproduction Development**, v.56, p.80-88, 2000.

ISACHENKO, V. et al. The open pulled straw vitrification of ovine GV-oocytes: positive effect of rapid cooling or rapid thawing or both? **Cryoletters,** v.22, p.157-162, 2001.

LANE, M. et al. Containerlless vitrification of mammalian oocytes and embryos. **Nature Biotechnology**, v.17, p.1234-1236, 1999.

LE GAL, F.; MASSIP, A. Cryopreservation of cattle oocytes: effects of meiotic stage, Cycloheximide treatment and vitrification procedure. **Cryobiology**, v.38, p.290-300, 1999.

LEHMKUHL, R.C. et al. Viabilidade de oócitos bovinos mantidos em líquido folicular. **ARS Veterinária**, v.18, p.273-279, 2002.

MARTINO, A, et al. Effect of chilling bovine oocytes on their developmental competence. **Molecular Reproduction and Development,** v.45, p.503-512, 1996a.

MARTINO, A. et al. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biology of Reproduction**, v.54, p.1059-1069, 1996b.

MEZZALIRA, A. et al. Vitrificação de oócitos bovinos em micropipetas de vidro. **Arquivos da Faculdade de Veterinária da UFRGS**, v.27, p.262, 1999. (Abstract).

MEZZALIRA, A. et al. Vitrificação de oócitos e embriões bovinos produzidos *in vitro* e expostos à citocalasina B. **Brazilian Journal of Veterinary Research and Animal Science**, v.39, p.260-265, 2002.

NOWSHARI, M.A.; BREM, G. Effect of freezing rate and exposure time to cryoprotectant on the development of mouse pronuclear stage embryos. **Human Reproduction**, v.16, p.2368-2373, 2001.

OTOI, T. et al. *In vitro* fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. **Cryobiology**, v.32, p.455-460, 1995.

OTOI, T. et al. Cryopreservation of mature bovine oocytes following centrifugation treatment. **Cryobiology**, v.34, p.36-41, 1997.

PARKS, J.E.; RUFFING, N.A. Factors affecting low temperature survival of mammalian oocytes. **Theriogenology,** v.37, p.59-73, 1992.

RUFFING, N.A. et al. Osmotic behavior, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages. **Cryobiology**, v.30, p.562-580, 1993.

SAUNDERS, K.M.; PARKS, J.E. Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. **Biology of Reproduction,** v.61, p.178-187, 1999.

SHAW, J.M. et al. Fundamental cryobiology of mammalian oocytes and ovarian tissue. **Theriogenology,** v.53, p.59-72, 2000.

THIBIER, M. More than half a million bovine embryos transferred in 2002. **Embryo Transfer Newsletter**, v.21, p.12-19, 2003.

VAJTA, G. et al. Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. **Cryo-Letters,** v.18, p.191-195, 1997.

VAJTA, G. et al. A new way to avoid cryoinjuries of mammalian ova end embryos: the OPS vitrification. **Molecular Reproduction and Development, v.**51, p.53-58, 1998.

VIEIRA, A.D. et al. Calves born after open pulled straw vitrification of immature oocytes. **Cryobiology,** v.45, p.91-94, 2002.

YANG, B.C. et al. Effect of freezing of immature and mature bovine oocytes on their development to blastocyst in vitro. In: WORLD CONFER ON ANIMAL PROD, 8., 1998, Seoul. **Proceedings...** Seoul: s/ed, 1998. V.2, p.950-951.

ZERON, Y. et al. The effect of liposomes on thermotropic membrane phase transitions of bovine spermatozoa and oocytes: implications for reducing chilling sensitivity. **Cryobiology**, v.45, p.143-152, 2002.