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# VITRIFICATION OF BOVINE IVP EMBRYOS: AGE OF EMBRYOS AND EXPOSURE TIME TO CRYOPROTECTANT INFLUENCE VIABILITY

## *(Vitrificação de embriões bovinos produzidos in vitro: A idade dos embriões e o tempo de exposição aos crioprotetores influenciam a viabilidade)*

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**RESUMO –** Avaliou-se diferentes tempos de exposição e concentrações de crioprotetores na vitrificação de embriões bovinos PIV. No primeiro experimento, foram utilizados blastocistos do dia 7 (Bx-D7). No tratamento 1 (T1), 82 embriões foram expostos por 1 min. à solução de equilíbrio (SE1 = 10% EG + 10% dimetilsulfóxido (DMSO), seguido da exposição por 20 segundos à solução de vitrificação (SV1 = 20% EG + 20% DMSO). No Tratamento 2 (T2) 84 embriões foram expostos por 3 minutos à SE2 (8,25% EG + 8,25% DMSO), seguido de 45 segundos na SV2 (16,5% EG + 16,5% DMSO). No segundo experimento adotou-se os mesmos procedimentos do primeiro, porém com Bx D8. A remoção dos crioprotetores foi executado em duas etapas de cinco minutos, em 0,3 e 0,15M de sacarose. Os embriões foram incubados por 72 horas, avaliando-se as taxas de re-expansão e eclosão (12 e 72 horas, respectivamente). No primeiro experimento, a taxa de re-expansão no T1 (91,6%) foi superior a do T2 (82,0%) ($p<0,05$), porém as taxas de eclosão de 49,6% no T1 e 54,0% no T2, foram semelhantes ($p>0,05$). No segundo experimento, as taxas de re-expansão não diferiram entre T1 e T2 (65,8 e 68,7% respectivamente), porém a taxa de eclosão do T1 (51,7%) foi superior a do T2 (33,2%) ($p<0,05$). Conclui-se que a idade do embrião pode influenciar a viabilidade de embriões vitrificados e que a redução na concentração de crioprotetores com aumento do tempo de exposição possibilita idêntica viabilidade na vitrificação de embriões PIV D7, porém não é eficiente na vitrificação de embriões PIV D8.

**Palavras chave**: Embriões PIV, vitrificação, OPS, criopreservação.

**ABSTRACT –** Different exposure times and cryoprotectant concentrations were evaluated in vitrification of D7 and D8 IVP bovine embryos. In the first experiment, D7 expanded blastocysts (Bx) were used. In Treatment 1 (T1) 82 embryos were exposed for 1 minute to an equilibrium solution (ES1 = 10% EG + 10% DMSO), followed by 20 seconds exposure to vitrification solution (VS1 = 16.5% EG + 16.5% DMSO). In Treatment 2 (T2) 84 embryos were exposed for 3 minutes to ES2 (8.25% EG + 8.25% DMSO), followed by 45 seconds exposure to VS2 (16.5% EG + 16.5% DMSO). Embryos were loaded in OPS with 5µL VS, and plunged into liquid nitrogen. The second experiment used D8 Bx as previously described. Cryoprotectant removal was performed in two steps, in 0.3 and 0.15M sucrose gradients for 5 minutes each. Embryos were then incubated for 72 hours, and re-expansion and hatching rates evaluated at 12 and 72 hours, respectively. In the first experiment, re-expansion rate in T1 (91.6%) was higher ($P<0.05$) than in T2 (82.0%), while hatching rates (49.6% and 54.0% in T1 and T2, respectively) did not differ ($P>0.05$). In second experiment, re-expansion rates did not differ between T1 and T2 (65.8% and 68.7%) respectively, while hatching rate in T1 (51.7%) was higher ($P<0.05$) than in T2 (33.2%). In conclusion, embryo age may influence the viability of blastocysts after vitrification procedure, while reduction on cryoprotectants concentration associated with increase in exposure time did not affect the viability of D7 IVP bovine vitrified embryos. However, it was not efficient for D8 embryos vitrification.

**Key words:** IVP embryos, vitrification, OPS, cryopreservation.

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## Introduction

Advancements on in vitro production experiments have increased bovine embryo offer. These knowledge advancements brought advantages to obtain genetically superior or biologically important animals. The excess of embryos determined by increased in vitro production has led to many studies about new preservation methodologies. THIBIER (2003) demonstrated in a 2002 world survey that over 50% of bovine embryos transferred were previously cryopreserved, being 39.300 in Brazil. This technology is well established for in vivo produced embryos, but not for those produced in vitro (IVP). IVP embryos are more sensitive to cryopreservation, mainly when slow freezing method is used (MERTON *et al.* 2001). International Embryo Transfer Society data retrieval annual report demonstrated that about 19% of bovine IVP embryos were frozen around the world in 2002, and 4.2% in South America only (THIBIER, 2003), demonstrating the need for new studies in this area. Ultra-rapid freezing and vitrification are more indicated for cryopreservation of high lipid content structures like oocytes (VIEIRA *et al.* 2002), early stage embryos and IVP embryos (VAJTA *et al.* 1998), because these methods allow high freezing rates. Even for in vivo produced embryos, similar viability has been evidenced with vitrification and conventional freezing methods (ISHIMORI *et al.*, 1993; LOPATÁROVÁ *et al.*, 2002). Researches is being directed to in vitro produced embryos cryopreservation (VAJTA *et al.*, 1997; BAUTISTA and KANAGAWA, 1998; SOMMERFELD and NIEMANN, 1999; KAIDI *et al.*, 2000; MERTON *et al.*, 2001, MEZZALIRA *et al.*, 2001), with the purpose of simplifying protocols steps and/or obtaining higher viability. The variation observed in different studies evidenced that vitrification methodology is not definitive yet. Variation in viability is occasioned by simple component or protocol modifications. Solutions composed by the cryoprotectants ethylene glycol (EG) and dimethyl sulfoxide (DMSO) have showed the best results in literature until now, but there are no studies comparing solution concentrations and exposure times. Lower concentrations and longer exposure times appear to be more efficient for swine embryos and bovine oocytes. According to the exposed, it is convenient to investigate the influence of different exposure times and cryoprotectant concentrations on vitrification of IVP bovine embryos. The aim of this study is to evaluate the viability of bovine IVP expanded blastocysts (D7 or D8) vitrified with different cryoprotectant concentrations and exposure times.

## Materials and Methods

*Recovery and selection of oocytes:* Bovine ovaries were collected in a slaughterhouse and transported (physiological saline solution at 25-35ºC) for no more than 5 hours. Only follicles with 2-8mm diameter were aspirated with a 19g needle adapted to a vacuum pump, adjusted to 20mL/minute flow. Oocytes were searched in a 110mm Petry dish, maintained in centrifuged follicular fluid (LEHMKUL *et al.*, 2002), and selected by morphological criteria. Oocytes with compact cumulus cells layers and homogeneous cytoplasm were used in the experiments.

*In vitro Maturation:* Selected oocytes were submitted to a bath in holding medium (TCM-HEPES + 10% Estrum Mare Serum - EMS) and placed in 400µL of maturation medium (TCM-199 + Hepes 5.95mg/mL + Piruvic Acid 0.025mg/mL + FSH/ml 0.01UI + LH 0.5µg/mL + 10% EMS), in NUNC 4 well dishes. Incubation was done for 22-24 hours at 39ºC, 5%$CO_2$ and 95% humidity.

*In vitro Fertilization:* Bos taurus semen was thawed at 37ºC and the spermatozoa selected by swim-up procedure. A sample of 100µL of thawed semen was placed at the bottom of a pre-warmed tube, under 1mL of TALP-SPERM medium with 6mg/mL BSA. After one hour, the supernatant was taken and centrifuged at 800g for 5 minutes. The formed pellet was aspirated and adjusted to a final concentration of $1x10^6$ spermatozoa/mL. Maturated oocytes were placed in 400µL of TALP-FERT medium with 6mg/mL BSA and 30µg/mL heparin, 30µg/ml penicillinamine, 15µM hypotaurina and 1µM epinefrine and co-incubated during 22-24 hours.

*In vitro culture:* Presumptive zygotes were denuded in holding medium by vortexing agitation

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, MO, USA).

during 60 seconds, washed in holding medium again and placed in 400µL of SOFaaci (HOLM, *et al.*, 1999) with 5% EMS under 400µL of mineral oil. After 48 hours, cleavage rate was evaluated, and only cleaved embryos were maintained in culture. Embryos were then placed in a 100x110mm gas impermeable bag from a laminated foil (VAJTA *et al.*, 1997), which was filled with a gas mixture: $N_2$ 90%, $O_2$ 5%, and $CO_2$ 5% and incubated at 39ºC for additional 5 days. Blastocyst rate evaluation was performed on day 7 and on day 8. Expanded blastocysts (Bx) classified as excellent (grade 1) or Good (grade 2) were used in the experiments.

*Experiment I:* In the first experiment (7 replications) 245 day 7 Bx were allocated in 3 uniform groups and maintained in holding medium. The Control (not vitrified) group was composed by 79 embryos, and the remaining were vitrified in 2 treatments (T1 and T2). Eighty-two embryos (T1) were 1 minute exposed for 1 minute to equilibrium solution (ES1 = 10% EG + 10% DMSO) and just after, 20 seconds exposed for 20 seconds to vitrification solution (VS1 = 20% EG + 20% DMSO), while they were loaded in OPS (3 to 5 embryos) and immediately plunged in liquid nitrogen. Eighty-four embryos (T2) were 3 minute exposed for 3 minutes to ES2 (8.25% EG + 8.25% DMSO) and just after, 45 seconds exposed for 45 seconds to VS2 (16.5% EG + 16.5% DMSO), while they were loaded (3 to 5 embryos) in OPS and immediately plunged into liquid nitrogen.

*Experiment II:* In the second experiment (6 replications), 180 day 8 Bx were used. One group was not vitrified (n=59 control), and the remaining were vitrified in 2 treatments (T1 and T2). On T1, 61 Bx were 60 seconds exposed for 60 seconds to ES1 (10%EG + 10% DMSO), followed by 20 seconds exposure to VS1 (20% EG + 20% DMSO), loaded in OPS (3 to 5 embryos) and plunged into liquid nitrogen. On T2, 60 day 8 Bx were exposed for 3 minutees to ES2 (8.25% EG + 8.25% DMSO), 45 seconds exposed for 45 seconds to VS2 (16.5% EG + 16.5% DMSO), loaded in OPS (3 to 5 embryos) and plunged in liquid nitrogen.

*Re-warming and evaluation:* Re-warming procedure was similar for both experiments. The OPS was exposed to air (25ºC acclimatized room) for 4 seconds, immersed in a 0.3M sucrose solution, in TCM-HEPES + 10% (v/v) SEM, at 35°C for 5 minutes. Afterwards, embryos were placed into a solution with 0.15M sucrose for additional 5 minutes and finally placed in holding medium. For both experiments, embryos from all treatments were cultured in SOFaaci medium. Re-expansion rate was evaluated after 12 hours and hatching rate, after 72 hours. The experimental design was in blocks with treatments nested in the age of embryos (day 7 and day 8). The proportion of re-expansion and hatching were submitted to ANOVA, using SAS (SAS Institute, 1993). The significance level of 5% was considered, when discussing the results.

## Results and discussion

Average of re-expansion (77%) and hatching (47.1%) rates from both experiments demonstrated that the method is adequate for cryopreservation of day 7 and day 8 bovine IVP embryos. BALASUBRAMANIAN *et al.* (1998) showed the negative effect of cooling on IVP embryos of different developmental stages, evidencing inadequacies of slow freezing method. LOPATÁROVÁ *et al.* (2002) also evidenced this, obtaining higher surviving rates for vitrified IVP blastocysts, in comparison to slow freezing. In experiment I of this study, re-expansion rate obtained on T1 (91.6%) was higher than in T2 (82.0%) ($P<0,05$), while hatching rates were similar for T1 and T2 (49.6% and 54.0%), respectively. According to our result, both protocols tested proportionate satisfactory viability. In a study using day 7 embryos and the same methodology, LAZAR *et al.* (2000) obtained 74.6% of re-expansion and 46% of hatching rate, and MEZZALIRA *et al.* (2001) observed 86.2% of re-expansion and 47.1% of hatching rate, being these results similar to the ones obtained in this study. VAJTA *et al.* (1996), vitrifying day 7 embryos in normal straws obtained 84% and 69% of re-expansion and hatching rates, respectively, with a higher

hatching rate than the one observed in this experiment. In the present study day 8 embryos had a distinct behavior. They had an identical survival, characterized by re-expansion rates (TABLE 1), but a reduced hatching rate in T2 (33.2%) when compared to T1 (57.7%). This finding highlights the fact that even in lower concentrations longer exposure time to cryoprotectant decrease viability.

TABLE 1 – RE-EXPANSION AND HATCHING RATES OBSERVED IN DAY 7 VITRIFIED IVP BOVINE EMBRYOS.

| Treatment | N | Re-expansion % | Hatching % |
|---|---|---|---|
| T1 = 1 min / 20sec | 82 | 91.6[a] | 49.6[a] |
| T2 = 3min / 45sec | 84 | 82.0[b] | 54.0[a] |
| Control | 79 | - | 89.7 |

[a,b] Different letters at the same column means statistical difference ($p<0.05$).

Different authors have evidenced lower viability of day 8 vitrified embryos (VAJTA *et al.*, 1996; SAHA and SUZUKI, 1997; SOMMERFELD and NIEMANN, 1999 and LAZAR *et al.*, 2000). SAHA and SUZUKI (1997) suggest that late blastocysts (day 8) have lower quality because they present fewer cells. In spite of the fact that the cells of embryos were not counted in this study, and the experiments were conducted at distinct moments, the lower rates obtained for all groups from experiment II corroborates these observations. Results by KONG *et al.* (2000) suggest that hatching difficulties can explain day 8 embryos behavior, that even presenting identical re-expansion rates, had important difference on hatching rates. This can explain the results on experiment II (TABLE 2).

TABLE 2 – RE-EXPANSION AND HATCHING RATES OBSERVED IN DAY 8 VITRIFIED IVP BOVINE EMBRYOS.

| Treatment | N | Re-expansion % | Hatching % |
|---|---|---|---|
| T1 = 1 min / 20sec | 61 | 65.8[a] | 51.7[a] |
| T2 = 3min / 45sec | 60 | 68.7[a] | 33.2[b] |
| Control | 59 | - | 89.7 |

[a,b] Different letters at the same column means statistical difference ($p<0.05$).

These results, associated to SAHA and SUZUKI (1997) observations of higher viability presented on 3 steps cryoprotectant addition when compared to one step, suggest the need of specially adapted protocols for each type or/ and age of embryo. However the hatching rate observed in the control not vitrified group (89.7%) was higher than vitrified groups (49.6 and 54.0%) with day 7 embryos, and (51.7 and 33.2%) whit day 8 embryos, suggesting that the methodology still needs studies to improve its efficiency and to reduce its damage effect.

## Conclusion

Data obtained on this study leads to conclusion that embryo age may influence its viability after vitrification, and the reduction on cryoprotectant concentration associated to an increase in exposure time provides identical viability for vitrification of day 7 bovine IVP embryos. However, this is not efficient for day 8 vitrified IVP bovine embryos.

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