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Post-thaw viability of *in vivo*-produced canine blastocysts cryopreserved by slow freezing

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Abstract

The objectives were to evaluate the reexpansion blastocoele rate, post-thaw viability, and *in vitro* development of canine blastocysts cryopreserved by slow freezing in 1.0 M glycerol (GLY) or 1.5 M ethylene glycol (EG). Fifty-one *in vivo*-produced canine blastocysts were randomly allocated in two groups: GLY (n = 26) and EG (n = 25). After thawing, embryos from M0 were immediately stained with the fluorescent probes propidium iodide and Hoechst 33 342 to evaluate cellular viability. Frozen-thawed embryos from M3 and M6 were cultured in SOFaa medium + 10% FCS at 38.5°C under an atmosphere of 5% CO_2 with maximum humidity, for 3 and 6 days, respectively, and similarly stained. The blastocoele reexpansion rate (24 h after *in vitro* culture) did not differ between GLY (76.5%) and EG (68.8%). Post-thaw viable cells rate were not significantly different between GLY and EG (66.5 ± 4.8 and 57.3 ± 4.8, respectively, mean ± SEM), or among M0 (62.3 ± 5.7%), M3 (56.9 ± 6.0%), and M6 (66.5 ± 6.0%). In conclusion, canine blastocysts cryopreserved by slow freezing in 1.0 M glycerol or 1.5 M ethylene glycol, had satisfactory blastocoele reexpansion rates, similar post-thawing viability, and remained viable for up to 6 days of *in vitro* culture.

Keywords: Cryopreservation; Embryo viability; Blastocoele reexpansion; Glycerol; Ethylene glycol; Dog

1. Introduction

Assisted reproductive technologies during the 20th century have progressed much further in livestock than in companion animals, presumably due to the relative lack of commercial interest in the latter. However, during the last two decades, there were major advances in basic and applied research involv-

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ing dogs, due to their importance as companion animals in modern society, as well as the concern with preservation of species threatened with extinction [1,2].

Cryopreservation of canine embryos has potential for preserving the gene pool of this species and of specific breeds, with potential implications for use in conserving non-domestic canids. Furthermore, the dog can be a more relevant experimental model than mice due to their size, and similar physiology in drug and radiation reactions when compared to the human [3,4]. In addition, embryo cryopreservation allows the dissociation of time and/or space between embryo recovery and the transfer to recipient females, as well as it facilitates international transportation of embryos.

An embryo's capacity to survive cryopreservation depends on several factors, including species, stage of development, origin (*in vivo* or *in vitro*), cryopreservation methodology, cryoprotectant [5], and intracytoplasmic lipid content [6]. Post-thaw viability can be estimated with the blastocoel reexpansion rate, embryonic cleavage rate, *in vitro* hatching [7,8], as well as with fluorescent probes (e.g., Hoechst 33 342 and propidium iodide), which stain living and dead cells, respectively, and are useful to estimate the percentage of viable blastomeres [8,9].

There are limited reports regarding cryopreservation of canine embryos. Kim, et al. [10] cryopreserved canine embryos in glycerol by conventional slow freezing, but they did not verify post-thaw viability and did not obtain pregnancies after the transfer of the thawed embryos to recipient females. Conversely, Abe, et al. [6] obtained embryo viability rates of 50% for morulae and 40% for blastocysts and concluded that vitrification was a good method for cryopreserving canine embryos.

The objectives of this study were to evaluate the blastocoel reexpansion rate, post-thaw embryo viability, as well as *in vitro* development of canine embryos cryopreserved by slow freezing with 1.0 M glycerol or 1.5 M ethylene glycol as cryoprotectants.

2. Materials and methods

A 2 × 3 factorial experimental design was used to test two cryoprotectants (1.0 M glycerol and 1.5 M ethylene glycol) and three moments of post-thaw embryo evaluation (M0, M3, and M6). This study was approved by the "Federal University of Espirito Santo Ethical Committee on Use of Animals" (UFES, Brasil - Protocol no. 035/2010).

Fifty-one *in vivo*-produced embryos were recovered from 16 healthy donors (various breeds or cross-bred). Donors, which were 5 to 20 kg and 1 to 5 yrs old, were identified as proestrus on the basis of vulvar swelling, serosanguineous vaginal discharge, or both. After initial observations, every other day vaginal cytology was performed until 80 to 90% superficial cells were detected [11]. Bitches sexually receptive to a male were mated every other day, for three times, whereas those which reached a minimum of 80 to 90% of superficial cells on vaginal smears and were not sexually receptive were vaginally inseminated (same breeding schedule). Vaginal AI was done with a plastic disposable syringe and plastic catheter. After semen deposition, the bitches were held with their hindquarters elevated for 10 min. A fertile male dog ($>300 \times 10^6$ morphologically normal, motile sperm) was used for breeding.

Embryos were randomly allocated in two groups, 1.0 M glycerol (GLY, n = 26) and 1.5 M ethylene glycol (EG, n = 25). Cryopreserved embryos were thawed and assessed for viability using propidium iodide and Hoechst 33 342 staining immediately post thawing (M0; GLY = 8; EG = 9), 3 days (M3; GLY = 8; EG = 8) or 6 days after *in vitro* culture (M6; GLY = 9; EG = 8) in SOFaa + 10% FCS medium at 38.5°C under an atmosphere of 5% CO_2 with maximum humidity.

2.1. Embryo recovery

Embryo recoveries were performed by uterine flushing, using the *ex vivo* technique standardized in our laboratory [12], 12 days after first mating or AI. The anesthetic protocol used was 0.1 mg/kg IM acepromazine (Acepran 0.2%; Vetnil, Louveira, SP, Brasil), 7.5 mg/kg IM tiletamine/zolazepam (zoletil 50; Virbac Saúde Animal, São Paulo, SP, Brasil), and 5.0 mg/kg IV propofol (Propovan; Cristália, São Paulo, SP, Brasil) It was noteworthy that propofol was administered only after the uterus had been removed. Briefly, bitches were subjected to an ovariohysterectomy. After the uterus was removed, each uterine horn was flushed three times using a total of 30 mL of Dulbecco's PBS (Nutricell, Campinas, SP, Brasil) [12]. The uterine flushes were immediately recovered into petri dishes. After identification of embryos, they were transferred to disposable 35 × 10 mm petri dishes, in PBS with 0.4% BSA (Nutricell) manipulation medium, for embryo evaluation [13] under a stereomicroscope (magnification, 20–40x). The global embryo recovery rate was calculated as the number of structures collected divided by the number of CL present in the ovaries, multiplied by 100 [12].

2.2. Freezing and thawing procedures

Cryopreservation was performed by slow freezing, using a programmable freezer (TK 3000, TK tecnologia em Congelação, Uberaba, MG, Brasil). Grades I and II blastocysts [13] (n = 51) were washed 10 times in DPBS solution and transferred to 200 μL of 1.0 M glycerol (GLY, n = 26) or 1.5 M EG (n = 25) droplets and equilibrated for 10 min. The embryos were individually placed into the middle column of the three permeable glycerol-containing columns (GLY), or into the middle column between the 1.0 M sucrose columns (EG). The straws were then placed into the programmable freezing at −6 °C, and held at this temperature for 2 min (equilibrium period). Seeding was induced by touching the straws in lateral columns with LN_2-cooled forceps. Five min after seeding, cooling was continued at a rate of 0.6°C/min until −35 °C (for both groups). After reaching −35 °C, straws were held at this temperature for 5 min, the removed from the freezing apparatus and immediately submerged in LN_2.

For post-thaw evaluations, straws containing the embryos were held in air for 15 s and then plunged into a 37 °C water bath for another 15 s. The contents of the straws were emptied into a petri dish. The glycerol was removed in three steps. Embryos were held for 5 min in each glycerol-decreasing solution (6.6, 3.3, and 0.0%, respectively) containing 1.0 M sucrose, and then were washed in DPBS plus 0.4% BSA. The straws containing EG embryos were similarly thawed and washed in buffer solution, but without the cryoprotectant removal steps.

2.3. Post-thaw evaluation of embryo viability

Fifty-one embryos were evaluated as to post-thaw viability. Embryos from M0 were thawed and immediately stained with propidium iodide (PI; Sigma, Chemical Co, St. Louis, MO, USA) and Hoechst 33 342 (Sigma, Chemical Co.) for cellular viability evaluation; embryos from M3 and M6 were thawed, cultured *in vitro* for 3 or 6 days, respectively, and similarly stained. For staining, embryos were incubated for 30 s in DPBS with 0.4% BSA solution with 125 μg/mL PI, then transferred to a slide containing a droplet of 1 mg/mL Hoechst 33 342 and glycerol, and after 15 min, examined under fluorescence microscopy (Leica DM LB, with 535 and 617 nm filters). Blastomeres with an intact plasma membrane had a blue fluorescence and were considered live, whereas those with a disrupted plasma membrane stained by PI were considered dead. The number of viable and unviable cells was counted with the aid of ImageJ 1.43u (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Embryos with >50% live blastomeres were considered viable [7,14].

Twenty-four h after *in vitro* culture, embryos (GLY, n = 17, e.g., n = 16) were evaluated to determine blastocoel reexpansion rate. Embryo hatching was evaluated 3 and 6 days after *in vitro* culture.

2.4. In vitro culture (IVC)

Thawed-embryos were *in vitro* cultured at 38.5 °C under an atmosphere of 5% CO_2 with maximum humidity, for 3 or 6 days. The culture medium used was SOFaa with 10% FCS.

2.5. Plasma progesterone concentrations

Blood samples were obtained on Day 0 (first mating or AI) and on Day 12 by jugular venipuncture and immediately centrifuged (600*g* for 15 min). Plasma was stored in microvials and frozen (−20 °C) pending assay. Commercial solid phase radioimmunoassay kits (progesterone Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA, USA) were used as previously validated for canine plasma [15]. Samples were processed in duplicate (following all embryo recoveries). The intra-assay CV was 3.2% and sensitivity was 94.0% (0.03 ng/mL).

2.6. Statistical analysis

Data for blastocyst total cell number and viable cell rate were analyzed by ANOVA using the general linear model (GLM) procedure of SAS (SAS Institute, Inc., Cary, NC, USA). Re-expansion rate data were analyzed by logistic regression, using the LOGISTIC procedure of SAS. Sources of variation in the model included cryoprotectant (glycerol and ethylene glycol), post-thaw embryo evaluation moments (M0, M3 and M6), and all first-order interactions; all factors were considered fixed effects. The *arcsine* and logarithm transformation were applied to embryo percentage rate and blastocyst total cell number data, respectively. In the absence of significant interactions, only main effect means are presented. For all analyses, $P < 0.05$ was considered significant.

3. Results and discussion

Seventy-two embryos recovered from 16 female dogs, 51 were classified as blastocyst Grades I and II, and were used in the study. All other structures (unfertilized eggs, early embryos, morulae and Grade-III blastocysts) were discarded. A total of 125 CL were

identified, with an embryo recovery rate of 57.6%. Interestingly, the recovery rate in 7 of 16 (43.7%) animals was 100%, consistent with a previous report in female Labrador Retrievers [16]. However, there was a 50 to 90% recovery of the embryos in three dogs (18.7%), whereas in four others (25.0%), no embryo was recovered. The average embryo recovery rate in this study (57.6%) seemed lower than our previous report (81.0%) [12], but was similar to results obtained with silver foxes (66.0%) [17]. Furthermore, there was also a greater blastocyst recovery in this study regarding the number of recovered embryos, as previously described [12]. Factors, such as, endometrial cystic hyperplasia [18], variations in plasma progesterone concentrations [19], or the presence of the embryos in the uterine tubes, instead of the uterine horns [12] may have affected the recovery rate. Moreover, it was noteworthy that expanded blastocysts were not shrunken [16] after recovery or handling (Fig. 1A).

Plasma progesterone concentrations on the day of the first mating or AI and at embryo recovery were 4.57 ± 3.77 ng/mL and 28.56 ± 3.21 ng/mL, respectively. Therefore, we inferred that the females were mated or inseminated during their fertile period [18] and embryo recoveries were performed at the beginning of pregnancy [19].

The percentage of embryos with ruptured zona pellucida immediately after thawing, did not differ between GLY and EG groups [3/26 (11.5%) vs. 2/25 (8.0%), respectively; P = 0.6726]. These rates were consistent with a report by Van den Abbeel and Van Steirteghem [14], who obtained rates from 2.3 to 16.6% with human embryos cryopreserved by slow freezing. Zona pellucida lesions have been reported in various mammals [20–23]. Damage to the zona pellucida of cryopreserved embryos may occur due to several factors, including mechanical stress produced from non-uniform volume changes of the freezing medium during phase-change [22], and may negatively affect embryo viability. The possible causes of the zona pellucida lesions found in this work was not the object of the study, but the rates obtained were considered satisfactory [14,21].

The embryo reexpansion rate (%) after 24 h of *in vitro* culture did not differ between the GLY and EG groups [13/17 (76.5%) vs. 11/16 (68.8%), respectively, P = 0.6196]. Shu, et al. [7] obtained reexpansion rates of 48.0 and 50.0% in frozen human blastocysts on Days 5 and 6, respectively. These authors concluded that the fast reexpansion of the blastocoel (2–4 h of *in vitro* culture) has a direct relation with greater implantation and pregnancy rates, when compared to the reexpansion that occurred more slowly. Similarly, studies with consumption of glucose during reexpansion of bovine blastocysts demonstrated that reexpansion is a good morphologic marker for embryo selection [24]. In sheep, Leoni, et al. [25] evaluated the viability of blastocysts produced *in vitro* and vitrified, obtaining reexpansion rates of 55.0 to 87.0%, and also considered

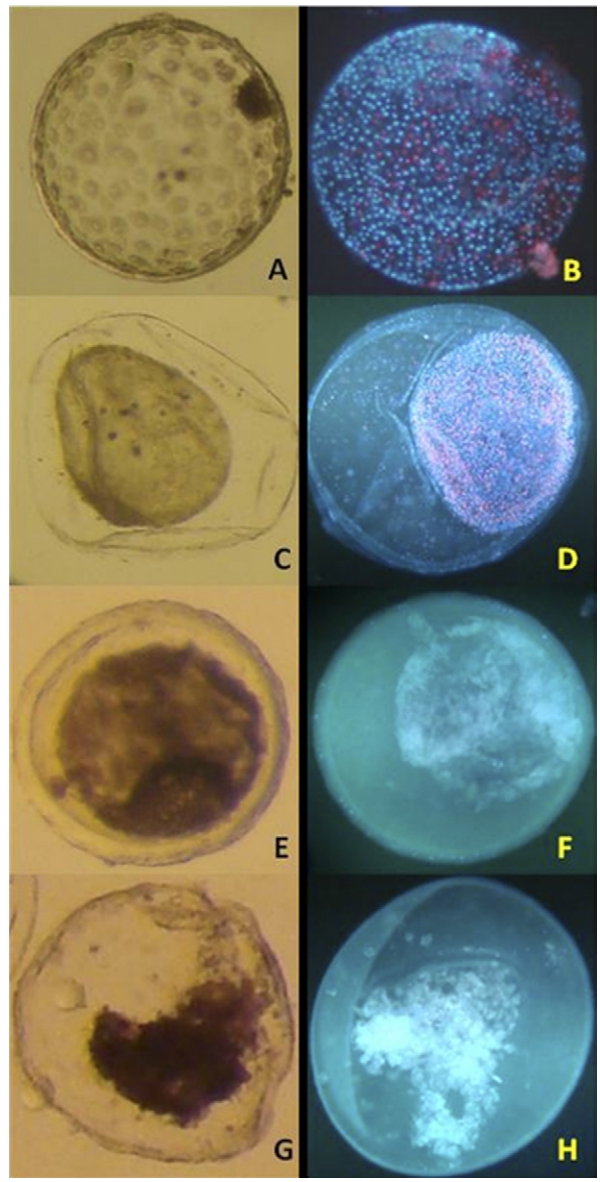

Fig. 1. Morphological appearance (A,C,E,G), and PI/Hoechst 33 342 staining (B,D,F,H) of fresh canine blastocysts (A,B) and frozen-thawed blastocysts (C,D immediately after thawing; E,F in vitro cultured for 3 d; G,H *in vitro* cultured for 6 d). The embryos were magnified (A,C,E,G ×10; D × 200; B,F,H ×400).

reexpansion as a factor determining embryo viability. Although no studies evaluating the reexpansion of cryopreserved canine embryos were found, the rates obtained in this study were considered satisfactory [7,24,25], and also reflected the percentage of embryos that remained viable *in vitro* for up to 6 days.

Surprisingly, none of the embryos hatched. However, similar results were obtained by Lindeberg, et al. [17], who did not observe any hatching in silver fox (Vulpes vulpes) embryos cultured *in vitro* for up to 3 wk. Since implantation of canine embryos occurs between 18 and 21 days after ovulation [26,27], and based on plasma progesterone concentrations, females were in their periovulatory period (approximately 2 days after the LH peak) [27] at breeding, at the end of culture of the M6 group, some of the embryos would be approximately 18 days old. However, hatching did not occur. The hatching process demands a lot from the embryo. Presumably, inadequate culture conditions or even "weakness" of the embryo were the main causes of failure to hatch [28]. Although the SOF medium used in this study is indicated for the initial culture of oocytes and embryos [29], perhaps its composition also contributed to the lack of hatching.

There was no significant difference on post-thaw embryo viability rates between groups (66.5 ± 4.8 and 57.3 ± 4.8 for GLY and e.g., respectively, $P = 0.2074$). Cocero, et al. [29] obtained better embryo viability rates after cryopreservation of ovine embryos with ethylene glycol instead of glycerol. Similar results were reported by Pantano, et al. [30], with a superiority of ethylene glycol in comparison with propylene glycol and glycerol for freezing mouse embryos. Conversely, Martinez and Matkovic [31] compared embryo development and hatching rates of ovine embryos, and did not find statistical differences between either cryoprotectant. In the dog, Kim, et al. [10] used glycerol as a cryoprotectant for freezing embryos by slow freezing, which after thawing were transferred to recipients, but no pregnancies were obtained (although post-thaw embryo viability was not assessed). To our knowledge, this is the first work to study the *in vitro* effects of glycerol and ethylene glycol in canine embryos, cryopreserved by slow freezing. Since glycerol has a higher molecular weight than ethylene glycol, it was removed by step-wise dilution method, and osmotic shock was prevented.

Blastocyst total cell number was similar in frozen-thawed embryos of both groups (311.2 ± 55.10 and 253.8 ± 55.10 for GLY and EG, respectively) ($P = 0.8550$). Furthermore, post-thaw embryo viability evaluation with PI and Hoechst 33 342 facilitated assessment of the percentage of living and dead blastomeres (Fig. 1). Although this method allows a good estimate of living and dead cells in the embryo, since dead cells are permeable to PI and living cells are stained by the Hoechst [8], an underestimation of dead cells may occur if they are surrounded by living cells [8].

Immediately after thawing, 62.3 ± 5.7% of the blastocyst cells in Group M0 were stained by Hoechst (viable cells), similar to that described by Abe, et al. [6], who evaluated the viability of vitrified canine embryos, immediately after warming, and obtained rates of 50% for morulae and 40% for blastocysts. However, unlike this study, in which the embryos were considered viable if they had more than 50% living blastomeres, those authors had a minimum threshold of 75% live cells. As previously mentioned, factors, such as freezing and warming rates may cause alterations in the plasma membranes after thawing [20,23]. Besides, large amounts intracellular lipids, which occur in canine embryos [4] may have contributed to the percentage of dead cells stained with PI immediately after thawing. Similar to swine [32], canine embryos usually have greater sensitivity to cryopreservation due to intracellular lipids.

Although no statistical difference was observed, the M6 group, thawed embryos cultured *in vitro* for 6 days (144 h), had 66.5 ± 6.0% of viable cells, almost 10% of more viable cells than the M3 group, with 56.9 ± 6.0% of viable cells. It is known that certain plasma membrane alterations resulting from cryopreservation are reversible, since most cells recover in a few hours [33,34]. This may have contributed to the satisfactory result obtained at the end of culture in this study.

The *in vitro* culture of the embryos in this study was performed in SOFaa medium. It is known that the maintenance of embryo viability *in vitro* may be affected by various factors, such as the culture medium, the donor's age, and even the addition of components, such as hormones and growth factors in the medium [29]. Although Lindeberg, et al. [17] successfully used TCM199 medium for *in vitro* culture of silver fox embryos, according to Hewitt and England [35], there is no difference between SOF and TCM199 for culture of canine oocytes. Thus, besides oocytes, the SOFaa medium was also apparently helpful for the *in vitro* culture of canine embryos, although no hatching occurred.

In conclusion, canine blastocysts cryopreserved in 1.0 M glycerol or 1.5 M ethylene glycol, by slow freezing, had satisfactory blastocoel reexpansion rates, sim-

ilar post-thaw *in vitro* viability and remained viable for up to 6 days *in vitro*.

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