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Comparison of different cryoprotectant regimes for vitrification of ovine embryos produced *in vivo*

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ABSTRACT

This study was aimed to compare different cryoprotectants for vitrification of sheep embryos produced *in vivo*. Blastocysts were obtained from superovulated Santa Ines ewes and randomized into three groups: conventional freezing using ethylene glycol (EG) (control group), vitrification with EG and dimethyl sulfoxide (DMSO vitrification), or vitrification with EG and dimethylformamide (DMF vitrification). All groups were analyzed for embryonic viability (propidium iodide staining), re-expansion rate after thawing (at morphological and ultrastructural levels) and pregnancy rate after embryo transfer (ET). Embryos of DMSO vitrification group showed lower cell viability (44.44%), compared to DMF group and control embryos (77.77% and 100%, respectively). The ultrastructural study showed similar cryopreservation damage among control and DMF embryos, and these were less damaged than DMSO vitrified embryos. Embryos vitrified with DMF had higher rates of re-expansion *in vitro* (53.33%) than DMSO (26.66%), and control (33.33%). After ET, similar pregnancy rates were obtained from all groups (DMF: 45%, DMSO: 30%, control: 40%). Collectively, DMF vitrification is more efficient than DMSO vitrification and is indistinguishable from conventional freezing of sheep embryos.

1. Introduction

Embryo cryopreservation was first described four decades ago (Whittingham et al., 1972; Wilmut, 1972). Currently, cryopreservation is a key procedure for embryo technologies for both commercial and research scenarios, allowing the conservation of biological material for long periods of time, while preserving cellular, genetic, and biochemical properties (Whittingham, 1980).

Two main cryopreservation methodologies have been described, namely slow conventional freezing and vitrification (Massip, 2001). Conventional freezing has been widely used for embryo cryopreservation of various species, including sheep (Bilton and Moore, 1976; Cognié et al., 2003). However, conventional freezing holds several limitations, such as need for sophisticated and expensive equipment, and time consuming protocols using slow freezing curves (Loutradi et al., 2008). In contrast, vitrification is characterized by simple and fast procedures, while dispensing expensive equipment (Vajta and Kuwayama, 2006; Dike, 2009). These advantages have made cryopreservation by vitrification an attractive approach to cell and tissue cryobanking under commercial and research settings (Szell et al., 1990; Vajta, 2000; Kuleshova et al., 2001).

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Vitrification of ovine embryos is as efficient as conventional freezing (Baril et al., 2001; Bettencourt et al., 2009), and using direct transfer (Isachenko et al., 2003), vitrification is advantageous to generate offspring (Green et al., 2009). Despite this, it remains controversial if vitrification affects the viability of sheep embryos, since different reports have found lower or similar pregnancy rates when compared to fresh embryos (Martinez and Matkovic, 1998; Dattena et al., 2000; Zhu et al., 2001; Papadopoulos et al., 2002; Green et al., 2009).

In order to improve sheep embryo survival and pregnancy rates after vitrification, different combinations of cryoprotectants and other protocol variations were tested (Leoni et al., 2002; Dattena et al., 2004; Shirazi et al., 2010). However, to our knowledge, dimethylformamide (DMF) has not been tested as a cryoprotectant for sheep embryo vitrification. Despite the poor results from initial attempt to use DMF for embryo cryopreservation (Kasai et al., 1981; Chen and Tian, 2005), the lower molecular weight of DMF compared to glycerol motivated recent investigations using DMF for semen cryopreservation aiming to reduce osmotic stress (Squires et al., 2004; Moustacas et al., 2011).

The objective of the present research was to compare two different vitrification protocols to conventional freezing using sheep embryos produced *in vivo*. Experimental groups were compared by embryo viability at morphological and ultra-structural levels, re-expansion rate of thawed embryos and pregnancy outcome after transfer of cryopreserved embryos to synchronized recipients.

2. Materials and methods

2.1. Chemicals

Chemicals were obtained from Sigma–Aldrich Chemical Company (Saint Louis, USA) unless otherwise indicated.

2.2. Experimental location

The experiment was performed at Research Station of Pendência, and at Research Station of Benjamin Maranhão, both research units are part of Paraíba State Agriculture Research Company (EMEPA), Brazil. Research was also conducted at Federal University of Pernambuco (UFPE), at Federal Rural University of Pernambuco (UFRPE), and at the *Center for Strategic Technologies of the Northeast* (CETENE), all located in Recife, Brazil.

2.3. Donor selection

All experimental procedures were conducted in accordance with local ethics review board on animal research. Thirty Santa Ines ewes, with average age of 3.2 years, with no reported reproductive problems, and with adequate nutritional (minimum body score of 2.0) and sanitary conditions were used. Animals were housed in a covered shed, fed with ray *ad libitum*, and concentrate supplement with 18% of crude protein, containing corn, soybean, wheat, and limestone. Animals had free access to water and mineral supplementation.

2.4. Estrous synchronization and FSH-treatment

Embryo donors had their estrous cycles synchronized by insertion of vaginal devices impregnated with 0.33 g of natural progesterone, controlled internal drug release (CIDR, Pfizer, Auckland, New Zealand), and considered it day 0 on protocol. On day 9, all CIDR were replaced by new devices, and were used until day 13. FSH-treatment was initiated on day 11 until day 15, using 252 mg of follicle stimulating hormone – pFSH (Folltropin-V, Bioniche, Ontario, Canada), divided in eight decreasing doses (four days), administered in 12 h intervals. Concomitant with removal of vaginal dispositive on day 13, 200 IU de equine chorionic gonadotropin (eCG) was administered (Folligon, Intervet, Boxmeer, Holland). Controlled natural mating was performed on day 14 with rams of proven fertility.

2.5. Embryo collection and evaluation

Embryos were collected on days 5.5 and 6.0 after estrus onset, aiming to recover embryos at developmental stages from morulae to expanded blastocyst. Animals were not fed 24 h before collection, and were anesthetized with 0.2 mg kg^{-1} xylazine chloride (Rompun, Bayer, São Paulo, Brazil) and 7.5 mg kg^{-1} ketamine chloride (Ketalar, Parke-Davis, Buenos Aires, Argentina). Embryo collections were performed by laparotomy, and both uterine horns were flushed with embryo collection medium Dulbecco's modified phosphate buffered saline (DPBS, Embriocare, Cultilab, Campinas, Brazil), supplemented with 1% fetal bovine serum (FBS) at 37 °C. Embryos were immediately identified and placed in holding medium (TQC Holding Plus, Nutricell, Bioniche, Athens, USA).

All embryos were scored by development stage and quality as described by the International Embryo Transfer Society – IETS (Stringfellow and Seidel, 1998): grade I (excellent), II (good), III (poor), and IV (dead or degenerated). Embryos scored as grade I and II were selected for cryopreservation.

2.6. Embryo cryopreservation

2.6.1. Conventional freezing – control group

Before freezing, embryos remained for 5 min in TqC Ethylene Glycol Freezer Plus solution on a heated stage at 39 °C (Nutricell, Bioniche, Athens, USA) and were loaded in 0.25 mL straws. Embryos were frozen using an automatic embryo freezer (TK 3000, Uberaba, Brazil). Placed in the embryo freezer, embryos were submitted to a freezing curve of −1.0 °C/min until −6 °C, starting from room temperature. When the temperature of −6 °C was reached, the process of freezing was stopped for 5 min to induce crystallization (*seeding*). Moreover, after waiting 10 min to reinitiate freezing, programming was reset to −0.5 °C/min until −32 °C. After 5 min stabilizing at final freezing temperature, embryo-containing straws were immersed in liquid nitrogen.

2.6.2. Vitrification in OPS (Open Pulled Straw) – DMSO and DMF groups

All vitrification solutions were prepared using a basal solution of Hepes containing-TCM-199 (M7653) (Nutricell, Bioniche, Athens, USA) supplemented with 20% FBS (Nutricell, Bioniche, Athens, USA) (holding medium). Embryos were initially kept in H-TCM for 5 min (Vajta, 2000). Immediately after, embryos of the dimethyl sulfoxide (DMSO) group were transferred to holding medium containing 10% ethylene glycol (EG) and 10% DMSO and transferred to a 20% EG + 20% DMSO + 0.5 M sucrose solution for 1 min each. Embryos of dimethylformamide (DMF) group were transferred to a 10% EG and 10% DMF solution for 1 min and moved to a 20% EG + 20% DMF + 0.5 M sucrose solution for an additional minute. After this, embryos from both groups were aspirated in 2 μL of their respective vitrification solution containing 0.5 M sucrose, containing 1 or 2 embryos and transferred by capillarity to OPS and identified properly. Immediately after, straws were transferred to liquid nitrogen and kept until further use.

2.7. Thawing of frozen embryos

Embryos were thawed by exposure of straws to room temperature for 10 s and immersion in water bath at 37 °C for 20 s. Straw content was deposited in a well of a four well dish containing holding medium, embryos were remained for 5 min, and were subsequently evaluated for morphology and quality.

2.8. Warming of vitrified embryos

Immediately after removal from liquid nitrogen, embryo-containing straws were held in air for 3 s, and the thinner tip was immersed in holding medium supplemented with 0.33 M sucrose. The cryoprotectant removal was performed in a four well dish containing holding medium supplemented with 0.33 M sucrose (well 1 and 2). Embryos were kept in wells (1 and 2) for 1 min each, transferred to well 3 containing H-TCM + 0.2 M sucrose for 1 min, and finally for 5 min, in H-TCM.