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Full-term potential of goat *in vitro* produced embryos after different cryopreservation methods

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ABSTRACT

Cryopreservation of preimplantation embryos represents a major challenge due to their shape and relatively large cells. Embryo source and cryopreservation method are key factors to cryotolerance efficiency and few reports have investigated more promising protocols for goat embryos. The study was aimed to compare different cryopreservation methods for goat *in vitro* produced (IVP) embryos. Goat blastocysts were subjected to conventional freezing (CF), Dimethyl sulfoxide vitrification (DMSO-V) and Dimethylformamide vitrification (DMF-V). Cryopreserved blastocysts were assessed for re-expansion, cell viability and *in vivo* development rates. Blastocyst re-expansion after cryopreservation was similar between groups, but cell viability was lower for DMF-V (32%) than CF (68%) and DMSO-V (60%). Pregnancy and delivery rates were similar for CF (60% and 50%) and DMSO-V (50% and 45%) and higher then DMF-V (20% and 15%), respectively. Finally, kidding rates were also indistinguishable for CF (40%) and DMSO-V (35%), but higher then DMF-V (12.5%). In conclusion, conventional freezing and vitrification using DMSO have similar efficiencies for cryopreservation of goat IVP embryos and cryoprotectant for vitrification affects its outcome.

1. Introduction

The efficiency of goat production systems is directly related to several factors such as genetic improvement program, nutrition regimen, sanitary practices and reproductive management [8]. Biotechnologies applied to goat reproduction, such as embryo *in vitro* production (IVP) and embryo cryopreservation, make goat production more efficient and profitable by allowing increased production of descendants from animals of high genetic merit.

Despite several advantages offered by *in vitro* embryo production (IVP) technology, IVP embryos display molecular and structural differences in comparison to their *in vivo* counterparts, such as increased incidence of apoptosis and lower cryotolerance [9]. Cryopreservation allows embryo storage for indefinite periods, avoiding efforts for estrus synchronization for both donors and recipient females. Moreover, embryo cryopreservation contributes to germplasm cryobanking and preservation of genetic variability, facilitating exchange of genetic resources of different breeds and species of economic potential, while minimizing risks of pathogen dissemination [26,27].

Embryos can be cryopreserved by conventional freezing or by vitrification [14,16]. Conventional freezing is characterized by progressive temperature lowering and usage of cryoprotectants of low toxicity and molecular weight [14]. In contrast, vitrification is defined by fast cryopreservation using cryoprotectants at high concentrations and immediate transfer of embryos to liquid nitrogen [21,25].

In cattle, molecular analysis has demonstrated increased viability of IVP embryos after vitrification [15,17,24]. Moreover, these results hold true even when applying vitrification of IVP embryos under field conditions [21]. In sheep, vitrified embryos

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generate similar pregnancy rates to those obtained by conventional freezing [2]. Despite the fact that it is a fast and simple procedure and does not require sophisticated equipment, vitrification for goat embryos remains in its infancy. Furthermore, assessment of vitrification efficiency has been mainly on *in vitro* evaluation of embryos obtained from FSH-treated females [1,3,10,11,30], although some reports described kidding rates after goat embryo vitrification [10,11].

Due to the limited number of investigations concerning the cryopreservation of caprine embryos, particularly of IVP embryos [29,31], the work was aimed to evaluate the viability cryopreserved embryos by conventional freezing and vitrification using embryo re-expansion and cellular integrity cellular as *in vitro* parameters and pregnancy and kidding rates rate as *in vivo* parameters.

2. Material and methods

2.1. Embryo in vitro production

2.1.1. Oocyte retrieval

Goat ovaries were obtained at slaughterhouses and were transported to the laboratory in 0.9% NaCl solution containing 30 μg mL^{-1} of gentamicin sulphate at 30 °C. *Cumulus*-oocyte complexes (COC) were recovered from 2 to 6 mm follicles with an 18 G needle in washing medium, consisting of 8.0 mg of sodium bicarbonate, 45.0 mg of glucose, 5.6 mg of sodium pyruvate, 11.9 mg of HEPES, 2.5 mg of gentamicin sulphate and 20.0 mg of polyvinyl alcohol (PVA) in 50 mL of TALP [7].

2.1.2. In vitro maturation (IVM)

Oocytes were selected based upon their morphology [6] and placed in maturation medium covered with sterile paraffin oil (Sigma-Aldrich). The maturation medium was TCM-199 with Earle's salts (Sigma-Aldrich) supplemented, with sodium bicarbonate 2.6 mL^{-1}, sodium pyruvate 50 μl mL^{-1}, 10 μg mL^{-1}, 1 mg mL^{-1} polyvinyl alcohol, gentamicin sulfate 50 mg mL^{-1} and 10% of sheep estrus serum. Furthermore, oocytes were incubated at 39 °C with 5% CO_2 at saturated humidity for 24 h.

2.1.3. In vitro fertilization (IVF)

Briefly, 0.1 mL of freshly collected semen was washed with 1.5 mL of modified defined medium (mDM) in conical tubes [13]. Viable sperm cells were selected by Percoll gradient [22], and after 15 min, 0.8 mL was aspirated from the upper layer and centrifuged at 350 g for 10 min. The pellet was supplemented with 200 μL of mDM containing 10 μg mL^{-1} of heparin.

Oocytes were assessed for *cumulus* expansion before IVF and COC that did not expand were discarded. Pools of 25 oocytes were transferred in an 100 μL droplet of mDM containing a final sperm suspension of 2.0×10^6 spermatozoa mL^{-1} under sterile paraffin oil. Gametes were co-incubated for 18 h [6].

2.1.4. In vitro embryo culture

After IVF, groups of 10 presumptive zygotes were gently denuded and transferred to KSOM droplets containing an oviduct cells monolayer (OCM) under paraffin oil at 39 °C in an atmosphere of 5% (v/v) CO_2 in humidified air [6]. After 48 h of culture, non-cleaved structures were removed and cleaved embryos were placed on new KSOM droplets with an OCM. Blastocyst development was recorded at day 8 after IVF.

2.2. Embryo cryopreservation

2.2.1. Conventional freezing

Embryos subjected to conventional freezing (CF) were kept in TqC Ethylene Glycol Freezer Plus solution (Nutricell, Bioniche, USA) for 5 min and loaded in pairs into 0.25 mL straws [2]. Embryos were frozen using an automatic freezer (TK 3000, Uberaba, Brazil). The freezing curve consisted of −1.0 °C per minute down to −6 °C and seeding was performed when the temperature reached −6 °C. After 10 min, freezing was resumed with programming reset to −0.5 °C per minute down to −32 °C. After stabilizing at −32 °C, straws were immersed in liquid nitrogen (−196 °C).

2.2.2. Vitrification in open-pulled straws (OPS)

Vitrification solutions were prepared using a solution of HEPES-containing TCM-199 medium supplemented with 20% FBS (H-TCM) [2]. Embryos were initially kept in H-TCM for 5 min [25]. Embryos from Dimethyl sulfoxide vitrification (DMSO-V) were transferred to an 10% ethylene glycol (EG) solution and 10% DMSO and transferred to an 20% EG + 20% DMSO + 0.5 M sucrose solution for 1 min each. Embryos from the Dimethylformamide vitrification (DMF-V) were transferred to a 10% EG and 10% DMF solution for 1 min and transferred to an 20% EG + 20% DMF + 0.5 M sucrose solution for an additional minute. Moreover, embryos from both groups were aspirated in pairs into 2 μL with the second vitrification solution by capillarity to OPS straws. Immediately afterwards, OPS were transferred to liquid nitrogen and kept until further use.

2.2.3. Thawing of frozen embryos

Embryos were thawed at room temperature for 10 s and immersed in a water bath at 37 °C for 20 s [2]. Thawed embryos were then deposited in warm H-TCM for 5 min and subsequently evaluated as described below.

2.2.4. Warming of vitrified embryos

After removal from liquid nitrogen, OPS straws were immersed in H-TCM with the thinner tip facing down for less than 3 s [2]. The cryoprotectant removal was performed in a four-well dish containing H-TCM medium supplemented with 0.33 M sucrose (wells 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 only.

2.3. Embryo evaluation

2.3.1. Embryo viability

Cellular viability was scored by propidium iodide staining, where membrane-lysed cells are selectively stained and considered unviable [2]. All blastocysts (n = 25 embryos per group) were cultured after thawing and warming for 10 min and incubated for 5 min in DPBS containing 1% BSA and 125 mg mL^{-1} of propidium iodide, and were further transferred to slides containing drops of DPBS with 100 mg mL^{-1} Hoechst 33342 solution. Cells that stained red or pink were considered unviable [2]. The total cell number in each embryo was determined by Hoechst 33342 staining (blue cells). Embryos with less than 50% of red or pink cells were considered viable. Stained embryos were examined on an inverted fluorescence microscope.

2.3.2. Re-expansion rates

Cryopreserved embryos were thawed or warmed as described above and morphologically evaluated before *in vitro* culture (IVC). Embryos (n = 15) were cultured in 400 μL of Synthetic Oviduct Fluid (SOF) medium (Nutricell, Bioniche, Athens, USA) under mineral oil in four well dishes, with 5% of CO_2 in air at 38.5 °C. Embryo survival was scored at 6, 12, and 24 h after IVC onset. Embryo viability was determined by blastocele expansion [2].