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Cryopreservation of *in vitro*–produced sheep embryos: Effects of different protocols of lipid reduction

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ABSTRACT

The low survival of sheep *in vitro*–produced (IVP) embryos after cryopreservation is a key limiting step to the widespread of this technology. In the present work, different approaches for enhancing cryosurvival of these embryos were compared: embryo delipidation by centrifugation in the absence or presence of cytochalasin D, a cytoskeleton stabilizer or by embryo culture in the presence of different doses of the *trans*-10 *cis*-12–conjugated linoleic acid isomer (CLA). Three experiments were conducted. In experiment 1, IVP blastocysts before vitrification were randomly distributed into four groups: control; centrifuged (cent), cytochalasin D (cyto-D), centrifuged + cytochalasin D (cent + cyto-D). In experiment 2, different doses of CLA (25, 50, and 100 μM) were supplemented during embryo culture before vitrification of blastocysts. A control group ran simultaneously. A third experiment was performed to compare both approaches from the previous ones but without the groups with the worst results (groups: control, cyto-D, cent + cyto-D, CLA25, CLA50). In all experiments, embryos integrity and reexpansion were assessed after warming and after 3 hours of culture. In experiment 1, the postwarming integrity rate was the lowest ($P < 0.05$) in embryos from the cent group (cent: 50.6 ± 10.3% vs. control: 74.6 ± 9.2%, cyto-D: 92.3 ± 9.7%, and cent + cyto-D: 90.5 ± 11.2%), whereas the best ($P < 0.05$) reexpansion scores were obtained in cent + cyto-D embryos (cent + cyto-D: 2.6 ± 0.28 vs. control: 1.8 ± 0.08, cent: 1.9 ± 0.2, and cyto-D: 1.8 ± 0.31). In experiments 2 and 3, higher ($P < 0.05$) cleavage rates were observed in CLA25 (50.9 ± 6.2% and 49.2 ± 5.6%, respectively) and CLA50 (48.9 ± 6.2% and 47.6 ± 5.6%, respectively) than those in the control (41.8 ± 6.1% and 40.4 ± 5.4%, respectively) group. In experiment 2, CLA100 presented the lowest ($P < 0.002$) Day-6 and -7 embryo production rate and quality. After warming, superior ($P < 0.02$) expansion scores were achieved in CLA25 (3.1 ± 0.29) and CLA50 (3.8 ± 0.17) than in the control (1.9 ± 0.10) group. Similar results were attained in experiment 3. However, although cent + cyto-D embryos showed higher ($P = 0.008$) postwarming expansion scores than the control (2.8 ± 0.29 vs. 1.9 ± 0.07) group, this score was lower ($P = 0.0009$) than that in CLA50 embryos (3.8 ± 0.17). In conclusion, our results showed that different protocols of lipid reduction can be successfully applied to improve the cryotolerance of IVP sheep embryos.

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1. Introduction

The cryopreservation of *in vitro*–produced (IVP) sheep embryos is a biotechnological tool that still achieves unsatisfactory results. Although acceptable embryo production rates can be obtained in sheep (cleavage rate: 55%–83%; blastocysts rate: 27%–53%), only 10% to 19.4% of lambs are born after transferring frozen–thawed IVP embryos [1–4]. Conversely, significantly higher pregnancy and lambing rates can be achieved of recipients bearing *in vivo*–derived cryopreserved embryos (50%–70.3% and 50%–62.9%, respectively [2,5]).

Many factors contribute to the low viability observed in cryopreserved preimplantation embryos produced by IVC techniques. According to several authors, the reduced quality of IVP embryos can be associated with oocyte quality but also with the composition of maturation medium [1,3,6,7]. Moreover, the quality and status (fresh or cryopreserved) of semen used for IVF is also important. Recent reports showed that oocyte fertilized by fresh semen clearly improved sheep embryo developmental competence by enhancing morphologic blastocyst quality [7]. Notwithstanding an increased lipid accumulation in IVP embryos has also been correlated to a reduced cryotolerance leading to lower pregnancy success [2,8–11]. This higher level of intracellular lipid content depends on culture conditions, being increased by serum-containing media [8,10,12]. Serum is useful in oocyte and embryo culture as a source of albumin that balances the osmolarity, acting as a free radical scavenger with an additional important nutritive role [13]. However, the fatty acids and lipoproteins of the serum seem to be the source of embryos' cytoplasmic lipids, hampering embryo quality [14,15], albeit the perturbations induced by the presence of serum in sheep embryo culture are higher before rather than after compaction [16].

Different strategies to enhance cryotolerance of embryos in sheep and other species were developed mainly through the decrease of their lipid content and/or the improvement of embryo cryopreservation procedures [17,18]. For instance in cattle, lipolytic agents or chemical delipidators have been successfully applied increasing cryotolerance of vitrified embryos (phenazine ethosulfate [19], *trans*-10 *cis*-12–conjugated linoleic acid [10,20], forskolin [21]). These chemicals that regulate metabolism were used to reduce embryo lipid content, inducing smaller lipid droplets and fat indexes, thus improving embryonic cryosurvival. Likewise Nagashima et al. [22] reported that the high lipid content of pig embryos was responsible for their chilling sensitivity and that delipidated embryos by centrifugation and microaspiration of polarized lipids became more tolerant to chilling. The lipid content was also pointed as responsible for the chilling and freezing sensitivity of IVP cattle embryos, and when lipid droplets were displaced by centrifugation (mechanical delipidation), their cryosurvival was improved [23–25]. To our knowledge, these techniques were not yet applied to sheep embryos.

Other approaches to improve embryo cryosurvival have been investigated. Cytoskeleton relaxant and stabilizers as cytochalasin B or D were previously used during cattle and pig embryos vitrification to prevent cellular disruption, specifically to the embryonic cytoskeleton during and after cryopreservation [26–28]. Dobrinsky et al. [29] have obtained an 82% birth rate in pig vitrified embryos using cytochalasin B. Moreover, the association of two strategies, cytoskeleton relaxants and centrifugation, was successfully attempted by different authors in cattle and pig IVP embryos [30,31].

The cryopreservation of sheep embryos is not as widely practiced as in cattle, and several methods need to be appraised [32]. Thus, the objective of the present research was to compare different approaches for enhancing cryosurvival of sheep IVP embryos: embryo delipidation by centrifugation in the absence or presence of a cytoskeleton relaxant or by embryo culture in the presence of different doses of the *trans*-10 *cis*-12–conjugated linoleic acid isomer (CLA). The study included three experiments with specific objectives: In experiment 1, the effect of mechanical delipidation through centrifugation in the presence or absence of cytochalasin D on sheep embryo cryosurvival was studied; in experiment 2, we induced embryonic chemical delipidation by using different doses of CLA (25, 50, and 100 μM) during embryo culture and their effects on embryo production and posterior cryosurvival were evaluated; in experiment 3, we compared both approaches, chemical and physical delipidation, used in previous experiments but without the groups presenting the worst results.

2. Materials and methods

All chemicals used were purchased from Sigma–Aldrich Chemical Co. (Sintra, Portugal) unless specified otherwise.

2.1. Experimental design

In the present work, different approaches for enhancing cryosurvival of sheep IVP embryos were evaluated in three experiments. In each experiment, embryos were produced in eight to nine sessions. On Days 6 and 7 (IVF = Day 0), embryos were evaluated for their developmental and morphologic status (grade 1, good to grade 3, poor) and then vitrified and warmed in open pulled straws (OPS; four sessions each). In all experiments, embryo viability after warming was evaluated by its integrity and reexpansion and after 3 hours of IVC.

2.1.1. Experiment 1

Before vitrification, IVP blastocysts of grade 1 (eight sessions) were randomly distributed into four groups as follows: (1) control group (n = 27), embryos were placed during 20 minutes in holding medium (HM); (2) centrifuged group (cent; n = 25), embryos were placed in HM for 10 minutes and then centrifuged at 15,000 × *g* for 10 minutes; (3) cytochalasin D group (cyto-D; n = 24), embryos were placed in 5 μg/mL cytochalasin D–containing medium for 10 minutes and then transferred to HM another 10 minutes; and (4) centrifugation + cytochalasin D group (cent + cyto-D; n = 22), embryos were placed in 5 μg/mL cytochalasin D–containing medium for 10 minutes and then centrifuged at 15,000 × *g* for 10 minutes in HM. After warming, embryo viability was evaluated.