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Improved embryonic cryosurvival observed after *in vitro* supplementation with conjugated linoleic acid is related to changes in the membrane lipid profile

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ABSTRACT

The aim of this study was to evaluate the effect of supplementing serum-containing media with a mixture of *cis*- and *trans-9,11*- and *-10,12*–conjugated isomers of linoleic acid (CLA) during different steps of the *in vitro* production (IVM, IVC, or IVM + IVC) of bovine embryos on their embryonic development, cryotolerance, and lipid profile. To evaluate the impact of the CLA on membrane lipids, such as phosphatidylcholine (PC) and sphingomyelin (SM), the embryos' lipid profiles were obtained using matrix-assisted laser desorption ionization mass spectrometry. The cleavage rates (78.6%–84.8%) and blastocyst development (44.8%–51.2%) remained unaltered. The postthawing reexpansion rates were higher ($P < 0.05$) when the CLA was added to the IVM medium (82.6%) or to the IVM + IVC medium (83.8%) than the control (69.3%) or IVC medium (63.0%). Changes in the blastocysts' lipid profile occurred when supplementation was restricted to the IVM or IVC medium. However, the most prominent effects of the CLA on the embryonic PC and SM profiles were observed when the supplement was added to IVM + IVC media, which was an increase in the level of highly unsaturated PCs containing 36 or 38 carbons, which are likely to contain CLA residues. These results showed that the molecular mechanism resulting in the improved cryosurvival, observed with CLA supplementation during bovine embryonic *in vitro* production, was related to the composition of structural lipids of cellular membranes and is dependent on the treatment length. Monitoring the lipid profile of embryonic membranes may improve the CLA supplementation strategy and facilitate the development of new IVC systems to improve the cryopreservation of bovine embryos and those of other domestic species.

1. Introduction

The sensitivity of *in vitro*–produced (IVP) bovine embryos to freezing or vitrification is the main obstacle to the expansion of cryopreservation technology [1]. The reduced cryotolerance of IVP embryos, particularly those cultured in serum-supplemented medium, has been correlated with an excessive accumulation of lipid droplets during *in vitro* embryonic development [2–4]. Although the mechanisms leading to lipid accumulation in embryos that were IVP remain unknown, it has been found that this phenomenon reduces the quality of the IVP embryos by increasing their sensitivity to oxidative stress and cryopreservation [3,5]. To avoid the undesired lipid accumulation, several studies

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attempted to replace the serum in the culture medium [6]. However, despite its potential detrimental effects on embryonic quality [7], serum contains substances that are beneficial for embryonic development, such as growth factors and chelators of heavy metals [8]. For this reason, it has been difficult to avoid or find a suitable substitute for serum in IVC media formulations, particularly for laboratories working with commercial entities.

To address the challenge of successful embryonic cryopreservation, two strategies can be pursued: developing better cryopreservation techniques or modifying the molecular composition of the embryos during IVC to render them more cryotolerant [2]. Optimization of cryotechniques has met with limited success, whereas changes in the *in vitro* production systems seem more promising and have already led to embryos of better quality and higher cryotolerance [2].

Emerging studies of changes in the *in vitro* production system have identified key molecules and the molecular mechanisms by which they modulate lipid uptake inhibition in adipocytes. One of the most promising of these biologically active molecules is the conjugated linoleic acid (CLA), which is a collective term for a mixture of positional and geometric isomers of linoleic acid (LA) in which the double bonds are conjugated in either the *cis* or *trans* configuration at positions *7,9-*, *8,10-*, *9,11-*, *10,12-*, or *11,13-* of the carbon chain [9]. Pariza et al. [10] reported that CLA (equal mixture of *trans-10,cis-12* and *cis-9,trans-11* isomers) reduced apolipoprotein B secretion in cultured human hepatoma HepG2 cells. The *trans-10,cis-12* octadecadienoic CLA (*10t,12c* CLA), which is one of the 28 CLA isomers, affects the lipid metabolism of adipocytes, reducing the uptake of free fatty acids (FAs) without increasing lipolysis [10]. Because of its ability to modulate lipid uptake, supplementation of serum-containing media for embryonic culture with *10t,12c* CLA increased the blastocyst cryosurvival rate at 24 hours of postwarming culture [11]. The molecular pathways by which *10t,12c* CLA increased embryonic survival after freezing–thawing processes remain to be elucidated, but the positive effect of *10t,12c* CLA on cryotolerance of IVP embryos was attributed to the reduction in their lipid content. A previous study also reported that supplementation with LA during bovine embryonic *in vitro* production system increased the membrane fluidity because of the direct incorporation of LA into the embryonic cellular membranes [12].

Cytoplasmic membranes are sensitive to low temperature [13], and their lipid composition is critical under such conditions because it dictates crucial physical and chemical properties, particularly membrane fluidity [14,15]. Phospholipids (PLs) are the most abundant type of lipid in eukaryotic cell membranes, and although their role in successful cryopreservation is still poorly understood [16], it was previously proposed that cytoplasmic membranes containing more unsaturated PLs suffer less osmotic stress because their ability to remain fluid at low temperatures [2]. Changes in the FA profile or changes in the molecular ordering of discrete lipid domains within a membrane can affect their fluidity and thus their ability to resist chilling [17]. Understanding the membrane lipid profile is therefore fundamental to fully comprehend and to overcome the great challenge of postcryopreservation embryonic survival.

Modifications in the composition of the structural lipids of membranes, which cannot be detected using cellular staining procedures, are readily detected using mass spectrometry (MS) [18–20]. The direct analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of single intact preimplantation embryos was initially standardized by Ferreira et al. [21]. This strategy provides reproducible and characteristic lipid fingerprints of single oocytes or preimplantation embryos in a procedure involving no extraction, no chemical manipulation, and no preseparation protocols [21]. Matrix-assisted laser desorption ionization MS is sufficiently sensitive to enable analysis of individual intact embryos and can be used to monitor changes in the membrane lipid composition in the early stages of preimplantation development, thus serving as a screening method for studies of the biology and biotechnologies of reproduction [20].

We hypothesized that the presence of *cis-* and *trans-9,11-* and *-10,12-* CLA isomers in serum-containing media would improve cryotolerance not only by decreasing lipid droplet accumulation but also by changing the PLs composition of the membranes of IVP embryos. In this study, we investigated the impact of CLA supplementation during different steps of bovine embryonic *in vitro* production on the developmental rates, cryopreservation, and the composition of the structural membrane lipids of the embryos.

2. Materials and methods

2.1. Reagents and media

Unless otherwise stated, the chemicals were purchased from Sigma (Sigma–Aldrich Corp., St. Louis, MO, USA). All of the reagents were tested for cell or embryonic culturing. Conjugated linoleic acid (a commercial mixture of *cis-* and *trans-9,11-* and *-10,12-* CLA isomers) was obtained from Sigma (ref. O5507) and was diluted in DMSO (the final concentration of DMSO was $<0.1\%$) to obtain a stock solution of 90 mM, which was aliquoted and stored at $-20\ ^{\circ}C$. At the moment of use, the CLA stock solution was diluted in maturation medium (B-199) and in culture medium (synthetic oviductal fluid [SOF]) to a final concentration of 100 μM of CLA.

The medium for IVM consisted of Tissue Culture Medium 199 (Gibco, Invitrogen Co., Grand Island, NY, USA) supplemented with 10% (v:v) fetal calf serum (FCS; Gibco, Invitrogen Co.), 0.2-mM sodium pyruvate, 25-mM sodium bicarbonate, 50 μg/mL of amikacin, 0.5 μg/mL of FSH (Folltropin-V; Bioniche Animal Health, Ontario, Canada), and 100 IU/mL of hCG (Vetecor; Hertape Calier). The fertilization medium consisted of Tyrode's albumin lactate pyruvate, as previously described [22], containing 0.2-mM sodium pyruvate, 6 mg/mL of fraction V FA-free BSA, 25-mM sodium bicarbonate, 13-mM sodium lactate, 50 μg/mL of amikacin, 40 μL/mL of PHE solution (final concentrations of 20-μM penicillamine, 10-μM hypotaurine, and 2-μM epinephrine), and 10 μg/mL of heparin. The embryonic culture (IVC) medium consisted of modified SOF, as