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High doses of lipid-core nanocapsules do not affect bovine embryonic development *in vitro*

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ABSTRACT

The improvement of *in vitro* embryo production by culture media supplementation has been a potential tool to increase blastocyst quality and development. Recently, lipid-core nanocapsules (LNC), which were developed for biomedical applications as a drug-delivery system, have demonstrated beneficial effects on *in vitro* embryo production studies. LNCs have a core composed of sorbitan monostearate dispersed in capric/caprylic triglyceride. Based on that, we firstly investigated if LNCs supplemented during *in vitro* oocyte maturation had affinity to the mineral oil placed over the top of the IVM media. Also, the effects of LNC supplementation in different concentrations (0; 0.94; 4.71; 23.56; 117.80 and 589.00 μg/mL) during the *in vitro* maturation protocol were evaluated in oocytes and blastocysts by *in vitro* tests. LNCs seemed not to migrate to the mineral oil overlay during the *in vitro* oocyte maturation. Interestingly, LNCs did not show toxic effects in the oocyte *in vitro* maturation rate, cumulus cells expansion and oocyte viability. The highest LNCs concentration tested (589 μg/mL) generated the lowest ROS and GSH levels, and reduced apoptosis rate when compared to the control. Additionally, toxic effects in embryo development and quality were not observed. The LNC supramolecular structure demonstrated to be a promising nanocarrier to deliver molecules in oocytes and embryos, aiming the improvement of the embryo *in vitro* development.

1. Introduction

Reproductive biotechnologies are essential tools for agricultural animal production, human infertility treatment and animal species preservation (Gianaroli et al., 2012; Holt et al., 2014; Knox, 2014). They enable to underlie the mechanisms of gametogenesis, fertilization and early embryo development providing significant advances in fertility related issues (Barkalina et al., 2016). However, due to the suboptimal culture conditions observed in *in vitro* culture systems, the development rates and embryo quality are still compromised in comparison to the *in vivo* embryo production (Agarwal et al., 2006; Pomar et al., 2005).

A potential alternative to improve the competence of *in vitro* produced embryos have been the supplementation of culture media with macromolecules, such as antioxidants and growth factors (Krisher and Prather, 2012; Remião et al., 2016; Tian et al., 2014; Yun et al., 2013). Additionally, to supply the lacking components that are associated with an abnormal embryonic development, a nanotechnology-based strategy have been used to enhance the delivery of different components into gametes and embryos (Barkalina et al., 2015, 2014; Komninou et al., 2016; Lucas et al., 2015; Remião et al., 2016).

The use of materials in nanoscale has brought the possibility of its application in many different areas of research (Sarikaya et al., 2003; S. Wang et al., 2014). Nanomaterials can be defined as materials that have one or more dimensions of the order of 100 nm (SCENIHR, 2007). However, it should be noted that depending of the field, this term could be used to cover a broader size range (Souto et al., 2016). The nanoparticle-mediated delivery could optimize the efficacy of many

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therapeutic agents by increasing their bioavailability and chemical stability (Ourique et al., 2011). The nano-sized platforms allow to modify the release and the distribution profile of drug and biodrugs, providing local or site-specific delivery, followed by rapid internalization through physiological uptake mechanisms (Uskoković, 2013).

Among the different nanoparticles developed for drug delivery purposes, the gold nanoparticles (Au-NP) have been the most used (Dreaden et al., 2012). However, recent studies demonstrated that Au-NP caused significant levels of cytotoxicity (Boyles et al., 2015; Chuang et al., 2013) and increased the susceptibility to cell death by apoptosis (Leite et al., 2015). As an alternative, the use of biodegradable polylactic acid nanoparticles (PLA-NP) and poly(epsilon-caprolactone)lipid-core nanocapsules (LNCs) are gaining attention due to their several benefits (da Luz et al., 2017; Torge et al., 2017; Ulery et al., 2011). Poly-lactic acid and poly(epsilon-caprolactone) polymers are highly processable; however, the poly-lactic acid degradation generates highly acidic products (Ulery et al., 2011). Lipid-core nanocapsules (LNCs), which are composed of a core of sorbitan monostearate dispersed in capric/caprylic triglyceride, and a polymeric wall of poly(epsilon-caprolactone) (PCL), have shown promising use as carriers for drug delivery (Coradini et al., 2015; de Brum et al., 2015; Bender et al., 2014; Pohlmann et al., 2013). These carriers successfully delivered drugs to target organs as liver, kidney and brain with no significant systematic toxic effects suggesting that LNC might be a safe and efficient delivery tool (Bulcão et al., 2013; Pohlmann et al., 2013).

Previous studies by our research group demonstrated that the addition of LNCs associated with different supplements during oocyte *in vitro* maturation and *in vitro* embryo culture increase embryo cleavage, blastocyst production and hatching rates. Also, it was observed a decrease in reactive oxygen species production (ROS), a downregulation of apoptosis-related genes and an upregulation of genes related to antioxidant defense (Komninou et al., 2016; Lucas et al., 2015; Remião et al., 2016). Interestingly, drug-unloaded LNCs, without the presence of the supplement, showed not to be completely inert demonstrating beneficial effects during the *in vitro* embryo production (Lucas et al., 2015; Remião et al., 2016).

Thus, considering the news findings about the LNCs, *in vitro* tests were performed for a better elucidation of the LNCs effects on oocyte *in vitro* maturation (IVM) and embryo development. Supplementation of IVM medium with different concentrations of LNCs was performed to test if it has beneficial or toxic effects.

2. Materials and methods

2.1. Experimental design

Based on our previous studies (Komninou et al., 2016; Lucas et al., 2015; Remião et al., 2016) three interdependent experiments were designed.

The experiment 1 determined if LNCs supplemented during *in vitro* oocyte maturation had affinity to the mineral oil placed over the top of the IVM media. The oil mineral overlay has been a standard component of *in vitro* embryo culture systems, currently used for pH and osmolality maintenance and as a physical barrier for microorganism contamination (Wale and Gardner, 2016). Four experimental groups were designed, following results from our earlier studies (Komninou et al., 2016; Remião et al., 2016): LNCs in the presence of a mineral oil overlay (LNCs/oil); LNCs without a mineral oil overlay (LNCs-no oil); control in the presence of a mineral oil overlay (Control/oil); and control without a mineral oil overlay (Control-no oil). Four independents replicates were done for each experimental group, totaling approximately 70 cumulus-oocyte complexes (COCs) per group. The effects in preimplantation embryos were evaluated on day 7 (D7) of the culture, at the blastocyst stage. Data from embryo development (cleavage and blastocyst formation rates) and total blastocyst cell number were collected.

In experiment 2, it was decided to maintain the mineral oil overlay and evaluated the LNCs effects in COCs during *in vitro* oocyte maturation. LNCs have already been shown beneficial effects (Lucas et al., 2015), so COCs were exposed to different LNCs concentrations in order to investigate its possible toxic effects. We designed five experimental groups: LNCs at 0.94 μg/mL (0.94 μg/mL LNCs group), 4.71 μg/mL (4.71 μg/mL LNCs group), 23.56 μg/mL (23.56 μg/mL LNCs group), 117.80 μg/mL (117.80 μg/mL LNCs group) and 589.00 μg/mL (589.00 μg/mL/LNCs group). A control group without LNCs was also tested. Cumulus cells expansion and maturation rate were determined in 5 replicates per group, and reactive oxygen species (ROS) production, glutathione (GSH) levels, and cell viability were determined in three replicates per group.

In experiment 3, the effects of LNCs on embryo development were evaluated. The experimental groups were the same as in the experiment 2 and the supplementation of LNCs was also in IVM period. Data of embryo development (cleavage and blastocyst rate) and number of cells per blastocyst were collected. Embryos were also analyzed by the TUNEL assay to assess apoptosis rate. The experiment was replicated three times.

2.2. Preparation and characterization of lipid-core nanocapsules (LNCs)

LNCs were prepared by interfacial deposition of preformed polymer method (Jäger et al., 2009; Venturini et al., 2011). Briefly, an organic phase (24 mL of acetone, 3.0 mL of ethanol) containing the polymer (PCL, 0.100 g), sorbitan monostearate (0.038 g) and capric-caprylic triglyceride (0.157 g) was stirring at 40 °C until dissolution of all components. A turbid solution was instantaneously obtained by injecting this organic phase into an aqueous phase (54 mL of water and 0.077 g of polysorbate 80). After 10 min, the organic solvent was eliminated and water partially evaporated under reduced pressure at 40 °C. The final volume was adjusted to 10 mL.

The physicochemical characterization of three LNCs batches was conducted by determining the following parameters: particle size distribution, mean particle size, polydispersity index, zeta potential and pH. To confirm that the particle population was exclusively at the nanoscale, each batch of formulation was characterized by laser diffraction (Mastersizer® 2000, Malvern Instruments, UK). The mean particle sizes were described as the volume-weighted mean diameter ($d_{4,3}$) and polydispersity by the span value, calculated by Eq. (1):

$$Span = \frac{d(0.9) - d(0.1)}{d(0.5)} \quad (1)$$

where d(0.9), d(0.1) and d(0.5) are the diameters at 90%, 10% and 50% of the cumulative distribution of diameter curve, respectively.

Particle size (z-average diameter), polydispersity index (PDI) and zeta potential were determined using a Zetasizer® (Nano-ZS ZEN 3600 model®, Malvern, UK). The nanoparticles were previously diluted (500 ×) in ultrapure water (particle size and PDI) or in 10 mM NaCl aqueous solution (zeta potential). The pH measurements were determined directly in the samples using a calibrated potentiometer (DM-22, Digimed, Brazil).

2.3. Oocyte collection

Bovine ovaries were collected in a local slaughterhouse (Pelotas, Rio Grande do Sul, Brazil) and transported in a thermal box to the laboratory. COCs were punctured from 2 to 8 mm follicles, using a sterile 18-gauge needle attached to a vacuum pump. The follicular fluid was washed with PBS (phosphate buffer saline) and filtered in an embryo collector filter (Nutricell, Campinas, São Paulo, Brazil). Only COCs with a homogeneous cytoplasm and at least three layers of surrounding cumulus cells were selected under a stereomicroscope for *in vitro* maturation (IVM).