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Effects of chitosan-coated lipid-core nanocapsules on bovine sperm cells

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ABSTRACT

Toxicology studies have a pivotal role for selection of new nanosystems. As lipid-core nanocapsules (LNC) rise as a potential system not only for drug delivery but also for immunotherapy and gene therapy, the demand for models of toxic screening increases, and sperm arises as a promising model due to the easiness to evaluate its viability parameters. LNCs were coated with chitosan, chitosan-coated lipid-core nanocapsules (LNC-CS), in order to modify the nanocapsule surface. We evaluated the toxicity of LNC and LNC-CS after incubation with bovine sperm in different concentrations (2.5%, 5%, 10%, 20%, 40% and 80%) (v/v) and periods of exposure (0 h and 1 h). CASA parameters and flow cytometry assays were performed to assess LNC and LNC-CS effects. The results corroborated with previous studies indicating that there is no toxicity from LNCs and LNC-CS below 40% (v/v) concentration.

1. Introduction

In the last decade, nanotechnology has played a prominent role in medicine due to its potential to produce new and more efficient tools for diagnosis, therapies and treatment of diseases (Mahapatro and Singh, 2011; Mora-huertas et al., 2010). Nanocapsules, regardless their composition, have been extensively used as a delivery system for a more efficient or site-specific molecule distribution, improving the solubility of insoluble or poorly soluble drugs (Dobrovolskaia et al., 2009; Schaffazick et al., 2005) and reducing the toxic effects. Recently, one type of polymeric nanocapsules, the lipid-core nanocapsules (LNC), has received increased attention due its great potential to drug delivery in different tissues (Oliveira et al., 2014).

LNC are core-shell structures, with an oily core surrounded by a polymeric wall, in which the oily core is formed by sorbitan monostearate and capric-caprylic triglyceride, structured as an organogel (Durli et al., 2013). Applications of LNC range from drug delivery, cancer immunotherapy and gene therapy (Pohlmann et al., 2013), in addition to the great cell uptake capacity of these nanocapsules (Fiel et al., 2014). In gene therapy, cationic polymers have been employed to modify the nanocapsules surface. The biodegradable, biocompatible, non-toxic and mucoadhesive polysaccharide extracted from crustacean shells, known as chitosan, has been one of the most promising material for nanocapsules development including a surface-coating on LNC due to its ability of binding covalently to DNA (Bender et al., 2012; Siqueira et al., 2011). Our group has successfully enhanced bovine oocyte maturation and embryonic development by supplementing the maturation medium with tretinoin-loaded and melatonin-loaded lipid-core (Komninou et al., 2016; Lucas et al., 2015; Remião et al., 2016). Furthermore, we previously determined that LNC and chitosan-coated lipid-core nanocapsules (LNC-CS) are human blood compatible (Bender et al., 2012).

Following the 3Rs principle (refinement, reduction, replacement) alternative toxicity screening strategies which provide the reduction in animal use have been developed and validated (Beker et al., 2012; Scholz and Genschow, 1999; Tessaro et al., 2015). Under the EU Integrated Project ReProTect (6th Framework Programme) (http://www.reprotect.eu); (Lorenzetti et al., 2011) a battery of *in vitro* tests have been implemented to study the reproductive toxicity. Some *in vitro* tests, such as the bovine oocyte maturation assay (bIVM) and the bovine *in vitro* fertilization assay (bIVF) were validated (Lazzari et al., 2008;

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Lorenzetti et al., 2011; Tessaro et al., 2015). As oocytes and embryos, the mammalian spermatozoa has been used efficiently as a biosensor for environmental and toxicological insults (Andersson et al., 2009; Mikkola et al., 2007; Peltola et al., 2004). Sperm parameters are required for the male reproductive success as egg penetration and fertilization. Any damage to the sperm motility, plasma membrane and DNA integrity could impair its reproductive capability (García-Vázquez et al., 2009).

Considering that lipid-core nanocapsules are promising intracellular carriers, the current study has investigated the action of LNC and LNC-CS on bovine sperm cells. The toxicity of different LNC and LNC-CS concentrations on sperm kinetics, membrane and acrosome integrity, mitochondrial activity, lipid perodoxitation and DNA damage are examined.

2. Materials and methods

2.1. LNCs synthesis

2.1.1. LNC and LNC-CS

The lipid-core nanocapsules in aqueous dispersion were prepared by a self-assembling mechanism. An organic phase (containing 25 mL of acetone, 0.038 g of sorbitan monostearate, 0.160 mL of capric-caprylic triglyceride and 0.100 g of PCL) was added to a lecithin solution in ethanol (0.600 g in 5 mL) under magnetic stirring at 40 °C. The organic phase was injected in an aqueous phase (50 mL of water and 0.077 g of polysorbate 80). After 10 min at 40 °C, the organic solvent was eliminated and water partially evaporated under reduced pressure (at 40 °C) to a final volume of 10 mL. This formulation was named LNC.

Regarding the cationic coating, 1 mL of 1% chitosan in 1% acetic acid aqueous solution was added (dropwise) to 9 mL of LNC. Thus, the formulation was stirred for 2 h to ensure complete interfacial reaction. This formulation was defined as LNC-CS.

2.1.2. Fluorescent-labeled LNC and LNC-CS

To obtain fluorescent-labeled LNC and LNC-CS, the same methodology above was used replacing 10% of polymer by rhodamine B-PCL conjugate. The rhodamine B was covalently bound to the hydroxyl end-groups of poly(ε-caprolactone) under inert atmosphere (argon) using carbodiimide as activating agent for the carboxylic acid conversion to ester (Weiss-angeli et al., 2012).

2.2. Physic-chemical characterization

2.2.1. Laser diffraction

Laser diffraction analysis was carried out using a Mastersizer® 2000 instrument (Malvern Instruments, Malvern, UK). Approximately 300 μL of each sample was added in the dispersion accessory (Hydro 2000SM Dispersion Unit) containing about 100 mL of distilled water (necessary to obtain obscuration levels between 2% and 8%). Background signal (distilled water) was measured before each analysis. For rhodamine B-labeled formulations (RhoB-LNC-CS and RhoB-LNC) the blue laser was turned off to avoid interference by molecular photon absorption.

2.2.2. Photon correlation spectroscopy

Photon correlation spectroscopy (or dynamic light scattering) analysis was performed in a ZetasizerNano ZS model ZEN 3600 instrument (Malvern Instruments, Malvern, UK). The formulations were diluted 500 times in pre-filtered ultrapure water (Millipore®, 0.45 μm).

2.2.3. Zeta potential

The zeta potential was evaluated by electrophoresis mobility in a ZetasizerNano ZS model ZEN 3600 (Malvern Instruments, Malvern, UK). The samples were diluted 500 times in 0.5 mmol mL^{-1} NaCl aqueous solution (pre-filtered through 0.45 μm filter, Millipore®).

2.3. Semen processing and experimental design

Frozen semen straws from five bulls were thawed in a water bath at 35 °C for 30 s and separately suspended in 2 mL of Opti-MEM I medium (Invitrogen, Carlsbad, CA, USA). The samples were centrifuged twice for 5 min at 200 × *g* to remove cryoprotectants from media. An aliquot of 25×10^4 sperm/mL from the pellet was added in each treatment and used in subsequent analysis.

To sperm kinetics analysis the samples from each bull were incubated with five concentrations of both LNC and LNC-CS: 2.5%, 5%, 10%, 20%, 40% and 80% (v/v). Spermatozoa were analyzed immediately after incubation (time 0 h) and after 1 h exposition (time 1 h). Two control groups, named M control, containing sperm suspended in medium, and W control, containing sperm suspended in water at 40% concentration were used. Incubation and analysis were performed in three independent replicates.

To examine the toxicity of different LNC and LNC-CS concentrations on membrane and acrosome integrity, mitochondrial activity, lipid peroxidation and DNA damage, samples were incubated with three concentrations of both LNC and LNC-CS: 10%, 20% and 40% (v/v). Spermatozoa were analyzed immediately after incubation (time 0 h) and after 1 h exposition (time 1 h). Only the M control was used for these analyses.

To evaluate LNC and LNC-CS incorporation in sperm cells, RhoB-LNC-CS and RhoB-LNC nanocapsules were used for incubation of spermatozoa in all concentrations (2.5%, 5%, 10%, 20%, 40% and 80% (v/v)), at 0 and 1 h. The incorporation was analyzed by flow cytometry and images were taken by confocal microscopy.

2.4. Sperm kinetics analysis

The sperm samples exposed by the different treatments were prepared in slides and analyzed by computer-assisted sperm analysis (AndroVision®, Minitube, Germany). To perform the evaluations, ten microscopic fields of each slide was recorded and investigated. The following parameters were analyzed: Total motility (%), progressive motility (%), distance average path (DAP, μm), distance curved line (DCL, μm), distance straight line (DSL, μm), velocity average path (VAP, μm/s), velocity curved line (VCL, μm/s), velocity straight line (VSL, μm/s), straightness [STR (VSL/VAP), %], linearity [LIN (VSL/VCL), %], wobble [WOB (VAP/VCL), %], amplitude of lateral head displacement (ALH , μm), and beat cross frequency (BCF, Hz).

2.5. Cytometry analysis

In regard to flow cytometry analysis, Attune Acoustic Focusing Cytometer® (Applied Biosystems, California, USA) was used. The samples were exposed to Hoechst 33342 (H33342, 14533, Sigma-Aldrich, St. Louis, USA) in concentration of 2 mM, 5 min before each analysis. The events were detected by fluorochrome with photomultiplier (Photomultiplier - PMT) VL1 (filter 450/40); except in DNA fragmentation analysis, when H33342 was not used. The green, orange and red fluorescences were analyzed by PMTs BL1 (530/30 filter), BL2 (575/24 filter) and BL3 (filter > 640) filters, respectively. Cytometer fluorescence stability was tested daily using standard beads (Invitrogen, Carlsbad, California, USA). The acquisition rate was 200 events/s totaling 20,000 events per sperm sample. All assays were done in three independent replicates. The results were analyzed using the *Attune Cytometric Software* V2.1. The non-sperm events from analysis by scatter plots of FSC × SSC (Petrunkina et al., 2005) and negative fluorescence of H33342 events (debris) were eliminated.

2.5.1. LNCs incorporation assay

The incorporation analysis of different concentrations of LNC and LNC-CS were implemented through RhoB-LNC and RhoB-LNC-CS. The sperm cells were classified as incorporated with LNC/LNC-CS when