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Cationic liposomes containing antioxidants reduces pulmonary injury in experimental model of sepsis
Liposomes antioxidants reduces pulmonary damage

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ABSTRACT

The intracellular redox state of alveolar cells is a determining factor for tolerance to oxidative and pro-inflammatory stresses. This study investigated the effects of intratracheal co-administration of antioxidants encapsulated in liposomes on the lungs of rats subjected to sepsis. For this, male rats subjected to sepsis induced by lipopolysaccharide from Escherichia coli or placebo operation were treated (intratracheally) with antibiotic, 0.9% saline and antioxidants encapsulated or non-encapsulated in liposomes. Experimental model of sepsis by cecal ligation and puncture (CLP) was performed in order to expose the cecum. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site. As an index of oxidative damage, superoxide anions, lipid peroxidation, protein carbonyls, catalase activity, nitrates/nitrites, cell viability and mortality rate were measured. Infected animals treated with antibiotic plus antioxidants encapsulated in liposomes showed reduced levels of superoxide anion (54% or 7.650 ± 1.263 nmol/min/mg protein), lipid peroxidation (33% or 0.117 ± 0.041 nmol/mg protein), protein carbonyl (57% or 0.039 ± 0.022 nmol/mg protein) and mortality rate (3.3%), p value <0.001. This treatment also reduced the level of nitrite/nitrate and increased cell viability (90.7%) of alveolar macrophages. Taken togheter, theses results support that cationic liposomes containing antioxidants should be explored as coadjuvants in the treatment of pulmonary oxidative damage.

1. Introduction

The imbalance of intracellular redox status affects the signaling pathways responsible for cell proliferation, immune responses, bioenergetic metabolism, gene expression and cell death. This imbalance plays an important role in the pathogenesis of acute lung injury induced by generalized infection (Crimi et al., 2006). The

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intracellular redox state of alveolar cells is a determining factor for tolerance to oxidative and pro-inflammatory stresses. Exposure to H_2O_2, tumor necrosis factor alpha (TNF-α) or lipopolysaccharides (LPS) results in the decrease of tumor growth factor beta-1 (TGF-β1), increased permeability of the alveolar-capillary barrier and increased cytokines (IL-1 and TNF-alpha)(Rahman et al., 2005).

A strategy to limit injury of lung tissue would be to restore the alveolar redox balance (Kawabata et al., 2002). Antioxidants can prolong the initiation phase or inhibit the propagation phase of reactive oxygen (ROS) and nitrogen species (RNS) and contribute to the prevention of pulmonary oxidative damage (Bhavsar et al., 2009; Mitsopoulos et al., 2008; Teixeira et al., 2008). Despite being part of the diet, most antioxidants have poor bioavailability due to one or more factors such as poor solubility, instability during storage, first-pass effects or enzymatic degradation by the gastrointestinal tract (Ratnam et al., 2006). Recently, the development of new liposomal systems (Ratnam et al., 2006) has been proposed in order to mitigate these losses when compared to conventional formulation systems (Epperly et al., 2002; Li et al., 2013).

Based on this finds, we proposed investigated the effects of co-administration of antioxidants encapsulated into liposomes as a co-therapy in the treatment of respiratory tract infections. Therefore, our aim was to analyse the effects of inhaled co-administration of ascorbic acid, alpha-tocopherol and *n*-acetycysteine (NAC) encapsulated in liposomes into acute lung injury induced by sepsis.

2. Methods

2.1. Preparation of liposomes

Positively charged liposomes were prepared by the method of hydration of a lipid film (Lira et al., 2009).Briefly, an assay was carried out with liposomes with 42 mM total lipids (phosphatidylcholine, cholesterol and stearylamine) at a molar ratio of 7:2:1 (w/w) to test the possibility of nanoencapsulating three antioxidants: vitamin C, N-acetylcysteine in the aqueous phase, and vitamin E in the organic phase. The following physicalchemical parameters of liposomal formulations were analyzed: pH, size (nm), polydispersity index (PDI), zeta potential (mV), molarity and stability of phase buffer. Then, to determine the best lipid concentrations of the organic phase of the liposomes, a complete 2^3 factorial design was performed with the following variables: phosphatidylcholine (0.179–0.298 mg), cholesterol (0.037–0.061 mg) and stearylamine (0.008–0.014 mg). In this assay, the following parameters were used for analysis of the results: size (nm), PDI, zeta potential (mV), pH and lipid peroxidation.

For the *in vitro* and *in vivo* studies the best formulation (F9) obtained from the factorial design described above was used. The formulation comprised the lipids (soybean phosphatidylcholine, cholesterol and stearylamine (in a molar ratio of 7:2:1) and 2 mg vitamin E dissolved in a mixture of $CHCl_3$:MeOH (3:1 v/v), under magnetic stirring. The solvents were removed under pressure over 60 min (37 ± 1 °C, 80 rpm), resulting in the formation of a thin lipid film (organic phase). This film was then hydrated with 10 mL phosphate buffer solution to 0.15 M (aqueous phase), pH 7.4, containing 5 mg of vitamin C and 1 mg of N-acetylcysteine(NAC), resulting in multilamellar liposomes (NAC/VitC/VitE-lipo) described as Lip AO. The liposomal suspension was then sonicated (Vibra Cell, Branson, USA) at 200 W and 40 Hz for 300 s to form small unilamellar liposomes (Lira et al., 2009).

2.2. Characterization of liposomes

The pH of the liposome dispersions was measured with a glass electrode and a digital pH meter (Bioblock Scientific 99.622, Prolabo, Paris, France) at room temperature. The size diameter and polydispersity index of the liposomes was measured by photon correlation spectroscopy (Beckman Coulter Delsa™ Nano S particle analyzer) (Lira et al., 2009). The measurements were performed at 25 °C with a fixed angle of 90° and the sizes were indicated as the average liposomal hydrodynamic diameter (nm). One hundred microliter (100 μL) liposomes were diluted in 1 mL of ultrapure water. The zeta potential of the liposomes was used to analyse the surface charge of the vesicles (Zetasizer Nano ZS analyzer, Malvern) (Lira et al., 2009).

Drug encapsulation efficiency was determined by the ultrafiltration/ultracentrifugation technique, using Ultrafree units (Millipore, USA, MW cut-off = 10,000 Da)(Cadena et al., 2013). Samples of liposomes (400 μL of formulation number 9) were inserted in filtration unit and submitted to ultracentrifugation at 8792*g* for 1 h, 4 °C (Eppendorf, model 5810R). Ten microliters of the sample that passed through the filter was diluted in 5 mL of methanol and the antioxidants encapsulated and non-encapsulated were quantified by UV spectrophotometry (λ_{NAC} = 203 nm; λ_{VitC} = 245 nm; λ_{VitE} = 210 nm). The contents of NAC, vitamin C and E were determined from standard curves prepared at concentratios form 0.25–1.5 μg/mL.

$$EE\% = ([\text{measured antioxidant content}] - [\text{unloaded antioxidant}]/[\text{measured antioxidant content}]) \times 100 \quad (1)$$

For the long-term and accelerated stability testing, the methodology as described by Lira et al. was used (Lira et al., 2009). After preparation, the samples of the liposomal suspension were subjected to centrifugation (3165*g* for 1 h at 4 °C) and horizontal mechanical stirring (180 beats/min for 48 h at 37 °C). To evaluate the long-term stability, the macroscopic appearance, pH change, particle size and polydispersity index (PDI) were monitored after preparation and during storage of lyophilized liposomal formulations at predetermined time intervals (0, 1, 3 and 9 days).

2.3. Experimental model of sepsis by cecal ligation and puncture

All experimental procedures involving animals were performed in accordance with the Brazilian College of Standards for Ethics in Animal Experimentation (COBEA) and approved by the Ethics Committee on Animal Experiments (CEUA) UFPE under letter 215/09, process n°. 23076.018913/2009-41.

Experimental model of sepsis by cecal ligation and puncture (CLP) was performed under aseptic conditions with male Wistar rats, aged 2–3 months. (n = 9 animals/group). Briefly, a 3-cm, midline laparotomy was performed in order to expose the cecum (Andrades et al., 2011). The cecum was tightly ligated with a 3.0 silk suture at its base below the ileocecal valve, and pierced with a 14 gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site. A sham operation (laparotomy and cecal display, without any manipulation) was performed as a control. The animals were resuscitated with a normal saline solution (30 mL/kg, subcutaneously) immediately and after 12 h. After surgery, animals received "basic support" (saline, 50 mL/kg, and ceftriaxone, 30 mg/kg, intraperitoneally every 24 h for 5 consecutive days).

2.4. Administration of antioxidants encapsulated in liposomes

All the animals (n = 9 animals/group) were anesthetized with ketamine and xylazine (80 mg/kg and 15 mg/kg, respectively, at a dose of 0.2 mL/100 g, 1:1 v/v) and given, intratracheally (i.t.), 20 μL of the following fresh formulations: empty liposomes;