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Effects of a lycopene-layered double hydroxide composite administration in cells and lungs of adult mice

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ABSTRACT

Lycopene is a natural compound with one of the highest antioxidant activities. Its consumption is associated with lower risks in lung cancer and chronic obstructive pulmonary disease, for example. Experimentally, a murine model demonstrated the ingestion of lycopene, which reduced the damage in lungs caused by cigarette smoke. Since lycopene is highly hydrophobic, its formulations in supplements and preparations for laboratory assays are based on oils, additionally, bioavailavility is low. We developed a lycopene layered double hydroxide (Lyc-LDH) composite, which is capable of transporting lycopene aqueous media. Our objective was to evaluate the cytotoxicity of Lyc-LDH and the intra-cellular production of reactive oxygen species (ROS) in J774A.1 cells. Also, *in vivo* assays were conducted with 50 male C57BL/6 mice intranasally treated with Lyc-LDH 10 mg/kg (LG10), Lyc-LDH 25 mg/kg (LG25) and Lyc-LDH 50 mg/kg (LG50) during five days compared against a vehicle (VG) and control (CG) group. The blood, bronchoalveolar lavage fluid (BALF) and lung tissue were analyzed. The results revealed that Lyc-LDH composite attenuated intracellular ROS production stimulated with lipopolysacharide. In BALF, the highest doses of Lyc-LDH (LG25 and LG50) promoted influx of macrophages, lymphocytes, neutrophils and eosinophils compared to CG and VG. Also, LG50 increased the levels of IL-6 and IL-13, and promoted the redox imbalance in the pulmonary tissue. On the contrary, low concentrations did not produce significative effects. In conclusion, our results suggest that intranasal administration of high concentrations of Lyc-LDH induces inflammation as well as redox status changes in the lungs of healthy mice, however, results with low concentrations open a promising way to study LDH composites as vehicles for intranasal administration of antioxidant coadjuvants.

1. Introduction

Lycopene is a carotenoid conferring the red color to some fruits like tomato, watermelon, papaya, and red guava [1]. Its molecular structure comprises 11 conjugated double bonds, enabling the high capability for singlet oxygen sequestration [2,3], thus being one of the most efficient among carotenoids [4]. Although some carotenoids including β-carotene and α-carotene, cryptoxanthin, lutein, and lycopene [5] are found in human tissues, the ingestion through the diet is essential since these carotenoids are not endogenously synthesized [6]. The best source of

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lycopene is tomato since it supplies around 30 mg of lycopene/kg and the sauce derivatives increase the content to 100 mg/kg) [7].

Several studies have already demonstrated the positive relationship between adequate intake of lycopene-containing foods and the prevention of chronic diseases [8]. For instance, one of the well know benefits of lycopene is related to the reduction of incidences and risk in prostate cancer as determined from epidemiological studies [9,10]. Another important case is found against chronic obstructive pulmonary disease (COPD) promoted by cigarette smoke and lung cancer where lycopene exerts antioxidant and anti-inflammatory effects either in its complete molecular form or derivative molecules such as the apo-10′-lycopenoic acid [11].

In experimental murine model, our research group reported the antioxidant and anti-inflammatory protection of lycopene diluted in vegetable oil administrated by orogastric gavage against the exposure of mice to cigarette smoke during five days [12]. Further, the experiment comprising 60 days demonstrated that the antioxidant and anti-inflammation markers were controlled with the lycopene consumption, whereas the pulmonary emphysema and the pulmonary parenchyma were alleviated [13]. These results motivated us to study the administration of lycopene through the airway and the effects on lungs of mice, however, as lycopene is highly hydrophobic, we needed a formulation that allowed for the preparing of aqueous solutions or dispersibility of lycopene in saline solution. Furthermore, intranasal administration was chosen because previous studies have shown that the intranasal route provides excellent local action results using bioactive compounds especially in small animals, while avoiding gastrointestinal or hepatic metabolization that could occur when using another route of administration [14–18].

Lycopene has been encapsulated to prevent isomerization and degradation caused by oxygen, light and bioavailavility could be improved [19]. Several systems and techniques are available for encapsulation, such as nano and microemulsions, nanostructured lipid carriers, nano-liposomes, and lipid-based nanocarriers [19]. Among the various possibilities, hybrid composites of layered double hydroxides (LDH) offer a strategy to develop water-soluble particles containing lycopene. LDHs are biocompatible and synthesized at low cost with environmentally low impact reagents [20]. A hybrid composite could present new properties that were not present in the initial separate components [21] as in the case of the lycopene- LDH (Lyc-LDH) composite.

Recently, Gutiérrez Galán et al. reported the dispersion of Lyc-LDH in saline solution and evaluated the effects of nanoparticles (Lyc-LDH) on markers of oxidative damage in rat liver [22]. The studies on the effects of this composite are still recent, therefore, the aim of this study was to evaluate the effects of the Lyc-LDH composite on J77A.1 macrophages and in the lung of C57BL/6 mice after intranasal administration in saline solution.

2. Method

2.1. Synthesis of the lycopene-LDH composite (Lyc-LDH)

The composite nanoparticles used in the *in vitro* and *in vivo* studies were synthesized and characterized according to the method described by González Rojas et al., [23] and were sent for our laboratory in vacuum tubes and protected from light to avoid oxidation of lycopene.

2.2. In vitro study

2.2.1. Antioxidant capacity

The scavenging of the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) radical was measured to determine the antioxidant capacity of Lyc-LDH as described by Rufino et al., [24]. We used concentrations of Lyc-LDH ranging from 7.5 to 80 μg/mL and quercetin (Sigma-Aldrich, San Luis, Missouri, USA) standards from 0.3 to 7.5 μg/mL. Initially, 120 μL of the Lyc-LDH or quercetin solutions were transferred to 96-well plates, then 100 μL of the DPPH (Sigma-Aldrich, San Luis, Missouri, USA) radical in 0.008% (m/v) ethanol (Dinâmica Química Contemporânea LTDA, Indaiatuba, São Paulo, BR) solution were added. The mixture was incubated during 30 min at room temperature in the dark. After incubation, reading was performed at 517 nm in a spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA).

The analyze was performed in triplicate. The ability to scavenge $DPPH\bullet$ radicals was obtained by: % Inhibition = [(A1 – A Sample) / A1] × 100, where A1 = control absorbance, A Sample = sample or standard absorbances. The expression of the results was reported as % of inhibition, thus obtaining the CE50, representing the concentration to reduce 50% of the initial DPPH after the reaction equilibrium [25].

2.2.2. Cell culture

Cytotoxicity of Lyc-LDH was studied using murine macrophages of the J774A.1 cell line. Cells were grown in DMEM medium mixed with 10% fetal bovine serum, Streptomycin (0.1 mg/mL) (Sigma-Aldrich, San Luis, Missouri, USA), Penicillin (100 U/mL) (Sigma-Aldrich, San Luis, Missouri, USA), Hepes sodium salt and $NaHCO_3$ (analytical grade) (Sigma-Aldrich, San Luis, Missouri, USA) in a cell culture bottle (Biofil-25 cm2) incubated at 37 °C in air atmosphere with 5% CO_2.

In order to renew the medium and ensuring cell viability, when cells reached 80–90% confluence, a wash was conducted using the phosphate buffered saline (PBS), dibasic anhydrous Na_3PO_4 (10 mM), and KH_2PO_4 (2 mM). To detach the cells from the bottom of the bottle, EDTA (0.02% Sigma-Aldrich, San Luis, Missouri, USA) and Trypsin (0.25% Sigma-Aldrich, San Luis, Missouri, USA) diluted in PBS was used for 5 min. Subsequently, the supplemented DMEM medium was added to neutralize EDTA-Trypsin and the contents of the bottle were discarded, then DMEM medium was added to complete 10 mL [25].

2.2.3. Assessment of cell viability

Cell viability was determined by measuring the MTT((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction. The viable cells present in the sample are capable of forming the formazan salt from MTT (Sigma-Aldrich, San Luis, Missouri, USA) reduction, allowing the analysis of cellular metabolic activity by spectrophotometry [26]. The cells were deposited in 96-well plates (2.5×10^4 cells per well) and mixed with different concentrations of Lyc-LDH (0.5; 1; 2; 4; 8; 10 and 25 mM). The cells were incubated for 3, 6 and 24 h.

After this period, the medium was replaced by another medium containing MTT (0.5 mg/ml), and the cells were incubated for 14 h. After incubation, the solution was removed, and Dimethyl Sulfoxide (Synth, Diadema, São Paulo, BR) was used to solubilize the crystals of Formazan. The VICTOR reader (Multidetector VICTOR™ X3, Perkin Elmer, Waltham, Massachusetts, USA) was used to analyze the absorbance at 570 nm [25,27].

2.2.4. Reactive oxygen species (ROS) production

Initially, 1×10^4 J774A.1 macrophages per well, were added in 96-well plates. Intracellular ROS formation was estimated by fluorescence after 24 h of culture, using 2,7-dichlorofluorescein diacetate (H2DCFDA), adapted by Halliwell and Whiteman [28] and Lebel, Ischiropoulos and Bondys [29]. H2DCFDA is cleaved by intracellular ones producing H2DCF, which is trapped inside the cells. Intracellular ROS oxidize H2DCF to form the highly fluorescent DCF compound. After treatment with Lyc-LDH at concentrations of 10 μM and 25 μM for 24 h, the medium was replaced with 150 μL of H2DCFDA (Sigma-Aldrich, San Luis, Missouri, USA) solution (2 μM) for 45 min. Then, the wells were washed with Ca^{2+} and Mg^{2+}-free Hank's saline solution (HBSS) three times, and the HBSS (Sigma-Aldrich, San Luis, Missouri, USA) was left on the third time. Fluorescence was monitored at times 0 h, 1 h, 2 h, 3 h, 4 h and 18 h after addition of the LPS solution at the final concentration of 1 μg/ml. The reading was performed in a microplate reader, at 495 and 530 nm wavelengths (excitation and emission) [25,27].

2.3. In vivo study

2.3.1. Animals

Fifty C57BL/6 male mice, aged 8 weeks and weighing between 20 and 25 g from the Animal Science Center, were used. The animals were fed a large quantity of water and standard balanced chow ad libitum and remained in an environment with controlled humidity (50 ± 10%), temperature (21 ± 2 h light/dark cycle), and luminosity (1 light/dark cycle). The procedures are performed following the Ethical Principles of Animal Experimentation established by the Committee on Ethics in the Use of Animals at the Federal University of Ouro Preto (CEUA-UFOP). The animals were randomly divided into 5 groups, as follows: a control group exposed to room air (CG); a vehicle group receiving intranasal saline (VG); one receiving 10 mg/kg intranasal Lyc-LDH (LG10); another one with 25 mg/kg of Lyc-LDH (LG25) intranasally and the fifth group, which received 50 mg/kg of Lyc-LDH (LG50) intranasally.

2.3.2. Intranasal instillation of Lyc-LDH

The animals were anesthetized, one by one, in chambers with 2% of isoflurane before intranasal instillation. While the animals were unconscious, the respective solutions were administered. Once daily for five consecutive days, 25 μL of the solution was administered into each nostril, resulting in a total volume of 50 μL per exposure [15,30].

2.3.3. Body mass

The evaluation of body mass was performed at the beginning of the experiment to determine the amount of Lyc-LDH that would be administered to each animal and at the end of the experiment before the collection of ventilatory parameters. For this, the animals were individually weighed on a digital scale (Marte science and industrial instrumentation ltda., São Paulo, SP, BR).

2.3.4. Ventilatory parameters

24 h after the experimental protocol, the ventilatory parameter was analyzed in 5 animals from each experimental group. Mice were anesthetized, one by one, using a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). Then, the trachea was accessed through an incision in the neck. Subsequently, the trachea was cannulated with a catheter (18G) attached to a 2 cm long tube in order to attach the animal to the flow head (MLT1L), which was connected to the small animal spirometer (ADInstruments, Bella Vista, NSW, Australia). The ventilatory parameter was recorded during 60 s, with 3 repetitions each. Four parameters were evaluated: tidal volume, respiratory rate and minute volume. All signals were amplified (Octal Bridge Amp model ML228; Powerlab 4/35 model ML870; AD Instruments) and collected with LabChart for Windows (version v8.1.11) [31].

2.3.5. Blood collection

After collecting the ventilatory parameters, the animals were euthanized using an overdose of sedative, a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). After euthanasia, a midline incision was made in the thorax of the animals to expose the rib cage. Then, a cardiac puncture (right ventricle) was performed through the third intercostal space. Blood aliquots were collected from each animal, using polypropylene tubes with anticoagulant (15 μL). Samples of about 0.2 mL were analyzed in the Bc2800 vet auto Hematology Analyzer (Mindray® Bio-Medical Electronics, Shenzhen, China) equipment [31].

Blood smear slides were produced for the differential leukocyte count. A sample with approximately 5 μL of blood was deposited on the end of the histological slide using an automatic pipette. In order to form a continuous film, an extension blade (positioned at 45°) was used to slide over the blade and spread the blood evenly. The staining was performed with a rapid kit (Renylab, Barbacena, Minas Gerais, BR). The analysis was performed through the relative count of 100 leukocytes on each slide. Leukocytes were differentiated into eosinophils, lymphocytes, monocytes, and neutrophils based on certain parameters (staining, nuclear and cytoplasmic morphology) performed by two researchers at two different times [25].

2.3.6. Bronchoalveolar lavage fluid (BALF) analysis

The trachea of the animals was cannulated with a catheter (18 G) connected to a 1 mL syringe to collect the bronchoalveolar lavage fluid (BALF). The lungs were washed twice with saline solution (0.9% NaCl), to recover a BALF volume between 1.5 and 2 mL, which was stored in sealed polypropylene tubes cooled down with ice (4 °C) to avoid cell lysis. To count the total and differential amount of the cells in the airways, the BALF was centrifuged at 4 °C and 3000 rpm (NT805 - Novatecnica, Piracicaba, São Paulo, BR) for 10 min. The pellet containing the leukocytes was immediately resuspended in 0.1 mL of saline and the supernatant was stored and frozen (−80 °C). Then, 20 μL of the resuspended solution was mixed with 180 μL of Turk's solution in a tube. An aliquot was transferred to the Neubauer chamber for total leukocyte count under an optical microscope. For differential counting, the samples were centrifuged in a Cyto-centrifuge (INBRAS health equipment, São Paulo, SP, BR). For differential cell count, 50 μL of BALF samples were centrifuged in a cytocentrifuge for 1 min at 1000 rpm. The slides were stained with the rapid staining kit (Renylab, Barbacena, Minas Gerais, BR) and used for the differential count of leukocytes in an optical microscope with oil immersion (100X magnification) counted by two researchers twice at different times [25,32].

2.3.7. Lung collection

After BALF collection, the thorax was opened to reach the right ventricle for perfusion with saline solution. Then, the left main bronchus was clamped. The left lung was perfused with buffered formalin under the constant pressure (25 cmH_2O) for 2 min. An inferior third of the left lung was removed and immersed in fixation solution for 48 h. After fixation, the lungs were processed and embedded in paraffin. Using a microtome, 5 μm thick sections were made in the paraffin-embedded tissue and stained with Hematoxylin and Eosin (H&E) techniques for pulmonary histoarchitecture analysis. The right lung was also removed and stored (at −80 °C) to perform biochemical analyses. For this, 100 mg of the lung tissue was homogenized in 1 mL of phosphate buffer (0.01 M; pH 7.8). The samples were centrifuged at 1300 rpm for 10 min (NT805 - Novatecnica, Piracicaba, São Paulo, BR) at 4 °C. The resulting supernatant was stored at −80 °C [25,32].

2.3.8. Analysis of antioxidant defense oxidative stress biomarker

In order to determine the profile of the antioxidant system, the pulmonary homogenate was used. Catalase enzyme (CAT) activity was determined using the method described by Aebi [33]. Such analysis is based on the consumption of hydrogen peroxide (H_2O_2) for the formation of water and oxygen. The reaction is verified by reducing the absorbance of H_2O_2 at 240 nm in a spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The decomposition of hydrogen peroxide was monitored using the molar absorption coefficient of 39.4 $M^{-1}cm^{-1}$. The activity of superoxide dismutase (SOD) was analyzed using the spectrophotometric method described by Marklund and Marklund [34], which starts from the capacity of SOD to inhibit the auto-oxidation of pyrogallol. For the analysis of the carbonylated proteins, the method described by Reznick and Packer [35] was used. The results of the biochemical analyses were corrected for the amount of protein determined by the Bradford method [36].

2.3.9. Stereological analyzes

The analysis of pulmonary histoarchitecture was performed through the alveolar septum volume density (Vv). For this, a test system was used consisting of a known area composed of 16 points, with the forbidden line still in the system, considered as a limit to prevent overestimation in the number of structures. Conducive to obtaining uniform and proportional lung samples, 20 random fields (in 40x magnification) of the slides were analyzed in the monitor coupled to the system.

The points (Pp) that reached the alveolar spaces (Vv[a]) and alveolar septa (Vv [sa]) were assessed according to the total number of points in the test system (Pt). Starting from the point count using Pt, the reference volume will be estimated. In regard to determining the volume density analysis of Vv[sa] and Vv[a], the total area of 1.94 mm^2 was analyzed in HE-stained sections [13,37,38].

2.4. Statistical analysis

Statistical analyses were performed with GraphPad Prism version 7. For parametric data, the one-way ANOVA test was used followed by Tukey's post-test, whereas the Kruskal Wallis test followed by the Dunn's post-test was used for non-parametric data. Data were expressed as mean ± standard deviation or as median and interquartile range (percentiles 25 and 75). In both cases, the significance level was determined when $p < 0.05$was used as a significance value.

3. Results

3.1. In vitro study

3.1.1. Antioxidant capacity of Lyc-LDH

The analysis of the nanoparticle antioxidant capacity by the DPPH method are shown in Fig. 1. No differences were observed in the DPPH radical inhibition capacity between the concentrations used. The obtained data were used to calculate the CE50, which suggests that to inhibit 50% of the DPPH radical, it would require 379.41 ug/ml (Fig. 1).

3.1.2. Assessment of cell viability

The MTT assay was performed to verify whether exposition to Lyc-LDH is cytotoxic to J774A.1 cells. At 3 and 6 h for the concentrations used, no change in the viability of cells incubated with the different solutions containing nanoparticles was observed (Fig. 2A-B). After 24 h incubation (ANOVA, $F = 6.24$, $p < 0.0001$) with Lyc-LDH, a lower percentage of viability was observed for cells incubated with 8 mM (91.51 ± 3.16), 10 mM (91.67 ± 2.67) and 25 mM (91.93 ± 4.19) compared to control (100.0 ± 3.02) (Fig. 2C).

3.1.3. Effect of Lyc-LDH on ROS production

The analysis of intracellular ROS production demonstrated that at the following analyzed times (0 h, 1 h, 2 h, 3 h, 4 h and 18 h) lipopolysaccharide stimulated the cells leading to an increase in ROS production when compared to control ($p < 0.001$). The nanoparticles alone did not promote increased ROS production when compared to the control for all analyzed times ($p > 0.05$). The administration of the nanoparticles to LPS-stimulated cells led to a decrease in ROS production at all times, initially the two concentrations of Lyc-LDH (10 and 25 μM) promoted a decrease in ROS production when compared to LPS-stimulated cells ($p < 0.0006$). However, for the other analyzed times (1 h, 2 h, 3 h, 4 h and 18 h), only the higher concentration promoted a reduction of intracellular ROS when compared to the control ($p < 0.01$) (Fig. 3).

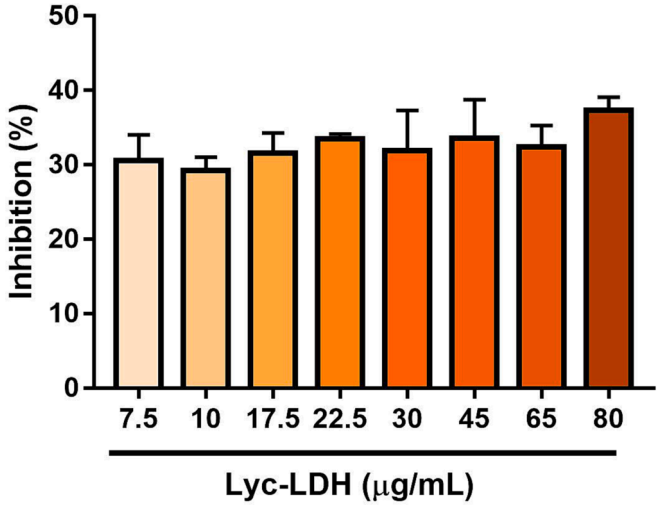

Fig. 1. Antioxidant capacity of Lyc-LDH by the DPPH method *in vitro*. The mean values ± standard deviation was obtained from three experiments. One Way ANOVA was followed by Tukey's post-test.

3.2. In vivo study

3.2.1. Ventilatory parameters and body mass

We observed that intranasal administration of Lyc-LDH did not promote changes in respiratory patterns, including respiratory rate, tidal volume, and minute ventilation compared to CG and VG. For body mass, there was also no statistical difference between the experimental groups (Table 1).

3.2.2. Hematological data

Table 2 shows the red blood cell, hemoglobin, platelets, total leukocytes, lymphocytes, and neutrophils values of the experimental groups. The animals that received intranasal administration of 50 mg/kg Lyc-LDH showed lower hematocrit (ANOVA, $p = 0.0097$, $F = 3.79$) compared to CG and VG ($p < 0.05$). Regarding the peripheral blood leukocyte count, a higher monocyte count (ANOVA, $p = 0.0224$, $F = 3.16$) was observed in the LG50 compared to CG and LG10 ($p < 0.05$). For the other cell types analyzed, no significant differences were observed between the groups.

3.2.3. Bronchoalveolar lavage fluid (BALF) analysis

The total and differential count of the BALF was performed to analyze the influx of inflammatory cells in the airways. There was an increase of inflammatory cells (ANOVA, $p < 0.0001$, $F = 16.49$ from GL50 and GL25 compared to the CG, VG and LG10 ($p = 0.001$). Also, in relation to the differential count, LG50 and LG25 had higher lymphocyte (Kruskal Wallis, $p < 0.0001$), neutrophil (Kruskal Wallis, $p < 0.0001$) and macrophage (ANOVA, $p < 0.0001$, $F = 10.98$) count compared to the CG and VG ($p < 0.05$). Regarding the eosinophil count (Kruskal Wallis, $p = 0.0021$), there was a higher cell count of LG50 compared to the CG, VG and LG10 ($p < 0.05$) (Table 3).

3.2.4. Analysis of redox status

The results demonstrate that the animals exposed to the highest lycopene concentrations (LG50) increased CAT activity (ANOVA, $p = 0.0112$, $F = 3.68$) compared to the CG. There was a lower SOD activity (ANOVA, $p = 0.0340$, $F = 2.86$) compared to the CG. The levels of protein carbonyl (ANOVA, $p < 0.0001$, $F = 10.32$) were higher in the LG50 compared to the other groups ($p < 0.001$). Significant differences were not observed in the levels of GSH/GSSG ratio among the groups (Table 4).

3.2.5. Inflammatory cytokine levels

The inflammatory markers TNF-α, IL-6, and IL-13 were determined in the BALF. Regarding the levels of TNF-α, no differences were observed among the groups. The intranasal administration with 50 mg/kg of Lyc-LDH promoted increased levels of IL-6 (ANOVA, $p < 0.0001$, $F = 25.84$) compared to the other experimental groups ($p < 0.0001$). The IL-13 levels (ANOVA, $p < 0.0001$, $F = 21.83$) were higher in the LG25 and LG50 compared to CG and VG ($p < 0.01$) (Table 5).

3.2.6. Stereological analyzes

The effects of Lyc-LDH administration in the lung tissue were evaluated by the stereological method. There was no significant variation in the volume density of alveolar air space (Kruskal Wallis, $p = 0.1083$) and volume density of the alveolar septa (Kruskal Wallis, $p = 0.2241$)

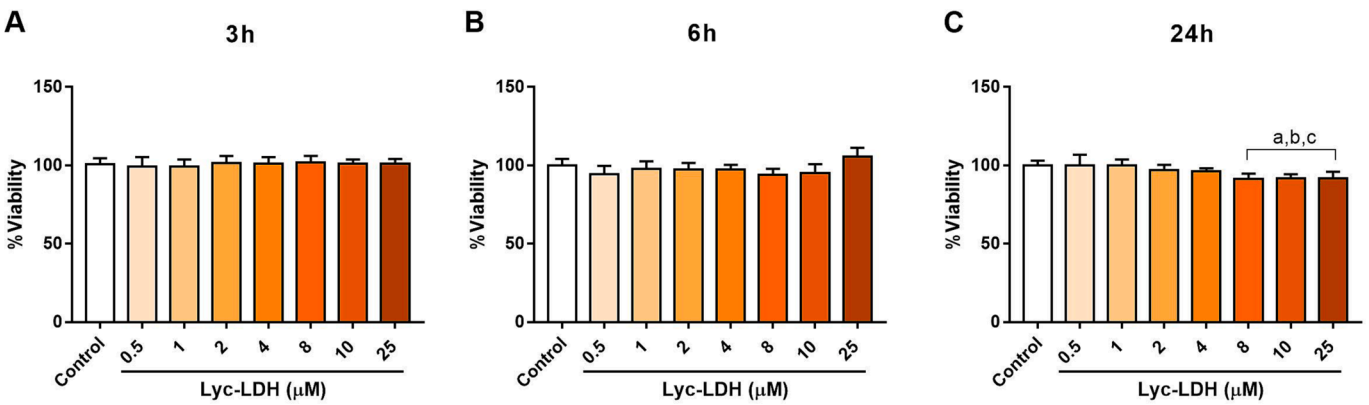

Fig. 2. Viability evaluation of J774A.1 cell exposed to Lyc-LDH. (A) 3 h, (B) 6 h, (C) 24 h after incubation with different Lyc-LDH concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 10.0 and 25.0 mM). The mean values ± standard deviation was obtained from three experiments. Analysis of Variance One Way ANOVA was followed by Tukey's post-test ($p < 0.05$). *a)* represents a significant difference compared to control; b) represents a significant difference compared to 0,5 μM; and c) represents a significant difference compared to 1 μM.

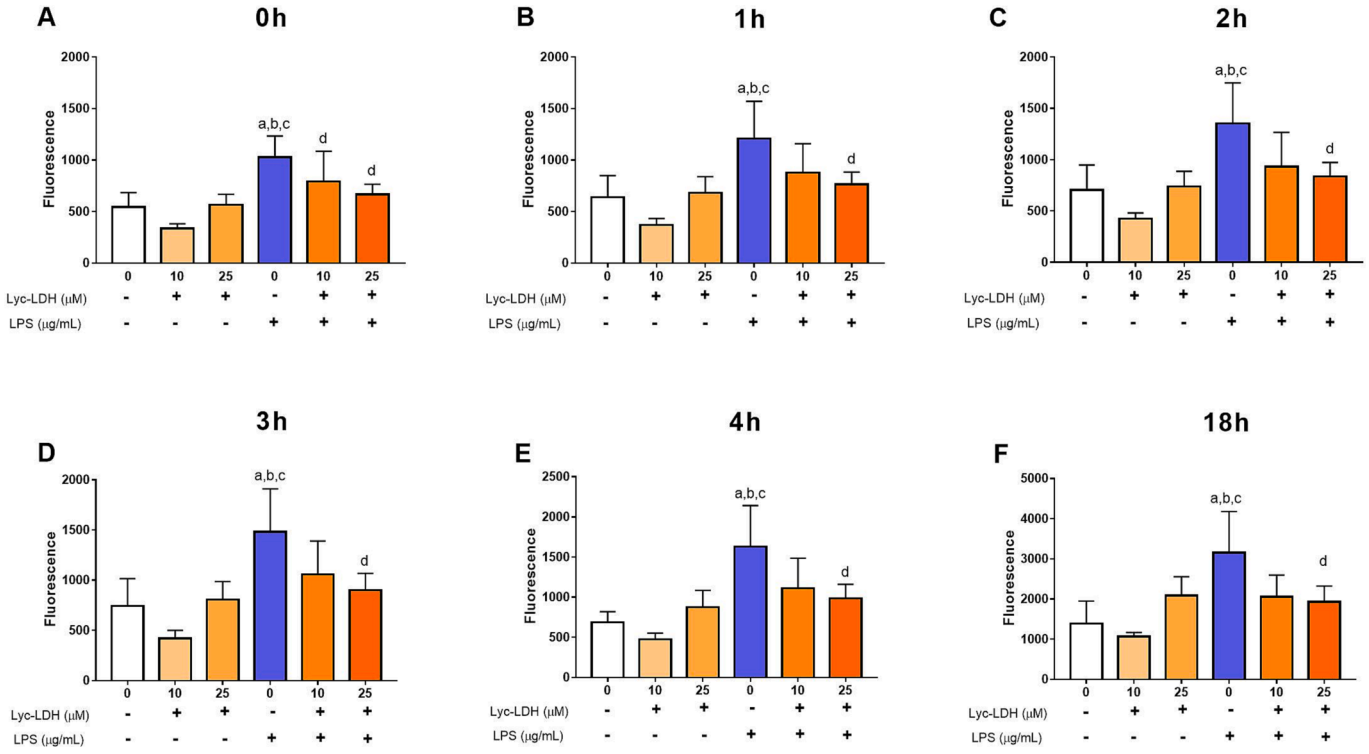

Fig. 3. Effect of Lyc-LDH on ROS production in J774A.1 cell. Quantitative analysis of ROS in J774A.1 cells administered with Lyc-LDH (10 and 25 μM) and/or LPS (1 μg/ml) after (A) 0; (B) 1 h, (C) 2 h, (D) 3 h, (E) 4 h, and (F) 18 h. The letter *a)* represents a significant difference when compared to control; b) represents a significant difference compared to Lyc-LDH 10 mM; c) represents a significant difference compared to Lyc-LDH 25 mM; and d) represents a significant difference compared to LPS. Data were analyzed by one-way ANOVA followed by Tukey's post-test and are expressed as mean ± standard error of the mean ($p < 0.05$).

among the experimental groups (Fig. 4).

4. Discussion

We evaluated the effects of the Lyc-LDH composite nanoparticles on J774A.1 macrophages and in the lungs of C57BL/6 mice. LDH nanoparticles represent a promising strategy for drug delivery and release due to the biocompatibility and high drug-loading capacity [39]. Our results show that Lyc-LDH nanoparticles in cell culture showed antioxidant effects, however, in the *in vivo* model, the higher concentration promoted an increase in oxidative damage, higher recruitment of inflammatory cells, as well as the production of inflammatory mediators in the lungs of the adult animals.

The analysis of the antioxidant capacity by the DPPH method showed that at different concentrations the nanoparticles presented a percentage of inhibition in the radical close to 30%, thus making approximately 370 μg/mL to inhibit 50% of the radical. Previously, Gozález-Rojas et al., evaluating the same nanoparticle observed higher antioxidant capacity by ABTS method [23]. Gutiérrez Galán et al., observed high total antioxidant capacity of nanoparticles containing lycopene when compared to nanoparticles without lycopene [22]. In our study, the low antioxidant capacity may be related to the technique used and the time used for the analysis. The nanoparticles release the lycopene slowly into the medium and the time used for the analysis was not enough to observe the effects of the carotenoid.

The analysis of cell viability was performed during three different

Table 1
Evaluation of lung function parameters in experimental groups exposed to Lyc-LDH.

	CG	VG	LG10	LG25	LG50
RR (breaths/ min)	174.0 ± 14.40	172.2 ± 21.43	170.8 ± 21.74	158.8 ± 10.13	170.8 ± 11.03
VT (mL)	0.31 ± 0.07	0.28 ± 0.04	0.34 ± 0.10	0.35 ± 0.08	0.31 ± 0.04
MV (mL/ min)	53.01 ± 6.42	47.38 ± 4.10	57.21 ± 8.21	55.64 ± 11.93	53.39 ± 7.34
Body mass (g)	22.23 ± 0.60	22.11 ± 0.68	21.94 ± 0.84	22.57 ± 0.50	22.18 ± 0.82

CG: control group; VG: vehicle group; LG10: 10 mg/kg of Lyc-LDH group; LG25: 25 mg/kg of Lyc-LDH group; LG50: 50 mg/kg of Lyc-LDH group; RR: respiratory rate; VT: tidal volume; MV: minute ventilation. The data were expressed as mean ± standard deviation. Analysis of Variance One Way ANOVA followed by Tukey's post-test, n = 5 animals per group ($p < 0.05$).

Table 2
Assessment of hematological parameters of experimental groups exposed to Lyc-LDH.

	CG	VG	LG10	LG25	LG50
Erythrocytes ($\times 10^6/mm^3$)	7.32 ± 0.46	7.61 ± 0.29	7.39 ± 0.36	7.32 ± 0.41	7.37 ± 0.31
Hemoglobin (g/ dL)	14.26 ± 0.60	14.14 ± 0.71	14.01 ± 0.39	13.83 ± 0.44	13.8 ± 0.53
Hematocrit (%)	32.31 ± 2.09	32.49 ± 2.09	30.61 ± 2.83	30.15 ± 3.82	28.56 ± 1.92 [a,b]
Platelets ($\times 10^3/mm^3$)	321.3 ± 67.05	286.5 ± 42.27	279.6 ± 27.22	283.7 ± 33.48	290.5 ± 28.82
Total Leukocytes ($\times 10^3/mm^3$)	1.54 ± 0.97	1.61 ± 0.58	1.87 ± 0.68	1.61 ± 0.50	1.67 ± 0.33
Monocytes ($\times 10^3/mm^3$)	0.12 ± 0.11	0.18 ± 0.11	0.11 ± 0.07	0.15 ± 0.05	0.26 ± 0.18 [a,c]
Lymphocytes ($\times 10^3/mm^3$)	1.29 ± 0.83	1.19 ± 0.51	1.43 ± 0.55	1.29 ± 0.46	1.12 ± 0.33
Neutrophils ($\times 10^3/mm^3$)	0.13 ± 0.07	0.17 ± 0.08	0.25 ± 0.18	0.17 ± 0.09	0.24 ± 0.19

CG: control group; VG: vehicle group; LG10: 10 mg/kg of Lyc-LDH group; LG25: 25 mg/kg of Lyc-LDH group and LG50: 50 mg/kg of Lyc-LDH group. (a) represents a significant difference compared with CG. (b) represents a significant difference compared with VG. (c) represents a significant difference compared with LG10. The data were expressed as mean ± standard deviation. Analysis of Variance One Way ANOVA followed by Tukey's post-test, n = 10 animals per group ($p < 0.05$).

times, we observed a decrease in cell viability only after 24 h of incubation using higher concentrations of nanoparticles (8, 10 and 25 μM). The results suggest that Lyc-LDH did not exert cytotoxic effects, at the concentrations used, which is different from the results observed for lycopene. Previously, our research group evaluated the cytotoxic effect of pure lycopene using the same cellular model, revealing that lycopene at high concentrations decreased the cell viability at all time points [13]. Although the antioxidant activity of Lyc-LDH by DPPH method was low, we evaluated the production of intracellular reactive oxygen species in J774A.1 cells. The analysis was performed at different times, and at all analyzed times, Lyc-LDH did not induce ROS production. On the other hand, LPS from gram-negative bacteria is capable of exerting toxic effects by activating macrophages and promoting increased production of reactive species, which is associated with down-regulation of antioxidant enzymes [40]. Our results corroborate previous findings of our research group that demonstrated increased production of ROS [25], however, in the cellular model LPS, it seems to be metabolized by cells and its toxic effects are observed in the short term [25]. In this study, when we associated LPS and Lyc-LDH, the production of ROS was lower when compared to LPS, especially at the highest concentration used and the antioxidant effect was observed at all times, including 18 h, hence suggesting that nanoparticles are not toxic and exerts an antioxidant effect.

A significant part of the *in vivo* studies performed the administration of lycopene orally, however, just a few studies investigated the effects of administration by alternative routes, such as intranasal [18]. This carotenoid previously exhibited antioxidant and anti-inflammatory activities in a murine model of exposed to cigarette smoke [12,13,38]. In

Table 4
Analysis of antioxidant defense and oxidative stress biomarkers in lung samples.

	CG	VG	LG10	LG25	LG50
SOD (U/mg protein)	42.5 ± 9.98	40.23 ± 15.4	37.59 ± 6.44	33.66 ± 9.83	28.55 ± 7.74 [a]
CAT (U/mg protein)	0.18 ± 0.01	0.25 ± 0.20	0.19 ± 0.09	0.39 ± 0.21	0.41 ± 0.23 [a]
Protein carbonyl (nmol/ mg protein)	22.13 ± 5.95	20.22 ± 5.58	18.01 ± 5.49	24.48 ± 8.86	40.13 ± 14.11 [a,b,c,d]
GSH/GSSG ratio	0.83 (0.72; 1.13)	0.76 (0.56; 1.00)	0.76 (0.51; 1.17)	0.8 (0.69; 1.10)	1.07 (0.86; 1.24)

CG: control group; VG: vehicle group; LG10: 10 mg/kg of Lyc-LDH group; LG25: 25 mg/kg of Lyc-LDH group; LG50: 50 mg/kg of Lyc-LDH group; SOD: superoxide dismutase enzyme; CAT: catalase enzyme. catalase; GSH: glutathione sulfide; GSSG: oxidized glutathione. (a) represents a significant difference compared with CG. (b) represents a significant difference compared with VG. (c) represents a significant difference compared with LG10. (d) represents a significant difference compared with LG25. SOD, CAT and protein carbonyl were expressed as mean ± standard deviation. Analysis of Variance One Way ANOVA followed by Tukey's post-test, n = 10 animals per group ($p < 0.05$). GSH/GSSG ratio were expressed in median and interval between quartiles (25th and 75th percentile) This data was analyzed by Kruskal-Wallis followed by the Dunn's post-test, n = 10 animals per group ($p < 0.05$).

Table 3
Profile of inflammatory cell in bronchoalveolar lavage after Lyc-LDH administration.

	CG	VG	LG10	LG25	LG50
Leukocytes ($\times 10^5$/mL)	4.00 ± 0.86	3.85 ± 0.73	3.5 ± 0.95	7.15 ± 2.41 [a,b,c]	8.62 ± 2.86 [a,b,c]
Macrophages (x10^5/mL)	3.53 ± 0.54	3.67 ± 0.78	3.19 ± 1.04	5.26 ± 1.28 [a,b,c]	5.39 ± 1.12 [a,b,c]
Lymphocytes ($\times 10^5$/mL)	0.02 (0.00–0.06)	0.06 (0.03–0.08)	0.32 (0.27–0.59)	0.85 (0.74–1.50) [a,b]	1.30 (1.03–2.4) [a,b]
Neutrophils ($\times 10^5$/mL)	0.00	0.00	0.08 (0.04–0.22)	1.51 (1.25–2.10) [a,b]	2.61 (1.79–3.07) [a,b,c]
Eosinophils ($\times 10^5$/mL)	0.00	0.00	0.00	0.00 (0.00–0.03)	0.02 (0.00–0,10) [a,b,c]

CG: control group; VG: vehicle group; LG10: 10 mg/kg of Lyc-LDH group; LG25: 25 mg/kg of Lyc-LDH group; LG50: 50 mg/kg of Lyc-LDH group. (a) represents a significant difference compared with CG. (b) represents a significant difference compared with VG. (c) represents a significant difference compared with LG10. Leukocytes and macrophages were expressed as mean ± standard deviation. Analysis of Variance One Way ANOVA followed by Tukey's post-test, n = 10 animals per group ($p < 0.05$). Lymphocytes, neutrophils and eosinophils were expressed in median and interval between quartiles (25th and 75th percentile). Data was analyzed by Kruskal-Wallis followed by the Dunn's post-test, n = 10 animals per group ($p < 0.05$).

Table 5
The inflammatory markers in experimental groups exposed to Lyc-LDH.

	CG	VG	LG10	LG25	LG50
TNF-α (pg/mL)	332.4 ± 25.04	358.8 ± 67.81	344.4 ± 47.53	348.6 ± 69.34	305.9 ± 23.81
IL-6 (pg/mL)	616.9 ± 75.45	644.1 ± 101.9	760.8 ± 74.08	749.4 ± 172.8	1093 ± 59.08$^{(a,b,c,d)}$
IL-13 (pg/mL)	334.4 ± 27.89	339.0 ± 17.35	377.6 ± 27.12	410.3 ± 61.67$^{(a,b)}$	482.7 ± 27.61$^{(a,b,c,d)}$

CG: control group; VG: vehicle group; LG10: 10 mg/kg of Lyc-LDH group; LG25: 25 mg/kg of Lyc-LDH group; LG50: 50 mg/kg of Lyc-LDH group; TNF-α: Tumor necrosis factor α. IL-6: interleukin 6; IL-13: interleukin 13. (a) represents a significant difference compared with CG. (b) represents a significant difference compared with VG. (c) represents a significant difference compared with LG10. (d) represents a significant difference compared with LG25. The data were expressed as mean ± standard deviation. Analysis of Variance One Way ANOVA followed by Tukey's post-test, n = 7–8 animals per group ($p < 0.05$).

addition, previous studies demonstrated that oral administration of lycopene reduced nephrotoxicity in mice by activating the Nrf2/HO-1 pathway [41], and prevented mycotoxin-induced acute toxic effects on hematological and reproductive parameters [42]. A recently published study investigated the action of lycopene on allergic rhinitis in mice with comparative administration of carotenoid via gavage versus intranasal drip [18]. They demonstrated that the suppression of nasal symptoms evidenced a local response after nasal administration, but significant systemic effects were observed only in the group that received lycopene administration at the highest doses by gavage [18]. This work is one of the first studies to examine the acute changes caused by intranasal administration of a lycopene nanoformulation in mice.

First, we evaluated the effects of Lyc-LDH on ventilatory parameters and body mass. The carotenoid protection against obesity is well documented through its ability to regulate signaling pathways in different tissues [43]. In this study, the treatment with the nanoparticles did not affect the final weight of the animals. Similar results have been previously demonstrated with the weight of the animals remaining unchanged, with no hypertrophy or catabolism in muscle and bone tissue observed in female mice that received the carotenoid [44]. There was no alteration in the ventilatory parameters on the experimental groups that received the nanoparticles in our experiment. These results agree with a previous study in humans, which demonstrated that daily doses of lycopene did not affect lung function after physical activity in young athletes [45].

Hematological parameters were also evaluated, and the LG50 showed lower hematocrit and increased peripheral blood monocyte counts. Lycopene is known to have a protective effect on hematological parameters at various concentrations after oral administration [42,46–48]. In this study, the increase in the monocyte count in peripheral blood suggests that 50 mg/kg of Lyc-LDH evokes an innate immune response probably by a reaction with the chemical composition or structure.

Previous studies using nanoparticles of similar composition not loaded with lycopene showed no cytotoxic effects on red blood cells [49]. However, inflammatory mediators can inhibit erythroid progenitors in the bone marrow of mice, impacting the production of mature red blood cells [50]. In the present study, the administration of the highest doses of nanoparticles may have caused cellular toxicity, which activated the immune response, and consequently, in inflammatory mediators, thus justifying the decrease in hematocrit observed. Although layered double hydroxide nanoparticles are considered to be of low toxicity when compared to other nanoparticles [51], there is an immunological relevance that must be considered before applying such a compound, given its ability to trigger an immune response.

There are techniques that have improved the stability and dispersity of nanoparticles. However, nanoparticles can promote inflammation due to their tendency to aggregate in biological environments [52]. Consequently, it initiates a systemic response from the release of

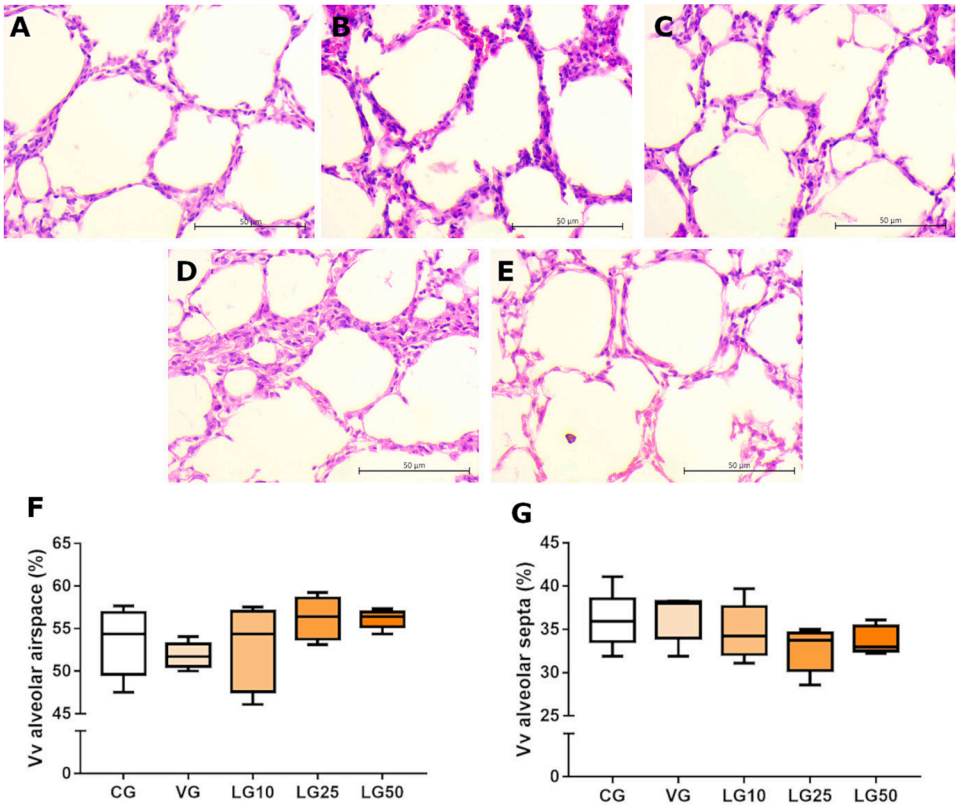

Fig. 4. Stereological analyzes of the lung parenchyma. (A) Control group (CG); (B) Vehicle group (VG); (C) 10 mg/kg of Lyc-LDH group (LG10); (D) 25 mg/kg of Lyc-LDH group (LG25), (E) 50 mg/kg of Lyc-LDH group (LG50), (F) Volume density of alveolar septa; (G) Volume density of alveolar airspace. The data presented in F and G were analyzed by Kruskal-Wallis followed by Dunn's post-test, and are expressed in median, minimum and maximum value. n = 5 animals per group ($p < 0.05$).

cytokines, triggering the inflammatory process, typically involving the generation of reactive oxygen species (ROS) [53]. The inflammatory process attracts the tissue defense cells to carry out phagocytoses, such as macrophages and neutrophils. We demonstrated that intranasal administration of Lyc-LDH at the highest concentrations (25 and 50 mg/kg) promoted an influx of inflammatory cells into the airways, specifically lymphocytes, neutrophils, and macrophages compared to controls. In addition, 50 mg/kg Lyc-LDH promoted greater recruitment of eosinophils to the airways. Eosinophils are key immune effector and inflammatory cells with diverse functions. These cells remain quiescent in the blood after exposure to pro-inflammatory mediators such as IL-13, which become partially active and can migrate to the site of inflammation. The activation of this cell is to the release of proteins regarding lung remodeling, reduced lung function, and increased risk of disease exacerbation such as chronic obstructive pulmonary disease [54]. The increase of inflammatory cells in the airways suggests that nanoparticles in high concentrations exert a toxic effect on the lungs by promoting inflammatory response. This is also reflected by the increase in the levels of inflammatory interleukins, such as IL-6 and IL13. The IL-13 is one of the main mediators in pulmonary pathophysiology, which is directly related in allergic asthma through its effects on monocytes and B lymphocytes [55]. Similarly, IL-6 is an important interleukin in the development of asthma [56]. These findings support the hypothesis that nanoparticles in high concentration triggered a pulmonary allergic process in the animals.

Natural products such as quercetin, curcumin, and lycopene are used for the treatment of various inflammatory diseases due to their bioactive compounds that exert pharmacological and antioxidant functions [57]. Lycopene is a potent antioxidant with *in vivo* and *in vitro* properties against protein, lipid and DNA oxidation [58]. One study demonstrated that lycopene pretreatment in human lymphocytes increased SOD and CAT activities after administration of ionizing radiation when compared to the control group [59]. Another study using type 2 diabetic rats showed that antioxidant activities (SOD, CAT, GSH) increased after lycopene administration, showing the role of this carotenoid against oxidative stress [60]. Protein oxidation generates the carbonyl group on the protein side chain and can occur through two main pathways: glutamine oxidation and α-amidation [61]. Protein oxidation was analyzed using the carbonyl protein marker [62]. The administration of Lyc-LDH in the highest concentration increased the carbonyl level of the protein. From this result, the Lyc-LDH composite can exert an effect opposite to that expected, causing oxidative stress in lung tissue when administered intranasally.

Catalase activity was higher in the LG50 compared to the control. This increase in CAT activity is due to the ability of Lyc-LDH to protect against oxidative stress by neutralizing superoxide radicals resulting from inflammation promoted by the intranasal route of administration. We can assume that administration with Lyc-LDH protects the lung tissue from the deleterious effects of hydroxyl radicals by increasing CAT levels. No differences in GSH/GSSG ratio were observed. As in previous work that evaluated the effects of lycopene on DNA damage in mice, we can also assume that CAT activity increased to compensate for the lower activity of the GSH-reductase enzyme [63]. SOD acts on superoxide radicals, reducing their intracellular levels. The reduction of SOD activity may be related to its role in neutralizing the excess of free radicals generated by the administration of Lyc-LDH.

In order to analyze changes in the lung parenchyma, we used stereological analysis. Although the highest concentrations of Lyc-LDH (25 and 50 mg/kg) administered intranasally promoted a local inflammatory reaction, influx of macrophages, neutrophils and eosinophils did not promote injury and remodeling in the lung tissue. Our results corroborate previous studies that demonstrated alveolar structure without differences between the control group and the lycopene-treated group [64].

Lyc-LDH represents an innovative strategy to administer lycopene, however, further studies are still needed to understand how these nanoparticles behave before using it as an antioxidant agent. Moreover, new studies evaluating the LDH nanoparticles with *in vivo* and *in vitro* models are necessary to verify if it exhibits oxidative character. While *in vitro* results demonstrate that Lyc-LDH nanoparticles have an antioxidant capacity, *in vivo* assays indicated that intranasal administration might not be adequate for concentrations above 25 mg/kg since Lyc-LDH is recognized by the immune system triggering a possible allergic response. On the contrary, the lowest concentration (10 mg/kg), showed no inflammatory or oxidative effects, and this is a recommended upper limit concentration if further studies are conducted.

Initially, most studies administer lycopene orally, it is possible that nanoparticles aid oral administration even at high concentrations, as gastrointestinal metabolism would slowly release lycopene to the organism and possible effects would be observed in various organs including the lungs.

5. Conclusion

In conclusion, our results suggest that intranasal administration at high Lyc-LDH concentrations plays inflammatory effects in the lungs of healthy mice that received the compound for a short period of time. However, new studies with chronic exposure to the nanoparticles are necessary to understand the inflammatory and oxidative mechanisms when intranasal administration is used, specially to verify if low concentrations could provide a range for administration in a safety manner.

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CRediT authorship contribution statement

Iriane Marques de Carvalho: Formal analysis, Writing – original draft. **Ana Beatriz Farias de Souza:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Investigation, Writing – review & editing. **Thalles de Feitas Castro:** Formal analysis, Investigation. **Pedro Alves Machado-Júnior:** Formal analysis, Investigation. **Tatiana Prata Menezes:** Formal analysis. **Andreia da Silva Dias:** Formal analysis. **Laser Antônio Machado Oliveira:** Formal analysis, Investigation. **Katiane de Oliveira Pinto Coelho Nogueira:** Formal analysis, Investigation. **André Talvani:** Resources. **Silvia Dantas Cangussú:** Writing – review & editing. **Gregorio Guadalupe Carbajal Arízaga:** Conceptualization, Methodology, Formal analysis. **Frank Silva Bezerra:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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