# Toxicology Research

Accepted Manuscript






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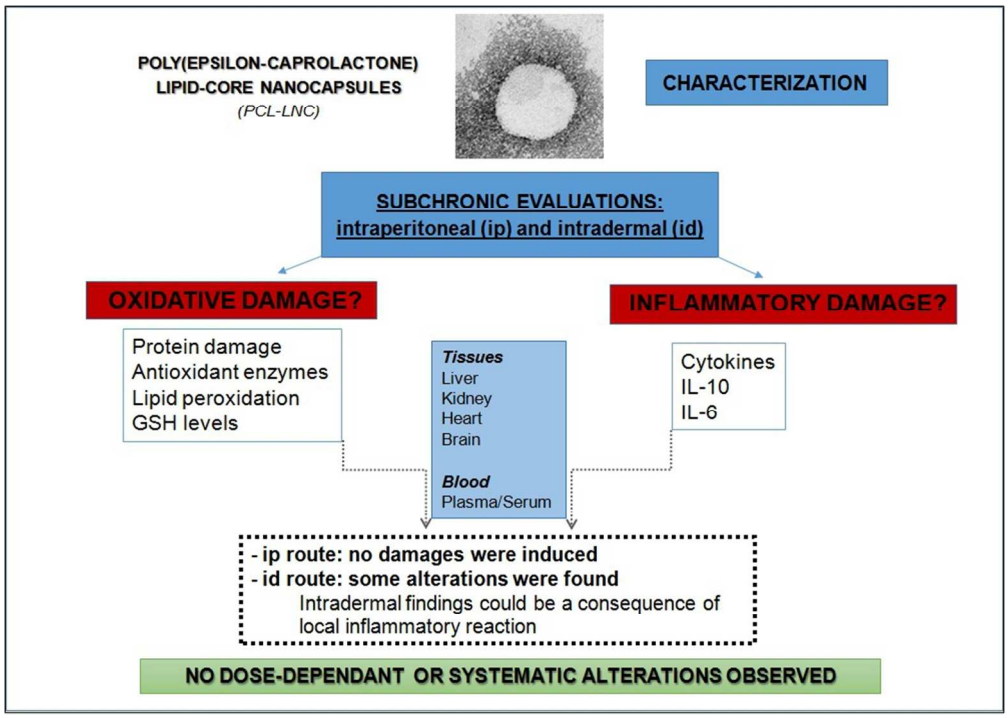


Graphical Abstract
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# DO POLY(EPSILON-CAPROLACTONE) LIPID-CORE NANOCAPSULES INDUCE OXIDATIVE OR INFLAMMATORY DAMAGES AFTER *IN VIVO* SUBCHRONIC TREATMENTS?

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Among toxicity mechanisms linked to nanoparticles (NPs), oxidative stress (OS) and inflammation are in general presumed to mediate their toxicological responses. In a previous toxicological screening, we evaluated if lipid-core nanocapsules (LNC) induced in vivo alterations, however no mechanisms of toxicity were determined. The present study aimed to investigate oxidative stress (OS) and inflammatory markers following poly(e-caprolactone) (PCL) LNC intradermal and intraperitoneal subchronic treatments. OS biomarkers and cytokines were analyzed in blood and/or tissue homogenates. We report that PCL-LNC did not induce lipid peroxidation in plasma, liver, kidney and cardiac tissues, except for the brain after id administration of the highest dose. In contrast, enhanced protein damage by carbonylation was found in the intermediate and highest ip doses and polysorbate 80 (PS80) group compared to saline group and also high protein nitrosylation in the highest id dose. In general, no important alterations were found in the activities of antioxidant enzymes SOD and CAT compared to controls. IL-10 levels were only decreased after the highest id dose and PS80-group compared to saline. Overall, the tested PCL-LNC, especially via ip, did not alter the oxidative status in a systematic repeated-dose approach, thus providing evidence for a safe use of these biodegradable PCL nanocapsules as systemic drug nanocarriers. However, intradermal results could be a consequence of local inflammatory reaction which resulted in modified oxidative status and inflammation, requiring further investigation or alternative routes of administration.

**Keywords:** biodegradable, LNC, oxidative status, inflammation, nanotoxicology, nanocapsule, drug delivery, intradermal, intraperitoneal

## 1. Introduction

Biomedical nanotechnology has been proposed for many interesting applications, but progress is hampered by a lack of understanding on how nanostructures interact with biological systems and the environment[1]. The potential inherent risks to any new technology should be investigated[2] and for that toxicological studies are critical to establish the full *in vivo* potential of nanotechnology, and more specifically nanomedicine[3].

Novel biodegradable polymer-based drug delivery systems have provided promising alternatives to improve therapeutic efficacy and pharmacokinetic parameters of several drugs[4]. As a result of its biocompatibility and biodegradability, poly(e-caprolactone) (PCL) has been extensively studied for controlled drug delivery and tissue engineering applications in several formulations[5]. Our research group has studied therapeutic applications of these nanocapsules, which have shown great potential as drug delivery systems for topical, oral or systemic applications[6,7,8,9,10,11,12]. They are promising nanocarriers and their toxicological profile is the subject of intensive research efforts in order to accelerate the LNC entrance into clinical trials.

The mechanisms of nanoparticles (NPs) toxicity is not completely clear, and it is possible that more than one mechanism is involved. Literature supports oxidative stress (OS) as an important mechanism involved[13]. Meanwhile, it remains to be established if the same mechanisms induced by NPs *in vitro* are also induced *in vivo*. There are many *in vitro* studies being conducted which suggest that NPs induce toxic responses through mechanisms such as particle breakdown and the subsequent release of toxic metals and the production of reactive oxygen species (ROS)[1]. On the other hand, it is still necessary to use animal models, which are the preferred systems to perform the toxicological evaluation of novel agents, to characterize their toxicities as well as to investigate potentially involved mechanisms.

Intracellularly, NPs may interact with cellular components, disrupt or alter cell function, or create ROS. Their interactions with mitochondria and cell nuclei are being considered as key sources of toxicity[14]. ROS and reactive nitrogen species (RNS) overproduction lead to cell injury through several mechanisms, including direct damage to DNA, lipid peroxidation (LPO) generation of vasoactive and proinflammatory molecules, and protein oxidation (primarily in sulfhydryl groups) leading to altered protein activity[15]. It has been suggested that NPs, because of their small sizes, could also act as haptens to modify protein structures, either by altering their function or rendering them antigenic, thus raising their potential for autoimmune effects[16].

Inflammation is controlled by a complex series of intracellular and extracellular events[17]. Altogether, according to Nel et al. (2006), specific biological and mechanistic pathways can be elucidated by *in vitro* studies under controlled conditions which, in conjunction with *in vivo* studies, could reveal a link between the mechanism of injury and the pathophysiological outcome in the target organ[18].

There are few studies about the potential *in vivo* toxicity of polymeric biodegradable nanocapsules or polymeric nanomaterials and the assessment of inflammatory or oxidative injuries. Although studies are conflicting regarding the magnitude and mechanisms of NPs toxicity, it is evident that some nanomaterials such as PCL, which were previously considered biocompatible due to the safety of the bulk material, may in fact be toxic[14]. In addition, degradability of the material is known to influence acute and long-term toxicity, once non-degradable nanomaterials can accumulate in organs and cells, generating detrimental cellular effects, similar to those of lysosomal storage diseases[19]; biodegradable nanomaterials may lead to unpredicted toxicity due to the formation of unexpected toxic degradation products[20].

Previously, we reported a systematic toxicological evaluation of the same poly(e-caprolactone) (PCL) lipid-core nanocapsules formulation in an animal model[21,22] by investigating markers of hemato-, hepato- and nephrotoxicity after the single and repeated doses, and no evidence of toxic effects was observed. However, some reports show that ROS overproduction and OS occur as an early event, leading to DNA and protein damages, lipid peroxidation, and also exacerbating the inflammatory response[23]. Therefore, it is essential to evaluate these adverse biological responses *in vivo* in addition to screening for toxic effects of NPs. To the best of our knowledge, possible effects involving mechanisms of OS and inflammation of LNC in animals have not yet been explored. The present study aimed to systematically investigate whether ip and id repeated-dose administrations of biodegradable PCL-LNC were able to modify oxidative status or induce inflammation in rats.

## 2. Materials and methods

*2a.* Preparation and characterization of lipid-core nanocapsules

Lipid-core nanocapsules were prepared by interfacial deposition[24]. At 40°C, PCL (0.25 g), capric/caprylic triglycerides (0.41 ml), and sorbitan monostearate (0.096 g) were dissolved in acetone (67 ml). In a separate flask, polysorbate 80 (0.193 g) was added to 132 ml of water. The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, acetone was eliminated and the suspensions were concentrated under reduced pressure. The final volume was adjusted to 25 ml and three different batches were prepared. Formulations of lipid-core nanocapsules were fully characterized regarding their physicochemical properties, whose results have been previously published[21,22]. Briefly, the physicochemical characterization was conducted by determining following parameters: mean

particle size, polydispersity index (PDI), zeta potential and pH, all of which evaluated as previously described[24]. Particle size, polydispersity index and zeta potential of the formulation were determined using a zetasizer (Nano-ZS ZEN 3600 model®, Malvern, UK). The samples were diluted without previous treatment in ultrapure water (MilliQ®) (particle size) or in 10 $mmol \cdot L^{-1}$ NaCl aqueous solution (zeta potential). Additionally, the surface area and the number of particles were calculated based on the mean particle diameter, showing values of 0.869 ± $0.07 \times 10^4 .cm^2 .ml^{-1}$ and 6.01 ± 0.24 x $10^{12}$ particles $cm^{-3}$, respectively, according to Jäger et al. (2009)[25]. Dynamic light scattering and electrophoretic mobility was used to analyze the nanometric population and determine the zeta potential, respectively. The pH value of the formulation was directly determined without sample treatment using a potentiometer (B474, Micronal S.A., Brazil). In addition, morphology and structure of the resulting LNC were observed with transmission electron microscope (TEM). Samples were diluted and deposited on specimen grid (Formvar-Carbon support film, Electron Microscopy Sciences). Subsequently, they were negatively stained with uranyl acetate solution (2% w/v). The analyses were carried out using a transmission electron microscope (TEM; JEM 1200 Exll, Japan) operating at 80 kV.

*2b.* Animals

All experiments were conducted using male adult Wistar rats (250–350 g) obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), located in Porto Alegre, Brazil. Animals were maintained at 22±2.8ºC under a 12/12 h light/dark cycle, receiving standard food and water *ad libitum*. The animals were looked after in accordance with the ‘‘Guiding Principles in the Care and uses of Animals’’[26] and the study was approved by the local ethics committee (Nr UP4482/10).

*2c.* Experimental protocol

Animals were randomly distributed into five groups (N=6 rats/group): control groups receiving saline solution 0.9%, or polysorbate 80 (PS80, 0.78%) and three groups receiving LNC formulations (low, intermediate or high doses). PS80, a nonionic surfactant, was used as control due to its presence to stabilize LNC in the formulation. Animals were submitted to subchronic (multiple doses) treatments by intraperitoneal and intradermal administration. For the intraperitoneal treatment, control group received saline solution (3ml/kg) or PS80 (3ml/kg), and the three other groups received poly(ε-caprolactone) nanocapsules (LNC) daily, during 28 days, being $6.01 \times 10^{12}$ (Group I), $12.02 \times 10^{12}$ (Group II) or $18.03 \times 10^{12}$ LNC particles $ml.kg.day^{-1}$ (Group III). For intradermal treatment, the rats were injected with no more than 0.5 ml in each paw, control groups received saline solution (0.9ml/kg) or PS80 (0.9ml/kg) and the doses of LNC were $1.8 \times 10^{12}$ (Group I), $3.6 \times 10^{12}$ (Group II), or $5.4 \times 10^{12}$ LNC particles $ml.kg^{-1}$ (Group III). After treatments, animals were euthanized, blood and tissue were collected for oxidative stress biomarkers, and cytokines quantification.

*2d.* Blood collection

After treatments, rats were sacrificed with an overdose of ketamine and xylazine anesthesia. Blood samples were collected from the vena cava and transferred to tubes (BD Vacutainer®) containing EDTA or heparin as anticoagulants and tubes without anticoagulants. Blood collected in EDTA tubes were centrifuged at 1500g for 10 min and the supernatant plasma was removed with care to avoid contamination with platelets. Plasma was obtained by centrifugation at 2000 g

for 10 min (hemolyzed plasma was discarded). Serum, plasma and total blood with heparin were stored in microcentrifuge tubes at -80ºC until analysis.

*2e.* Tissue preparation

Tissues (liver, kidney, cardiac and brain) were quickly removed and washed with saline solution to remove blood from tissues, then tissues were weighed, placed on ice, and homogenized in cold Tris-HCl (0.1 M; pH 7.4) for ALA-D, in cold PBS buffer (pH 7.4) for malondialdehyde (MDA) levels, SOD and CAT activities quantification. The homogenates were centrifuged at 3000g for 10 min to yield the low-speed supernatant fraction that was used for oxidative stress biomarker analyses.

*2f.* Oxidative status evaluation

*Lipid peroxidation (LPO)*

Lipid peroxidation (LPO) was determined by the measurement of malondialdehyde (MDA) by high performance liquid chromatography with visible detector (HPLC-VIS), as described by Grotto et al., (2007)[27] to plasma samples. For tissue homogenate, a volume was hydrolyzed by NaOH at 60°C for 30 min, followed by a deproteinization step; before injection into the chromatograph (WellChrom model, Knauer, Germany), the samples were extracted with n-butanol, according to Ribeiro et al., (2011)[28]. MDA levels are expressed as µmol/l.

*Protein oxidation*

Protein carbonyl levels (PCO): The formation of carbonyl groups was used as a parameter for oxidative damage to proteins. The protein carbonyl levels were determined using a

noncompetitive ELISA method according to Buss et al., (1997)[29] with some modifications. Total protein concentration in the serum was measured by Bradford method using bovine serum albumin as standard. Before measurement, serum samples were diluted with PBS to a normalized concentration of 4 mg protein/ml. Afterwards, the samples were derivatized with 2,4-dinitro-phenylhydrazine (DNPH) and incubated in Maxisorb multiwall plates (Nunc, Life Technologies, Germany) overnight at 4 ºC in the dark. Protein carbonyls were detected using a dinitrophenyl rabbit IgG-antiserum (Sigma, Deisenhofen, Germany) as the primary antibody and a monoclonal anti-rabbit immunoglobulin G peroxidase conjugate (Sigma) as the secondary antibody. Color development was performed with o-phenylenediamine and $H_2O_2$ and the reaction was stopped with $H_2SO_4$ after 15 min incubation at 37 ºC. The absorbance was measured using a microplate reader with a detection wavelength of 492 nm. Each sample was analyzed in triplicate. Serum protein carbonyl concentration was expressed as nmol carbonyl/mg protein.

Protein nitration: The levels of 3-Nitrotyrosine (3-NT) were determined using a noncompetitive ELISA method according to Weber et al., (2012)[30]. Total protein concentration in the plasma was measured by Bradford method using bovine serum albumin as standard. Before measurement, serum samples were diluted with PBS to concentration of 1 mg protein/ml. Afterwards, the samples were placed on the multiwall plates (Nunc Immuno 96 Microwell™ Maxisorp) with PBS. Each sample was analyzed in triplicate. The plate was incubated in the dark overnight on a shaker at 4 ºC. In the next day the solution was removed, the blocking solution was added and the plate was incubated for 2 h in the dark at room temperature. The plate was washed with 0.05% (v/v) Tween 20 in PBS, a polyclonal anti-nitrotyrosine primary antibody (Millipore) was added to wells and incubated for 2 h at 37 ºC. Then, a secondary antibody goat

anti-rabbit-IgG, HRP-conjugate (Millipore) was added and incubated for 1 h in dark at room temperature. Color was developed by adding o-phenylenediamine and $H_2O_2$ and the reaction was stopped with $H_2SO_4$ after 15 min. Absorbance was measured in a microplate reader at 492 nm. Serum 3-Nitrotyrosine concentration was expressed as pmol/mg protein

*Endogenous Antioxidants*

Quantification of reduced glutathione (GSH) in erythrocytes: The levels of GSH in red blood cells (RBC) were measured by high performance liquid chromatography (HPLC) with UV detection (WellChrom model, Knauer, Germany), described by Garcia et al., (2008)[31].

CAT activity: Catalase (CAT) activity was measured in 96-well microplates according to Aebi, (1984)[32], which is based on the decomposition of $H_2O_2$ by catalase. Enzymatic activity was evaluated by monitoring the rate of decrease in hydrogen peroxide ($H_2O_2$) absorbance in a microplate reader (SpectraMax M2e, Molecular Devices, USA) at 240 nm during 5 min with readings every 20s at 37ºC. CAT activity was expressed as CAT Units/mg protein.

SOD activity: Superoxide dismutase (SOD) activity was measured by the spectrophotometric method described by McCord and Fridovich, (1969)[33] in 96-well microplates, which is based on the inhibition of superoxide-dependent adrenaline auto-oxidation in a microplate reader (SpectraMax M2e, Molecular Devices, USA) at 480 nm during 15 min with reading every 20s at 32ºC. One unit of activity was defined as the amount of protein necessary to decrease the reference rate to 50% of maximum inhibition. SOD activity was expressed as SOD Units/mg protein.

ALA-D activity: δ-Aminolevulinate dehydratase (ALA-D) activity was assayed according to Sassa, (1982)[34], including some modifications. Blood and tissue homogenates enzyme activity was determined by measuring the rate of porphobilinogen (PBG) formation. The reaction was started by addition of the substrate (ALA) and then an incubation was carried out at 37 °C for 1h. Afterwards, the product PBG was determined using modified Ehrlich's reagent and absorbance was monitored at 555 nm, considering a molar absorption coefficient of 6.1 x $10^4$ $M^{-1}$ for the Ehrlich-PBG salt. δ-ALA-D activity are expressed as units/l.

Protein: The amount of protein in the assays of MDA, ALA-D, CAT, and SOD was assayed using the Lowry technique (Lowry et al., 1951)[35]. Total protein in the assays of PCO and 3-NT was assayed using Bradford method as cited earlier.

*2g.* Inflammatory markers

*Determination of cytokine levels in serum*

The blood samples were centrifuged at 1300g at 4°C for 10 min. The supernatant was rapidly frozen and stored at -80°C for later measurement of interleukin (IL)-6 and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits, according to the recommendations of the supplier (R&D Systems®).

*2h.*Statistics

Statistical analyses were performed by using SPSS 18.0 for Windows and GraphPad Software (San Diego, CA, USA). Results were expressed as mean ± standard deviation (SD) or standard

error of the mean (SEM). The statistical significance has been determined using one-way analysis of variance (ANOVA) followed by post-hoc test for multiple comparisons (Tukey's HSD test). Correlation tests were performed following the distribution of the variables. Associations between all predictors, and categorized variables were investigated using multiple linear regression analyses. The level of significance was considered $p<0.05$.

## 3. Results

*3a*. Lipid-core nanocapsules characterization

The characterization of lipid-core nanocapsules was briefly demonstrated in Table 1 as well as their spherical morphology (Fig. 1). The present physicochemical characteristics of the LNC batches are similar to those prepared for our previous toxicological study[21,22]. LNC showed monomodal size distributions and polydispersity indexes indicating narrow size distributions.

Table 1.

Characterization of lipid-core nanocapsules

| | |
|---|---|
| Polymer | Poly(ε-caprolactone) |
| Particle size | 245 ± 10 nm |
| Polydispersity index | 0.11 ± 0.02 |
| Zeta potential | -7.5 ± 0.8 mV |
| pH | 6.5 ± 0.2 |
| Superficial area | $0.869 \pm 0.07 \times 10^4 .cm^2 .ml^{-1}$ |
| Shape | Spherical |
| Number of particles per unit of volume *N* | $6.01 \pm 0.24 \times 10^{12}$ particles $cm^{-3}$ |

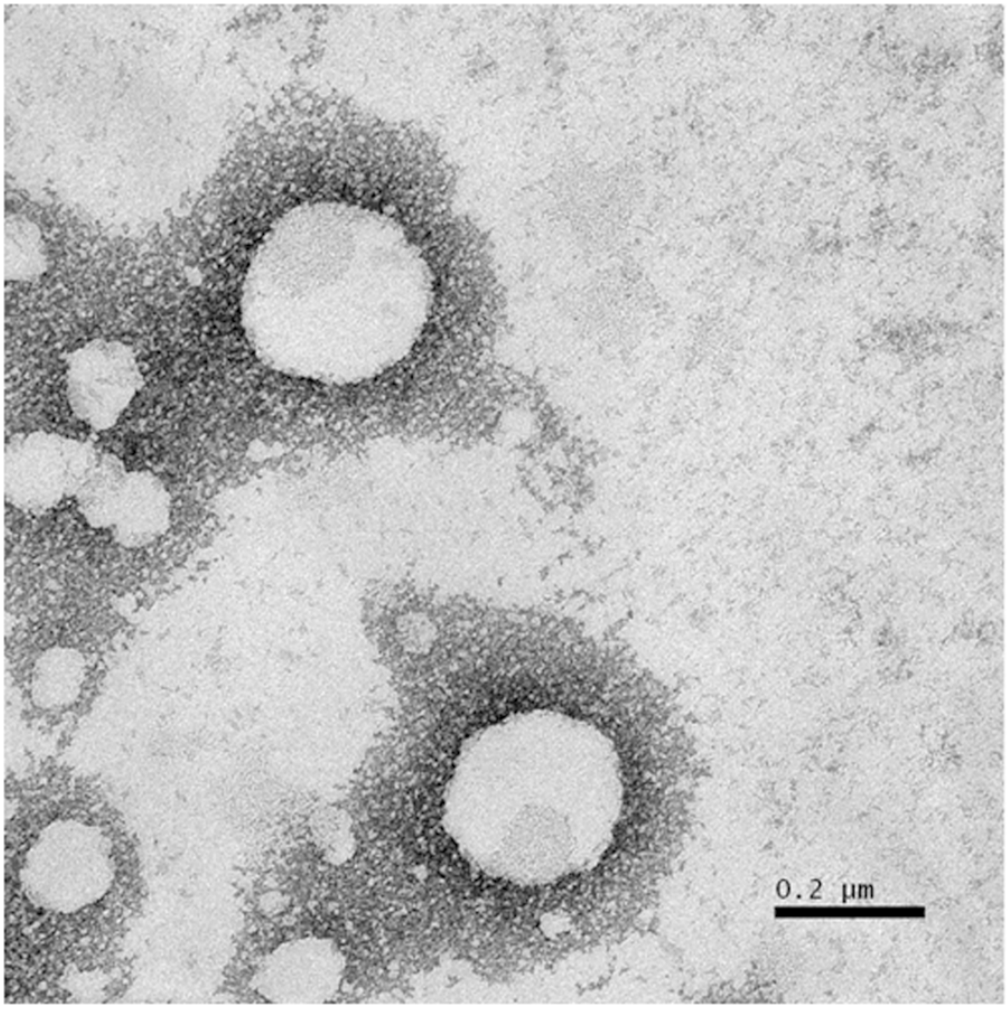


Fig. 1. Transmission electron microscope of LNC (bar = 200 nm).

*3b*. Biomarkers of oxidative stress and inflammation

Biomarkers of oxidative stress

Data from blood oxidative stress parameters after ip and id subchronic treatments are shown in Fig. 2 and Fig. 3, respectively.

Fig. 2 shows the results after ip treatment, in which the levels of plasmatic MDA were significantly reduced in Group II and III compared to saline control group ($p$<0.05). On the other hand, PCO levels were higher in LNC groups II and III compared to control ($p$<0.05) and similar to those found in the PS80 group. There are no statistical difference for enzymatic activities (SOD and CAT) as well as for GSH levels and ALA-D activities (data not shown).

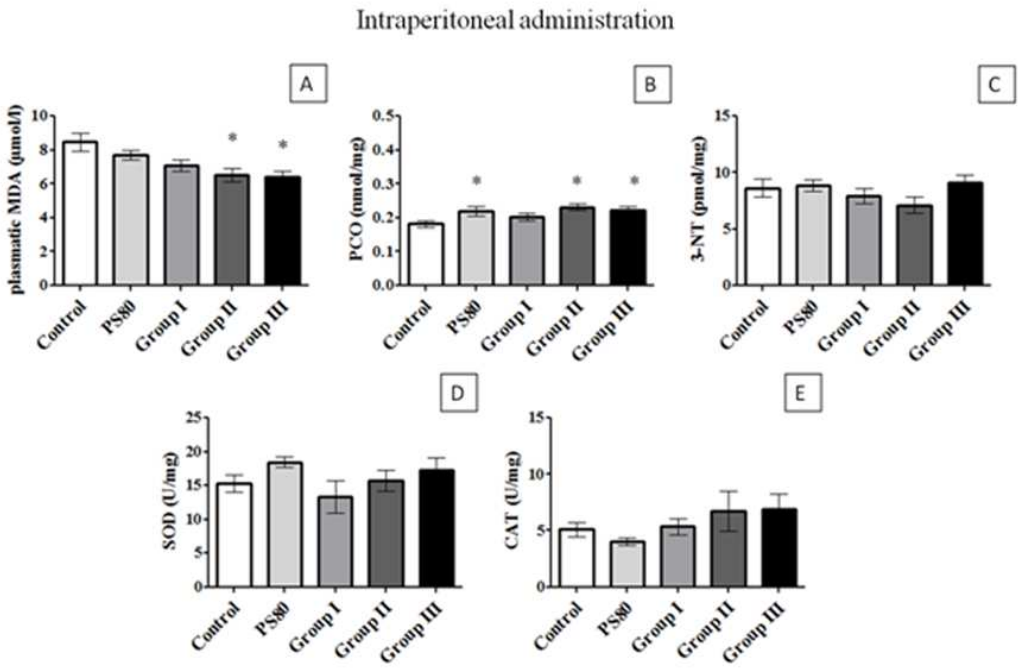


Fig. 2. Blood oxidative stress biomarkers after ip administration. Six rats per group received the following: Control – saline solution (3 ml/kg) or PS80 – polysorbate 0.78% (3 ml/kg); Group I – LNC ($6.01 \times 10^{12}$ LNC/kg); Group II – LNC ($12.02 \times 10^{12}$ LNC/kg); Group III – LNC ($18.03 \times 10^{12}$ LNC/kg) for 28 days. Abbreviations: MDA – malondialdehyde levels; PCO – protein carbonyl levels; 3-NT - 3-Nitrotyrosine levels; SOD – superoxide dismutase activity; CAT –

catalase activity. Values are expressed as mean ± SEM. Data were analyzed by ANOVA, followed by post-hoc comparisons (Tukey's test). *Significantly different from control ($p$<0.05).

After id treatment, protein damage biomarkers PCO and 3-NT were different among groups as demonstrated in Fig. 3. PCO levels were increased in PS80 compared to control ($p$<0.05), and reduced in LNC groups compared to PS80 ($p$<0.05). In addition, 3-NT levels along with SOD activity were statistically higher only in Group III compared to controls (saline or PS80), $p$<0.05.

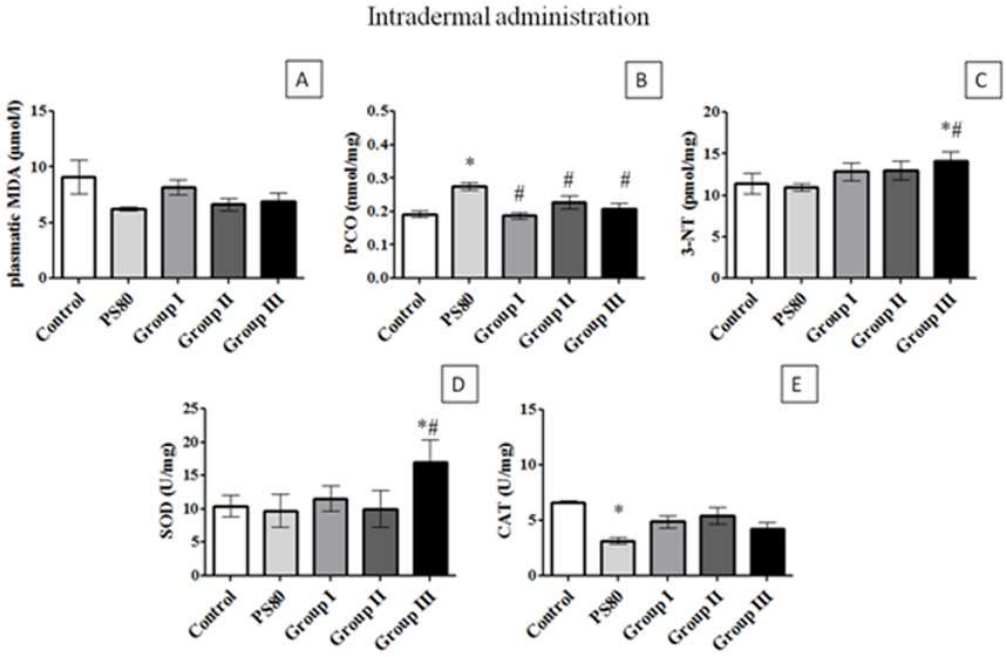


Fig. 3. Blood oxidative stress biomarkers after id administration. Six rats per group received the following: Control – saline solution (0.9 ml/kg) or PS80 – polysorbate 0.78% (0.9 ml/kg); Group I – LNC ($1.8 \times 10^{12}$ LNC/kg); Group II – LNC ($3.6 \times 10^{12}$ LNC/kg); Group III – LNC ($5.4 \times 10^{12}$ LNC/kg) for 28 days. Abbreviations: MDA – malondialdehyde levels; PCO – protein carbonyl levels; 3-NT - 3-Nitrotyrosine levels; SOD – superoxide dismutase activity; CAT – catalase activity. Values are expressed as mean ± SEM. Data were analyzed by ANOVA, followed by post-hoc comparisons (Tukey's test). *Significantly different from control ($p$<0.05); #Significantly different from PS80 ($p$<0.05).

As shown in Fig. 4, hepatic MDA levels were significantly decreased in all LNC-treated groups ($p$<0.05) after ip treatment. Subsequent analysis of CAT activity also demonstrated significant decreases in these groups compared to control and PS80 group ($p$<0.05). Liver ALA-D activity was similar among groups and did not show statistical differences, $p$>0.05 (data not shown). Intradermal injection did not affect hepatic markers of oxidative stress. Lipoperoxidation markers and antioxidant enzymes were similar among groups (Fig. 4D, 4E, 4F)

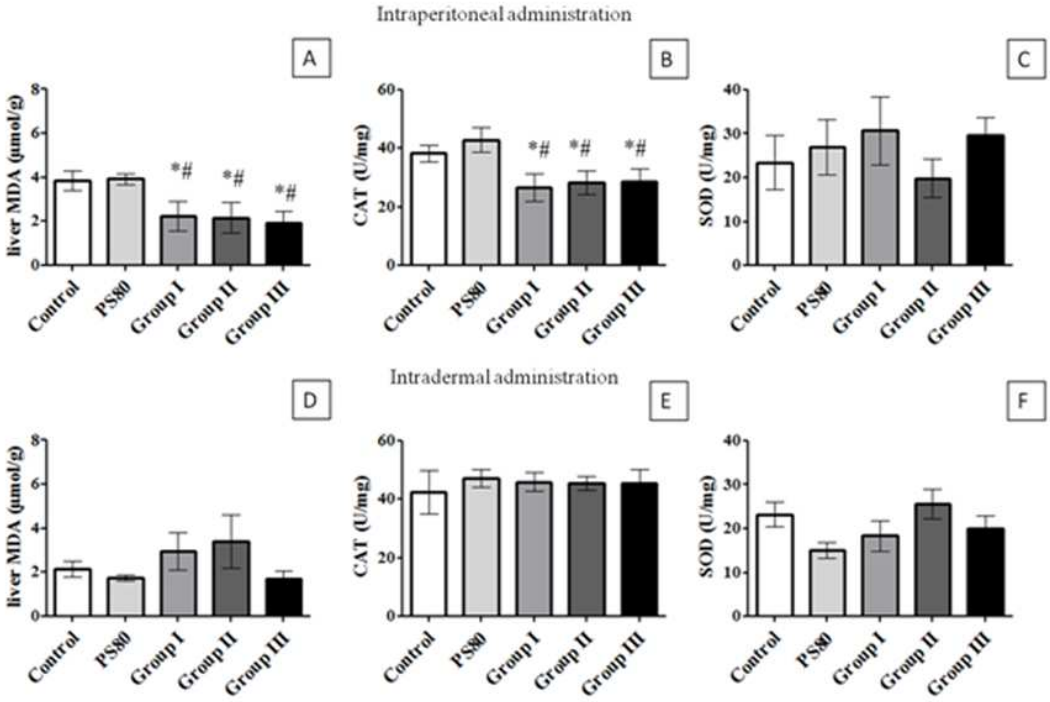


Fig. 4. Hepatic oxidative stress biomarkers after ip and id administration. Five rats per group received by ip route (A, B, C) the following: Control – saline solution (3 ml/kg) or PS80 – polysorbate 0.78% (3 ml/kg); Group I – LNC ($6.01 \times 10^{12}$ LNC/kg); Group II – LNC ($12.02 \times 10^{12}$ LNC/kg); Group III – LNC ($18.03 \times 10^{12}$ LNC/kg) and by id route (D, E, F): Control – saline solution (0.9 ml/kg) or PS80 – polysorbate 0.78% (0.9 ml/kg); Group I – LNC ($1.8 \times 10^{12}$ LNC/kg); Group II – LNC ($3.6 \times 10^{12}$ LNC/kg); Group III – LNC ($5.4 \times 10^{12}$ LNC/kg) during 28 days. Abbreviations: MDA – malondialdehyde levels; SOD – superoxide dismutase activity; CAT – catalase activity. Values are expressed as mean ± SEM. Data were analyzed by ANOVA,

followed by post-hoc comparisons (Tukey's test). *Significantly different from control ($p$<0.05); #Significantly different from PS80 ($p$<0.05).

Different from hepatic analyses, markers of oxidative stress measured in kidney were similar among groups after ip administration ($p$>0.05). Nevertheless, after id administration, PS80 group presented higher activities of SOD and CAT compared to saline group ($p$<0.05). SOD activity was also higher in Group III after the same treatment (Fig. 5E, 5F). Kidney ALA-D activity was also similar among groups and did not show statistical differences, $p$>0.05 (data not shown).

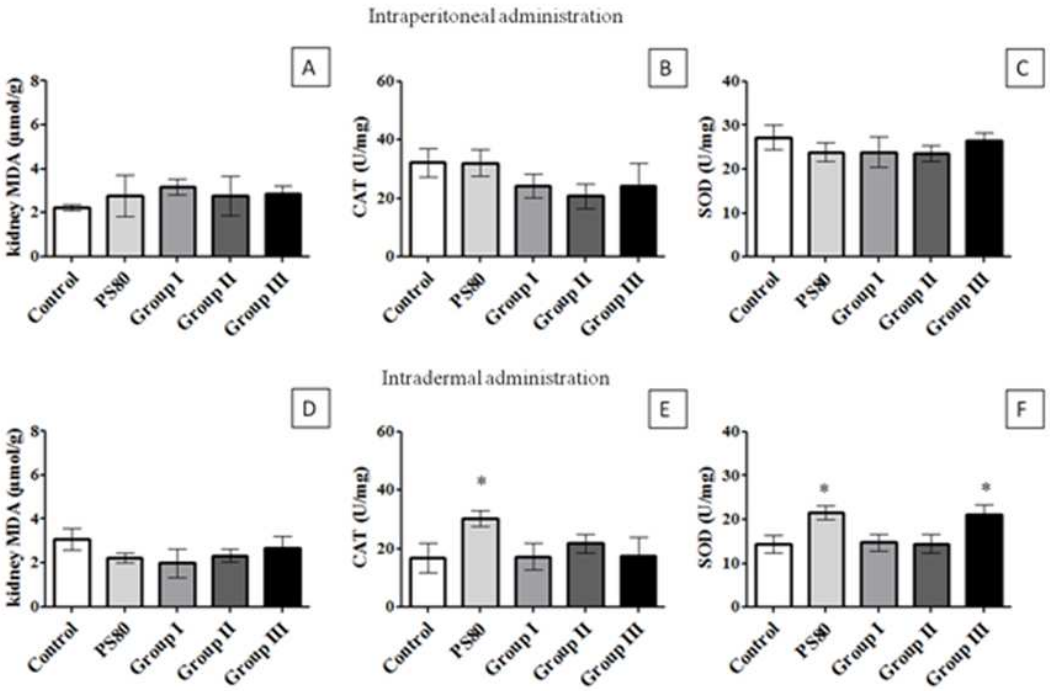


Fig. 5. Kidney oxidative stress biomarkers after ip and id administration. Five rats per group received by ip route (A, B, C) the following: Control – saline solution (3 ml/kg) or PS80 – polysorbate 0.78% (3 ml/kg); Group I – LNC ($6.01 \times 10^{12}$ LNC/kg); Group II – LNC ($12.02 \times 10^{12}$ LNC/kg); Group III – LNC ($18.03 \times 10^{12}$ LNC/kg) and by id route (D, E, F): Control –

saline solution (0.9 ml/kg) or PS80 – polysorbate 0.78% (0.9 ml/kg); Group I – LNC ($1.8 \times 10^{12}$ LNC/kg); Group II – LNC ($3.6 \times 10^{12}$ LNC/kg); Group III – LNC ($5.4 \times 10^{12}$ LNC/kg) during 28 days. Abbreviations: MDA – malondialdehyde levels; SOD – superoxide dismutase activity; CAT – catalase activity. Values are expressed as mean ± SEM. Data were analyzed by ANOVA, followed by post-hoc comparisons (Tukey's test).*Significantly different from control ($p$<0.05).

Analyses of cardiac tissue lysates are demonstrated in Fig. 6. No significant changes were observed after ip treatment in MDA levels and SOD activity, only in CAT activity which was significantly decreased in Groups II and III compared to other groups ($p$<0.05). After repeated id injections, the LPO biomarker and antioxidant enzymes activities in PS80 and LNC groups were found to be within similar ranges to saline.

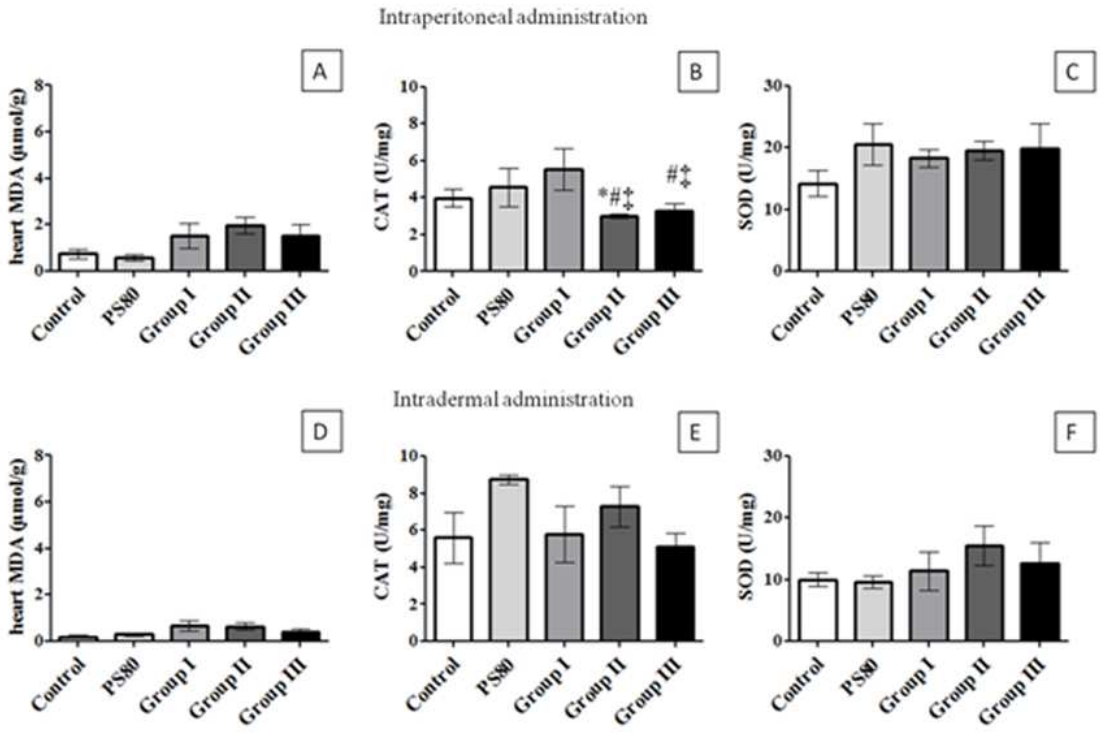


Fig. 6. Cardiac tissue lysate oxidative stress biomarkers after ip and id administration. Five rats per group received by ip (A, B, C) the following: Control – saline solution (3 ml/kg) or PS80 –

polysorbate 0.78% (3 ml/kg); Group I – LNC ($6.01 \times 10^{12}$ LNC/kg); Group II – LNC ($12.02 \times 10^{12}$ LNC/kg); Group III – LNC ($18.03 \times 10^{12}$ LNC/kg) and by id route (D, E, F): Control – saline solution (0.9 ml/kg) or PS80 – polysorbate 0.78% (0.9 ml/kg); Group I – LNC ($1.8 \times 10^{12}$ LNC/kg); Group II – LNC ($3.6 \times 10^{12}$ LNC/kg); Group III – LNC ($5.4 \times 10^{12}$ LNC/kg) during 28 days. Abbreviations: MDA – malondialdehyde levels; SOD – superoxide dismutase activity; CAT – catalase activity. Values are expressed as mean ± SEM. Data were analyzed by ANOVA, followed by post-hoc comparisons (Tukey's test). *Significantly different from control ($p<0.05$); #Significantly different from PS80 ($p<0.05$); †Significantly different from Group III ($p<0.05$) and ‡Significantly different from group I ($p<0.05$).

Fig. 7 depicts brain tissue MDA levels and CAT and SOD activities. MDA levels were increased only after id treatment, in PS80 and Group III (Fig. 7D), while CAT activity did not change after both treatments ($p>0.05$). SOD activity was increased only after ip treatment, in Groups I and II compared to PS80 ($p<0.05$). PS80 presented a decreased enzymatic activity (Fig. 7C) compared to control ($p<0.05$).

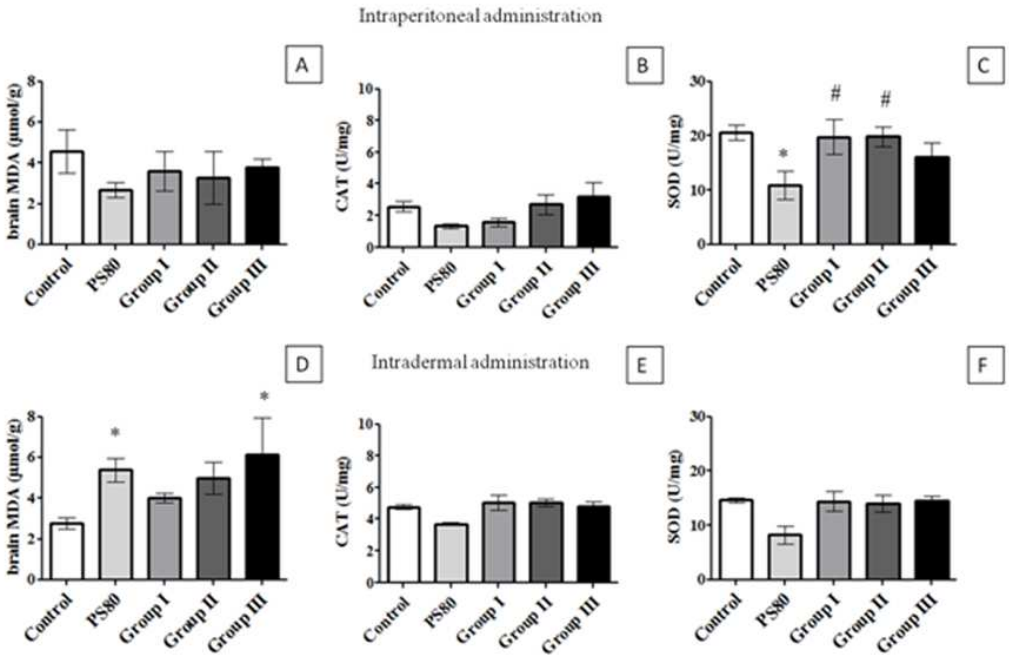

Fig. 7. Brain oxidative stress biomarkers after ip and id administration. Five rats per group received by ip (A, B, C) the following: Control – saline solution (3 ml/kg) or PS80 – polysorbate 0.78% (3 ml/kg); Group I – LNC ($6.01 \times 10^{12}$ LNC/kg); Group II – LNC ($12.02 \times 10^{12}$ LNC/kg); Group III – LNC ($18.03 \times 10^{12}$ LNC/kg) and by id route (D, E, F): Control – saline solution (0.9 ml/kg) or PS80 – polysorbate 0.78% (0.9 ml/kg); Group I – LNC ($1.8 \times 10^{12}$ LNC/kg); Group II – LNC ($3.6 \times 10^{12}$ LNC/kg); Group III – LNC ($5.4 \times 10^{12}$ LNC/kg) during 28 days. Abbreviations: MDA – malondialdehyde levels; SOD – superoxide dismutase activity; CAT – catalase activity. Values are expressed as mean ± SEM. Data were analyzed by ANOVA, followed by post-hoc comparisons (Tukey's test). *Significantly different from control ($p<0.05$); #Significantly different from PS80 ($p<0.05$).

*3c*. Markers of inflammation

To determine whether LNC elicits an acute immunological response, the secretions of pro-inflammatory cytokine IL-6 together with anti-inflammatory cytokine IL-10 were analyzed. Rats were treated as described and analyses were performed 24 hours after the last day of treatment. After ip treatment, no statistical differences among groups were observed for both pro-inflammatory and anti-inflammatory cytokines, as noted in Fig. 8 A and B. After id injection, the expression of anti-inflammatory cytokines in Groups I and II was found to be within similar production ranges compared to saline while from PS80 and Group III were significantly decreased compared to control ($p<0.05$); IL-6 serum levels were similar among groups as depicted in Fig. 8.

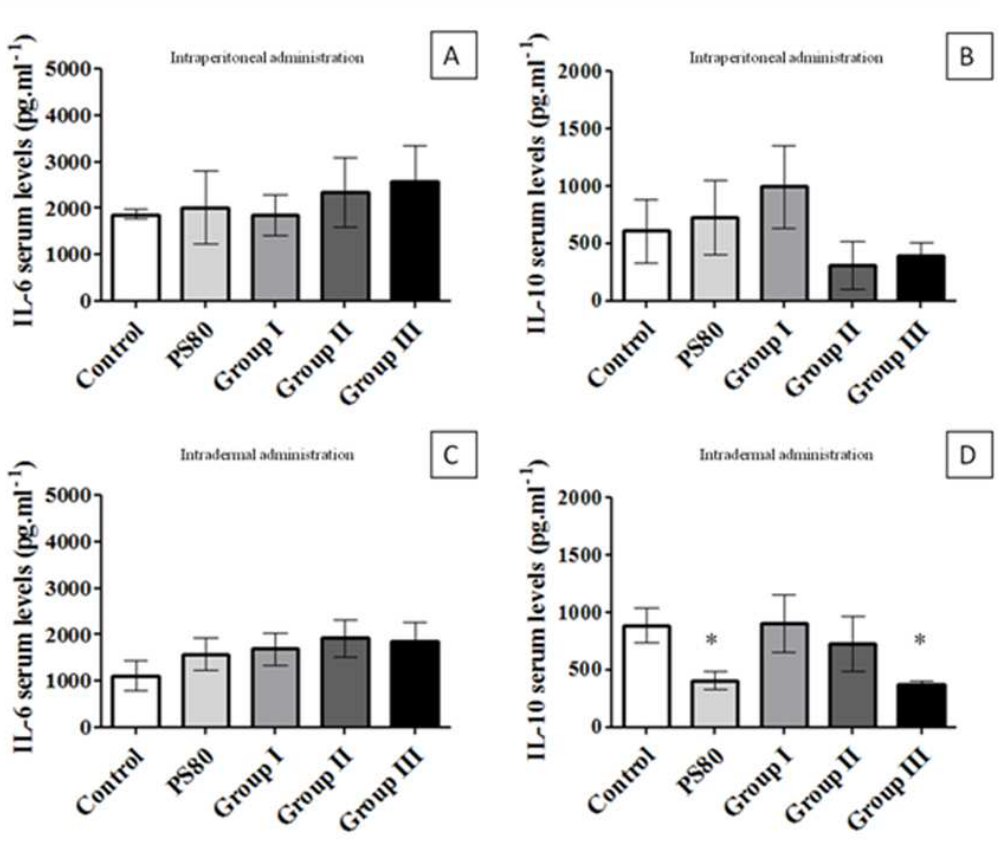


Fig. 8. Cytokine production profile in serum after 24 hours of the last (after 28th) day of ip (A and B) and id (C and D) administration of nanoparticles. Values are expressed as mean ± SD, n=4. Data were analyzed by ANOVA, followed by post-hoc comparisons (Tukey's test).* Significantly different from control ($p$<0.05).

*3d*. Correlation among biomarkers

Oxidants/inflammation markers versus antioxidant/anti-inflammatory markers correlation coefficients of rats treated by the intraperitoneal route are demonstrated in Table 2. In relation to the intradermal treatment, few correlations between biomarkers were found. Among the id associations, liver MDA was positively correlated to liver SOD (r= 0.5, $p$<0.05) whereas 3-NT, a biomarker of protein damage, was negatively correlated to the anti-inflammatory cytokine (r= -0.6, $p$<0.05).

Table 2.

Correlation coefficients among biomarkers (oxidant or inflammatory marker versus antioxidant or anti-inflammatory markers) after repeated intraperitoneal administration.

| | Blood SOD | Liver SOD | Kidney SOD | Cardiac SOD | Brain SOD | Blood CAT | Liver CAT | Kidney CAT | Cardiac CAT | Brain CAT | ALA-D Activity | Reat % ALA-D | Blood GSH | Serum IL-10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plasma MDA | - | 0.30# | - | 0.40# | 0.40# | - | - | 0.56* | - | - | -0.40* | 0.99* | - | - |
| Liver MDA | - | - | - | - | - | - | 0.70* | 0.53# | - | - | - | - | - | - |
| Kidney MDA | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Cardiac MDA | - | - | | 0.50# | - | - | - | - | - | - | - | - | -0.48# | - |
| Brain MDA | - | - | - | - | 0.60* | - | - | - | - | - | -0.80* | - | - | - |
| Plasma PCO | 0.42* | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Plasma 3-NT | - | - | - | - | 0.36# | - | - | - | - | - | - | - | 0.30# | - |
| Serum IL-6 | - | | 0.50* | - | - | - | - | - | - | - | - | - | - | - |

* Significant correlation between variables, $p<0.05$; # $p=0.05$.

## 4. Discussion

The physicochemical and biochemical properties of NPs, e.g. surface properties, charge, size or the adsorption of biological components, are important factors mediating their interactions with cells, including cell-stress reactions and the biological characteristics of the particular cells[18,36]. Polymeric NPs may also be internalized by cells and their polymer components may be found in different cellular localizations. Cellular oxidative stress and the production of ROS upon cell exposure to NPs have been shown to be a common property of NPs, relevant to assess their potential negative effects on cell functions[37].

Several studies have been carried out to determine the influence of NPs on the oxidation of lipids and proteins or DNA damage[37,38,39,40]. However, as cited before, there are rare studies demonstrating the response of OS biomarkers after exposure to polymeric nanocapsules, especially *in vivo* studies. In this context, there is a current need for animal models based on the assessment of toxicity elicited by biodegradable polymeric nanoparticles. In the present study, inflammatory and oxidative stress biomarkers were analyzed in order to assess the mechanisms involved after administration of LNC in repeated doses and through different routes, as already demonstrated for other NPs.

Regarding plasmatic biomarkers, which are shown in Fig. 2A, we observed after ip administration a decrease in lipoperoxidation in LNC groups. Interestingly, the low MDA levels results are in agreement with a previous study from Külkamp et al., (2011)[41], who studied the extent of lipid peroxidation through *in vitro* TBARS measure of a formulation of lipoic acid–loaded LNC. Furthermore, protein carbonyls were increased in LNC groups compared to saline, but were similar to PS80 group, despite the decrease in lipid damage also observed, suggesting a partial antioxidant effect in relation to protein damage. This increase could be due to the

presence of polysorbate 80 in the LNC formulation. In addition, 3-NT, another protein damage biomarker which is released to the blood after proteins are degraded, was not changed, probably as a consequence of the short 3-nitrotyrosine plasma half-life of 1-2 hours or transitory levels due to protein degradation, repair or clearance[42,43].

Both antioxidant enzymes SOD and CAT were also increased in the same groups (Group II and III) (Fig. 2B, 2C, 2D and 2E). After analyzing oxidant versus antioxidant correlations, it was possible to observe an increase in PCO plasma levels with the increase of SOD activity (r=0.42; $p<0.05$), and after a linear regression analysis (b=0.142; $p<0.001$), this correlation was also revealed, confirming a counterbalance of SOD antioxidant enzyme (Table 2), and suggesting that protein carbonylation was influenced by an increase in hydrogen peroxides ($H_2O_2$), and that SOD activity was increased due to an endogenous compensatory mechanism to balance hydrogen peroxide levels. Differently from PCO results, after linear regression analyses, the influence of MDA levels by antioxidants was not confirmed by the model ($p>0.05$). Our results, however, do not demonstrate a systematic oxidative stress, once no increase in lipid peroxidation was found and only protein carbonyls increased in groups II and III, similarly to PS80, but no alterations in protein 3-nitrotyrosine levels were observed.

The antioxidant effect of the formulation with 10 mg/ml PCL was significantly higher than all others, and an increase in the polymer contents in the formulations significantly and progressively enhanced the antioxidant activity up to this concentration[41]. In addition, we also measured GSH levels, the main non-protein thiol species involved in antioxidant cellular defense against the toxic effects of ROS[31], which were similar among groups, as well as ALA-D, an enzyme of the heme-biosynthesis pathway, essential for all aerobic organisms, which has also been used as a marker of OS because it is highly sensitive to−SH oxidation by pro-oxidant

elements leading to reduced enzyme activity[44]. There is speculation involving the antioxidant capacity of these LNC, but studies to confirm such capacity are still required.

Liver, kidney, cardiac and brain tissues of all animals were investigated for three OS parameters, levels of the lipid peroxidation byproduct MDA, and activities of the antioxidant enzymes CAT and SOD. After ip treatment, tissue analyses of LPO biomarker demonstrated that only liver MDA levels were significantly decreased compared to both groups (saline solution and PS80), $p<0.05$. These results are consistent with the blood MDA levels, which were also reduced in these same groups. As observed in the blood correlation analyses, correlations of liver MDA levels to antioxidant biomarkers did not remain after linear regression analyses ($p>0.05$). Kidney, heart and brain presented similar MDA levels among groups to ip treatment.

Unfortunately, we did not measure protein damage levels in tissues, but plasmatic quantification provided us with an idea of the PCO and 3-NT balance in the organism. In addition, CAT activity was decreased in liver of LNC-treated groups. This decrease could be in order to counterbalance some injury. Cardiac tissue CAT activity was also reduced in Groups II and III. SOD activity was decreased in brain of PS80 group compared to saline, leading to higher SOD activities in LNC groups compared to PS80. After correlation analyses, a positive correlation was found between brain MDA levels and brain SOD activity, and inversely with ALA-D activity, as shown in Table 2; these correlations were confirmed after linear regression analysis (brain MDA vs. brain SOD b=0.7; $p<0.01$) and (brain MDA vs. ALA-D b=-0.84; $p<0.01$).

Fenández-Urrusuno et al., (1997)[39] studied the uptake of polymeric nanoparticles by Kupffer cells in the liver and demonstrated modifications in hepatocyte antioxidant systems, probably due to the production of ROS. Similarly to the present study, the authors state that the

depletion of antioxidants was not extensive enough to initiate hepatocyte damage, since no changes in lipid peroxidation and reversible alterations were observed.

Bulcão et al., (2013)[21] demonstrated that liver parameters including serum levels of albumine (ALB), total protein, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were not significantly different in any of the groups tested in the acute and subchronic toxicity studies. Kidney markers were also quantified, such as blood urea nitrogen (BUN), creatinine (Cr), uric acid (UA), and early kidney injury markers as microalbumin (MA-U) for glomerular dysfunction evaluation and N-acetyl-beta-D-glucosaminidase (NAG) activity for renal tubule dysfunction. All parameters tested were similar among groups, and the lack of tissue damage was corroborated by the absence of histopathological alterations and relative weight changes in both organs. Cardiac and brain tissues did not present histopathological alterations and relative weight changes in the same study; few laboratorial parameters were measured to evaluate alterations in heart and brain, only enzymes LDH, which is not specific for cardiac dysfunction and butyrylcholinesterase (BuChe), which has been found in many tissues, including brain; however, they did not indicate any alterations in all animals tested[21].

After id treatment, protein damage biomarkers were altered compared to control or PS80. PCO levels were increased in PS80 group when compared to control (Fig. 3). All rats that received LNC presented lower PCO levels compared to PS80; on the other hand, 3-NT were increased in Group III compared to both controls (saline and PS80), similarly to the SOD activity, which was high in the same group (Group III).

Despite the increased levels of protein damage biomarkers, it was not sufficient to irreversibly unbalance the oxidant/antioxidant system, once antioxidant enzymes were not depleted and were altered probably to counteract possible damage to cells.

Tissues from id treatments presented unaltered levels of the three OS biomarkers analyzed in any of the groups tested, except for kidney and brain which presented alterations in PS80 group with increased CAT and SOD activities in kidney and increased MDA levels in brain, $p<0.05$. Along with these alterations, rats from Group III, which received the highest concentration of PS80 in formulation, presented high levels of SOD activity in kidney and high levels of MDA in brain and the same results were found in the PS80 group for both organs. A previous study from our group indicated that among several biochemical parameters analyzed, the majority was unaltered in all tested groups, except for the PS80 group, which presented few alterations[22]. Moreover, as cited in the same study, it is also important to emphasize that PS80 is permitted for intradermal and intravenous injection and that the polysorbate coating in nanocapsules do not have exactly the same properties of PS80, used as control, justifying the use of LNC by id route.

It has been established that phagocytosis by macrophages is accompanied by an enhanced secretory activity. During phagocytosis a number of agents involved in the killing of pathogens (i.e. reactive oxygen intermediates (ROIs), reactive nitrogen intermediates, cytokines or enzymes) are secreted by macrophages[39]. Further, it is well known that, depending on the setting, the degradation of biodegradable materials may result in a localized inflammatory response. This inflammatory response is often the result of accumulated degradable byproducts that induce cellular OS[45]. Therefore the present study also focused on the

mechanisms of NPs on inflammation markers, analyzing the cellular response and focusing on the generation of OS.

Calarco et al. (2013)[40] suggested that ROS production by polyethylenimine NPs (PEI-PLGA) and acetylated polyethylenimine NPs (AcPEI-NP) treatment should be a cell autonomous phenomenon, since they use an *in vitro* cell system. Additionally, an *in vitro* study with different cell lines (RAW 264.7, A549, HepG2, A498 and Neuro 2A) and four polymeric nanocapsules prepared with four distinct polymers (including the PCL utilized in this study) showed no cytotoxicity up to 48h of treatment at all concentrations, however a decrease in cell viability was observed after 72h up to a concentration of 100 µg/mL of polymeric nanoparticles. Besides, ROS and TNF-α production were increased at high concentrations and IL-6 and reactive nitrogen species (RNS) were not affected. These data suggested that ROS and TNF-α production are more sensitive and can be useful to predict potential toxic/inflammatory effects of polymeric nanoparticles when compared to IL-6 and RNS[46].

Other investigators have also demonstrated that nanoparticles may elicit an inflammatory response that in turn contributes to intracellular ROS production[47]. Inflammatory cytokine (IL-6) and anti-inflamatory IL-10 results also demonstrated no significant differences among groups after ip treatment (Fig. 8A, 8B). Recently, we reported similar and reduced levels of high-sensitivity C-reactive protein (*hs*-CRP) and complement component C3 after ip administration[21], which are indicators of the inflammatory process. In another study, we also determined *hs*-CRP and C3, both with low levels in LNC-treated groups by id route, acute and repeated doses (unpublished data). However, in the current study, IL-10 levels were diminished in PS80 and Group III after id treatment (Fig. 8D), which is in accordance to our previous study, in which alterations on laboratorial parameters were due to the presence of PS80 (Bulcão et al. 2014).

After correlation analyses, an inverse correlation between 3-NT and IL-10 was found and confirmed after linear regression (b=-0.53; $p<0.001$); in this case, it is possible that increased 3-NT levels influenced a decreased of anti-inflammatory IL-10, indicating a strong relation between these mechanisms.

Further, an increased ROS production results in oxidative stress when cells fail to compensate the increased ROS and consequently fail to maintain or restore normal physiological redox-regulated functions, leading to toxicological outcomes, such as DNA damage and expression of inflammatory cytokines; however that was not observed in the present study since antioxidant defenses were slightly different comparing saline solution, polysorbate and LNC groups, thus preventing oxidative damage, e.g. lipoperoxidation, in all tissues and blood samples, except in the brain after id administration.

In summary, herein we evaluated for the first time the potential effect of LNC in oxidative biomarkers and inflammatory markers showing that there was no systematic induction of oxidative stress or inflammation, but a few isolated and not dose-dependent alterations in the animals, and often comparable to the effects of PS80. In addition, the observations of some oxidative and inflammatory changes in the id route require further studies, due to the association with inflammatory response by this route of administration and also by the use of PS80.

## Conclusions

The present results on lipoperoxidation, protein damage and inflammation indicated neither a systematic nor a dose-dependent alterations in the LNC-treated rats. Liver, kidney and cardiac tissues did not demonstrate oxidative unbalance following ip administration as well as inflammatory alterations. On the other hand, some blood or tissue alterations were observed after

id administration of LNC in relation to oxidative and inflammatory biomarkers, which may be related to the intrinsic oxidative unbalance triggered by administration through the intradermal route, and not properly due to the LNC exposure thus requiring additional studies for that specific purpose, in addition to the use of PS80 for this specific route of administration. Furthermore, these findings are in agreement with our earlier results of no significant toxicological responses after acute and subchronic studies, with the implement of demonstrating that no oxidative stress mechanisms are involved with the ip administration of LNC nanocapsules. In conclusion, we observed that LNC did not systematically induce oxidative damage or inflammation after ip administration, suggesting that LNC have potential safety and still remain as promising drug nanocarriers.

**Acknowledgements** This research was financially supported by FAPERGS (PRONEX-nr. 08/09; PRONEM, Edital Pesquisador Gaúcho (12/1925-5)). The authors thank the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) and Instituto de Cardiologia/Fundação Universitária de Cardiologia (IC/FUC). Garcia SC, Guterres SS and Pohlmann AR. are recipients of CNPq Research Fellowships.

**Declaration of interest** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper

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