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Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Toxicology Research

ARTICLE

Evaluation of potential acute cardiotoxicity of biodegradable nanocapsules in rats by intravenous administration

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The nanotoxicology aims to study the safety of nanomaterials, especially towards human exposures. Biodegradable polymeric nanocapsules have been indicated as potential drug carriers applicable for treating several pathologies. Thus, the objective of this study was to evaluate the potential cardiotoxicity of biodegradable lipid-core nanocapsules (LNC) containing poly(ε-caprolactone). Nanocapsules were characterized and the acute toxicity evaluation was conducted in Wistar rats. Two control groups (saline and tween/glycerol) were utilized, and three treated groups were chosen for low, intermediate and high doses: 28.7 x 10^{12} (LNC-1), 57.5 x 10^{12} (LNC-2) and 115 x 10^{12} (LNC-3), expressed as number of nanocapsules *per milliliter/Kg*. Blood pressure measures were performed in non-anesthetized animals by caudal plethysmography. The electrocardiographic (ECG) and echocardiographic analyses were carried out after anesthesia by isoflurane in two moments, previously to treatment and after 14 days. Blood was collected 24 hours and 14 days after treatment. Biochemical and histopathological analyses were performed. During the evaluation period, no deaths, weight loss or clinical signs were observed. Post-treatment systolic pressures (24h and 14 days) were significantly increased in comparison to pre-treatment in both control groups and treated groups, suggested as a possible consequence of the infused volume. Serum sodium, potassium, aspartate aminotransferase and alkaline phosphatase, as well as, hematological parameters were within reference values established for rats. ECG showed no indications of cardiotoxicity. Despite of the echocardiograms, no alterations in the ejection fraction were found as indicators of cardiotoxicity. Cardiac histopathology also demonstrated no alterations. Therefore, the present results on acute evaluation after i.v. administration, by slow infusion, showed potential safety once no cardiotoxic effects by ECG, echocardiographic, arterial pressure, biochemical and histopathological analyses were found.

Keywords: nanotoxicology, biodegradable polymeric nanocapsules, cardiotoxicity, ECG, echocardiography.

Introduction

Cardiotoxicity consists in events that leads to total or partial loss, with reversible or irreversible consequences in cardiac function that might progress to heart failure and cardiovascular death.[1, 2] From 1988 to 2008 cardiotoxicity was the main responsible for recalls of the pharmaceutical industry, putting at risk the public health and thus impairing financially the pharmaceutical industry.[3]

Biomedical nanotechnology is a promise to reduce the toxicity, since nanodelivery systems promote specific target for drugs, decrease of doses and number of administrations. This is especially helpful in cancer therapy, where new molecules developed with high technology, specificity and low solubility can be delivered and act directly in tumor cells.[4, 5] On the other hand; the potential risk promoted by the unknown interactions of nanoparticles (NPs) should be investigated.[6, 7]

NPs toxicity is related to the physicochemical characteristics of the particle such as size, shape, surface charge (zeta potential), solubility, surface modifications, release of ions, contamination, besides the ability of deposition and translocation to others sites.[6, 8] Moreover, it is known that the composition of NPs also plays an important role in the level of toxicity. Metal NPs have a tendency to bioaccumulation,[9-11] while carbonaceous NPs might induce an inflammatory response.[12] Toxicity may result from the metabolism of the components used in the composition of NPs, which can eventually generate ROS.[13] For this reason, studies evaluating the behavior of different kinds of NPs, such as polymeric, are needed.

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Currently, nineteen clinical trials with nanotechnological products are occurring in the world according to data of U.S. National Institutes of Health,[14] clearly demonstrating the interest of the pharmaceutical industry on this new technology. These trials mainly focused on respiratory systems, coronary stenosis, hormonal reposition, cancer and neurodegenerative disease.

Although the use of NCs is promising, there is a need for toxicological safety assessment. Some of the biomedical NPs developed to date have showed a dose-dependent toxicological response, generally causing more harmful effects at high doses.[15, 16] According to the document FDA-2010-D-0530,[17] the FDA considers that the current methodologies to ensure the safety of chemicals are sufficient to classify the safety of nanomaterials, however, it emphasizes that the application of nanotechnology can result in different attributes from those of conventionally manufactured products, requiring new or modified methodologies.

Due to their advantages and physicochemical characteristics, polymeric lipid-core nanocapsules (NCs) have shown to be promising for drug delivery[18] and studies using these NCs have shown their ability to slow the release of encapsulated drugs, biocompatibility and biodegradability.[19, 20] *In vivo* studies with lipid-core nanocapsules of poly(ε-caprolactone) demonstrated non-toxic results in acute and subchronic toxicological tests by intraperitoneal and intradermal administration,[21, 22] requiring more specific investigations, such as the assessment of cardiotoxicity.

Regarding the route of administration in the development of toxicity it is noteworthy that oral, intradermal and intraperitoneal routes have a limited absorption by their nature. The LNCs absorption by the intraperitoneal route may take days and by oral can have large losses due to interaction with gastro-intestinal tract.[23] So, the intravenous route allows immediate availability of the NPs in the bloodstream at a known and controlled rate, being a good model for the assessment of acute and systemic toxicity.[24]

Nowadays there was an increase in nanotoxicology studies[25]. Classical cardiotoxicity of drugs is depending of number of administration, high doses, infusion rate, of multiple drugs, and kidney and liver preexisting disease.[1] In this line, it is important to investigate if polymeric NPs can interact and produce cardiotoxicity. Therefore, the aim of this study was to evaluate the acute cardiotoxicity of biodegradable lipid-core nanocapsules of poly(ε-caprolactone) in Wistar rats after IV administration.

Materials and methods

Chemical and reagents

Span 60® (sorbitan monoesterate), poly(ε-caprolactone) and glicerol were supplied by Sigma-Aldrich (Strasbourg, France), Caprylic/capric triglyceride (CCT) and Polysorbate 80 were obtained from Delaware (Porto Alegre, Brazil). All other solvents and chemical used were analytical grade.

Lipid-core nanocapsules preparation

Lipid-core NCs were prepared as previously described.[26] Briefly, an organic phase containing poly(Ɛ-caprolactone) (0.1 g), caprylic/capric triglyceride (0.16 g), sorbitan monostearate (0.038 g) was dissolved in acetone (27 mL) and stirred at 40°C until dissolution of all components. The organic phase was injected into an aqueous phase containing polysorbate 80 (0,078 mg) dispersed in ultrapure water (53 mL) using a funnel and magnetic stirred for 10 minutes. After, the acetone solvent and water excess were evaporated under reduced pressure rotatory evaporator at 40 °C, then 0.245 g of glycerol were added and the volume was completed to 10 mL/Kg.

Physicochemical characterization of the lipid-core nanocapsules

Particle size distribution, z-average, polydispersity index (PDI), zeta potential and pH were determined as previously described.[20] Z-average, polydispersity index and zeta potential of the formulation were determined using a Zetasizer®nano-ZS ZEN 3600 model (Malvern, UK). The samples were diluted (500x) without previous treatment in water (MilliQ®) (particle size) or in 10 mmol•L-1 NaCl aqueous solution (zeta potential). Mean particle size distribution and specific area were determined by laser diffraction (LD), analyzed by Mastersizer® 2000 (Malvern Instruments, UK). Diameters were expressed by the corresponding volume of the sphere D[4,3] and volume distribution diameter by the span value previously described were Span = d(0.9) – d (0.1)/d(0.5) and d(0.9), d(0.1) and d(0.5) diameter are 90%, 10% and 50% of the cumulative distribution of diameter, respectively.[26]

Surface area was obtained with relation of specific area and volumetric fraction of nanocapsules suspension. Particle number density was determined by turbidimetry according.[26] The suspension was analyzed using a Cary 50 UV-Vis spectrophotometer (Varian, USA) with wavelength (395 nm). The pH value of the formulation was directly determined without sample treatment using a potentiometer (Micronal B-474). All experiments were conducted with 3 batch for each sample.

Animals

The male Wistar rats weighing 305 ± 28 g and aged 6-8 weeks were conditioned in propylene cages, being 4 to 5 animals per cage. In order to reduce stress and mimic the natural habitat, the boxes contained 1 metallic igloo 18x9x19 cm[27, 28]. The temperature was controlled between 22 ± 2 °C, light / dark cycle of 12 hours (7 AM to 7 PM) and relative humidity around 60 %. All procedures were approved by the local Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA) register No.130279. The protocol used in the experimental design were based on Organization for Economic Co-Operation and Development (OECD)[24,29] and previous works from the group.[21,22] This study followed the recommendation of Canadian Council on Animal Care,[30] and Brazilian law 11.794/08.[31]

Determining dose

In our previous works to toxicological evaluation in acute treatment, the maximum dose was chosen from maximum volume per kg in accordance to the route of administration.[21,22] The doses, showed in Table 1, were determined from the maximum volume per Kg by i.v. administration, in acute treatment, according to Diehl

et al.[32] with modification. Because, following the flow rate 2 mL/hour the maximum volume per Kg did not cause death by acute lung edema was 10 mL/Kg. After that, it was determined a medium and a low volume, respectively 5 and 2.5 mL/Kg. The concentration was expressed (numbers of nanocapsules per mL/Kg and m^2/kg).

Experimental

The animals were anesthetized with isoflurane 2.5% at a 0.5 L/min constant O_2.[33] The tail vein was cannulated using a flexible 22G catheter and the infusion was performed using an infusion pump Infusomat® Compact B. Braun (Melsungen, Germany) with a flow rate of 2 mL/hour. The animals received intravenous 0.9% saline (saline group), 38 mg/dL Tween solution with glycerol (PS80 group). The biodegradable lipid-core nanocapsules (LNC1-3) were administrated in different volumes of infusion: 2.5 mL/kg (LNC1 group), 5 mL/kg (LNC2 group) and 10 mL/kg (LNC3 group). All animals received a final volume of 10 ml/kg which was completed with saline when necessary[32]. The experimental design of this study, represented in Figure 1, shows the moments that the biochemical analysis, echography, electrocardiogram (ECG) and pressure evaluations were performed.

Behavior, clinical signs and mortality

After single dose administration all animals were observed and the follow signals were noted: pain, piloerection, droopy eyelid, activity in cage, anxiety, tone, seizures, tremor, paralysis of limbs, eye color, tears, salivation, urination, defecation, diarrhea, respiratory rate and death. Animals were observed for 1 min at 10, 20, 30, 60, 120, 240, 360 min and 24 and 48 hours after acute treatment. After 24 hours, body temperature was also measured by inserting a digital thermometer into the rectum (1 cm) using lidocaine gel as local anesthetic.

Body and heart weight

The body weights were noted each 24 h during every experiment day. Fourteen days after the treatment, the rats were euthanized under anesthesia (isoflurane 80%, 0.5 L/min) and were also necropsied. Blood was drawn from the vena cava for hematology and laboratorial analyses with potassium EDTA and without anticoagulation, respectively. After euthanasia, the heart was removed, washed in cold saline and weighed. The relative heart weight was calculated as follows: relative organ weight = (organ weight/body weight x 100).[21]

Heart damage markers in blood

The measure of cardiac damage was assessed by the laboratory biomarkers troponin I, which was evaluated by chemiluminescence Centaur XP (Siemens Healthcare Diagnostics Inc, Tarrytown U.S.A.), sodium and potassium, determined by ion selective electrode ADVIA 1800 (Siemens Healthcare Diagnostics Inc, Tarrytown U.S.A.), aspartate transaminase (AST) assessed by kinetic UV ADVIA 1800 (Siemens Healthcare Diagnostics Inc, Tarrytown U.S.A.) and alkaline phosphatase evaluated by kinetic colorimetric ADVIA 1800 (Siemens Healthcare Diagnostics Inc, Tarrytown U.S.A.). The biochemical parameters were assessed in serum 24 hours and 14 days after the acute treatment.

Hematological analyses

The markers selected were red blood cell count (RBC), hemoglobin, hematocrit (PVC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), white blood cell count (WBC), granulocytes, lymphocytes and monocytes that were assessed using ABX Micros 60 (ABX Diagnostics, Montpellier, France) after 14 days of the acute treatment.

Histopathological examination

After euthanasia the heart and aorta were dissected out and fixed in 10% buffered formalin and embedded in paraffin. The slices were stained with hematoxylin and eosin stain (HE), Picro Sirius stain (PI) and Prussian blue on service of pathology of HCPA. To get better details of structures a polarized light microscope Zeiss Axioskop 40 was used (Carl Zeiss Microscopy, Thornwood, USA).

Echocardiographic assessment

The evaluation of cardiac remodeling by echocardiography was performed before the administration (basal) and after 13 days of acute treatment. It was assessed *in vivo* under anesthesia using an echocardiograph machine (EnVisor, Philips Systems - Andover, USA), with a transducer 12-3 MHz and depth of 2 cm. Images from left parasternal window (longitudinal and transverse) were taken. The linear measurements taken from images obtained by M-mode were: LV diameters at end-diastole (LVEDD) and end-systole (LVESD).[34] The ejection fraction (%) (LVEF) was calculated using the equation: $LVEDD^3$ - $LVSD^3$ / LVEDD3 x100. Shortening fraction (%) was estimated by the equation: (LVEDD - LVESD) / LVESD x 100.[35] The echocardiographic operator was blind to the groups.

Electrocardiogram (ECG)

The measurements were performed using the Biopac MP100 (Biopac Systems, Inc., Santa Barbara, USA) device with software for signal capture (AcqKnowledge 4.1 Biopac Systems, Inc., Holliston, USA), and later analysis of the measures by ADInstruments LabChart 7 for ECG software Adinstrument (Sydney, Australia). Gold-plated acupuncture needles were used to get the better electrical signals in previously described ECG points.[36] The ECG captures were performed prior the i.v. treatment (basal) and one day before the euthanasia (13 days). The acquisition time was 5 minutes. The QT-interval duration (QTc) was corrected by formula QT/(RR/100).[36]

Blood pressure assessment

Heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were determined by tail cuff plethysmography (Insight®, Ribeirão Preto, Brazil). Before the experiments all animals were acclimatized four times. Three measurements of blood pressure were made the day prior of acute treatment (basal), 24 hours and 14 days after. HR, SBP, and DBP were recorded by the device's software after each measurement.

Statistical analyses

The data were analyzed using SPSS (Statistical Package for the Social Sciences, version 18) and GraphPad Prism (GraphPad Software,

Table 1. Amount of LNC administrated by i.v. administration, acute treatment, following flow rate of 2 mL/hour.

	Saline	PS80	LNC1	LNC2	LNC3
Volume injected					
LNC Groups	-	-	2.5 mL/kg	5 mL/kg	10 mL/kg
Saline	10 mL/kg	-	7.5 mL/kg	5 mL/kg	-
PS80	-	10 mL/kg	-	-	-
Concentration of LNC injected					
LNC/kg	-	-	$28.7x10^{12}$	$57.5x10^{12}$	$115x10^{12}$
Surface area received					
m^2/kg	-	-	3.40	6.80	13.60

*Male Wistar rats weighting 305 ± 28 g.
**Amount of LNC per milliliter: 11.5 ± 0.42 x 10^{12}.
***Surface area per m^2/mL: 1.36 ± 0.01.

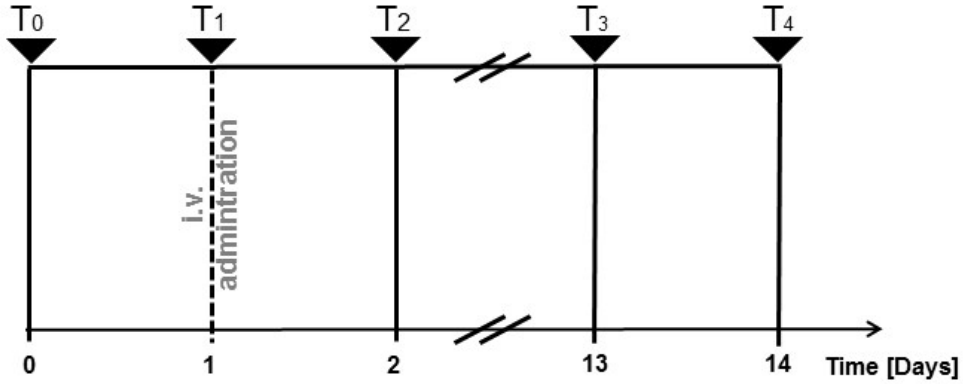

Figure 1. Experimental design for acute i.v. administration. Chronological graphic of experimental design of this study: Initially, the basal measures of echography, ECG and pressure were performed before intravenous administration, being the basal assessment (T_0). The i.v. administration, acute treatment, following 2mL/hour, was performed in the first day (T1). Vital signals were observed during 24h after the administration (between T_1 and T_2). At the end of 24 hours, the blood pressure was measured and then, the body temperature was checked after local rectal anesthesia. Additionally, the first blood sampling was collected, by orbital plexus, for biochemical analysis (T2). Blood pressure, ECG and echography were evaluated thirteen days after the acute treatment (T_3). Finally, the last blood sample for biochemical and hematological analysis was collected and euthanasia was performed, being heart removed, weighed and fixed to histopathology (T_4).

Inc.). Data are presented as mean ± standard error of the mean. For troponin I and hematology analysis it was used one-way ANOVA, followed by Tukey's post hoc test. To analyse the alterations of biochemical analysis, body and heart weight, blood pressure, echocardigram and ECG, the Generalized Estimating Equations (GEE) was used. Correlation tests were performed according to Pearson's or Spearman's rank following the variables distribution. Values of $p \leq 0.05$ were considered significant.

Results

Preparation and characterization of lipid-core nanocapsules

The nanocapsules formulations were prepared as previously reported[26] and the physicochemical characterization is briefly demonstrated (Table 2). After preparation, the z-average was 181.13 ± 2.8 nm. The suspensions showed monomodal size distributions and the SPAN was around 1.3 indicating narrow size distributions (Fig. 2). The zeta potential value was −7.8 ± 1.4 mV and the pH values were around 5.82 ± 0.2. The number of particles was 11.5 ± 4.21 x 10^{12} particles per cm^3. The LNC surface area was 0.869 ± 0.07 × 104 $cm^2.ml^{-1}$. The specific area was 45.66 m^2/g and pH was maintained at 5.82.

Observations of clinical and pathophysiological signs

No change was observed in clinical signs, as piloerection, salivation, tremors, seizures, ptosis, tearing, deaths, among others, as well as there was no alteration in body temperature after 24h of the treatment ($p>0.05$).

Body and heart weight

No change was observed in body weight and relative heart weight after the administration of the treatments as showed in Fig. 3 and Table 3, respectively.

Table 2 Physiochemical Characterization of Nanocapsules.

Characteristics	
d[4,3] (nm)	158.77 ± 1.53
SPAN	1.34 ± 0.01
Z- average (nm)	181.13 ± 2.83
PDI	0.09 ± 0.02
Zeta Potential mV	-7.84 ± 1.44
Surface area (m^2/mL)	1.36 ± 0.01

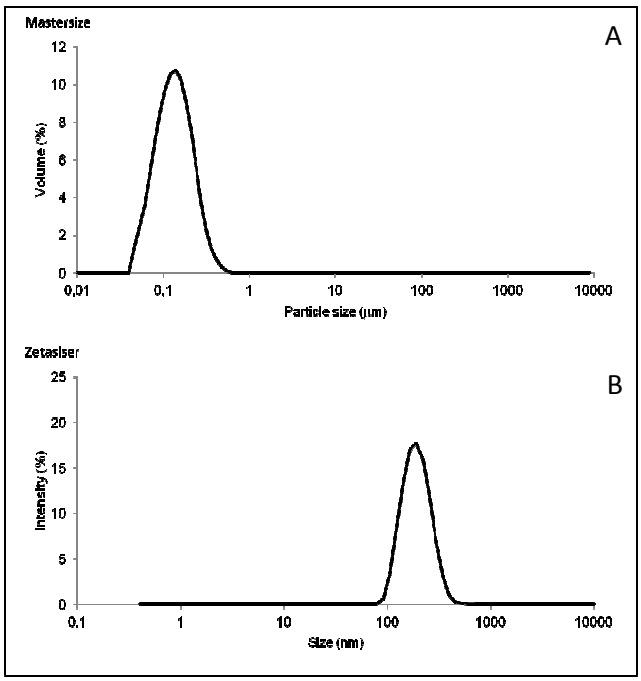

Figure 2. Nanocapsules distribution. (A) Granulometric profile (laser diffraction) and (B) Polydispersity (dynamic light scattering).

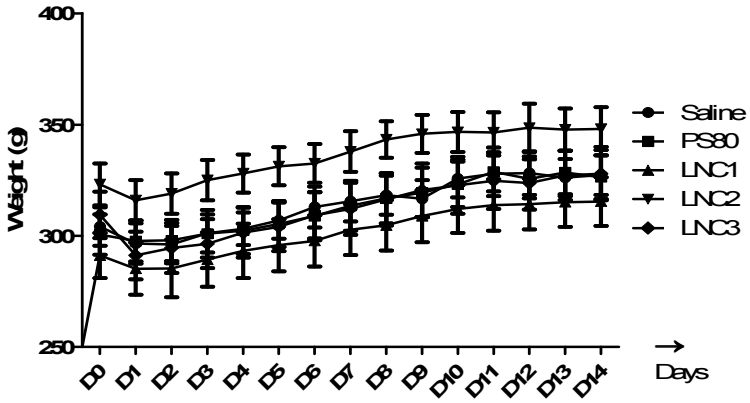

Figure 3. Body weight gain during the single dose experiment. No statistical difference was observed among the groups after 14 days of treatment (p>0.05). Data were analyzed by Generalized Estimating Equations.

Table 3. Relative heart weight in rats treated with LNC or vehicle by i.v. route.

Group	Heart weight (%)
Saline	0.29 ± 0.01
PS80	0.29 ± 0.01
LNC1	0.28 ± 0.01
LNC2	0.29 ± 0.01
LNC3	0.28 ± 0.01

No statistical difference was found between groups (p>0.05).

The results are showed as mean ± SEM and were analyzed by ANOVA Oneway.

Biochemical markers

All groups of treated Wistar rats presented baseline cTnI levels, being 0.01 ± 0.01 ng/mL in saline group, 0.01 ± 0.01n g/mL in PS80 group, 0.02 ± 0.02 ng/mL in LNC1 group, 0.02 ± 0.01 ng/mL in LNC2 group and 0.03 ± 0.03 ng/mL in LNC3 group without statistical difference ($p>0.05$; Fig. 4). As shown in Fig. 5, the levels of potassium were within the reference values for Wistar rats[37], however it were decreased in the group LNC3 compared to saline, PS80 and LNC1 group at 24 hours. On the other hand, LNC2 group showed reduced potassium levels compared only to PS80. AST, ALP and sodium showed no statistical difference among the groups, but AST was lightly above the reference values at 24 hours while ALP and sodium were in accordance to references values for rats at the two moments of measure.[37]

When compared the biochemical markers between 24 hours and 14 days after the treatment has been done, it was possible to observe a reduction in AST and ALP levels in all study groups ($p<0.05$). Also, a decrease in the potassium levels in PS80, LNC1 and LNC2 group was found within this time interval ($p<0.05$).

Hematological analyses

Representative hematological results are presented in Table 4. It was found significant difference to RBC parameter in the LNC3 group versus saline and LNC2 groups. Moreover, HCT values were significant lower in the LNC3 group than LNC2 group ($p<0.05$). Additionally, no significant alterations were observed in WBC, HGB, MCV, MCH, MCHC, RDWCV, RDWSD, PLT, MPV, PDW, PCT, PLCC and CSFP. All results were within the reference values for rats, except the HCT and MPV.[37]

Macroscopic and histopathological evaluations

The macroscopic observation of the heart and aorta showed normal morphology, color and size. No signs of ischemia or other pathological processes were found. The HE staining showed no heart remodeling process, but rather normal morphology. However, small spaces between cells were noted in the groups PS80, LNC1, LNC2 and LNC3, especially in outlying heart tissue near to blood vessels, suggesting possibly, a light edema process (Fig. 6), because in fact, it did not find edema by increase of weight of the hearts. But it was not the result of fibrosis, which was confirmed by PI staining and no hemorrhagic sign was observed by Prussian blue staining.

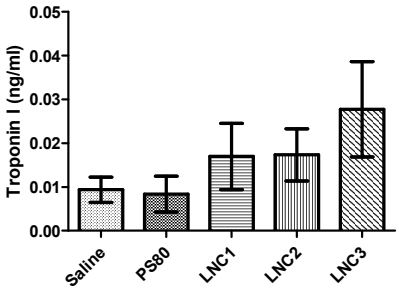

Figure 4. Troponin I evaluated after 24 hours. No statistical difference was found among groups ($p>0.05$). The results are showed as mean ± SEM and were analyzed by ANOVA Oneway.

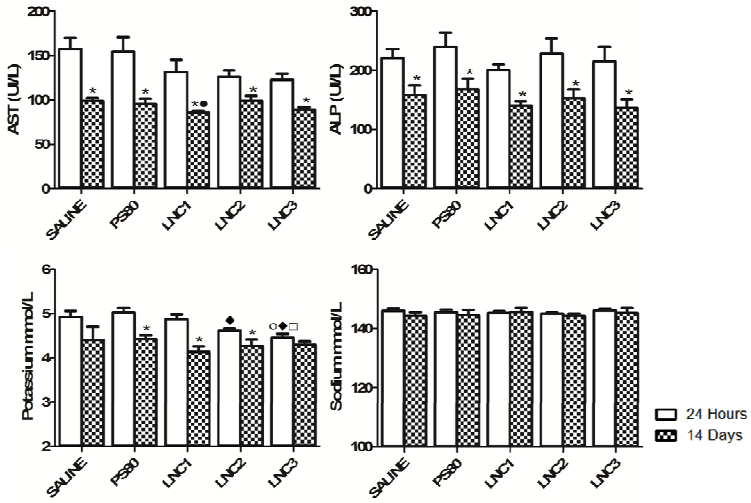

Figure 5. Heart damage markers in blood measured at 24 hours and 14 days after the acute treatment. * p< 0.05 compared to values of 24 hours of its own group; ○ p< 0.05 compared to Saline group 24 hours; ● p< 0.05 compared to Saline group 14 days; ♦ p< 0.05 compared to PS80 group 24 hours; □ p< 0.05 compared to LNC1 group 24 hours. Reference values: AST: 39 to 111 UI/L; ALP: 16 to 302 UI/L; Sodium: 135 to 146 mmol/L; Potassium: 4 to 5.9 mmol/L.[27] Data were analyzed by Generalized Estimating Equations.

Table 4. Hematological parameters after 14 days of acute treatment.

Parameter	Saline (n=8)	PS80 (n=8)	LNC1 (n=9)	LNC2 (n=9)	LNC3 (n=8)	Reference[27]
WBC (10^3/µL)	9.04 ± 0.69	8.90 ± 0.38	9.14 ± 0.55	8.87 ± 0.35	9.59 ± 0.84	1.96 – 8.25
RBC (10^6/ µL)	7.37 ± 0.07	7.08 ± 0.12	7.04 ± 0.11	7.33 ± 0.04	6.84 ± 0.08 ○▲	7.62 – 9.99
HGB (g/dL)	13.97 ± 0.15	13.60 ± 0.21	13.72 ± 0.12	14.02 ± 0.12	13.49 ± 0.15	13.7 – 17.6
HCT (%)	37.89 ± 0.39	36.94 ± 0.68	37.31 ± 0.46	38.34 ± 0.43	35.89 ± 0.45▲	39.6 – 52.5
MCV (fL)	51.41 ± 0.28	52.19 ± 0.51	53.10 ± 0.59	52.33 ± 0.67	52.48 ± 0.55	48.9 – 57.9
MCH (pg)	18.97 ± 0.20	19.18 ± 0.18	19.53 ± 0.21	19.14 ± 0.18	19.73 ± 0.21	17.1 – 20.4
MCHC (g/dL)	36.89 ± 0.26	36.85 ± 0.27	36.77 ± 0.32	36.58 ± 0.25	37.60 ± 0.27	32.9 – 37.5
RDW (%)	12.54 ± 0.17	12.76 ± 0.43	12.57 ± 0.20	12.51 ± 0.13	12.43 ± 0.09	11.1 – 15.2
PLT (10^3/µL)	713.29 ± 11.75	720.75 ± 27.39	694.33 ± 20.86	757.89 ± 35.49	713.75 ± 26.75	638 – 1177
MPV (fL)	5.53 ± 0.09	5.63 ± 0.12	5.63 ± 0.07	5.59 ± 0.11	5.64 ± 0.08	6.2 – 9.4
PDW	14.89 ± 0.05	14.88 ± 0.05	14.93 ± 0.03	14.86 ± 0.05	14.94 ± 0.05	11.1 – 15.2
PCT (%)	0.39 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	0.42 ± 0.01	0.40 ± 0.01	-
PLCC (10^9/L)	38.29 ± 2.81	40.13 ± 3.79	39.33 ± 2.46	41.22 ± 1.93	40.38 ± 2.17	-
PLCR (%)	5.39 ± 0.44	5.64 ± 0.58	5.68 ± 0.35	5.62 ± 0.50	5.71 ± 0.42	-

○ p< 0.05 compared to Saline group.
▲ p< 0.05 compared to LNC2 group.
The results are showed as mean ± SEM and were analyzed by ANOVA Oneway.

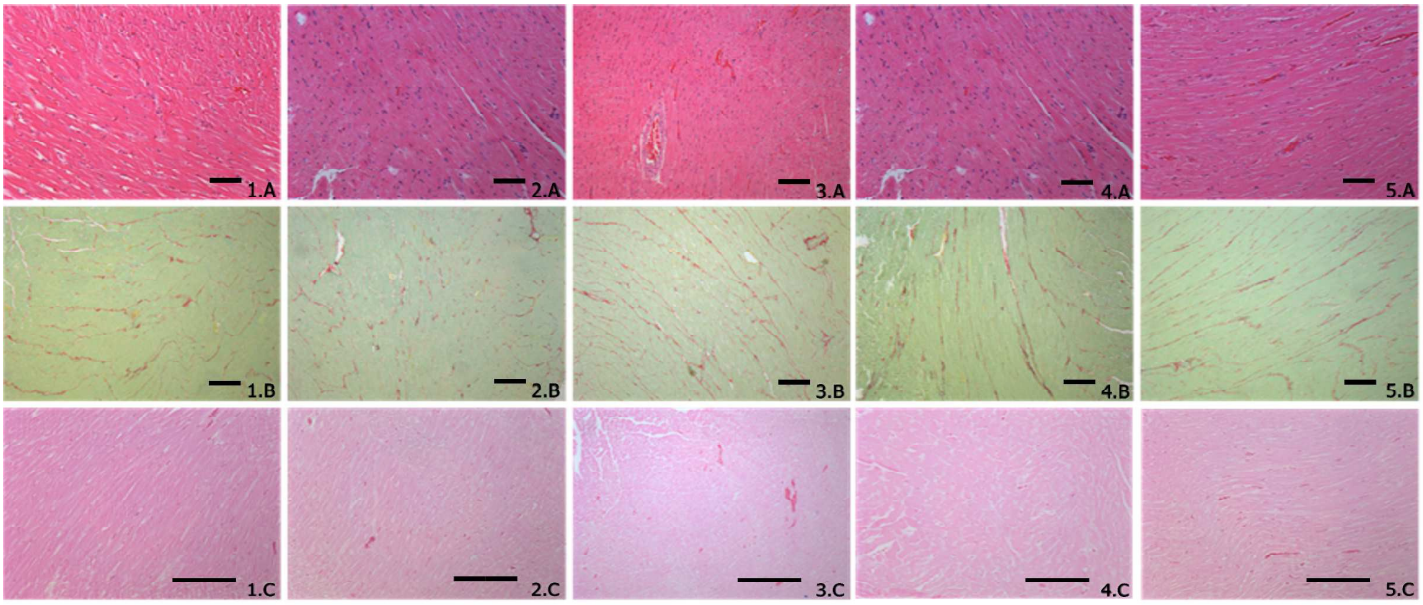

Figure 6. Histopathological morphology of heart tissue. (A) HE staining (100x); (B) PI staining (100x); (C) Prussian blue staining (200x); (1) Saline; (2) PS80; (3) LNC1; (4) LNC2; (5) LNC3. Discrete congestion process was observed in the groups PS80, LNC1, LNC2 and LNC3. Black bars on the inferior right corner of each picture are equal to 100 µm.

Echocardiographic findings

In the echocardiographic evaluation, the changes were assessed prior to treatment (basal) and after 14 days of the acute administration (Table 5). There was an increase in systolic diameter after 14 days compared to baseline in the LNC3 group ($p<0.05$). The same happened to diastolic diameter in saline, LNC1 and LNC3 groups. In addition, after 14 days the measures of diastole and systole left ventricle anterior wall thickness have changed only in LNC1 group. The systole left ventricle posterior wall thickness (LVPWTs) had significant increase in the PS80 and LNC3 groups after 14 days treatment compared to basal levels, but when analyzing the diastole left ventricle posterior wall thickness (LVPWTd) only LNC1 group presented significant increase. However, the ejection fraction was higher only in the saline group after 14 days of the acute treatment ($p<0.05$), and do not indicate classical cardiotoxicity. Similarly, the shortening fraction was significant higher after 14 days from treatment in the saline and LNC2 groups.

Electrocardiogram (ECG)

Electrocardiogram parameters were collected before and 14 days after the treatment. Administration of PS80 and LNC2 caused a slight delay in heart electrical conductance, since larger QRS times could be seen in both groups at day 14th. ST segment decreased in the saline and LNC1 groups ($p<0.05$), while other parameters had no significant differences (Table 6).

Blood Pressure

Regarding blood pressure, particularly systolic pressure, differences were found only in the times 24 hours and 14 days after the acute treatment in comparison with basal time of the saline, PS80, LNC1, and LNC3 groups. In addition, the heart rate decreased in the LNC1 and LNC2 groups after 14 days compared to basal time measure (Fig. 7). However, there were no differences between groups comparing them at the same moment of measurement.

Discussion

In nanotoxicology, the concept from Paracelsus about toxic effect of a substance was expanded because it is not important only quantify the “nanomaterials”, same that expressed in number of particles, mass, volume or surface area, but also the composition (what is the nanomaterial; its format) and its size, being all essentials to the development of the potential toxicological effects. Thus, it is possibly to infer that in nanotoxicology the toxic effects starting of a tridimensional (3D) system and it is not unidimensional.

Recent studies, evaluating the cardiotoxicity of nanomaterials, have reported close relationship between the composition, size, dose, permeation ability and bioaccumulation to cardiotoxicity events.[38-40]

Metal nanoparticles such as gold NPs, especially with sizes smaller than 50 nm, have permeation and bioaccumulation in cardiac tissue.[10] Abdelhalim demonstrated, that after infusion of 50 µL of gold nanoparticles in rat, were observed cardiac congestion, blood viscosity changes, bleeding and

vacuolization.[40] Leifert et al demonstrated alteration in QT interval prolongation in mouse by 50 mg/kg of gold nanoparticles administrated.[41]

Single wall carbon nanotubes have been reported to induce aortic intima and mitochondrial DNA damage, being responsible for caspase-3 activation, the worsening of atherosclerotic plaques and increase in expression of inflammatory genes and adhesion molecules.[15, 42] Since the damage has occurred, even in other organs, there is a release of cytokines that can reach the heart by the systemic circulation, inducing cardiotoxicity[38] through vascular dysfunction, thrombotic events[15, 42] and changes in the control of the autonomous system by decreasing the number of sequences baroreflex.[43]

Indeed, there are no studies of cardiotoxicity of biodegradable lipid-core nanocapsules of poly(ε-caprolactone) in the literature. There is a study using poly-ε-caprolactone but it is not LNC.[44]

The LNCs used in this study are similar to those previously studied by our group[21] with the same chemical composition differing only by having glycerol as isotonizing agent, with a relatively smaller size and a larger number of NCs per milliliter. This study, in turn, intends to elucidate one scenario of total availability of the formulation through intravenous administration, characteristic of this route.

In relation to the dose, it is important to compare with pre-clinical studies using therapeutic applications. Thus, the doses to potential treatment in different pathological conditions however, by i.p. route, varied of 0.1 ml/day until 2.4 ml/day.[45-47] On the other hand, the present study was performed by i.v. route and the doses varied of 0.9 until 3.5 ml. In this way, it is possible infer that higher doses than therapeutic proposes, considering the volume and the route, were performed as classically it is realized in toxicological studies.

Classic cardiotoxicity induced by anthracyclines, through repeated doses in short time or high single doses, is initially characterized by symptoms like tiredness, fatigue and digestive symptoms such as anorexia, abdominal distension and diarrhea.[1, 48] In the present study, within first 24 hours vital signs were observed without any events of diarrhea, altered motor behavior or fever. Likewise, all groups had weight gain during the fourteen days of experiment, without signs of anorexia.

In the present study, the hematological parameter, leukocyte count (WBC) did not differ among the studied groups. This finding is unlike from that found in a previous study with intraperitoneal (i.p.) administration,[21] which showed an increase of monocyte count in all LNC-treated groups in acute treatment, probably demonstrating a sign of proinflammatory exposure. However, regarding the red series, it was found a significant reduction in red blood cell count (RBC) in the group treated with the greatest number of NCs (LNC3) compared to the saline group, but it was in the range of normal values and did not indicate any disturbance. This difference can be explained by the inherent characteristics of the i.v. administration, once the direct contact between NCs and red cells may lead to a discreet hemolysis.[49] Bender et al. related *in vitro* hemolytic findings after addition of 10% of LNC (v.v) in blood.[49]

Regarding the biochemical results, except to AST in PS80 and saline groups, all results were within of reference values. It is known that in case of tissue damage the levels of AST or ALT are more elevated compared with the reference values. This is not observed because the increase after 24 hours was 35% above of superior limit (111 UI/L). Moreover, after 14 days all results are within reference intervals. In this line, it is possible to infer that the LNC did not damage the enzymes AST and ALT. As well as, it did not induce important alteration to serum sodium and potassium with pathological reflex in the present model.

Furthermore, despite certain fluctuations in the levels of troponin I among the experimental groups, there was no significant difference for this parameter that is a specific marker for cardiac injury, considered the gold standard for the evaluation of cardiotoxicity.[50, 51] Besides, studies evaluating the cardiotoxicity of doxorubicin found TnI concentrations higher than 0.07 ng/ml.[52] In addition, studies of cardiotoxicity in rabbits and rats without any evidences of heart diseases found serum baseline levels of 0.033 ng/mL to rats [53] and 0.03 ng/mL to rabbits.[54, 55]

With respect to potassium levels, the PS80, LNC1 and LNC2 groups, presented higher values 24 hours after acute treatment compared to their results at 14 days, when the values reached the same level to all groups. It was also observed that the group whose potassium concentrations remained at baseline levels was the LNC3 group, which received only LNCs during the treatment. However, these values were within the reference values. Further studies are needed, nevertheless, this finding suggests that the poly(ε-caprolactone) LNC treatment did not affect the potassium electrolyte balance.

In this line, the histological analysis showed no characteristic cardiotoxic damage on heart tissue after 14 days of the acute exposure. Just discrete edema process, a hemodynamic event, on heart tissue was noted, mainly on minor peripheral vessels, without any response or consolidated damage. Studies with induced cardiotoxicity often find cardiomyocellular vacuolation, perivascular and interstitial fibrosis, congestion, hemorrhage, infiltration of leukocytes, degeneration in myocytes and nuclear material clumping.[40, 52, 55, 56]

The international cardiology society guidelines cite the importance of identification of the left ventricular ejection fraction (LVEF) that is the most common method to screening of toxic effects on the heart.[2] The LVEF near to 90% represents a normal function of heart and when decreased to less than 50% indicates cardiac insufficiency.[57] Additionally, the guidelines of the Brazilian Society of Cardiology define as cardiotoxic effect a decrease of 10-20% in ejection fraction after administration of acute dose or high doses.[1] According to the present results, the echocardiographic alterations do not mean damaged or expressive cardiac remodeling of ventricles during the experiments of this study. Moreover, the heart weight was

similar to all groups, which is consistent with histopathological findings showing absence of fibrosis or remodeling processes.

On the other hand, the heart tissue is peculiar, the most part of internal structures, as ventricles, are directly irrigated by circulating blood.[58] Thereby, the size of NCs is directly proportional to the input capacity in cardiac tissue, thus, gold nanoparticles with size less than 50nm have been found on heart after i.v. acute treatment, while nanoparticles bigger than 100 nm, like polymeric NCs used in this study, were rarely detected.[10] Further nanotoxicological studies are needed to verify possible methodological interferences, however, *in vitro* models need recreate the complex geometric structure to simulate the heart tissue and generate reliable results.[59, 60]

Table 5. Ecocardiogram findings prior (Basal) and after 14 days of the acute treatment in the different treated groups.

Parameter	Saline (n=8)		PS80 (n=8)		LNC1 (n=9)		LNC2 (n=9)		LNC3 (n=8)	
	Basal	14 days	Basal	14 days	Basal	14 days	Basal	14 days	Basal	14 days
Diastolic diameter (mm)	0.67 ± 0.06	0.74 ± 0.07*	0.73 ± 0.05	0.74 ± 0.03	0.69 ± 0.05	0.74 ± 0.06*	0.74 ± 0.06	0.77 ± 0.07	0.67 ± 0.03	0.78 ± 0.03*
Systolic diameter (mm)	0.34 ± 0.04	0.32 ± 0.08	0.33 ± 0.04	0.35 ± 0.08	0.30 ± 0.11	0.38 ± 0.05	0.37 ± 0.09	0.32 ± 0.10	0.32 ± 0.03	0.35 ± 0.06*
LVAWTd (mm)	0.19 ± 0.09	0.22 ± 0.08	0.18 ± 0.11	0.22 ± 0.08	0.14 ± 0.03	0.24 ± 0.07*	0.21 ± 0.08	0.25 ± 0.07	0.23 ± 0.06	0.22 ± 0.09
LVAWTs (mm)	0.18 ± 0.06	0.21 ± 0.07	0.19 ± 0.07	0.17 ± 0.09	0.22 ± 0.07	0.13 ± 0.04*	0.18 ± 0.07	0.16 ± 0.09	0.17 ± 0.06	0.18 ± 0.08
LVPWTd (mm)	0.13 ± 0.02	0.14 ± 0.03	0.12 ± 0.02	0.13 ± 0.02	0.12 ± 0.01	0.13 ± 0.02*	0.14 ±0.03	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
LVPWTs (mm)	0.28 ± 0.04	0.29 ± 0.08	0.26 ± 0.02	0.29 ± 0.03*	0.28 ± 0.04	0.27 ± 0.04	0.30 ± 0.05	0.30 ± 0.04	0.25 ± 0.04	0.29 ± 0.04*
Ejection fraction (%)	86.65 ± 3.52	91.64 ± 4.49*	91.00 ± 2.22	88.29 ± 7.03	90.45 ± 7.91	86.58 ± 3.88	86.93 ± 7.79	91.66 ± 5.63	88.85 ± 4.71	90.11 ± 4.48
Shortening fraction (%)	49.18 ± 4.29	57.69 ± 8.53*	55.46 ± 3.72	53.01 ± 10.42	57.30 ± 13.47	49.26 ± 5.03	50.99 ± 10.12	58.77 ± 11.27*	52.60 ± 6.75	54.72 ± 7.00

* p< 0.05 compared to basal values of its own group. (LVAWTd) diastolic left ventricle anterior wall thickness; (LVAWTs) systolic left ventricle anterior wall thickness; (LVPWTd) diastolic left ventricle posterior wall thickness; (LVPWTs) systolic left ventricle posterior wall thickness. The data were analyzed by Generalized Estimating Equations.

Table 6. Electrocardiogram changes evaluated prior (Basal) and after 14 days of the acute treatment in the different treated groups.

Parameter	Saline (n=8)		PS80 (n=8)		LNC1 (n=9)		LNC2 (n=9)		LNC3 (n=8)	
	Basal	14 days	Basal	14 days	Basal	14 days	Basal	14 days	Basal	14 days
RR Interval (ms)	148.13 ± 13.81	132.41 ± 54.19	146.71 ± 17.80	154.29 ± 8.53	150.88 ± 12.21	153.50 ± 5.57	145.05 ± 17.41	160.21 ± 14.51	150.93 ± 11.19	154.90 ± 11.12
Heart Rate (BPM)	408.26 ± 37.48	398.23 ± 25.54	414.41 ± 51.19	390.15 ± 21.01	400.14 ± 2.06	391.33 ± 14.18	418.38 ± 44.56	377.46 ± 32.73	399.51 ± 27.85	389.31 ± 28.15
Segment PR (ms)	42.57 ± 3.32	36.80 ± 15.03	42.96 ± 2.55	43.36 ± 4.47	46.52 ± 5.95	44.67 ± 4.37	43.97 ± 3.36	45.42 ± 5.10	43.08 ± 3.47	40.80 ± 3.68
P Wave (ms)	15.53 ± 2.23	12.96 ± 5.42	16.10 ± 3.19	17.68 ± 4.84	16.65 ± 3.51	17.41 ± 3.99	15.31 ± 3.78	17.57 ± 4.01	15.49 ± 3.80	16.33 ± 4.61
QRS Complex (ms)	19.06 ± 1.44	18.09 ± 7.37	18.65 ± 1.89	20.24 ± 1.75*	19.72 ± 1.59	20.48 ± 0.63	20.26 ± 1.60	21.78 ± 1.17*	18.99 ± 1.46	19.85 ± 0.76
QT interval (ms)	53.54 ± 8.98	45.97 ± 18.79	56.29 ± 12.01	55.77 ± 8.31	58.25 ± 13.68	56.30 ± 6.92	59.59 ± 9.84	55.01 ± 2.94	55.60 ± 13.55	57.52 ± 8.67
T peak (ms)	26.91 ± 10.40	14.60 ± 6.35*	26.44 ± 15.70	22.03 ± 10.64	28.76 ± 10.31	22.54 ± 5.48*	24.36 ± 6.60	20.40 ± 3.24	26.46 ± 16.28	23.06 ± 9.70
ST segment (volts)	-0.18 ± 0.50	-0.52 ± 0.27*	-0.02 ± 0.78	-0.16 ± 0.43	0.27 ± 0.51	-0.16 ± 0.27*	0.13 ± 0.62	-0.24 ± 0.45	-0.20 ± 0.37	-0.24 ± 0.39
T wave (volts)	0.38 ± 0.37	0.17 ± 0.27	0.50 ± 0.81	0.38 ± 0.50	0.79 ± 0.50	0.39 ± 0.18	0.71 ± 0.57	0.39 ± 0.24	0.27 ± 0.42	0.33 ± 0.36
QTc (ms)	44.12 ± 7.72	42.77 ± 3.10	46.66 ± 9.98	44.97 ± 7.02	47.44 ± 10.90	45.43 ± 5.40	49.87 ± 9.66	43.56 ± 2.83	45.57 ± 12.34	46.29 ± 7.07

* p< 0.05 compared to basal values of its own group. The data were analyzed by Generalized Estimating Equations.

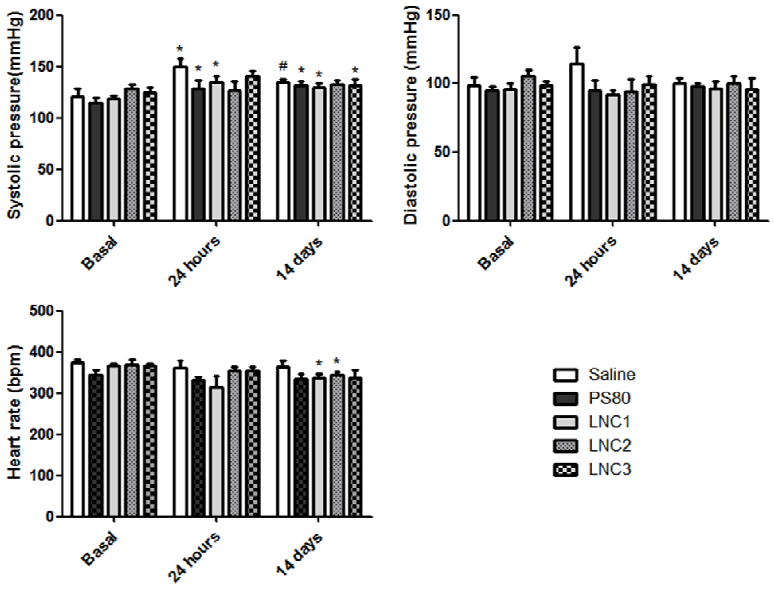

Figure 7. Blood pressure evaluation at three moments: Before the treatment (Basal), 24 hours and 14 days after the acute treatment. * p< 0.05 compared to basal values of its own group. # p< 0.05 compared to values of 24 hours of its own group. The data were analyzed by Generalized Estimating Equations.

Drugs with high ability to induce cardiotoxicity promote electrophysiological changes, especially after acute administration and in high concentrations.[1, 61] Physiologically the electrophysiological changes occur in the ventricular repolarization in greater proportion, due to interaction of drugs, hormones, cytoquines and peptides.[62] When this occurs, it is usually observed a ventricular fibrillation, sinus tachycardia and QT interval prolongation.[61] Gold nanoparticles have been related to interact with ventricle ionic channels causing QT interval prolongation.[41] Therefore, the QT interval corrected for heart rate (QTc) is the most appropriate parameter to evaluate this type of change.[63] In this study, no electrophysiological changes consistent with classic cardiotoxicity were observed when compared the measures obtained 14 days after the treatment to basal measures. Additionally, it is known that arrhythmias in intoxications are dependent, in most of cases, of abnormal impulse conduction, abnormal impulse formation and triggered activity, besides to be influenced by acid-base and electrolyte imbalances hypotension and hypoxia conditions [64], events that were not related in this study.

Increase in systolic blood pressure without diastolic pressure alterations are related to pathophysiological changes in the vascular intimae, particularly in the aortic diameter and aortic knuckle.[65] The mechanism of this process is related to breakage of elastin fibers present in the vessels[65] and it has been found usually in aging and diseases in which there is increased stiffness of the arterial wall.[66, 67] In the present study the blood pressure results were within the reference values for rats[25] and the increase in average systolic pressure at the different times maybe could be related to the large volume infused in the animals, since most of the groups showed an increase in the values. Therefore, further studies with larger assessment of hemodynamic and biochemical markers are needed.

Conclusions

In acute cardiotoxicity evaluation, during the whole observation period, the rat treated groups with LNC did not demonstrate alteration on electrocardiographical and ecocardiographical analyses compared with control groups. Additionally, did not found important difference on biochemicall and hematological analysis, as well as, by histophalogical evaluation. Thus, from the cardiac viewpoint the present findings support the conclusion that biodegradable lipid-core nanocapsules of poly(ε-caprolactone) are safe in Wistar rats, after acute single intravenous administration.

Acknowledgements

This work was supported by FAPERGS, CNPQ and FIPE-HCPA. R. Fracasso is the recipient of a CAPES master scholarship. S.S. Guterres, N. Clausell, A.R. Pohlmann and S.C. Garcia are recipients of CNPq Research Fellowship.

Conflict of interest

All authors declare that there are no conflicts of interest.

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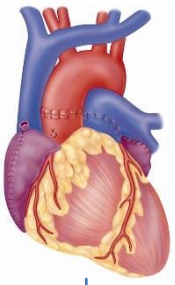
NANOTECHNOLOGY

DRUG DELIVERS

NANOCAPSULES

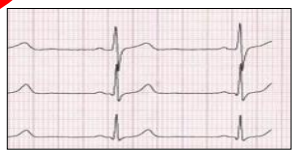
POTENTIAL
NEW TREATMENT

WITHDRAWAL MEDICAMENT
OF THE MARKET

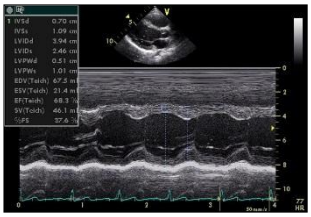


CARDIOTOXICITY

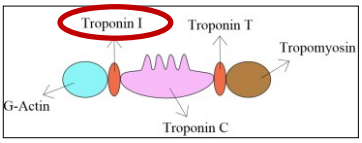
Wister rats
i.v. route



Electrocardiography



Echocardiography



Troponin I levels