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Acute and Subchronic Toxicity Evaluation of Poly(ε-Caprolactone) Lipid-Core Nanocapsules in Rats

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Owing to concerns over the effects of the physicochemical properties of nanoparticles and their interaction with biological systems, further investigation is required. We investigated, for the first time, the toxicity of lipid-core nanocapsules (LNCs) containing a polymeric wall of poly(ε-caprolactone) and a coating of polysorbate 80 used as drug delivery devices (~245 nm) in Wistar rats after single- and repeated-dose treatments. The suspensions were prepared by interfacial deposition of the polymer and were physicochemically characterized. Toxicological effects were determined after single doses of 18.03, 36.06, and 72.12 × 10^{12} LNC/kg and repeated doses of 6.01, 12.02, and 18.03 × 10^{12} LNC/kg for 28 days by ip administration. The results for both the treatments showed no mortality or permanent body weight changes during the experiments. A granulomatous foreign body reaction was observed in the liver and spleen of higher dose groups in acute and subchronic treatments. Most of the hepatotoxicity and nephrotoxicity markers were within the reference values and/or were similar to the control group. However, a slight alteration in the hematologic parameters was observed in both the studies. Thus, to verify a possible methodological influence, we performed an *in vitro* test to confirm such influence. These findings are in agreement with earlier reports regarding no appreciable toxicity of biodegradable polymeric nanoparticles, indicating that LNC might be a safe candidate for drug delivery system. Furthermore, the results presented in this study are important for health risk assessment and to implement strategies for testing biodegradable polymeric nanoparticles.

***Key Words:* *in vivo* test; nanotoxicity; polymeric; biodegradable; nanotoxicology.**

Nanocarriers may alter the physicochemical properties of xenobiotics, resulting in pharmaceutical changes in stability, solubility, and pharmacokinetic disposition (Vega-Villa *et al*., 2008). Interestingly, pharmaceutical sciences are using nanoparticles to reduce toxicity and side effects of drugs and until recently did not realize that carrier systems themselves can impose risks to the patient (De Jong and Borm, 2008). However, as with any new technology, the identification of potential health risks is a prerequisite for a proper assessment of the usefulness and safety of new chemicals, materials, and products that may be developed. The unusual or new properties of nanomaterials (NM) are predominantly associated with their nanometer-scale size, structure-dependent electronic configurations, and an extremely large surface area-to-volume ratio relative to large-sized chemicals and materials.

Recently, biodegradable nanoparticulate systems have been receiving considerable attention as potential drug delivery vehicles (Maham *et al.*, 2009; Ren *et al.*, 2011; Wang and Ho, 2010). Polymeric nanoparticles are vesicular or matricial colloids containing a polymer as a domain in the system, whereas nanocapsules are vesicular carriers comprising an oil core surrounded by a polymeric wall (Couvreur *et al*., 2002). A new type of nanoparticle recently developed is the lipid-core nanocapsule (LNC), which is structured by the dispersion of solid lipid (sorbitan monostearate) and liquid lipid (caprylic/capric triglyceride [CCT]) in the core enveloped by a polymeric wall (Fig. 1) (Jäger *et al*., 2007; Venturini *et al*., 2011). Several

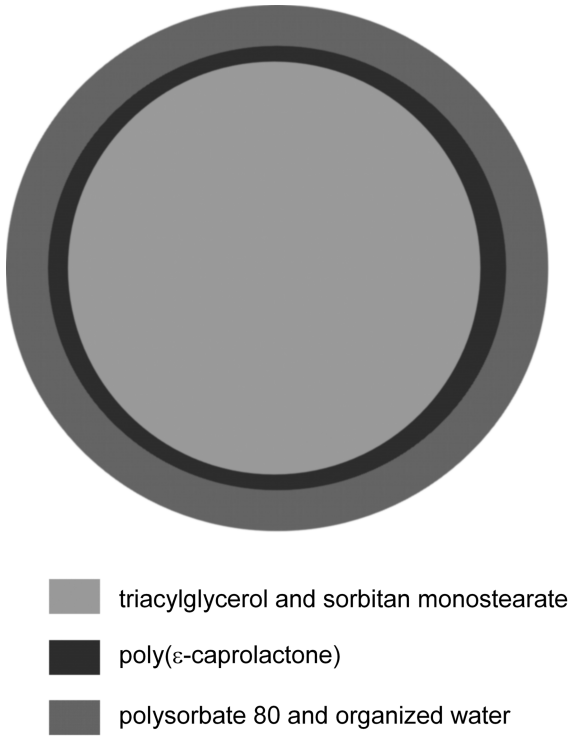

FIG. 1. Scheme of the LNC.

studies have reported the use of LNC, prepared with poly(ε-caprolactone) (PCL) and polysorbate 80 (PS80), presenting promising results in the field of drug delivery, including anti-inflammatory indomethacin (Bernardi *et al*., 2008, 2009a, b), antimalarial quinine (Haas *et al*., 2009), and antioxidant trans-resveratrol (Frozza *et al*., 2010). Also, there are some molecules that have been successfully incorporated into PCL nanoparticles to increase their therapeutic value, including tamoxifen (Shenoy and Amiji, 2005), saquinavir (Shah and Amiji, 2006), and insulin (Damgé *et al*., 2007). PCL is suitable for controlled drug delivery due to a high permeability to many drugs, excellent biocompatibility, and its ability to be fully excreted from the body once bioresorbed. Nevertheless, biodegradation of PCL is slow, compared with other polymers (Woodruff and Hutmacher, 2010). In addition, nanocapsules containing PS80 can be used as long circulation delivery systems because PS80 can delay opsonization; consequently, the nanocapsules remain in the blood for a long time before being taken up by cells of the mononuclear phagocytic system (Bender *et al*., 2012; Soppimath *et al*., 2001).

The ability of NM to interact with biological systems in adverse ways is creating a new field of knowledge known as nanotoxicology (Oberdörster *et al*., 2007), which emphasizes the relationship between the physical and chemical properties of nanostructures with induction of toxic and biological responses (Fischer and Chan, 2007). The need for this area of investigation became apparent after the intensive expansion of nanotechnology (Vega-Villa *et al*., 2008).

Even though it is extremely important to elucidate the toxicity of polymeric nanocapsules, there are a few articles related to their overall behavior. Till date, little is known about the possible toxicity and safety of polymeric nanocapsules. Standard *in vitro* and *in vivo* toxicological studies have not been carried out in detail, and data on their toxicity are scarce. To our knowledge, no extensive research has been conducted to analyze the toxicity of nanocarriers *per se*. Huang *et al.* (2010) performed a study on acute and genotoxic effects of iv administration of copolymers and NM containing PCL in rats, which did not show any adverse effect. In a previous work, Fang *et al.* (2009) focused mainly on the acute organ toxicity of BALB/c mice by sc injection of triblock copolymer containing PCL, and the formulation was thought to be nontoxic. *In vitro* and *in vivo* toxicological investigations conducted in mice showed that nanospheres containing PCL and methoxy poly(ethylene glycol) (MePEG) did not show signals of toxicity by ip administration (Kim *et al*., 2003). The author suggested that these diblock copolymeric nanospheres were a biocompatible drug delivery system. Meanwhile, no study has investigated the toxicity of nanocapsules produced with PCL, which present different physicochemical properties that change their possible toxicity mechanisms.

The aim of this study was to investigate the possible toxic effects, after ip injection, of LNC after single- and repeated-dose treatments in animal model. The safety evaluation of these nanocapsules is extremely important for its application in drug delivery system and for most of the biodegradable polymer-derived nanocarriers. Therefore, the results presented in this study are important for health risk assessment and to implement strategies for testing biodegradable polymeric NM.

MATERIALS AND METHODS

Materials

Span 60 (sorbitan monostearate) was supplied by Sigma-Aldrich (Strasbourg, France), and PCL (M_w = 50,000) was supplied by Capa (Toledo, Ohio). CCT and polysorbate 80 were obtained from Delaware (Porto Alegre, Brazil). All other chemicals and solvents were of analytical or pharmaceutical grade. All reagents were used as received.

LNC Preparation

LNC was prepared as previously described (Venturini *et al*., 2011). PCL (0.2490 g) and sorbitan monostearate (0.096 g) were dissolved in acetone (67 ml at 40°C) to form the diffusing phase. CCT (412 µl) was then added to this phase. This organic phase was injected into a dispersing phase containing PS80 (0.1926 g) dissolved in water (132 ml) at 40°C. A turbid dispersion was obtained instantaneously, which was kept under magnetic stirring for 10 min. The solvent of the diffusing medium was eliminated, and the suspension was concentrated under reduced pressure at 40°C. The final volume was adjusted to 25 ml in a volumetric flask. The suspensions were prepared in three different batches.

Physicochemical Characterization of the Formulations

Laser diffraction. The granulometric profiles of LNC were analyzed by laser diffraction using a Mastersizer 2000 (Malvern Instruments, UK). The laser diffraction particle size analysis is a standard method (ISO13320) using the Mie theory recommended for the analysis of particles ranging from 0.1 to 3000 µm. The values of D[4,3] and D[3,2] refer to the volume-weighted mean

diameter and the surface-weighted mean diameter, respectively. In addition, the size distribution is expressed by the SPAN value, calculated by Equation 1

$$\text{Span} = \frac{d(0.9) - d(0.1)}{d(0.5)} \quad (1)$$

Here, d(0.9), d(0.1), and d(0.5) are the diameters at 90, 10, and 50% of the cumulative size distributions based on volume, respectively.

Dynamic light scattering and electrophoretic mobility. Dynamic light scattering and electrophoretic mobility were used to analyze the nanometric population and determine the zeta potential, respectively. Measurements of the mean diameter (*z*-average diameter) and zeta potentials were carried out using a ZetaSizer ZS (Malvern, UK) at room temperature (25°C) by previously diluting the samples in MilliQ water and 0.01 mol/l NaCl aqueous solution, respectively (for details, see Supplementary data).

Nanoparticle tracking analysis. A sample was introduced into a scattering cell by a focused laser beam using a Nanosight LM10TM (NanoSight, UK). The sample was first diluted 5000-fold with ultrapure water (MilliQ water). The video acquisitions were performed for 120 s and analyzed using the NanoSight Nanoparticle Tracking Analysis (NTA) Software "NTA 2.0 Build 0122." The values of gain and shutter were 98 and 253, respectively. The analysis conditions to process the video records were an automatic detection threshold, and values of gain, minimum particle size expected, blur, and brightness were selected as 1, 100 nm, 3 × 3, and −0, respectively.

Multiple light scattering. LNC was investigated by multiple light scattering (MLS) using Turbiscan Lab equipment (Formulaction, France) (Mengual *et al*., 1999). The equipment consists of a detection head that moves up and down along a cylindrical cell. The detection head is composed of a pulsed near-infrared light source (λ = 880 nm) and two synchronous detectors. The transmission detector (at 180º) receives the light, which goes through the sample, whereas the backscattering detector (at 45º) receives the light scattered backward by the sample. The system detector scans from the bottom to the top of the sample, simultaneously acquiring data every 40 µm. The samples (20 ml) were poured into the optical cells without any dilution and analyzed at 25ºC for 3 h.

pH measurements. The pH values of the suspensions were measured without previous dilution using a calibrated potentiometer B474 (Micronal, Brazil) at 25°C.

Transmission electron microscopy. The LNC shape was analyzed by transmission electron microscope (JEM 1200 Exll) operating at 80 kV. The 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% wt/vol).

Turbidimetry. Turbidity technique was used to determine the concentration of LNC in the suspensions (Jäger *et al*., 2009; Poletto *et al*., 2008; Zattoni *et al*., 2004). The formulations were diluted (MilliQ water) and analyzed at 380 nm using a spectrophotometer (UV-1601PC, Shimadzu, Japan). To the calculus of *N* values, the mean diameters obtained by dynamic light scattering were used. The purpose of determining the number of particles per milliliters (particle number density, *N*) was to access the surface area of the nanocapsules (S) in a determined volume of suspension (Jäger *et al*., 2009).

In vivo *Studies*

Animals. Male Wistar rats weighing 280 ± 37 g and aged 6–8 weeks, obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), were used. The animals were housed in 47- × 34- × 18-cm polyethylene cages (four animals per cage) at 22 ± 0.1°C controlled temperature, humidity around of 60% with light and dark cycles (12:12 h), and food and water were provided *ad libitum*. For urine analyses, the animals were housed individually for 12 h in four different periods in metabolic cages with controlled food and water. All the experiments were performed according to the "Principles of Laboratory Animal Care" (National Institutes of Health, 1985). The protocol was approved by the local Ethics Committee of Universidade Federal do Rio Grande do Sul (No. 18427) and Fundação Universidade de Cardiologia do Rio Grande do Sul (No. 4482/10) and fulfilled all local regulations for research involving experimental animals. The research was conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology. Considering the reduction in the number of animals in biological studies and the ethical aspects, we did not use female rats in these studies.

Study design. Acute and subchronic toxicity studies were conducted based on Organization for Economic Co-Operation and Development (OECD, 1995, 2001) protocols and French Agency for Safety of Health Products (AFSSAPS, 2009, 2011) recommendations. Additionally, we included early renal damage markers (N-acetyl-beta-D-glucosaminidase [NAG] and microalbumin [MA]), an inflammatory marker (*high-sensitivity* C-reactive protein [*hs*-CRP]), and an immunological marker (complement component C3) in the study to better evaluate the safety of this new formulation. The parenteral route of administration was chosen considering a future application of these LNCs, and the ip route was elected given the limitations of the iv route in repeated-dose administration studies. This kind of formulation does not allow the preparation of different concentrations, and hence, we had to prepare the doses by volume.

For single-dose toxicity test, the experiment was conducted by distributing the rodents randomly into groups of 6–9 rats each, with ip administration of saline solution (12 ml/kg) and PS80 at a concentration of 0.78% (12 ml/kg; controls), and 18.03×10^{12} (Group I), 36.06×10^{12} (Group II), and 72.12×10^{12} LNC/kg (Group III) of PCL nanocapsules (LNC). The animals were observed for 1 min at 10, 20, 30, 60, 120, 240, and 360 min and at 24 and 48 h to detect possible gastrointestinal, respiratory, and neurological alterations. The specific monitored signs were alterations in locomotor activity, stimuli reaction, piloerection, ptosis, salivation, gasping, tearing, tremors, writhing, and seizures. The number of deaths and weight were registered every day for 14 days. After the 14th day, the rats were euthanized and also necropsied.

For subchronic toxicity evaluation, the experiments were conducted by distributing the rodents randomly into groups of six rats each, which were treated ip with saline solution (3 ml/kg) and PS80 in a concentration of 0.78% (3 ml/kg; controls), and 6.01×10^{12} (Group I), 12.02×10^{12} (Group II), and 18.03×10^{12} LNC/kg (Group III) of PCL nanocapsules (LNC) every day for 28 days. The number of deaths and weight were registered every day for 28 days. After the 28th day, the rats were euthanized and also necropsied. The study design for each treatment is presented in Supplementary figure S9. PS80, a nonionic surfactant, employed to stabilize the LNC in the formulation, was used as control in both the treatments. The amount of LNC formulation administered in acute and subchronic experiments is given in Table 1.

Relative weight of organs. The organs such as liver, spleen, kidney, heart, brain, and lung were excised and weighed accurately. The relative weight of these organs was calculated as follows: organ relative weight = (organ weight/body weight × 100).

Histopathological analyses. The following organs were dissected out for histopathological analysis: liver, spleen, kidney, heart, and brain. All the samples were fixed in 10% buffered formalin and were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and analyzed by a pathologist under light microscopy.

Scanning electron microscope. The polymeric agglomerate was examined under a scanning electron microscope (SEM; JEOL Scanning Microscope, JSM-5800). The samples were analyzed after they had been gold sputtered.

Biochemical parameters in blood and urine. Blood was drawn for biochemical analysis (heparin collection tube) using blood (vena cava) collection technique for hepatic and kidney function. Also, lipid profile and blood sugar were assessed using a biochemical auto analyzer (Labmax 240 Labtest Diagnóstica SA, Brazil). Butyrylcholinesterase activity (BuChE) in serum was determined by Doles (Doles reagents, Goiânia, GO, Brazil) commercial kits using a spectrophotometer (Biospectro SP-220). For urine collection and more information, see Supplementary data.

Microalbumin and NAG activity. Kidney function in the urine samples was evaluated by determining the levels of MA with immunoassay method

TABLE 1
Amount of LNC Formulation Administrated in Rats During Acute (Single Dose) and Subchronic (28 Days) Treatments

		Volume of administration (ml/kg)	Concentration of PCL (mg/kg)	Concentration of LNC* (LNC/kg)
Acute	Control	12	—	—
	PS80	12	—	—
	Group I	3	30	18.03×10^{12}
	Group II	6	60	36.06×10^{12}
	Group III	12	120	72.12×10^{12}
Subchronic	Control	3	—	—
	PS80	3	—	—
	Group I	1	10	6.01×10^{12}
	Group II	2	20	12.02×10^{12}
	Group III	3	30	18.03×10^{12}

Note. LNC containing PCL as polymer; *amount of LNC: $6.01 \pm 0.24 \times 10^{12}$ LNC/ml.

using Ebram kits (Ebram Produtos Laboratoriais Ltda, São Paulo, SP, Brazil) in a Cobas Mira instrument (Roche). NAG activity was determined spectrophotometrically based on the protocol developed by Horak *et al*. (1981).

Serum complement C3 and* hs-*CRP. A quantitative method for the measurement of complement C3 (C3; limit of detection: 0.4 mg/dl) and *hs*-CRP (limit of detection: 0.05 mg/l) in serum was employed using Ebram kits (Ebram Produtos Laboratoriais Ltda) in a Cobas Mira instrument (Roche). Before subchronic treatment, an aliquot of blood was also drawn from the tail vein for *hs*-CRP and C3 quantification.

Hematological parameters. For hematology analysis, blood was collected in potassium EDTA tubes. The selected markers 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), white blood cell count (WBC), and granulocytes, lymphocytes, and monocytes assayed using an ABX Micros 60 (ABX Diagnostics, Horiba-ABX, France).

In Vitro *Hematological Tests*

The hematologic assay was performed by adding 2 and 10% (vol/vol) of LNC suspension (similar to those concentrations used in Wistar rats and based on previous work by Bender *et al*. (2012)) to the rat whole blood, collected in EDTA anticoagulant tubes; run immediately after collection and within 2 h after collection; and incubated at 37ºC. Triplicate samples were prepared for each condition. Before and after incubation, the samples were measured for RBC, hemoglobin, PVC, MCV, MCH, MCHC, RDW, PLT, MPV, WBC, granulocytes, lymphocytes, and monocytes using an ABX Micros 60 (ABX Diagnostics).

Statistical Analyses

Statistics were performed using SPSS 18.0 for Windows and GraphPad Software (San Diego, CA). The results were expressed as mean ± SD. The statistical significance was determined using one-way ANOVA followed by *post hoc* for multiple comparisons (Tukey's HSD test). The level of significance was taken as $p < 0.05$.

RESULTS AND DISCUSSION

The toxicity of nanocarriers is a critical factor in evaluating the potential of new drug delivery systems. However, there are no harmonized standards for assessing the toxicity of nanoparticles even after they enter the blood stream or after topical or ocular application. Although the various toxicological aspects and the diversity of the NM assessed, they are just beginning to be explored, many deleterious effects have been documented (Li and Chen, 2011; Mutlu *et al*., 2010). This study was conducted to investigate the acute and subchronic toxicity of LNC in rats after ip administration based on the OECD (1995, 2001) protocols and AFSSAPS (2009, 2011) recommendations. Thus, we compared three LNC doses, with a saline solution group as control and one group receiving PS80 0.78%, to verify the influence of PS80 located on the LNC surface, evaluating the short- and long-term toxicological effects after proper characterization of the suspensions.

Physicochemical Characterization of Nanocapsule Formulations

First, the suspensions were prepared through the interfacial deposition of the polymer (Jäger *et al*., 2009; Venturini *et al*., 2011), which has been widely used for the production of nanocapsules. The LNC has been observed as a white bluish liquid, with pH value and zeta potential of 6.5 ± 0.2 and -7.5 ± 0.8 mV, respectively. Laser diffraction and dynamic light scattering analysis of three batches of LNC showed monomodal particle size distributions (Fig. 2) with appropriate values of media diameter, span, and polydispersity index (Table 2). These two techniques provided complementary results in terms of particle size characterization. Laser diffraction technique is extremely versatile for covering a wide range of diameters. Importantly, this technique is not specific for particles of diameter smaller than the wavelength of the light source, which requires the use of complementary technique such as dynamic light scattering (Müller-Goymann, 2004). Therefore, the main focus of this technique, mostly used to analyze nanoscale formulations, is to prove the absence of larger particles (Keck and Müller, 2008), whereas the dynamic light scattering is a technique that uses the interaction of particles with light to determine the particle diameter in nanometer scale (Gaumet *et al*., 2008).

NTA is a new method for direct and real-time analysis of nanosystems in liquids. Based on Brownian motion, nanocarriers are detected in real time by a CCD camera, with

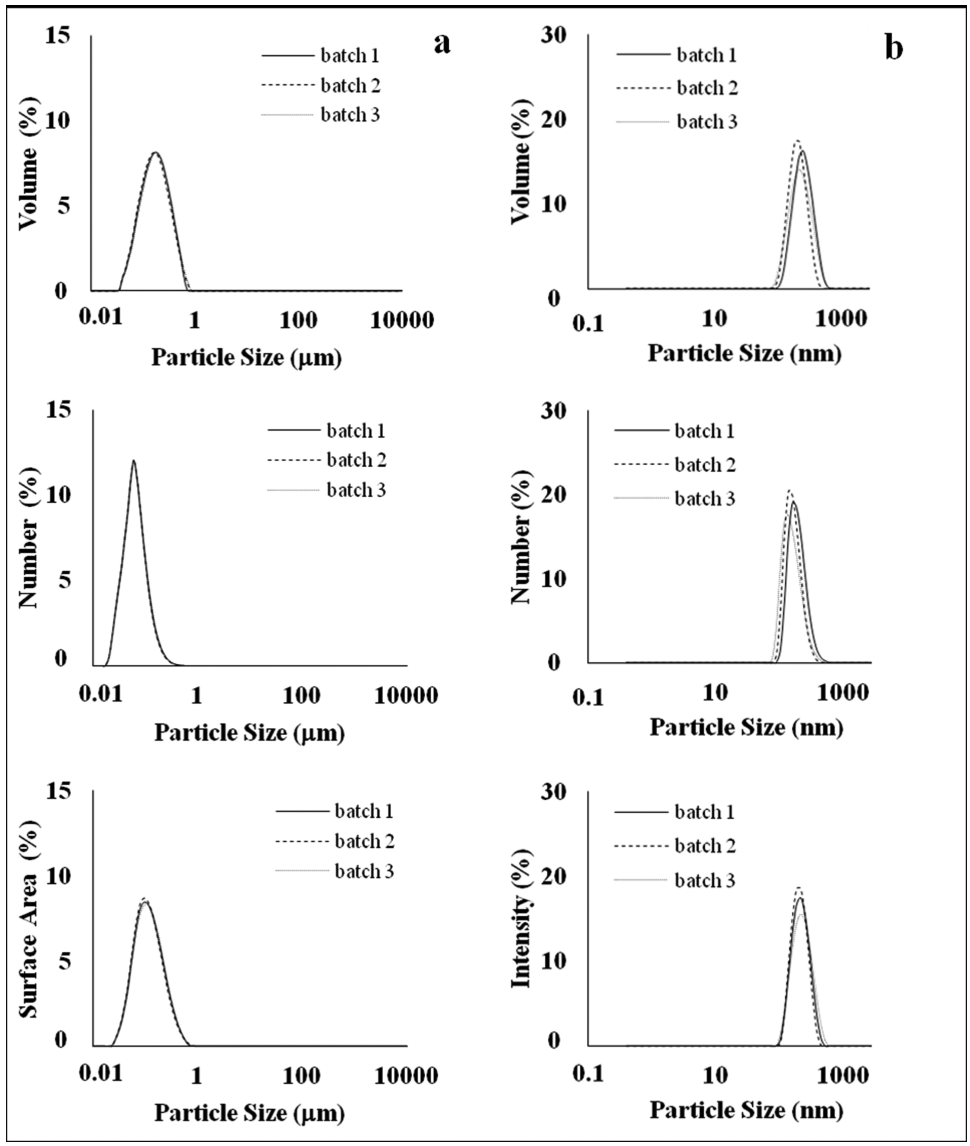

FIG. 2. (a) Size distribution profiles by laser diffraction of three batches for the LNC. (b) Size distribution profiles by dynamic light scattering of three batches for the LNC.

TABLE 2
Physicochemical Characterization of Nanocapsules' Formulations

	Particle size					
Formulation	D[4,3]	d(0.1)	d(0.5)	d(0.9)	Span	D[3,2]
Laser diffraction						
LNC	219 ± 7	79 ± 3	183 ± 7	413 ± 13	1.82 ± 0.04	147 ± 5
	Diameter (nm)	PdI	Peak 1	Width (nm)		
Dynamic light scattering						
LNC	215 ± 4	0.11 ± 0.02	239 ± 9 (100%)	83 ± 11		

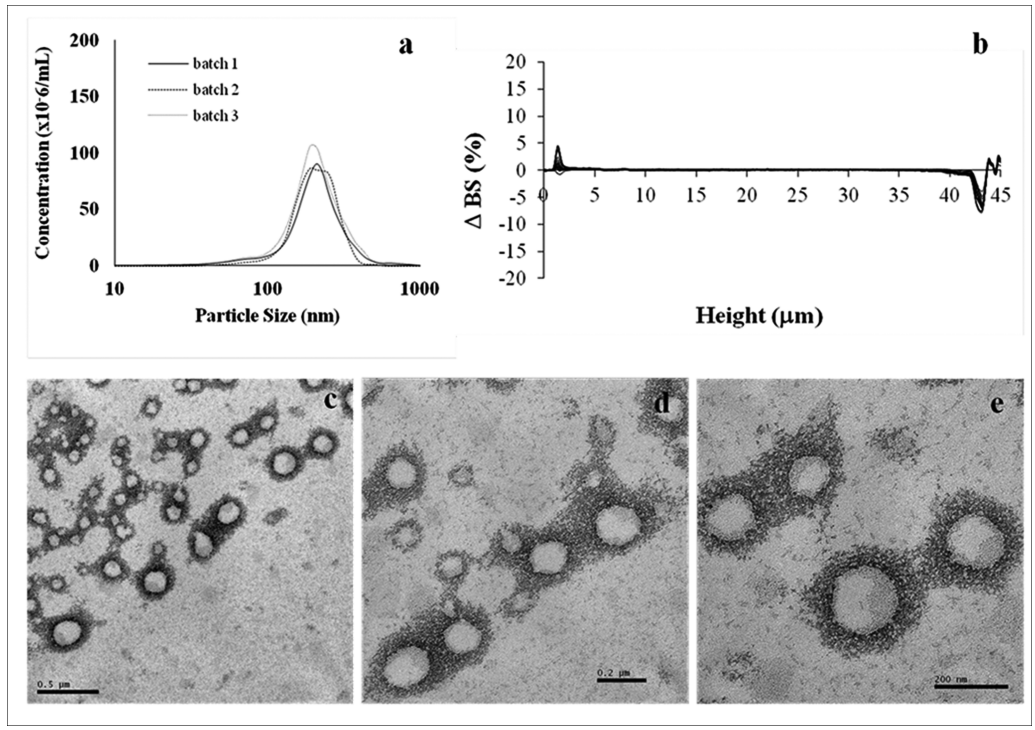

FIG. 3. (a) LNC size distribution by NTA. (b) Relative backscattering profile for LNC. (c) Transmission electron microscope of LNC at 50,000× (bar = 0.5 µm) (d) at 100,000× (bar = 200 nm), and (e) at 150,000× (bar = 200 nm).

single particles being simultaneously, but separately, visualized (Filipe *et al*., 2010; Malloy and Carr, 2006). When measured by the tracking analysis (Nanosight LM10), the LNC showed a mean particle diameter of 245 ± 12 nm. Figure 3a shows the size distribution of three different batches of LNC, where d0.1 was 151 ± 3, d0.5 was 227 ± 3, and d0.9 was 357 ± 34. Colloidal systems are complex and have a tendency to present the phenomenon of destabilization, affecting the homogeneity of the system. MLS is a technique that can be employed without dilution of the sample, providing information about the destabilization phenomenon of disperse systems as a function of time (Mengual *et al*., 1999). Therefore, MLS was performed for LNC, and the transmission signal was nil (data not shown). Thus, the relative backscattering profiles (BS) indicated very stable LNC (Fig. 3b). LNC showed only a slight tendency of sedimentation, presenting negative backscattering variation (ΔBS) at the top of cell (clarification of 7.5%) and positive ΔBS lower than 5% at the bottom. These results agree with the previous data, which presented a formulation of LNC containing indomethacin ethyl ester (Venturini *et al*., 2011), because the composition of those colloids render them denser than the aqueous phase. The lack of creaming indicates that the sample was not contaminated with nanoemulsion droplets, suggesting that only LNCs were formed. The photomicrographs (Figs. 3c–e) show spherical-shaped nanocapsules. In previous reports, formulations containing sorbitan monostearate and low concentration of CCTs presented spherically shaped particles (Jäger *et al*., 2009). The number of particles per unit of volume *N* was calculated, and the value was $6.01 \pm 0.24 \times 10^{12}$ particles/cm^3. The LNC surface area was $0.869 \pm 0.07 \times 10^4$ cm^2/ml[1].

In Vivo *Studies*

According to Nel *et al*. (2006), when *in vivo* toxicity is observed, it may also be appropriate to proceed with studies that formerly assess the absorption, distribution, metabolism, and elimination of NM. As these materials have the potential to spread beyond the portal of entry, it is important to assess systemic responses. The various interactions of NP also need to be considered through a range of possible pathways toward target organs (Maynard *et al*., 2011).

One of the major challenges in nanotoxicology is choosing the dose. We chose the dose for pilot toxicity study using a volume of administration 10 times higher than the volume used on previous studies using these nanocapsules with therapeutic applications (1 ml/kg) (Bernardi *et al*., 2008, 2009a, b; Frozza *et al*., 2010). Moreover, according to Hulin (2002), the maximum volume allowed for ip administration is 5 ml (Hulin, 2002). Taking into account that the highest dissolvable and dispersible concentration of PCL is 10 mg/ml, by limiting the change of polymer concentration per volume, we decided to administer a volume near the maximum permitted for ip administration and 10 times higher than the dose used for therapeutic purpose in

order to evaluate the safety of these nanocapsules. In pilot study, Wistar rats were euthanized 48 h after single administration of 72.12×10^{12} LNC/kg (dose is expressed by number of LNC per kg of rat). Subsequently, in acute toxicity treatment, we used the same dose used in pilot, but the rats were euthanized 14 days later according to OECD acute toxicity protocol (OECD, 2001). The subchronic dose was chosen based on acute treatment.

Clinical Evaluation in Acute Toxicity Test

The five groups did not exhibit any clinical abnormality (see details in Materials and Methods section), except for piloerection observed in PS80 and LNC-treated groups after administration, which remained for 4 h of the observation period. Also, abdominal contortions were observed in the highest dose group, which disappeared within 2–3 h. With these exceptions, no outward behavioral abnormalities were noticed during the 2-week posttreatment period. Deaths were not observed in any of the tested doses; therefore, it was not possible to estimate the DL50 level. Both piloerection and abdominal contortion could be attributed to pain-related behaviors after ip administration, probably as a result of osmotic disequilibrium, and not owing to the volume of administration because the rats that received the saline solution were administered the same volume as that used in PS80 and Group III (12 ml/kg). Meanwhile, these symptoms were temporary, and these problems may probably be associated with ip administration.

Relative Consumption and Body Weight Trends

Relative body weight was significantly different among the groups in acute treatment (Fig. 4a). When compared with the controls, water and food consumption were significantly decreased only in Group III following dosing ($p < 0.05$), and one day after in acute treatment, but all the groups were similar thereafter ($p > 0.05$). Further, the amounts of urine and feces were also reduced in this group only on the day after single administration (data not shown). This difference found in relative body weight, with decreasing in water and food consumption could be, in part, also attributed to transient behavior related to pain after acute ip administration, as mentioned earlier. In the subchronic study, Group III had higher weight loss, compared with the control ($p < 0.05$) until the 5th day, returning to normal levels thereafter (Fig. 4b), which is in agreement with the group that received the same dose (18.03×10^{12} LNC/kg) in acute treatment. However, changes in water and food consumption and amount of urine and feces did not occur in subchronic treatment (data not shown). An interesting point is that after the initial shock and disruption of increase in mass, the rate of weight increased the same as the control in subchronic treatment. Also, it can be noted that after 5 days of subchronic treatment, there was a slight decrease in body weight gain in the group treated with PS80, compared with Groups I and II. However, these changes in the relative weight of the rats, treated with LNC and PS80, are not so alarming because all rats had their body mass increased after a while in both the studies.

Relative Weight of Organs

After 2 (acute) and 4 weeks (subchronic), the rats were euthanized, and the organs and tissues were rapidly dissected out and weighed. No obvious differences were found in relative weight of the organs among the five groups in acute treatment (Supplementary table S4). However, a significant increase was observed in relative weight of spleen in Group III, compared with the control and PS80 in subchronic treatment (Fig. 5). Furthermore, necropsy of the animals at the end of treatment did not show any macroscopic changes in liver, kidneys, heart, brain, and lungs (data not shown).

Histopathological Analyses

No relevant histopathological changes were observed in any of the organs analyzed, with an exception for liver and spleen in high dose–treated rats (Group III), following acute (Supplementary fig. S10) and subchronic (Fig. 6) treatments. A multifocal granulomatous foreign body reaction on the serosal surface of spleen and liver was found (Fig. 6) probably because of the high volume administrated and the high levels of LNC per milliliter. Granulomatous foreign body refers to a specific pathological term for an immunological response to a foreign material, which is not accompanied by necrosis or apoptosis. No other microscopic findings, such as parenchymal inflammatory infiltration, necrosis, apoptosis, or vacuolation, were observed in these organs. Further investigation was carried out to explain these results, which showed an agglomeration of LNC in the abdominal cavity of Wistar rats that received the highest dose in acute and subchronic treatments (Figs. 6e and f). After SEM analysis, we determined the melting point of PCL, which ranged between 56ºC and 58ºC. This accumulation could also probably explain the clinical symptoms cited earlier, such as abdominal contortions and alterations in body weight, including a decrease in food consumption and the amount of feces and urine after administration of the suspensions. In addition, increase in weight of spleen could be due to the peritoneal inflammatory response. Granulomatous reaction was observed in 90% of the rats treated with high doses in both the experiments.

Biochemical Analyses in Blood and Urine

Hepatic markers. Reduction in liver function and cholestasis can be assessed by the substances produced by the liver. In this study, liver function was evaluated with serum levels of albumine (ALB), total protein, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). No significant difference in any of the hepatic markers analyzed in the acute and subchronic toxicity studies was found (Supplementary table S5). Owing to high standard deviation, no significant difference could be observed, neither for the highest nor for the lowest dose. Liver is a potential target organ for NM (Oberdörster *et al*., 2005; Sadauskas *et al*., 2007); however, hepatotoxicity studies on polymeric nanoparticles are limited. Importantly, a previous work by McClean *et al*. (1998) clarified that NM are

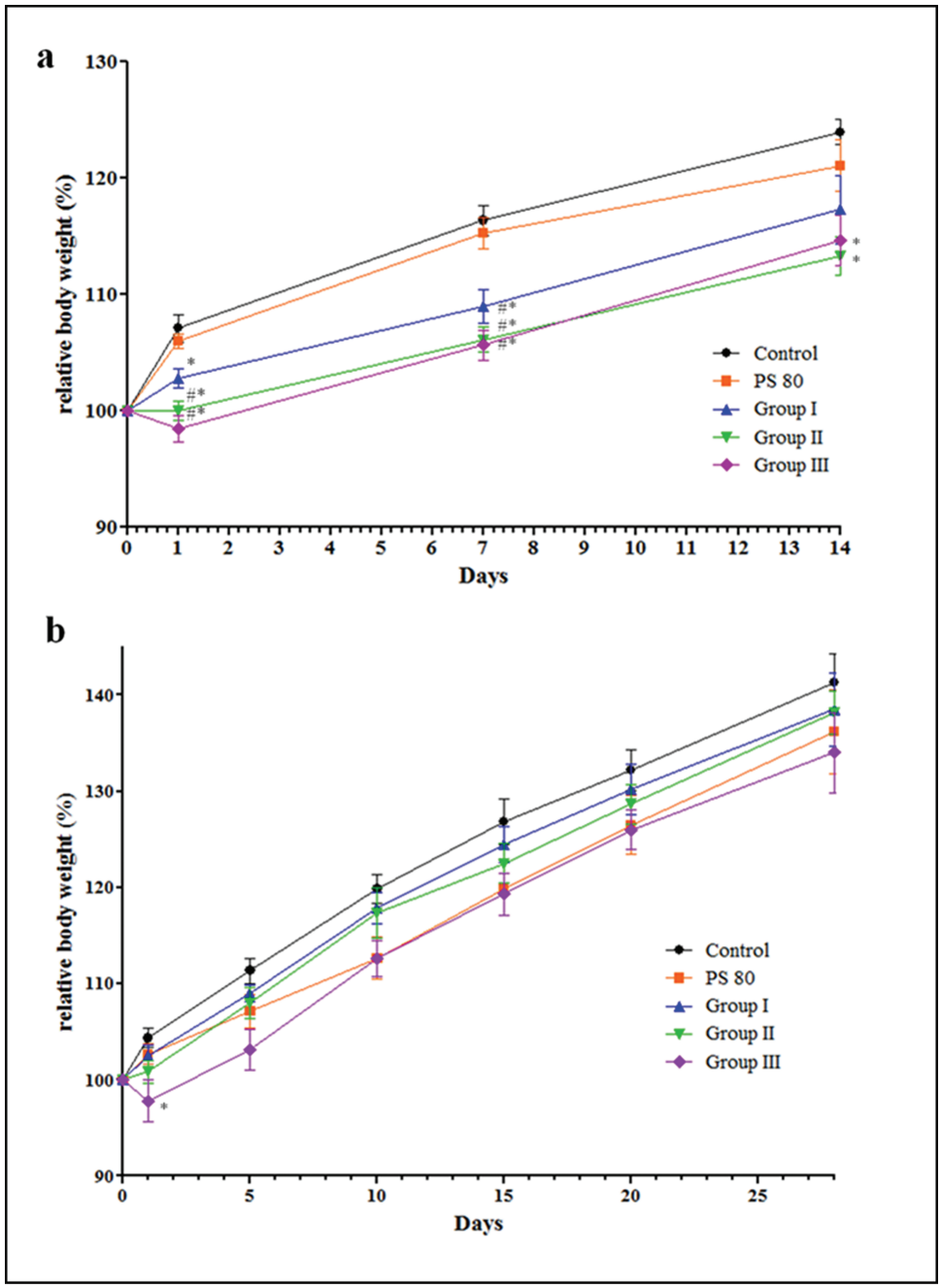

FIG. 4. Relative body weight gain during (a) acute and (b) subchronic treatments. (a) Six rats per group received the following: Control—saline solution (12 ml/kg) and PS80: polysorbate 0.78% (12 ml/kg); Group I—LNC (18.03×10^{12} LNC/kg); Group II—LNC (36.06×10^{12} LNC/kg); Group III—LNC (72.12×10^{12} LNC/kg) single dose by ip. The weight gain was observed on days 0, 1, 7, and 14. (b) Six rats per group received the following: Control—saline solution (3 ml/kg) and PS80: polysorbate 0.78% (3 ml/kg); Group I—LNC (6.01×10^{12} LNC/kg); Group II—LNC (12.02×10^{12} LNC/kg); Group III—LNC (18.03×10^{12} LNC/kg) for 28 days by ip. The weight gain was observed every day for days 0, 1, 5, 10, 15, 20, and 28. Data were analyzed by repeated measures one-way ANOVA. *Significantly different from the Control group, with $p < 0.05$. #Significantly different from PS80 group, with $p < 0.05$.

taken up by the cells of the reticuloendothelial system mainly located in the liver and spleen, following their evaluation of the binding, uptake, and absorption of polylactide micro- and nanoparticles in Caco-2 monolayers and in ileal tissue and gut-associated lymphoid tissue of anesthetized rats and rabbits. Furthermore, it has been reported that this uptake by hepatocytes and Kupffer cells may chronically accumulate and induce toxicity (Sahu and Casciano, 2009). Most of these studies are about metal-based nanoparticles and carbon nanotubes, indicating that oxidative stress plays a major role in the hepatotoxicity

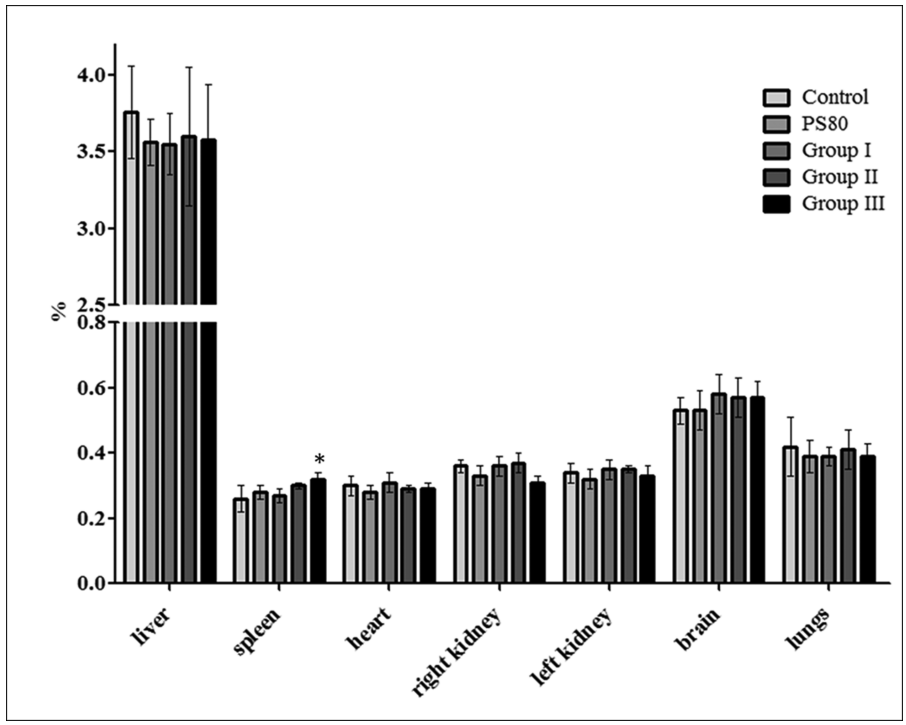

FIG. 5. Relative weight of organs in subchronic toxicity evaluation ($n = 6$). The values are expressed as mean ± SD. Data were analyzed by ANOVA, followed by *post hoc* comparisons (Tukey's test). *Significantly different from control ($p < 0.05$); #Significantly different from PS80 ($p < 0.05$).

of the NM (Patlolla *et al*., 2010; Sayes *et al*., 2005). In our study, enzymatic and nonenzymatic markers of liver damage were not statistically different among the groups, and all of them, even presenting slight alterations, were within the reference values for Wistar rats (Giknis and Clifford, 2008). These findings are in agreement with the histopathological results.

Kidney markers. As nanoparticles circulate, it is likely that they will pass through the complex vasculature of the renal system in the kidneys (Sahu and Casciano, 2009). In a previous work by Frozza *et al*. (2010), the efficiency of the LNC (prepared with PCL and PS80) encapsulating trans-resveratrol versus free trans-resveratrol was evaluated, along with their distribution in rat tissues. Evidence generated from this study showed that significantly higher trans-resveratrol concentrations were transported by LNC to all organs analyzed, compared with free trans-resveratrol, and that the highest concentration of LNC after 14 days of treatment via ip administration was found in kidney followed by liver. To evaluate possible nephrotoxicity of LNC, we measured classic kidney markers, such as BUN, Cr, and UA. No significant differences for these usual kidney markers were found in acute or subchronic treatments in any of the tested groups (Supplementary table S6). Urine was collected in metabolic cages for a period of 12 h (see details in Materials and Methods section and in Supplementary data). Qualitative and semiquantitative urine analyses in acute treatment showed a pH between 7.2 and 7.4 for the five tested groups and specific gravity of around 1.02. In subchronic study, the values for pH were between 7.3 and 7.8, and the specific gravity was around 1.02.

After determining the usual kidney marker results within the reference values, we decided to measure early kidney injury markers (Supplementary fig. S11). We measured urinary microalbumin (MA-U) for glomerular dysfunction evaluation and NAG activity for renal tubule dysfunction. After single- and repeated-dose treatments, MA-U and NAG activities were found to be similar among the groups, and no statistical differences were found. Considering the usual kidney marker results, tubular and glomerular early kidney marker endpoints, and absence of histopathological change (data not shown), it can be concluded that the LNC did not cause appreciable nephrotoxicity under the conditions of these experiments.

Other Biochemical Analysis

Glucose, lipid contents, and BuChE. Blood glucose and lipid contents did not indicate any significant difference at any of the doses in both the experiments (Supplementary table S7). We observed a tendency of decreasing levels of TAG and LDL-C in LNC-treated groups in acute treatment. On the other hand, in subchronic treatment, only Group III presented low levels of these markers. To further study the biochemical mechanism of LNC, BuChE was determined. BuChE has been found in many animal tissues, and it plays a role in the metabolism of lipids and LDL-C. For instance, when the liver is subjected to injury, BuChE activity is significantly elevated, leading to impairment

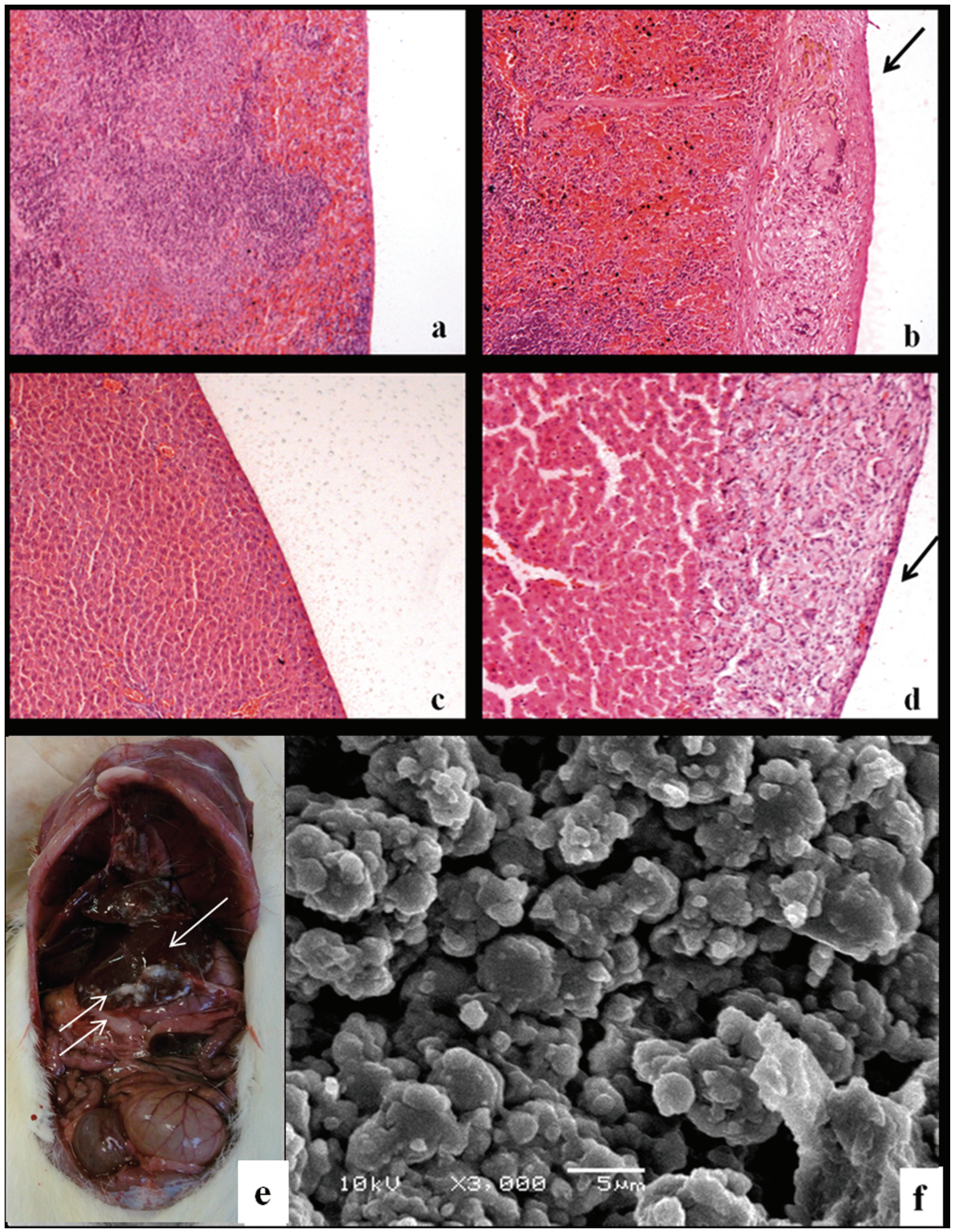

FIG. 6. Representative photomicrographs using hematoxylin and eosin staining of spleen control group (a—100×) and Group III (b—200×); liver control group (c—100×) and Group III (d—200×) in subchronic treatment (n = 4); black arrows indicate a granulomatous foreign body reaction. Acute treatment presented the same histopathological alterations (see Supplementary fig. S10). The LNC agglomeration in abdominal cavity is indicated by white arrows. (e) Wistar rats, 2 days after ip administration of a single dose (72.12×10^{12} LNC/kg), confirmed with (f) SEM analysis. The frequency of granulomatous body was 90% of Group III animals in both the treatments.

in the metabolism of lipids and LDL-C (Ma *et al*., 2009). In both the studies, even slightly decreased BuChE activity was not statistically different among the five groups tested (data not shown). These results are in agreement with those obtained for lipid profile, indicating that LNC did not cause metabolism imbalance of blood lipids and sugars in Wistar rats.

Serum complement C3 and hs-*CRP.* The acute phase response is the nonspecific early response of an organism to infection and inflammation and would be useful for predicting the risk of exposure to NM and their probable toxicities (Higashisaka *et al*., 2011). In our screening, we evaluated C3 for immunologic and *hs*-CRP for inflammatory disturbance

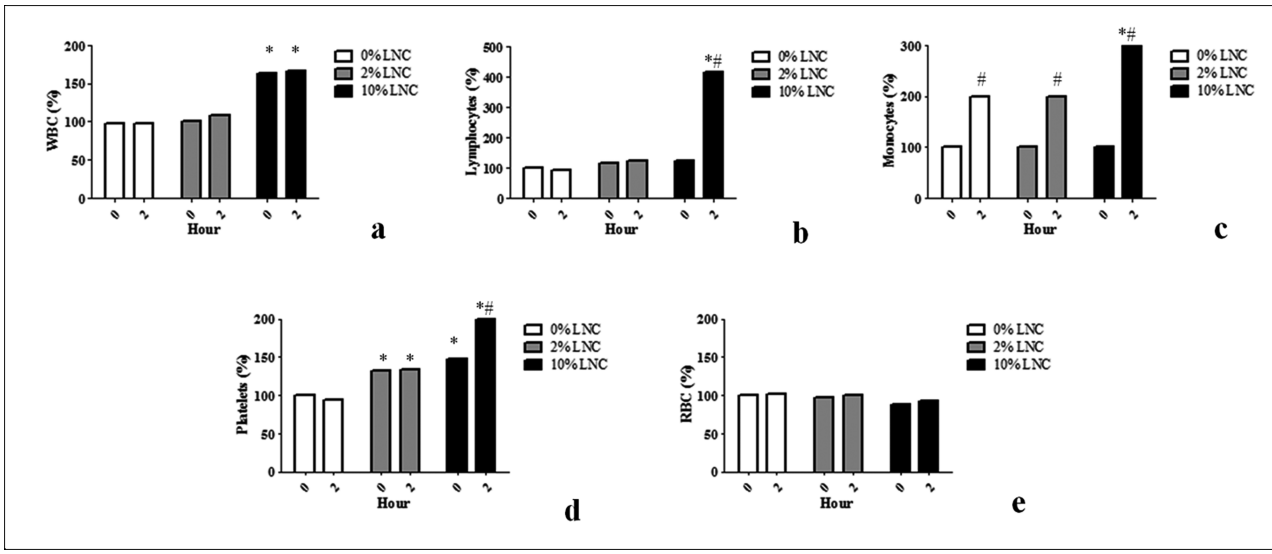

FIG. 7. Tests in blood samples with 0, 2, and 10% (vol/vol) of LNC suspensions ($n = 3$) before (0) and 2 h after blood collection. (a) White blood cells, (b) lymphocytes, (c) monocytes, (d) platelets, and (e) RBC. The data were analyzed by ANOVA followed by *post hoc* comparisons (Tukey's test). *Different from 0% LNC ($p < 0.05$); #different from time (0) ($p < 0.05$).

evaluation. The complement is formed by a group of proteins present in the blood, which interact to defend the body against cells recognized as foreign bodies (Salvador-Morales *et al.*, 2006). The activation of complement is one of the pathways by which nanoparticles may activate the immune system (Sahu and Casciano, 2009; Salvador-Morales *et al.*, 2006). C3 is a marker of immunotoxicity, and previous studies have demonstrated its interaction with nanoparticles in biological systems (Higashisaka *et al.*, 2011; Salvador-Morales *et al.*, 2006). Thus, following inflammatory stimuli and in response to proinflammatory cytokines and complement activation products, the liver secretes a large amount of CRP into the circulation (Szalai and McCrory, 2002). In this study, we determined the CRP levels through *hs*-CRP assay, which is a quantitative analysis to detect very low levels of CRP in blood. *hs*-CRP presented levels below the cutoff limit of 0.05 mg/l (data not shown) for all the groups in both acute and subchronic experiments. The C3 levels decreased in Group II, compared with the other groups in acute treatment, but the quantification was only done after 14 days of single-dose administration. On the other hand, in subchronic treatment, we compared the results obtained after 28 days with basal levels. Similar to acute treatment, Group II presented 26% of decrease, compared with the basal level, and PS80-treated group showed a decrease of only 5% (Supplementary table S8). A decreased C3 level indicates that the alternative complement pathway was activated, which is associated with some disturbance in the complement cascade, necessitating investigation of C3/C3b ratio obtained from crossed immunoelectrophoresis. Nevertheless, nanoparticles designed for systemic administration should be tested for the tendency of activation of the complement system; subsequently, this result can give an indication about which mechanism could be involved.

Hematological Parameters

Among histopathology parameters, the hematology data is a direct reflection of the possible tissue injury caused by the tested compound (Dandekar *et al.*, 2010). Representative hematology results are presented in Table 3. There were certain fluctuations among the groups tested, but they were in the range of normal values and did not indicate any disturbance. In accordance with other studies, increased RBC levels could reflect their overproduction in response to tissue injury (Dandekar *et al.*, 2010). It is well known that erythrocytes occupy a larger volume fraction of the blood than mononuclear phagocytic cells; hence, an injected nanoparticle is likely to interact with RBCs prior to an encounter with other immune cells (Dobrovolskaia *et al.*, 2008). However, these findings can also be interpreted as incidental, neither indicating a trend in toxicity nor falling outside of the expected range for Wistar rats (Dandekar *et al.*, 2010; Hauck *et al.*, 2010).

In relation to white blood cells, only monocyte count was increased in all LNC-treated groups in acute treatment ($p < 0.05$). According to Shaw *et al.* (2008), increased monocyte fraction might be a sign of proinflammatory or other toxic exposures. Besides, monocytes are phagocytic and take up certain nanoparticles more than many other cell types. Moreover, NM have been shown to cause pleiotropic effects on immune cells that are very sensitive to the NM' composition and surface (Dobrovolskaia and McNeil, 2007). Meanwhile, further studies are needed to verify whether the changes in the monocytes level are associated with other immune or adverse phenotypes (Shaw *et al.*, 2008) or if there is some methodological influence. Thus, to assert if there was an influence of the methodology in hematological results, we performed an *in vitro* test using the same cell counter equipment routinely used to assay complete blood counting.

TABLE 3
Hematological Parameters of the Five Study Groups in Acute and Subchronic Treatments

	Control	PS80	Group I	Group II	Group III
Acute					
RBC	5.9 ± 0.7	5.6 ± 0.5	6.8 ± 0.3[a,b]	6.4 ± 0.4	6.5 ± 0.6
Hemoglobin	13.25 ± 0.8	13.28 ± 0.9	13.13 ± 0.3	13.20 ± 1.0	13.6 ± 0.7
Hematocrit	35.7 ± 2.3	35.8 ± 2.6	35.4 ± 0.8	35.3 ± 2.8	36.7 ± 2.1
MCV	60.9 ± 7.3	64.0 ± 4.6	52.0 ± 3.5[a,b]	55.0 ± 3.9	56.9 ± 5.3
MCH	22.5 ± 2.7	23.7 ± 1.7	19.2 ± 1.3[a,b]	20.4 ± 1.4	21.1 ± 1.9
MCHC	37.5 ± 4.0	37.8 ± 2.9	36.2 ± 2.8	36.8 ± 3.4	37.6 ± 3.9
RDW	13.3 ± 0.4	13.1 ± 0.2	13.1 ± 0.0	13.3 ± 0.3	13.3 ± 0.3
WBC	7.7 ± 1.3	6.4 ± 1.0	7.6 ± 1.3	7.8 ± 2.4	6.6 ± 1.8
Granulocytes	1.12 ± 0.3	0.95 ± 0.2	1.30 ± 0.3	1.37 ± 0.3	1.13 ± 0.5
Granulocytes (%)	12.7 ± 2.6	13.3 ± 2.7	15.3 ± 3.3	16.8 ± 3.5	14.9 ± 4.3
Lymphocytes	6.22 ± 0.8	5.07 ± 0.8	5.66 ± 1.0	5.72 ± 2.1	4.98 ± 1.2
Lymphocytes (%)	81.3 ± 3.0	80.2 ± 3.5	75.2 ± 3.9	73.5 ± 4.7	75.9 ± 3.7
Monocytes	0.40 ± 0.1	0.37 ± 0.2	0.63 ± 0.1	0.70 ± 0.3[a,b]	0.53 ± 0.2
Monocytes (%)	5.9 ± 1.3	6.4 ± 2.0	9.3 ± 1.6[a,b]	9.6 ± 2.4[a,b]	9.1 ± 2.3[a,b]
Platelets	692 ± 33	703 ± 74	607 ± 68	680 ± 77	744 ± 113
MPV	6.9 ± 0.6	6.7 ± 0.3	6.9 ± 0.1	7.5 ± 0.6	6.9 ± 0.7
PDW	7.3 ± 1.2	7.5 ± 2.5	6.7 ± 0.7	6.5 ± 1.4	7.2 ± 0.5
Subchronic					
RBC	5.4 ± 0.2	6.3 ± 0.5	6.4 ± 0.4[a]	5.7 ± 0.2	5.5 ± 0.6
Hemoglobin	14.3 ± 0.4	13.2 ± 0.8	13.8 ± 0.7	13.7 ± 1.0	13.4 ± 0.6
Hematocrit	38.7 ± 0.8	35.5 ± 1.97	37.3 ± 1.7	37.0 ± 3.0	36.4 ± 1.5
MCV	71.1 ± 1.6	56.2 ± 5.9[a]	58.2 ± 2.3[a]	64.6 ± 5.2	65.8 ± 6.6
MCH	26.3 ± 0.6	20.8 ± 2.2[a]	21.6 ± 0.8[a]	24.0 ± 1.9	24.4 ± 2.4
MCHC	38.0 ± 1.1	36.4 ± 4.8	38.1 ± 2.6	37.4 ± 3.7	37.7 ± 4.9
RDW	12.8 ± 0.1	13.4 ± 0.1	13.4 ± 0.5	13.9 ± 0.3[a]	13.9 ± 0.3[a]
WBC	7.0 ± 1.6	7.4 ± 1.5	9.2 ± 0.9	8.8 ± 1.7	7.8 ± 1.2
Granulocytes	0.83 ± 0.2	1.05 ± 0.4	0.96 ± 0.2	1.10 ± 0.4	0.96 ± 0.2
Granulocytes (%)	10.9 ± 3.2	13.1 ± 4.5	9.3 ± 2.0	10.7 ± 3.5	11.2 ± 1.4
Lymphocytes	5.90 ± 1.5	6.00 ± 1.3	7.84 ± 0.8	7.34 ± 1.3	6.33 ± 1.0
Lymphocytes (%)	83.4 ± 5.3	80.9 ± 6.1	85.3 ± 2.9	83.5 ± 3.7	81.7 ± 1.8
Monocytes	0.33 ± 0.1	0.40 ± 0.1	0.46 ± 0.1	0.44 ± 0.8	0.50 ± 0.2
Monocytes (%)	5.6 ± 2.0	5.9 ± 1.6	5.3 ± 1.1	5.6 ± 0.5	6.9 ± 0.7
Platelets	719 ± 58	748 ± 198	700 ± 31	710 ± 61	739 ± 37
MPV	6.6 ± 0.1	7.4 ± 0.5	7.4 ± 0.2	7.5 ± 0.3[a]	7.2 ± 0.4
PDW	8.1 ± 0.6	5.6 ± 2.0	6.6 ± 2.0	6.6 ± 0.9	7.1 ± 0.7

Notes. RBC (10^6/mm^3); hemoglobin (g/dl); hematocrit (%); MCV (fl); MCH (pg); MCHC (g/dl); RDW (%); WBC (10^3/mm^3); granulocytes (10^3/mm^3); lymphocytes (10^3/mm^3); monocytes (10^3/mm^3); platelet (10^3/mm^3); MPV (μm^3); PDW (%), platelet volume distribution width. Data were analyzed by ANOVA followed by *post hoc* comparisons (Tukey test). Values are expressed as mean ± SD (n = 6). Different letters within the line indicate significant differences among groups ($p < 0.05$): [a]different from control, [b]different from PS80.

Importantly, toxicological assessment of the nanoparticles provides new challenges to how sciences traditionally conduct toxicity researches. Some of these challenges are interference of nanoparticles with *in vitro* toxicity assays, cellular uptake and visualization, proper characterization, agglomeration, etc. (Dhawan and Sharma, 2010; Doak *et al*., 2009; Kroll *et al*., 2009). One of the major challenges is also the methodological influence, mostly due to particular properties—nanoparticles interfere with normal test systems; they may interfere with fluorescence or visible light absorption detection systems, cause increased catalytic activity due to enhanced surface energy, interfere with adsorption capacity, and interact with a variety of indicator dyes employed in commonly used cytotoxicity assays (Dhawan and Sharma, 2010; Doak *et al*., 2009; Kroll *et al*., 2009; Monteiro-Riviere *et al*., 2009), which has been frequently documented in the literature (Boverhof and David, 2010; Dhawan and Sharma, 2010).

In Vitro *Hematological Analysis*

Hematology is typically performed on automated analyzers, after collection of whole blood. Cell populations are typically distinguished using volume cutoffs to indicate cell type, which are sorted after volume classification and counted. The cells are measured as the resistance through an aperture, similar to the counting and sizing done in flow cytometry (Marquis *et al*., 2009). As mentioned previously, we decided to investigate whether LNC interferes with blood counting analysis using cell counter equipment. Based on a previous work (Bender *et al*., 2012), we added 2 and 10% (vol/vol) of LNC suspension on rat whole blood (for details, see Materials and Methods section).

The results obtained for these tests are presented in Figure 7. White blood cells were increased by around 10 and 65% when adding 2 and 10% of formulation, respectively. There was also a slight increase 2 h after adding LNC. Likewise, lymphocyte count was 15 and 23% higher following addition of 2 and 10% of LNC, respectively. After 2 h, there was an increase of 325% in the test containing 10% of LNC, compared with blood without formulation. Monocytes, which were higher in acute toxicity test, were two times higher after 2 h in blood without LNC and with 2% of LNC. When adding 10% of the formulation, the number of monocytes increased three times after 2 h. The number of platelets increased by around 50% when adding 2 and 10% of LNC and by about 100% after 2 h, compared with 0 and 10% of LNC. Finally, the level of RBC almost did not change in these tests, remaining similar to the blood sample without LNC before and after 2 h. It is important to note that the concentrations of LNC suspensions in blood were chosen based on an acceptable volume for iv bolus administration (lower concentration, 2%, vol/vol) and intermediate concentration used in rats (acute toxicity test), and the higher concentration of 10% vol/vol was an excess. These results demonstrate that such interference could exist, especially with the addition of 10% of suspension, although more tests are required. Alternatively, reference manual RBC and white blood cell counts and white blood cells proportional counts could be performed according to standard protocols using visual enumeration of cells, for example, in a Neubauer counting chamber. In this regard, new methods for the hematological assessment after exposure to nanoparticle will become attractive in the future to obtain relevant and reliable results in studies performed *in vivo*.

CONCLUSION

This study aimed at evaluating the toxicological effects of PCL LNC *per se* after short- and long-term administration in Wistar rats. In acute toxicity test, during the whole observation period, the group of rats treated with LNC had no toxic effects and no death occurred. In both the studies, rats treated with LNC did not show significant difference compared with those of control and PS80-treated groups in blood biochemical markers. Moreover, histopathological findings could help to explain the nonappearance of changes in laboratory parameters of the highest dose group (Group III), except for increased relative weight of spleen. High-dose groups exhibited fewer changes, compared with Groups I and II; this may probably be due to the LNC accumulation in the abdominal cavity, which may have impaired the absorption of the nanocapsules. These results emphasize the importance of finding the right dose that should mimic the actual quantity of the nanoparticles exposure in humans and should not exceed a limit that enhances agglomeration, as observed in the highest dose of this study. Although an *in vitro* analysis to verify methodological influence in hematological parameters was needed, our current data suggest no obvious signals of toxicity in hematological parameters in all LNC-tested groups; meanwhile, it is important to investigate the possible inflammatory mechanisms that could be involved. In addition, preliminary *in vitro* results can help in choosing appropriate methods and equipments for complete blood counting.

Furthermore, these findings are in agreement with earlier reports about no appreciable toxicity of biodegradable polymeric nanoparticles as it is the first study to evaluate nanocapsules that have different physicochemical properties. Besides, the amount of material typically used as a drug carrier is much lower than the ones used in this study, and even the high doses in our tests did not cause any significant systemic toxic effects. The results indicate that LNC might be a safe candidate for drug delivery system.

Nevertheless, this *in vivo* screening might be used to further refine future nanotoxicity studies with successes or failures, because any effort on nanotoxicology might be useful to inform about toxicity, associated risk assessment, and risk management policy for future protocols.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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