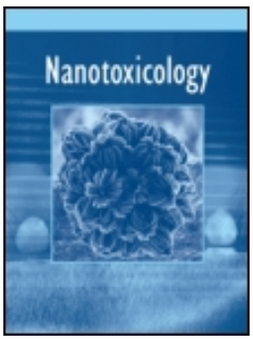

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: https://www.tandfonline.com/loi/inan20

Solid lipid nanoparticles induced hematological changes and inflammatory response in mice

Adny Henrique Silva, Claudriana Locatelli, Fabíola Branco Filippin-Monteiro, Betina G. Zanetti-Ramos, Aline Conte & Tânia Beatriz Creczynski-Pasa

To cite this article: Adny Henrique Silva, Claudriana Locatelli, Fabíola Branco Filippin-Monteiro, Betina G. Zanetti-Ramos, Aline Conte & Tânia Beatriz Creczynski-Pasa (2014) Solid lipid nanoparticles induced hematological changes and inflammatory response in mice, Nanotoxicology, 8:2, 212-219, DOI: 10.3109/17435390.2013.782076

To link to this article: https://doi.org/10.3109/17435390.2013.782076

View supplementary material

Accepted author version posted online: 02 Mar 2013.
Published online: 26 Mar 2013.

Submit your article to this journal

Article views: 192

View Crossmark data

Citing articles: 12 View citing articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=inan20

Nanotoxicology, March 2014; 8(2):212–219
© 2014 Informa UK, Ltd.
ISSN: 1743-5390 print / 1743-5404 online
DOI: 10.3109/17435390.2013.782076

informa
healthcare

Solid lipid nanoparticles induced hematological changes and inflammatory response in mice

Adny Henrique Silva[1], Claudriana Locatelli[2,4], Fabíola Branco Filippin-Monteiro[1], Betina G. Zanetti-Ramos[3], Aline Conte[2,4], & Tânia Beatriz Creczynski-Pasa[1]

[1]*Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, P.O. Box 476, Florianópolis, SC, Brazil,* [2]*Curso de Farmácia, Universidade do Oeste de Santa Catarina, Videira, SC, Brazil,* [3]*Nanovetores-Encapsulados de Alta Tecnologia, SC 401, km1, ParqTecAlfa, Florianópolis, SC, Brazil and* [4]*Universidade do Alto Vale do Rio do Peixe, Caçador, SC, Brazil*

Abstract

Solid lipid nanoparticles (SLNs) are an alternative drug delivery system compared to emulsions, liposomes and polymeric nanoparticles. Due to their unique sizes and properties, SLNs offer possibility to develop new therapeutic approaches. The ability to incorporate drugs into nanocarriers offers a new prototype in drug delivery that could be used for drug targeting. However, toxicity of these new formulations has not been investigated thus far. In this study, we carried out an *in vivo* toxicity study. For that mice were divided into three groups and treated intraperitoneally with triestearin-based SLNs (TN), natural wax-based SLNs (VN) or vehicle for 10 days. After that, necropsies, histopathological and hematological analysis, as well as hepatic and renal functions were performed. Our results indicated that both TN and VN were absorbed post-exposure and induced an inflammatory response in adipose tissue. However, histopathological analysis demonstrated the absence of toxicity in both treated groups. In addition, the body weights were similar among the groups and low toxicity was also indicated by the unchanged serum biochemical parameters. This study provides a preliminary data for toxicological studies of two different SLNs in long-term *in vivo* exposure. However, further studies should be conducted in order to investigate the inflammatory response in order to establish the safety of these SLNs.

Keywords: hepatic and renal function, *in vivo* studies, solid lipid nanoparticles, toxicity

Introduction

Recently, there has been an enormous expansion of research on potential applications of nanotechnology in medicine, including the use of materials structured on a nanometer scale in the delivering and targeting of pharmaceutical products (Brigger et al. 2002; Fadeel & Garcia-Bennett 2010; Faraji & Wipf 2009), and applications in the medical imaging (Cormode et al. 2010). The combination of active ingredients of known pharmacological activity with systems that can enable them to change and adapt to different physicochemical properties without changing their mechanism of action is an attractive alternative for the treatment of many diseases. The current interest in nanostructured systems reflects the need to release the drug in specific areas of the body, with the aim of increasing their therapeutic index while reducing their side effects. In addition to these benefits, the materials at nanosize scale are also capable of protecting the drug against enzymatic degradation, or even chemical or immunological alterations. The use of nanoparticles as drug carriers may also reduce drug toxicity, thus improving its bioavailability and could help increase adherence to prescribed therapeutic treatment (Brigger et al. 2002; Fadeel & Garcia-Bennett 2010).

Different materials used in the production of nanostructured systems are under investigation for their potential use as drug carriers and drug systems (Moghimi et al. 2005). Materials structured on a nanometer scale have different physicochemical properties compare with the same materials on larger scale. The small size, surface area, chemical composition, surface structure, solubility and shape make these formulations very interesting for application in different areas. However, some apparent advantages of such structures may trigger non-desirable effects with potential impact on the public health as well as on the environment (Aillon et al. 2009; Fadeel & Garcia-Bennett 2010; Rico et al. 2011; Semete et al. 2010b; Stern & McNeil 2008). It is assumed that due to the smaller size of nanostructured systems, which is similar to many biological molecules such as proteins, they can easily cross barriers in cells and organelles (Fadeel & Garcia-Bennett 2010). The design of such structures is determinant for their behavior of these materials *in vitro* and *in vivo* (Gratton et al. 2008).

Correspondence: Tânia Beatriz Creczynski-Pasa, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, P.O. Box 476, Florianópolis, SC, 88040-900, Brazil. Tel: +55 48 3721 2212. Fax: +55 48 3721 2212. E-mail: tania.pasa@ufsc.br, taniabcp@gmail.com

(Received 29 November 2012; accepted 28 February 2013)

Among the nanostructured systems, solid lipid nanoparticles (SLNs) have been widely used in medicine, dermocosmetics and cosmetics (Pardeike et al. 2009), which developed formulations for chemotherapy (Chattopadhyay et al. 2007; Joshi & Müller 2009; Wang & Thanou 2010), oral and eye medications (Müller et al. 2006); gene therapy (Kwon et al. 2008) and imaging applications. SLNs are composed by a lipid matrix, solid at body temperature, biocompatible and biodegradable (Joshi & Müller 2009) and usually between 50 and 1000 nm in size. They are prepared using fatty acids, typically combinations of small glycerides and waxes, which are stabilized by biocompatible anionic or cationic surfactants (Wong et al. 2007). However, the clinical use of such formulations requires toxicological risk assessment.

The physical state of the matrix lipid have a pronounced effect on the *in vitro* cell viability, where the crystalline form of SLNs can present high cytotoxicity when compared to some nanoparticle formulations in a liquid form (Petersen et al. 2011b). The internalization and toxicity of different nanoparticles seam to have a strong dependence on particle size and shape (Brown et al. 2007; Gratton et al. 2008).

Though many SLNs did not show risk assessment of their potential toxicological effects in previous studies (Joshi & Müller 2009; Liu et al. 2008; Müller et al. 2006; Yuan et al. 2010), the composition of nanoparticles used in the experiments vary in their nature, percentage and method of preparation (Petersen et al. 2011b). The literatures showing lack of toxicity of SLNs might be due to the differences between the methods and cell lines used in the investigation of toxicity, and the fact that in many cases, it was the drug that was being evaluated and, not the nanoparticles themselves. In this respect, our previous report (Silva et al. 2012) had shown that the cytotoxicity of a series of formulations (named F1 to F8) was dependent on the nature and concentrations of the molecules (lipids or surfactants) used to prepare the nanoparticles. High toxicity of SLNs was observed with sodium dodecylsulphate (SDS) as a surfactant. Based on these results, SLNs based on tristearin (TN) and SLNs based on the natural wax isolated from *Virola surinamensis* (VN) were chosen for *in vivo* studies. Therefore, this study sought to investigate the effects of these SLNs formulations after daily intraperitoneal administration in mice. Based on the results observed, an explanation concerning the influence of composition and SLNs toxicity is proposed.

Methods

Materials

Two types of lipid were employed in nanoparticles preparation: TN (Dynasan 118, Sasol, Germany) and a vegetal wax isolated from *Virola surinamensis* was purchased from Naturais da Amazônia (Belém, PA, Brazil). The surfactants: polyssorbate 80 (Tween 80), SDS and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

SLNs preparation

TN nanoparticles were prepared as previously described (Silva et al. 2012). Briefly, TN was solubilized in chloroform/methanol (1:1). The solvents were removed under dry nitrogen flow and the lipid layer was melted at 73°C (5°C above the lipid melting point, which is 68°C). An aqueous phase was prepared by dissolving Tween 80 (1%) in ultrapure water (MilliQ – Millipore®) to produce 20 ml of the preparation and heated to the molten lipid layer. The hot aqueous phase was added to the lipid phase and subjected to ultrasonic irradiation (Branson Ultrasonics, Danbury, CT, USA) for 2 min at 20 W. TN nanoparticles were cool down to room temperature. VN was prepared as described above, except for the use of organic solvents. The lipids used to prepare SLNs were chosen due to their potential use as drug nanocarries, and since these raw materials were already approved for the use in the cosmetic industry.

SLNs characterization

The average particle size/distribution and zeta potential were determined by dynamic light scattering (DLS) and laser-Doppler anemometry, respectively, using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), equipped with 173° scattering angle. The measurements were made at 25°C after appropriate dilution of the samples in distilled water. The size distribution of particles was given by the polydispersity index (PDI) (being 0.05 and 0.7 the extremes of PDI index possible in DLS analysis, according to the equipment supplier). To measure the zeta potential (Table I), samples were placed in the electrophoretic cell where a potential of ±150 mV was applied. Data were calculated from the mean of electrophoretic mobility using Smoluchowski's equation. In addition, the particle shape was visualized by transmission electron microscopy (JEM-1011 TEM, Tokyo, Japan). The samples were diluted with purified water (1:10) mounted on cooper grids and dried at room temperature. The samples were stained with uranyl acetate 1% for further examination. The microscope was operated at an accelerating voltage of 100 kV. For morphological analysis, the TEM was operated in bright field mode with magnification above 10,000×.

In vitro hemolysis

The hemolytic potential of SLNs was evaluated according to Wang et al. (2009), with modifications. Briefly, blood samples were obtained from healthy male swiss albino mice (6–8 weeks old) exsanguinated by cardiac puncture under halothane anaesthesia and blood was collected into test tubes containing sodium citrate. The erythrocytes were immediately separated by centrifugation at 2000*g* for 5 min and washed three times with 0.9% saline solution. Erythrocytes collected from whole blood were resuspended in 0.9% saline solution. Immediately thereafter, aliquots of the dispersions of SLNs in saline (1, 4 and 8% (w/v)) were incubated with an aliquot of the erythrocytes suspension for 1 h at 37°C with gentle tumbling. After incubation, samples were centrifuged for 5 min at 2000*g*, the supernatants were then collected and centrifuged for 1 h at 10,000*g*. Hemolysis was determined by measuring the absorbance of the supernatant at 405 nm, and water was used as a hypotonic solution for the positive control. A hemolysis ratio less than 5% is normally tolerable for intravenous administration (Rao & Sharma 1997; Wu et al. 2010).

Table I. Characterization of SLNs.

SLN	Lipid	Surfactant	Size (nm)	PDI	Zeta potential (mV)
VN	*Virola surinamensis*	Tween 80	135 ± 2.5	0.17 ± 0.08	–30
TN	Tristearin	SDS	116 ± 3.5	0.21 ± 0.05	–39

Animal care and treatment

Male swiss albino mice (6–8 weeks old) were maintained at 23 ± 2°C with relative humidity of 50–60% under a 12:12 h light:dark cycle with food and water *ad libitum*. Prior to performing the experimental procedures, mice were matched for body weight (25–30 g). Animals used in this study were handled in accordance with The Use on the Principles of Animal Care, previously approved by the Ethics Committee for Animal from University of Contestado (Opinion number 137/08). The animals were divided into three groups ($n = 6$ each): control, which received only the vehicle (saline), VN-treated and TN-treated groups, which received 2 mg/kg/day of each formulation. SLNs and saline were administered through intraperitoneal (i.p.) injection daily for 10 days. The administration was performed intraperitoneally because: (i) the SLNs composition is mainly lipid and it may be degraded when administered orally; (ii) this route mimics the i.v. route since this region of the body is rich vascularized and (iii) the i.p. route is one of the most effective way of dispensing test-drugs into animals under experimentation in a short term-procedure.

In vivo toxicological studies

After administration of SLNs, animals were examined daily. Individual body weights were recorded at the beginning and at the end of the treatment. Mean of the body weight gained was calculated for each group and the weight of the animals' organs (heart, liver, spleen, lung, kidney, stomach and brain) was also measured immediately after euthanasia. Adiposity was determined by the Lee index (cubic root of body weight in grams divided by the naso-anal length in millimetres multiplied by 10^4) (Bernardis & Patterson 1968).

Necropsy and histopathological analysis

At day 11, the animals were killed by cervical dislocation. Organs and tissues (heart, liver, spleen, lung, kidney, stomach, brain and adipose tissue) were carefully examined macroscopically and then weighed. For adipose tissues, abdominal, subcutaneous and epididymal fat were removed and weighed. Histological analysis was carried out on the organs and tissues from the control and SLNs-treated groups. The organs were fixed (4% PBS-formaldehyde), trimmed, processed, embedded in paraffin, sectioned (5 μm diameter), placed on glass microscope slides and stained with hematoxylin and eosin (H&E). Tissues sections were observed under a microscope at a magnification of 400×. In addition, blood was collected for biochemical and hematological analyses.

Hepatic and renal function analysis

Blood samples were collected at day 11, centrifuged at 400*g* for 10 min at room temperature and serum was separated for alanine (ALT) and aspartate (AST) aminotransferases, total protein, albumin, cholesterol and triglycerides. Renal function was assessed by urea and creatinine concentrations. Commercially available kits (Labtest Diagnóstica SA, Brazil) were used, according to the methodology described by the manufacturer (technical semi automated biochemical apparatus Thermo Plate® Analyser).

Hematological analysis

Hematological parameters such as red blood cells (RBC), white blood cells (WBC), lymphocytes and neutrophils count were evaluated according to the method of Garg & Goyal (1992). Hemoglobin, hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were determined according to Pari & Murugavel (2005).

Statistical analysis

The results were presented as mean ± SD ($n = 6$). Data was compared by one-way ANOVA, followed by Bonferroni's test. $P < 0.05$ was taken as statistically significant.

Results

SLNs characterization

Particles size and distribution (PDI) were determined by photon correlation spectroscopy (PCS), as well as SLNs composition and zeta potential are presented in Table I. As observed, the formulations presented diameter of 116 nm for TN and 135 nm for VN and the PDI values were 0.21 and 0.17, respectively (Table I). The values of PDI indicate a relative low polydispersity for both formulations (Supplementary material). Moreover, the TN showed a negatively charged surface with Tween 80, a non-ionic surfactant, while a decrease in the zeta potential was observed (from –30 mV to –39 mV) with VN, due to the presence of surfactant on the surface of negatively charged particles. To obtain more information about size and morphology, TEM analysis was performed. Through the TEM images, it was possible to determine and correlate size with DLS. It was observed that the majority of the particles are roughly spherical in shape with smooth surfaces (Figure 1 and Supplementary material).

In vitro hemolysis test

Hemolysis was an important factor in the evaluation of the biocompatibility of SLNs. The hemolytic properties of SLNs were investigated with three different concentrations of SLNs, 1, 4 and 8% (w/v of distilled water). As shown in Figure 2, TN and VN induced a high hemolytic ratio at all tested concentrations. In the present investigation, hemolytic ratios caused by of TN and VN at the lowest concentration of 1% were 82 ± 10% and 114 ± 8%, respectively ($n = 3$). When the final concentration was 4%, only TN presented a ratio below 90%.

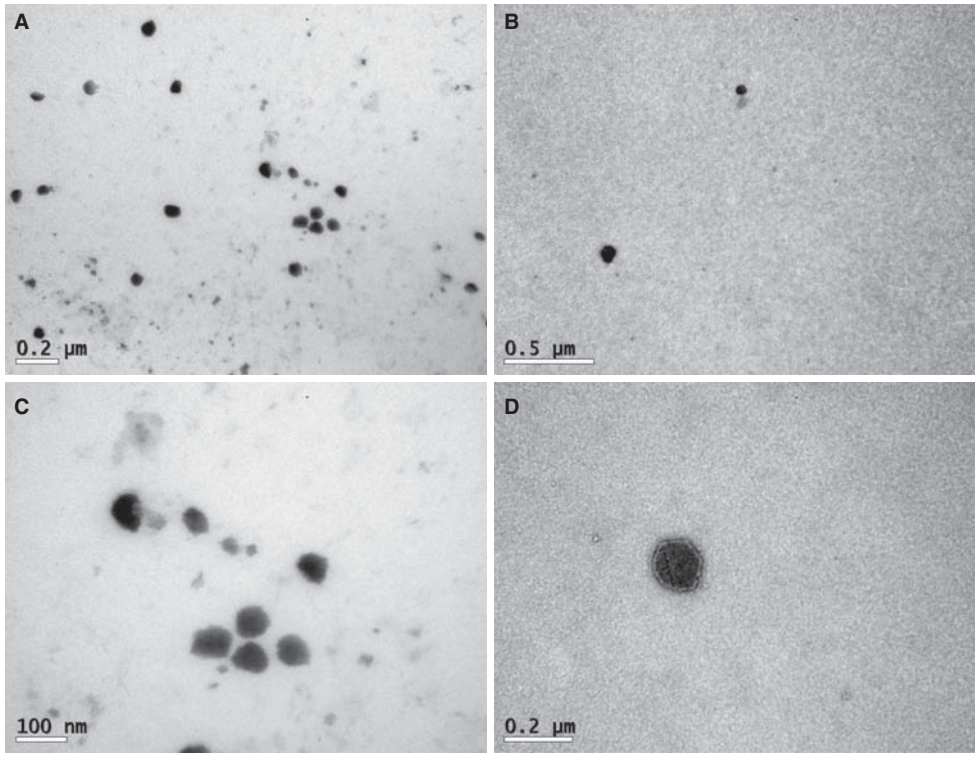

Figure 1. Transmission electron microscopic (TEM) micrograph of (A, C) TN and (B, D) VN.

In vivo toxicological studies

The dose of SLNs (2 mg/kg/day weight body) was determined from our previous *in vitro* cytotoxicity studies on cell culture models (Silva et al. 2012). The dosage was further confirmed as appropriate for this study because of the hemolysis results wherein SLNs at 1% concentration induced a significant lysis in erythrocyte suspensions.

The i.p. administration was elected to prevent possible toxic effects since hemolysis results showed great damage to erythrocytes. The animals tolerated well the i.p. injections and death was not observed throughout the treatment. However, a significant increase in body weight was observed in mice treated with TN and VN (Figure 3C). Macroscopically, an alteration in fat deposition was observed in the SLNs-treated groups. Both TN and VN induced a considerable increase in epididymal and subcutaneous fat; however, visceral adipose tissue was reduced in TN-treated group (0.0137 ± 0.0009 g/g). Conversely, VN-treated animals presented an increased in visceral fat deposition (0.01925 ± 0.0009 g/g vs. control 0.0165 ± 0.001 g/g). There was no difference in Lee index among the groups.

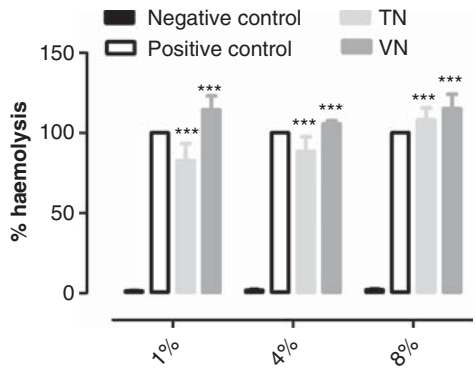

Figure 2. Hemolysis ratio under SLNs exposure. The results were expressed as mean ± SEM ($n = 3$). Hemolysis with water was considered 100%, ***$p < 0.001$.

Necropsy and histopathological analysis

In order to assess possible tissue damage, inflammation or lesions due to the treatment with SLNs, histological examination of kidney, intestine, lung, stomach, liver, heart, brain, spleen and abdominal fat was performed. Sections of the organs appeared to be within normal histological limits in all the treated mice (data not shown) except for abdominal fat where focal mononuclear inflammatory infiltrate was observed in all the treated animals (Figure 4).

Hepatic and renal function analysis

To determine if the SLNs produce hepatic and renal toxicity, various typical biochemical markers (e.g., ALT, AST, urea, creatinine, total protein and albumin) were assessed. Such markers are considered serum indicators to quantitatively assess liver and kidney function, as well as hepatocellular injury. The results were described in Table II. Although a significant increase in urea ($p < 0.05$) was observed in the animals treated with TN, which may be suggestive of kidney injury, non-significant differences were observed between the SLNs-treated and the control group for the other serum indicators after 10 days of exposure.

Hematological analysis

To evaluate the toxicity produced by SLNs exposure, hematological profiles of the animals were determined and

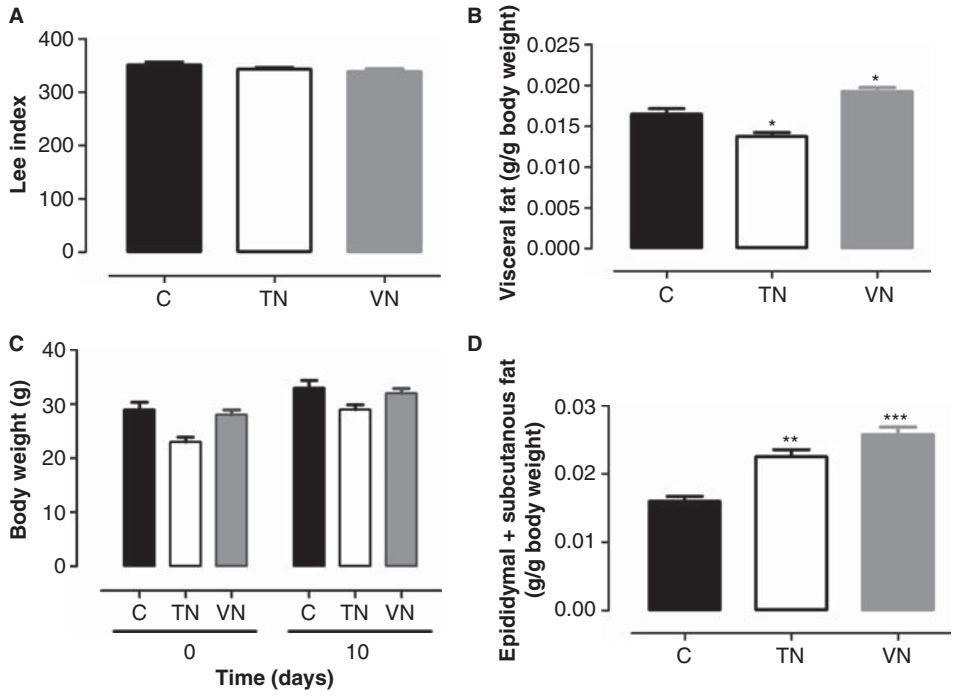

Figure 3. Effect of SLNs treatment on Lee index (A), accumulation of visceral fat (B), body weight (C), epididymal and subcutaneous fat (D). The results are expressed as mean ± SEM ($n = 6$), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

compared with respective values obtained for the control group. Table III shows the result obtained for the hematological analysis. The treatment with TN nanoparticles caused changes in several hematological parameters with significant increase in the total number of RBC ($p < 0.05$), MCV ($p < 0.05$) and neutrophil ($p < 0.05$). Animals treated with VN and TN had an increase in the percentage of neutrophils (VN-group, 20.83 ± 2.88%, TN-group 26.15 ± 1.80% vs. control 14.51 ± 2.13%).

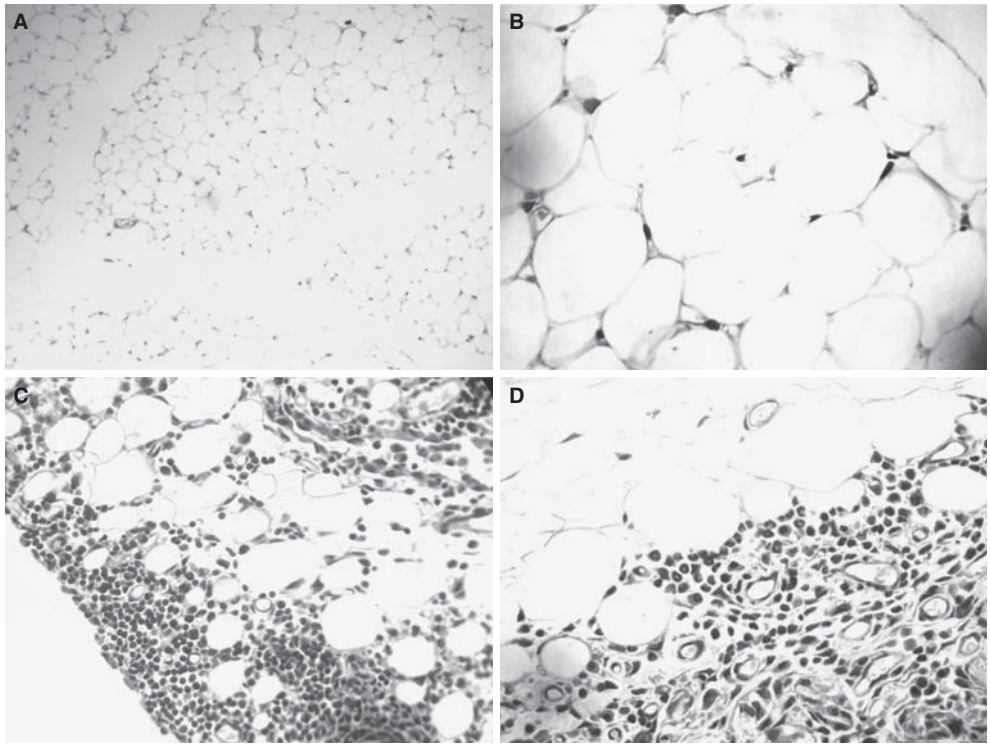

Figure 4. Histological analysis of abdominal fat after treatment with TN and VN. Tissues were stained as indicated in 'Materials and methods'. (A) Images (10×) and (B) (40×) showing the microscopic appearance of the abdominal fat of the animals control, and (C/D) image (40×), the focal mononuclear inflammatory infiltrate of the animals treated with TN and VN, respectively.

Table II. Biochemical parameters regarding liver and kidney injure in the serum of mice treated with 2 mg/kg/day of SLNs.

Parameters	Control	TN	VN
ALT (UI/L)	111 ± 9	87 ± 6.10	93 ± 15
AST (UI/L)	101 ± 10	97 ± 5.6	70 ± 9
Albumin (g/dl)	2.30 ± 0.05	2.24 ± 0.068	2.38 ± 0.091
Total protein (g/dL)	5.07 ± 0.19	4.93 ± 0.3	5.4 ± 0.14
Urea (mg/dL)	62 ± 5	72 ± 3.77*	62 ± 2.67
Creatinine (mg/dL)	0.48 ± 0.08	0.44 ± 0.06	0.39 ± 0.008
Triglycerides (mg/dl)	97 ± 19	83.21 ± 5.74	72.81 ± 6.3
Total Cholesterol (mg/dl)	91 ± 12	98 ± 2.54	104 ± 5.35

*$p < 0.05$ versus control.

Discussion

In this study, the sub-acute toxicity of SLNs (2 mg/kg/day) in mice was investigated following 10-days of exposure period. The results revealed that SLNs promoted inflammation in the adipose tissue and increased visceral and subcutaneous fat deposition in animals treated with TN and VN with a concomitant systemic inflammatory response due to related hematological factors such as increment in white blood cell count.

The hemolysis test was performed in order to evaluate the hemocompatibility of the formulations and the influence of these nanoparticles on the membranes of erythrocytes. SLNs have been extensively used in intravenous drug delivery systems, encapsulated with anticancer agents (Wan et al. 2008; Wong et al. 2006), imaging agents (Müller et al. 1996), anti-HIV (Dianzani et al. 2006), anti-rheumatoid (Olbrich et al. 2004) and antiparasitics (Manjunath & Venkateswarlu 2005). Thus, SLNs may be in direct contact with blood cells, suggesting that evaluation of hemolytic capacity of these materials is an important data (Wu et al. 2010). Our findings indicated that both formulations were very harmful to erythrocyte's membranes, when the formulations were tested *in vitro*. Subsequently, *in vivo* study was conducted using i.p. administration.

To determine the toxicity of TN and VN, animals were treated with both formulations for 10 days and the body weight, hematological, biochemical and histopathological analyses were conducted. The animals were weighed at the beginning of the experiment and 24 h after the last exposure (day 11). No death was observed in the exposed animals during the whole treatment period and a necropsy with histological assessment was performed. It was observed that there was a significant increase in body weight, which varied between the groups, however, Lee index, a simple estimation of body fat, was similar among the exposed groups. It was also observed that there was a significant deposition of visceral and subcutaneous fat as well as an intense inflammatory infiltration in animals treated with SLNs. The accumulation of visceral and subcutaneous fat and further pathological alterations could be attributed to two factors: (a) slow degradation of the lipid matrix used in SLNs (Weyhers et al. 2006) and (b) route of administration used (i.p), which may favor local inflammation and further accumulation of visceral and subcutaneous fat. In addition, the inflammatory reaction observed might the result of the exposure of the animals to the lipid matrix and the surfactants used in the formulation of the SLNs. Conversely, a study (Schöler et al. 2002) has shown that SLNs formulated with stearic acid or dimethyl-dioctadecylammonium bromide (0.01% concentration) also induced a local inflammation favoring fat deposition in adipose tissue.

Our *in vivo* observations are consistent with previous studies, which showed that different SLNs can act as immunomodulators inducing the activation of IFN-α after subcutaneous injection thereby improving lymphatic absorption (Nguyen et al. 2012; Qi et al. 2012). The i.p. administration of lactic co-glycolic acid (PLGA) nanoparticles was also capable of inducing expression of proinflammatory cytokines (including IL-2, IL-6 and TNF-α) in plasma and peritoneal fluid of rats (Semete et al. 2010a). Park and Park (2009) also have shown that single treatment with silica nanoparticles (50 mg/Kg, i.p) activated peritoneal macrophages, increased the levels of IL-1β and TNF-α, and the release of nitric oxide, as well as the expression of inflammation-related genes. The authors also suggested that pro-inflammatory response might have relation with the increased ROS generation. Likewise, our study showed analogs results with noticeable inflammation in adipose tissue, suggesting that SLNs might exert sub-chronic toxicity since the exposure period was 10 days with repeated doses.

SLNs are particulate materials within the range of the sizes of viruses and large proteins, and consequently may induce an inflammatory response (Pantic 2011). VN and TN were prepared with two distinct lipids, with sizes ranging from 116 to 135 nm obtained by DLS measurements. The characterization by TEM revealed quasi round-shaped

Table III. Hematological parameters after treatment with 2 mg/kg/day of SLNs.

Parameters	Control	TN	VN
Erythrocytes (/mm^3)	5 937 000 ± 457	7 615 000 ± 113*	5 582 000 ± 366
Leukocytes (/mm^3)	7 691 ± 689	5 450 ± 334	5 108 ± 500
VCM (fL)	71 ± 3	63 ± 2*	73 ± 2
HCM (pg)	23 ± 3	21 ± 4	25 ± 3
CHCM (%)	31 ± 2	33 ± 4	34 ± 3
Hemoglobin (g/dL)	14 ± 2	16 ± 3	14 ± 3
Hematocrit (%)	43 ± 2	48 ± 6	41 ± 7
Neutrophil (%)	14.51 ± 2.13	26.15 ± 1.80*	20.83 ± 2.88*
Mononuclear (%)	85.66 ± 3.07	73.66 ± 2.89	78 ± 4.16
Eosinophil (%)	1 ± 1	1 ± 1	1 ± 1
Basophil (%)	2.5 ± 1	0 ± 0	0 ± 0

*$p < 0.05$ versus control.

nanoparticles with sizes in a similar range obtained by DLS. It is possible that the nature and the size of the nano or micro structured materials may alter the mechanism of toxicity, but certainly toxicological evaluation is always necessary. It has been reported that particles ranging from 1000 to 4000 nm induced high levels of the neutrophil chemoattractant MIP-2 than the control group (Samuelsen et al. 2009), while nanoparticles ranging from 60 to 200 nm induced the production of other factors leading to neutrophil infiltration. Particularly, one possible factor might be the complement peptide C5a, which is to play a role in the innate immune response (Samuelsen et al. 2009). It also has been proposed that reactive oxygen species (ROS) generated by these nanoparticles might induce the formation of C5a. Our previous work confirmed that these nanoparticles induced the production and release of ROS as a primary mechanism of cytotoxicity (Silva et al. 2012). However the induction of ROS generation might be due to the facility inherent to the particles to enter into the cells, given their size at nanomolar range, which enables unique interaction with biological materials such as key proteins of the cellular pathways (Li et al. 2008). Other important physicochemical characteristic, which might be related to the toxicity of particulate structures, is their morphology and or crystal form (Petersen et al. 2011a). To obtain conclusive results on this subject with SLNs studied here would be necessary to evaluate them thermogravimetricaly, because there are some limitations inherent to TEM analysis due to the heat generation by the equipment during the procedure. Given these reasons, as well as the fact that the formulations induced hemolysis and an evident inflammatory response, sounded to us that it is not worthy further characterization on this aspect.

Interestingly, although an intense inflammation was observed in the adipose tissues, the other organs of the exposed mice appeared with normal aspect in comparison with the control group. Histological evaluation was carried out in order to find possible initial tissue damages, especially in the liver and in the lung. These organs are important components of the reticuloendothelial system and thus are involved in the clearance of nanoparticles (Okuda et al. 2006). Additionally, due to their vascularization, these organs are likely targets for the location of SLNs after intraperitoneal injection. In this study, TN and VN did not promote tissue damage or inflammatory infiltrates neither in the lungs nor in the livers of the exposed mice. However, an increase in white blood cells was observed in animals treated with TN and VN suggesting an increase in the activity of the immune system due to such exposure. These data corroborate the findings of inflammatory response in adipose tissue suggesting a state of sub-acute inflammation. The neutrophil infiltration observed in our work, although not yet understood, was also observed in other studies (Barlow et al. 2005; Samuelsen et al. 2009).

For further assessment of toxicity, biochemical and hematological parameters were monitored to determine whether or not the SLNs caused inflammation or alterations in lipid metabolism, thus acting as indicators of a toxic exposure. Blood analysis only revealed minimal abnormality in kidney function as evidenced by elevated urea levels only in TN-treated group. Urea is a metabolite associated with functionality of the kidneys and some fluctuation may result from various processes. However, the increased urea serum levels observed (15%) (Table II) may be an indicative of a discrete renal toxicity (Lasagna-Reeves et al. 2010). Besides that no additional alterations were observed in the other biochemical parameters assessed. The absence of a more expressive hematological change might be associated with the short exposure period with SLNs. A similar study showed that 7 days of treatment with poly (lactic-co-glycolic acid) (PLGA) nanoparticles did not cause any lesion or inflammation assessed by histopathology analysis (Semete et al. 2010a, b). A similar finding of Stebounova et al. (2011) showed that mice sub-acutely exposed to silver nanoparticles by inhalation (3.3 mg/m^3, 4 h/day to 10 days) a minimal pulmonary inflammation or cytotoxicity was observed, demonstrating that long-term exposure studies are necessary to evaluate possible effect in other organs.

Additionally, our study, like other sub-chronic evaluations concerning nanoparticles of a varied composition, was no longer enough to assess carcinogenesis, mutagenesis or even neurotoxicity.

Conclusions

Here, we reported an *in vivo* exposure to two different SLNs in mice. Overall, animals survived without any observational evidence of toxic effect, except for an inflammatory response in adipose tissue. Though the exact mechanism responsible for this response is not clear at present, studies have suggested the effect of SLNs components at the adipose tissue sites. This kind of nanoparticle should be theoretically biocompatible by the nature of the lipids, but surprisingly we faced with an inflammation process, allowing us to conclude that this study deserves attention since it provides basis for further toxicological studies of SLNs in long-term *in vivo* exposure as well as to investigate the profile of the inflammatory response.

Acknowledgments

This study was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and a PNPD-fellowship for Fabíola B. F. Monteiro, PhD and a doctoral REUNI-fellowship for Adny Henrique Silva, MSc from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), and grants from FAPESC (Fundação de Amparo à Pesquisa de Santa Catarina).

Declaration of interest

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

References

Aillon KL, Xie Y, El-Gendy N, Berkland CJ, Forrest ML. 2009. Effects of nanomaterial physicochemical properties on in vivo toxicity. Adv Drug Deliv Rev 61(6):457–466.

Barlow PG, Donaldson K, MacCallum J, Clouter A, Stone V. 2005. Serum exposed to nanoparticle carbon black displays increased potential to induce macrophage migration. Toxicol Lett 155 (3):397–401.
Bernardis LL, Patterson BD. 1968. Correlation between 'Lee index' and carcass fat content in weanling and adult female rats with hypothalamic lesions. J Endocrinol 40(4):527–528.
Brigger I, Dubernet C, Couvreur P. 2002. Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev 54(5):631–651.
Brown SC, Kamal M, Nasreen N, Baumuratov A, Sharma P, Antony VB, et al. 2007. Influence of shape, adhension and simulated lung mechanics on amorphous silica nanoparticle toxicity. Adv Powder Technol 18(1):69–79.
Chattopadhyay P, Shekunov BY, Yim D, Cipolla D, Boyd B, Farr S. 2007. Production of solid lipid nanoparticle suspensions using supercritical fluid extraction of emulsions (SFEE) for pulmonary delivery using the AERx system. Adv Drug Deliv Rev 59(6):444–453.
Cormode DP, Jarzyna PA, Mulder WJM, Fayad ZA. 2010. Modified natural nanoparticles as contrast agents for medical imaging. Adv Drug Deliv Rev 62(3):329–338.
Dianzani C, Cavalli R, Zara GP, Gallicchio M, Lombardi G, Gasco MR, et al. 2006. Cholesteryl butyrate solid lipid nanoparticles inhibit adhesion of human neutrophils to endothelial cells. Br J Pharmacol 148(5):648–656.
Fadeel B, Garcia-Bennett AE. 2010. Better safe than sorry: understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. Adv Drug Deliv Rev 62(3):362–374.
Faraji AH, Wipf P. 2009. Nanoparticles in cellular drug delivery. Bioorg Med Chem 17(8):2950–2962.
Garg DK, Goyal RN. 1992. Hematological and hepatotoxic effects of silken styles of corn in albino-rats. J Appl Toxicol 12(5):359–363.
Gratton SE, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, et al. 2008. The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci USA 105(33):11613–11618.
Joshi MD, Müller RH. 2009. Lipid nanoparticles for parenteral delivery of actives. Eur J Pharm Biopharm 71(2):161–172.
Kwon SM, Nam HY, Nam T, Park K, Lee S, Kim K, et al. 2008. In vivo time-dependent gene expression of cationic lipid-based emulsion as a stable and biocompatible non-viral gene carrier. J Control Release 128(1):89–97.
Lasagna-Reeves C, Gonzalez-Romero D, Barria MA, Olmedo I, Clos A, Sadagopa Ramanujam VM, et al. 2010. Bioaccumulation and toxicity of gold nanoparticles after repeated administration in mice. Biochem Biophys Res Commun 393(4):649–655.
Li N, Xia T, Nel AE. 2008. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 44(9):1689–1699.
Liu J, Gong T, Fu H, Wang C, Wang X, Chen Q, et al. 2008. Solid lipid nanoparticles for pulmonary delivery of insulin. Int J Pharm 356 (1-2):333–344.
Manjunath K, Venkateswarlu V. 2005. Pharmacokinetics, tissue distribution and bioavailability of clozapine solid lipid nanoparticles after intravenous and intraduodenal administration. J Control Release 107(2):215–228.
Moghimi SM, Hunter AC, Murray JC. 2005. Nanomedicine: current status and future prospects. FASEB J 19(3):311–330.
Müller RH, Maaβen S, Weyhers H, Specht F, Lucks JS. 1996. Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles. Int J Pharm 138(1):85–94.
Müller RH, Runge S, Ravelli V, Mehnert W, Thünemann AF, Souto EB. 2006. Oral bioavailability of cyclosporine: solid lipid nanoparticles (SLN) versus drug nanocrystals. Int J Pharm 317(1):82–89.
Nguyen DN, Mahon KP, Chikh G, Kim P, Chung H, Vicari AP, et al. 2012. Lipid-derived nanoparticles for immunostimulatory RNA adjuvant delivery. Proc Natl Acad Sci USA 109(14):E797–E803.
Okuda T, Kawakami S, Maeie T, Niidome T, Yamashita F, Hashida M. 2006. Biodistribution characteristics of amino acid dendrimers and their PEGylated derivatives after intravenous administration. J Control Release 114(1):69–77.
Olbrich C, Gessner A, Schröder W, Kayser O, Müller RH. 2004. Lipid–drug conjugate nanoparticles of the hydrophilic drug diminazene—cytotoxicity testing and mouse serum adsorption. J Control Release 96(3):425–435.
Pantic I. 2011. Nanoparticles and modulation of immune responses. Sci Prog 94(Pt 1):97–107.
Pardeike J, Hommoss A, Müller RH. 2009. Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. Int J Pharm 366(1-2):170–184.
Pari L, Murugavel P. 2005. Role of diallyl tetrasulfide in ameliorating the cadmium induced biochemical changes in rats. Environ Toxicol Pharmacol 20(3):493–500.
Park EJ, Park K. 2009. Oxidative stress and pro-inflammatory responses induced by silica nanoparticles in vivo and in vitro. Toxicol Lett 184 (1):18–25.
Petersen S, Steiniger F, Fischer D, Fahr A, Bunjes H. 2011a. The physical state of lipid nanoparticles influences their effect on in vitro cell viability. Eur J Pharm Biopharm 79(1):150–161.
Petersen S, Steiniger F, Fischer D, Fahr A, Bunjes H. 2011b. The physical state of lipid nanoparticles influences their effect on in vitro cell viability. Eur J Pharm Biopharm 79(1):150–161.
Qi J, Lu Y, Wu W. 2012. Absorption, disposition and pharmacokinetics of solid lipid nanoparticles. Curr Drug Metab 13(4):418–428.
Rao SB, Sharma CP. 1997. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J Biomed Mater Res 34(1):21–28.
Rico CM, Majumdar S, Duarte-Gardea M, Peralta-Videa JR, Gardea-Torresdey JL. 2011. Interaction of nanoparticles with edible plants and their possible implications in the food chain. J Agric Food Chem 59(8):3485–3498.
Samuelsen M, Nygaard UC, Løvik M. 2009. Particle size determines activation of the innate immune system in the lung. Scand J Immunol 69(5):421–428.
Schöler N, Hahn H, Müller RH, Liesenfeld O. 2002. Effect of lipid matrix and size of solid lipid nanoparticles (SLN) on the viability and cytokine production of macrophages. Int J Pharm 231(2): 167–176.
Semete B, Booysen L, Lemmer Y, Kalombo L, Katata L, Verschoor J, et al. 2010b. In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine 6(5):662–671.
Semete B, Booysen LI, Kalombo L, Venter JD, Katata L, Ramalapa B, et al. 2010a. In vivo uptake and acute immune response to orally administered chitosan and PEG coated PLGA nanoparticles. Toxicol Appl Pharmacol 249(2):158–165.
Silva AH, Filippin-Monteiro FB, Mattei B, Zanetti-Ramos BG, Creczynski-Pasa TB. 2012. In vitro biocompatibility of solid lipid nanoparticles. Sci Total Environ 432C:382–388.
Stebounova LV, Adamcakova-Dodd A, Kim JS, Park H, O'Shaughnessy PT, Grassian VH, et al. 2011. Nanosilver induces minimal lung toxicity or inflammation in a subacute murine inhalation model. Part Fibre Toxicol 8(1):5.
Stern ST, McNeil SE. 2008. Nanotechnology safety concerns revisited. Toxicol Sci 101(1):4–21.
Wan F, You J, Sun Y, Zhang X-G, Cui F-D, Du Y-Z, et al. 2008. Studies on PEG-modified SLNs loading vinorelbine bitartrate (I): Preparation and evaluation in vitro. Int J Pharm 359(1–2):104–110.
Wang JJ, Liu KS, Sung KC, Tsai CY, Fang JY. 2009. Lipid nanoparticles with different oil/fatty ester ratios as carriers of buprenorphine and its prodrugs for injection. Eur J Pharm Sci 38(2):138–146.
Wang M, Thanou M. 2010. Targeting nanoparticles to cancer. Pharmacol Res 62(2):90–99.
Weyhers H, Ehlers S, Hahn H, Souto EB, Müller RH. 2006. Solid lipid nanoparticles (SLN)–effects of lipid composition on in vitro degradation and in vivo toxicity. Pharmazie 61(6):539–544.
Wong HL, Bendayan R, Rauth AM, Li Y, Wu XY. 2007. Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles. Adv Drug Deliv Rev 59(6):491–504.
Wong HL, Rauth AM, Bendayan R, Manias JL, Ramaswamy M, Liu Z, et al. 2006. A new polymer-lipid hybrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug-resistant human breast cancer cells. Pharm Res 23(7):1574–1585.
Wu W, Chen B, Cheng J, Wang J, Xu W, Liu L, et al. 2010. Biocompatibility of Fe3O4/DNR magnetic nanoparticles in the treatment of hematologic malignancies. Int J Nanomedicine 5:1079–1084.
Yuan H, Zhang W, Du YZ, Hu FQ. 2010. Ternary nanoparticles of anionic lipid nanoparticles/protamine/DNA for gene delivery. Int J Pharm 392(1-2):224–231.

Supplementary material available online

Supporting information