Article

Toxicology and Industrial Health

Toxicology and Industrial Health
2017, Vol. 33(2) 147–158

DOI: 10.1177/0748233715613224
journals.sagepub.com/home/tih
SAGE

Visceral fat increase and signals of inflammation in adipose tissue after administration of titanium dioxide nanoparticles in mice

Adny H Silva[1], Claudriana Locatelli[2], Ubirajara PR Filho[3], Bruna F Gomes[4], Rui M de Carvalho Júnior[1], Jefferson S de Gois[1], Daniel LG Borges[1] and Tânia B Creczynski-Pasa[1]

Abstract

Titanium dioxide nanoparticles (TiO_2 NP) are present in several daily use products, and the risks associated with their bioaccumulation must be stablished. Thus, an evaluation of several toxicological-related effects was conducted after intraperitoneal injection of TiO_2 NPs in mice. Mice were divided into two groups, which received 2 mg kg^{-1} day^{-1} of TiO_2 NPs or vehicle saline. Assessments of body and organ weight as well as biochemical, hematological, and histopathological analyses were performed in order to evaluate adverse effects. The results showed that treatment resulted in an increased visceral and abdominal fat deposition, as well as a mononuclear inflammatory infiltrates in the abdominal fat tissue. The TiO_2 NPs induced significant decrease in the weight gain and splenomegaly. Additionally, TiO_2 NP-treated mice showed altered hematological parameters and significant liver injuries, which were characterized by histopathological and biochemical changes. Our results also indicated that TiO_2 NPs were absorbed and significantly accumulated in the spleen, liver, and kidney. These results showed the ability of TiO_2 NPs to infiltrate different organs and to induce inflammation and liver and spleen damage with visceral fat accumulation. The data obtained are useful for the governmental authorities to legislate and implement regulations concerning the use and the production of this kind of material that might be hazardous to the living beings, as well as to the environment.

Keywords

Nanotoxicology, titanium accumulation, inflammation, toxicity, *in vivo*

Introduction

The use of nanoparticles (NPs) in several areas, including communication, engineering and medicine has increased due to the physical and chemical features that are unique to nanostructured materials, such as size, shape, and surface area (Janer et al., 2014; Jin et al., 2008; Liu, 2006). However, due to the small size of NPs, that is similar to many biological molecules, they may easily pass through the tissues, cells and organelles, leading to potential toxic effects, which cannot be ignored (Chen et al., 2006).

Titanium dioxide (TiO_2) is a white odorless powder, which naturally exists, in the anatase, rutile, and brookite mineral forms. It is able to absorb ultraviolet radiation and reflect visible light and is often used as a white pigment in a wide range of products such as paints, paper, plastic, ceramic, sunscreen, cosmetics, pharmaceutical, and food additives (Daughton and

[1]Universidade Federal de Santa Catarina, Trindade, Florianópolis, SC, Brasil
[2]Universidade do Oeste de Santa Catarina, Videira, SC, Brasil
[3]Universidade de São Paulo, São Carlos, SP, Brasil
[4]Instituto de Química de São Carlos, São Carlos, SP, Brasil

Corresponding author:
Adny H Silva, Campus Universitário, Universidade Federal de Santa Catarina, Trindade, 88040900 Florianópolis, SC, Brasil.
Email: adnyh@yahoo.com.br

Ternes, 1999; Jin et al., 2008; Liu et al., 2009; Wang et al., 2007). In addition to toxicity, failures or accidents during the production of nanomaterials can generate the release of a significant amount of NPs to the environment (Moore, 2006). For instance, a study has shown the accumulation of most metal-based NPs in plants as well as carbon nanotubes and fullerene (Rico et al., 2011). TiO_2 is probably already present in the environment, although the current levels are unknown. Estimations of TiO_2 levels in the aquatic environment, based on modeling approaches, have suggested concentrations as high as 0.7–16 μg L^{-1} in water (Mueller and Nowack, 2008; Scown et al., 2009).

Although many researchers have demonstrated that TiO_2 NPs have toxic activity (Huang et al., 2009; Park et al., 2008; Pujalté et al., 2011; Soto-Alvaredo et al., 2014; Trouiller et al., 2009; Turkez, 2011), misinterpretation occurs, especially due to the fact that some toxicological assays used to determine the behavior of this material *in vitro* suffer interference from metallic NPs. Our previous *in vitro* studies (data not shown) with TiO_2 NPs demonstrated increased viability in Vero and MDCK cells after 24 h of incubation with NPs, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red viability assays. According to Wang and collaborators, MTT and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*h*-tetrazolium-5-carboxanilide (XTT) assays, when used to determine the toxicity of titanium (Ti) NPs, can result in inaccurately and overestimated cell toxicity in viability assays (Wang et al., 2011). Conversely, some misinterpretation may happen using the lactate dehydrogenase (LDH) assay, as shown by Han and colleagues (Han et al., 2011). In this work, it was demonstrated that NPs of copper, silver and Ti could interfere in the viability assay, resulting in LDH inactivation by adsorption of NPs on LDH molecules. Despite of the controversies in the literature concerning the biocompatibility of TiO_2 NPs, the use of this kind of NPs in different areas is still very common (Devanand Venkatasubbu et al., 2013; West, 1984). Thus, it is necessary to understand the mechanisms that determine the behavior of NPs, not only for their development but also in a tentative to predict the toxicological responses related to nanomaterials as well as their extent in living organisms and in the environment in general (Asare et al., 2012; Fadeel and Garcia-Bennett, 2010).

As an attempt to circumvent the interference in the viability assays and to bring new prospects to this theme, an *in vivo* model was used in this work to evaluate the potential hazard of a TiO_2 NP formulation, or perhaps its biocompatibility.

Materials and methods

NPs characterization

Commercial TiO_2 NPs were obtained from Sigma-Aldrich (St Louis, Missouri, USA). The NPs were a mixture of anatase and rutile TiO_2, with particles of mean diameter around 100 nm, based on the analysis report of the supplier (Sigma-Aldrich). To check the size and the shape of TiO_2 NPs, they were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The structure, composition and optical properties of NPs were analyzed using X-ray diffractometry (XRD) and Raman spectrometry. For DLS analysis, powdered TiO_2 NPs were suspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (10 mmol L^{-1}) already adjusted to pH 7.5 with a sodium hydroxide (NaOH) solution. The suspension was sonicated for 1 h before initiating the analysis. Particle size determination was carried out using a Zetatrac DLS system (Microtrac, Montgomeryville, Pennsylvania, USA), which utilizes a diode laser emitting at 780 nm. The run time and number of runs used for each sample measurement were 60 s and 3 s, respectively. For TEM evaluation, NPs were dispersed by dip coating on silicon dioxide (SiO_2)/silicon (Si) (100), held a cross section of the film by ultramicrotome, and the slices were deposited on copper coated with carbon and palladium. High-resolution TEM analysis was performed with a JEOL (JEM2100, Japan) TEM operated at an accelerating voltage of 200 kV and equipped with a LaB_6 electron gun. Before the *in vivo* studies, NPs were suspended in sterile water and sonified for 1 h.

The structure, composition, and optical properties of NPs were analyzed using an X-ray powder diffractometer and the Raman spectra of the TiO_2 powder were obtained with an LSI Dimension P-2 Raman spectrometer (Lambda solution, Seattle, Washington, USA) equipped with a 750 nm red laser and charge coupled device detector. The powder were recorded in 2θ range of 10–70 at 0.5 degree min^{-1} with a Rigaku Rotaflex RU-200 X-Ray diffractometer (Japan) using copper K_α X-ray radiation at 1.540 A°. The relative crystallite size was estimated using Scherrer formula (equation 1) based on the

broadening of diffraction peak at a 2θ value of 48.28, which corresponds to (200) reflection of anatase.

$$D = \frac{0.9\lambda}{B\cos\theta_B}, \quad (1)$$

where, D is the crystallite size (nm), λ is the X-ray wavelength (1.540 Å), and B is the peak width (radians) at half the peak height and θ_B is the Bragg angle obtained by diving by 2 the 2θ value of the corresponding peak (i.e. $\theta = 2\theta/2$).

Raman spectra of the TiO_2 powder were obtained with an LSI Dimension P-2 Raman spectrometer (Lambda solution) equipped with a 750 nm red laser and CCD detector. The laser power was adjusted to 150 mV, and an integration time of 20 s and 20 frames per measurement were used as the spectra acquisition parameters.

In vivo toxicological assays

Animal care and treatment. Swiss albino male mice (6–8-weeks old) were maintained at 23 ± 2°C and relative humidity of 50–60% under a 12-h light/12-h dark cycle, given food and water *ad libitum*. Prior to the experimental procedures, the mice were matched for body weight (25–30 g). The experimental procedures were previously approved by the Ethics Committee for Animal Use (137/08). The weight-matched animals were divided into two groups (6 individuals each): the control group, which received only the vehicle (saline) and the treated group, which received 2 mg kg^{-1} day^{-1} of TiO_2 NPs. The solutions were administered intraperitoneally daily for 10 days.

In the Organization for Economic Co-operation and Development (OECD) Guideline, it is suggested that the doses used to investigate the toxicity of chemicals must be between 5 and 5000 mg kg^{-1}. Thus, the dose used was chosen based on the OECD guideline and (i) *in vitro* studies of TiO_2 NP toxicity in cell culture; (ii) the maximum tolerated dose to mice that cannot be higher than 10% body weight; and (iii) on the main goal of this study, since higher doses lead to aggregation and poor absorption, preventing the systemic toxicity testing. Furthermore, the period of 10 days for the treatment was chosen to characterize this study as acute (OECD, 2001).

General examination. After administration of TiO_2 NPs, the animals were examined daily. They were weighed at the beginning and at the end of the treatment (Lasagna-Reeves et al., 2010). On the final day of the treatment, the animals were euthanized by cervical dislocation after ether anesthesia, blood was collected and selected organs (heart, liver, spleen, lung, kidney, stomach, brain, and adipose) were removed and weighed for the analysis of morphological changes. Tissues were fixed in 4% phosphate-buffered saline–formaldehyde (Sigma-Aldrich Corporate) and processed for histopathology. Blood was used to evaluate hepatic, renal, and hematologic parameters.

Hepatic and renal function analysis. Blood samples were centrifuged at 800 × *g* for 10 min at room temperature, and serum was separated to measure the alanine transferase (ALT) and aspartate aminotransferase (AST) activities, total protein, albumin, urea, and creatinine. Commercially available kits (Labtest Diagnóstica SA, Lagoa Santa, MG, Brazil) were used for biochemical assays, and performed as per manufacturer's instructions (technical semi-automated biochemical analyzer Thermo Plate® Analyzer).

Hematological and histopathological analysis. Hematological parameters such as red blood cell (RBC) number, white blood cell (WBC) number, lymphocyte, and neutrophil counts were evaluated according to a previously published method (Garg and Goyal, 1992). The serum content of hemoglobin, hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were determined according to reported method (Pari and Murugavel, 2005).

Tissues were processed by standard histopathological techniques. Fixed tissues were processed through dehydration in graded alcohol series, clearing in xylene and embedding in paraffin blocks. The embedded tissues were sectioned into 5 μm thickness and stained with hematoxylin and eosin. Tissue sections were observed in a microscope at a magnification of 400×.

Titanium content analysis. Samples of each organ were lyophilized and subsequently weighed directly on polyfluoroalcoxy (PFA) flasks. Afterward, 3 mL of nitric acid, which had been previously purified by double sub-boiling distillation in a quartz still (Kürner Analysentechnik, Rosenheim, Germany), 3 mL of deionized water (Milli-Q, Millipore, Bedford, USA) and 2 mL of high-purity H_2O_2 (Vetec, São Paulo, Brazil) were added and the mixture was subject to a microwave-assisted digestion program using an Ethos Plus microwave oven equipment (Milestone, Sorisole, Italy). The final solutions were diluted according to their Ti concentrations prior to inductively coupled

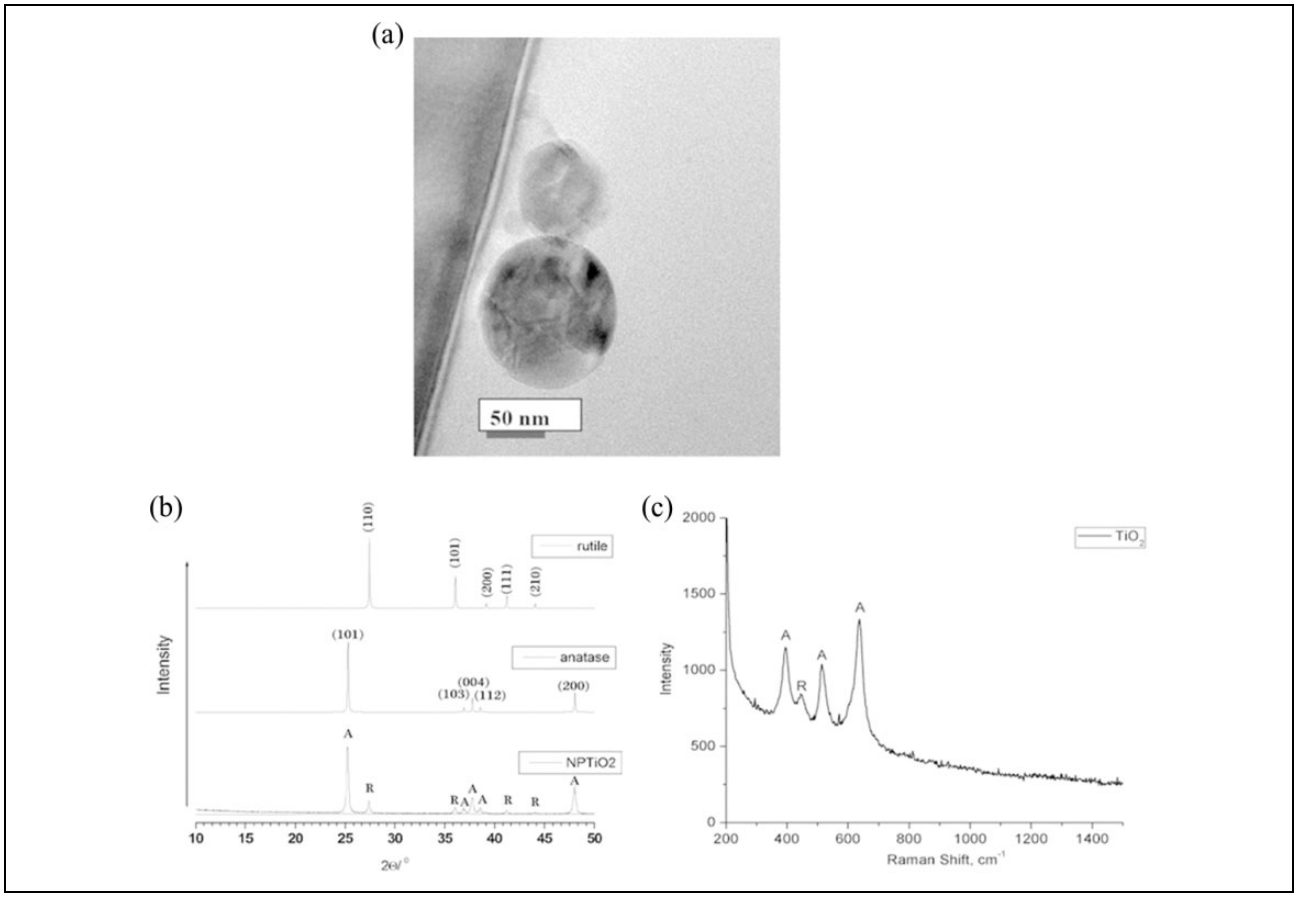

Figure 1. Characterization of TiO_2 NPs using high resolution transmission electron micrography of TiO_2 NPs. (a), X-ray power diffractometry (b) and Raman spectra of the TiO_2 powder (c). TiO_2 NPs: titanium dioxide nanoparticles.

plasma mass spectrometry (ICP-MS) analysis. The measurements were carried out using a NexION 300D inductively coupled plasma mass spectrometer (Perkin Elmer-Sciex, Thornhill, Canada); argon 99.996% (White Martins, São Paulo, Brazil) was used as the plasma and nebulizer gas. Rhodium (10 μg L^{-1}) was used as internal standard and recovery tests were performed to confirm the absence of interfering (recoveries obtained ranged from 92 to 114%). The detection limit for Ti obtained as 3 times the standard deviation of 10 readings of a blank solution was 0.3 μg g^{-1} (ppm). The method was proven to be free of spectroscopic interfering (Martin-Camean et al., 2014) monitoring the isotopes.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM) ($n = 6$) and data were compared by one-way analysis of variance, followed by Bonferroni's test; $^*p < 0.05$ was taken as statistically significant.

Results

NPs characterization

DLS was used to characterize the TiO_2 NP size and surface charge (zeta potential). The average size of TiO_2 NP was 118 ± 30 nm and the surface was negatively charged by −36.82 mV (data not shown). The image obtained using TEM of two isolated NPs on SiO_2/Si (100) is shown in Figure 1(a). The larger particles have a diameter of about 125 nm, confirming the results obtained by DLS measurements. Figure 1(b) illustrates the XRD patterns of TiO_2 NP samples. Analysis revealed that TiO_2 NP samples are fully crystalline with anatase:rutile ratio of 7:3. The Raman spectrum of the TiO_2 NP (Figure 1(c)) displays the characteristic peaks of anatase and rutile, and no peak was observed of brookite or amorphous Ti.

In vivo *assays*

General examination. TiO_2 NPs were administered intraperitoneally since this body region is highly

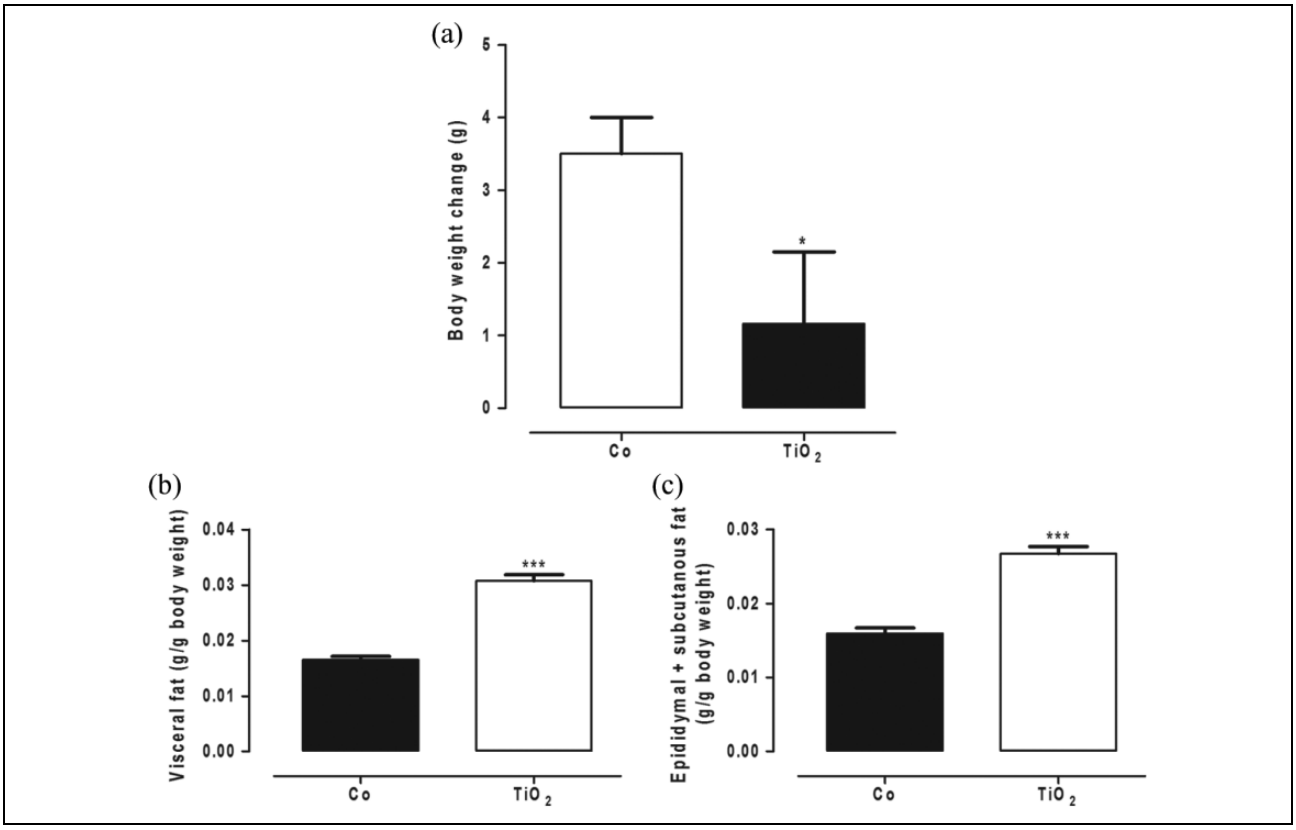

Figure 2. Effects of TiO_2 NP treatment on body weight change (a), accumulation of visceral fat (b) and epididymal and subcutaneous fat (c) compared with animals without treatment (Co). The results are expressed as mean $\pm$ SEM ($n = 6$), *$p < 0.05$ and ***$p < 0.001$. TiO_2 NPs: titanium dioxide nanoparticles; SEM: standard error of mean.

vascularized and mimics the intravenous route. Additionally, the intraperitoneal route is one of the most effective ways of dispensing test drugs into animals under experimentation, in a short term procedure. All animals tolerated the intraperitoneal injections of NPs, and no deaths were registered during the treatment. No changes were observed in the animal's behavior or in water and food intake. A significant decrease in body weight was observed in mice treated with TiO_2 NPs (Figure 2(a)), followed by a significant fat deposition in visceral, epididymal, and subcutaneous adipose tissue. The visceral fat deposition in TiO_2 NP-treated animals increased 0.0143 ± 0.0006 g g^{-1} while that of epididymal + subcutaneous did 0.0108 ± 0.0009 g g^{-1} compared with control animals (Figures 2(b) and (c)). The organs were also weighed separately and weights of spleen and kidneys showed significant difference (Table 1).

Hepatic and renal function analysis. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), albumin and total protein, which are considered markers associated with liver function, were evaluated. Likewise, in order to evaluate kidney functionality, urea and creatinine levels were also determined. The results obtained are given in Table 2 and no difference was observed in creatinine, albumin and protein levels, as well as the enzyme ALT. However, a slight increase in AST (almost 1.4-fold increment) and decrease in urea were observed in mice treated with TiO_2 NPs ($p < 0.05$).

Hematological and histopathological analysis. In order to evaluate the toxic effects resulting from the administration of TiO_2 NPs, hematological profiles of the animals were determined and compared with those obtained for the control group; the results are given in Table 3. The treatment with TiO_2 NPs induced changes in several hematologic parameters, with significant decrease in the total number of erythrocytes, leukocytes, and percentage of hematocrit and increase in the percentage of basophiles.

Histological examination of kidney, lung, stomach, liver, heart, brain, spleen, and adipose tissue was carried out. It was possible to observe fine vacuolar degeneration of the hepatocytes in 85% of the animals

Table 1. Organ weight of mice after treatment with 2 mg kg^{-1} day^{-1} of TiO_2 NPs. Results are presented as mean ± SEM.

Organ weight (g)	Control	TiO_2 NP-treated
Spleen	0.182 ± 0.012	0.285 ± 0.008[a]
Brain	0.374 ± 0.021	0.365 ± 0.009
Stomach	0.373 ± 0.008	0.357 ± 0.027
Lung	0.254 ± 0.019	0.264 ± 0.011
Liver	2.052 ± 0.139	2.149 ± 0.061
Heart	0.207 ± 0.023	0.186 ± 0.011
Kidney	0.252 ± 0.021	0.211 ± 0.011[a]

TiO_2 NPs: titanium dioxide nanoparticles; SEM: standard error of mean.
[a]$p \leq 0.05$.

Table 2. Biochemical parameters (regarding liver and kidney injury) concentration in the serum of mice treated with 2 mg kg^{-1} day^{-1} of TiO_2 NPs. Results are presented as mean ± SEM.

Parameters	Control	TiO_2 NP-treated
ALT (IU/L)	111 ± 9	119 ± 9.2
AST (IU/L)	101 ± 10	140 ± 7.19[a]
Albumin (g/dL)	2.3 ± 0.05	2.3 ± 0.08
Total protein (g/dL)	5.1 ± 0.19	5.2 ± 0.35
Urea (mg/dL)	62 ± 5	47 ± 2.23
Creatinine (mg/dL)	0.5 ± 0.08	0.5 ± 0.01

TiO_2 NPs: titanium dioxide nanoparticles; SEM: standard error of mean; ALT: alanine aminotransferase; AST: aspartate aminotransferase.
[a]$p \leq 0.05$.

Table 3. Hematological parameters concerning mice treated with 2 mg kg^{-1} day^{-1} of TiO_2 NPs. Results are presented as mean ± SEM.

Parameters	Control	TiO_2 NP-treated
Erythrocytes (/mm^3)	5,937,000 ± 457	4,833,000 ± 255[a]
Leukocytes (/mm^3)	7,691 ± 689	3,758 ± 236[a]
MCV (fl)	71 ± 3	66 ± 2
MCH (pg)	23 ± 3	20 ± 1
MCHC (%)	31 ± 2	31 ± 1
Hemoglobin (g/dL)	14 ± 2	10 ± 2
Hematocrit (%)	43 ± 2	32 ± 3[a]
Neutrophil (%)	14.5 ± 2.1	17 ± 2
Mononuclear (%)	86 ± 3	74 ± 2
Eosinophil (%)	1 ± 1	1 ± 1
Basophil (%)	2.5 ± 1	9 ± 1[a]

TiO_2 NPs: titanium dioxide nanoparticles; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.
[a]$p \leq 0.05$.

(Figure 3(d)) and hyaline degeneration of the smooth muscle of the stomach in 50% of the animals (Figure 3(e)). Sections of the abdominal fat showed mononuclear inflammatory infiltration extended to the cytoplasm as well as change in the color of fat tissue (Figure 3(f)).

All animals treated presented peri- and intrapancreatic inflammatory reaction and inflammatory infiltration in the renal calyx. Other changes were not observed in the kidney, lung, heart, brain, and spleen analyzed (data not shown).

Titanium content analysis. The concentration of Ti in heart, kidney, liver, spleen and lung of mice is given in Figure 4. Titanium was accumulated in kidney, liver, and spleen, suggesting that intraperitoneally administrated TiO_2 NPs were absorbed by systemic circulation, distributed, and accumulated into tissues, mainly in the spleen.

Discussion

The results obtained demonstrated relevant aspects related to the toxicity of TiO_2 NPs *in vivo*. After intraperitoneal treatment with TiO_2 NPs, there was an increase in visceral and subcutaneous fat deposition, in addition to mononuclear inflammatory infiltration in adipose tissue. Apart from these results, TiO_2 NPs also caused decreased body weight, which is considered a biomarker of toxicity (Klement et al., 2000; Thiagarajan et al., 2013), splenomegaly, changes in hematological parameters, and changes in biochemical parameters (with liver damage and vacuolar degeneration of hepatocytes).

Adipose tissue is considered a metabolically active endocrine organ that activates different inflammatory pathways as pro-inflammatory cytokines and oxidative stress. In this study, fat deposition in visceral area and mononuclear infiltration were found in adipose tissue of TiO_2 NP-treated mice. Two possible explanations for the observed results are (i) a systemic inflammation triggered by the NPs could induce the abdominal inflammation and fat increase; or (ii) simply as a consequence of the local injury related to the administration pathway. Furthermore, the change in the fat color, evidenced in histopathological analysis, can be related with: (i) the presence of Ti in the abdominal fat, presented as brown after tissue stained for histopathological analysis; and (ii) a "browning process", which consists in the appearance of brown-like adipocytes in white adipocytes, which are described to be related with many other causes, but not

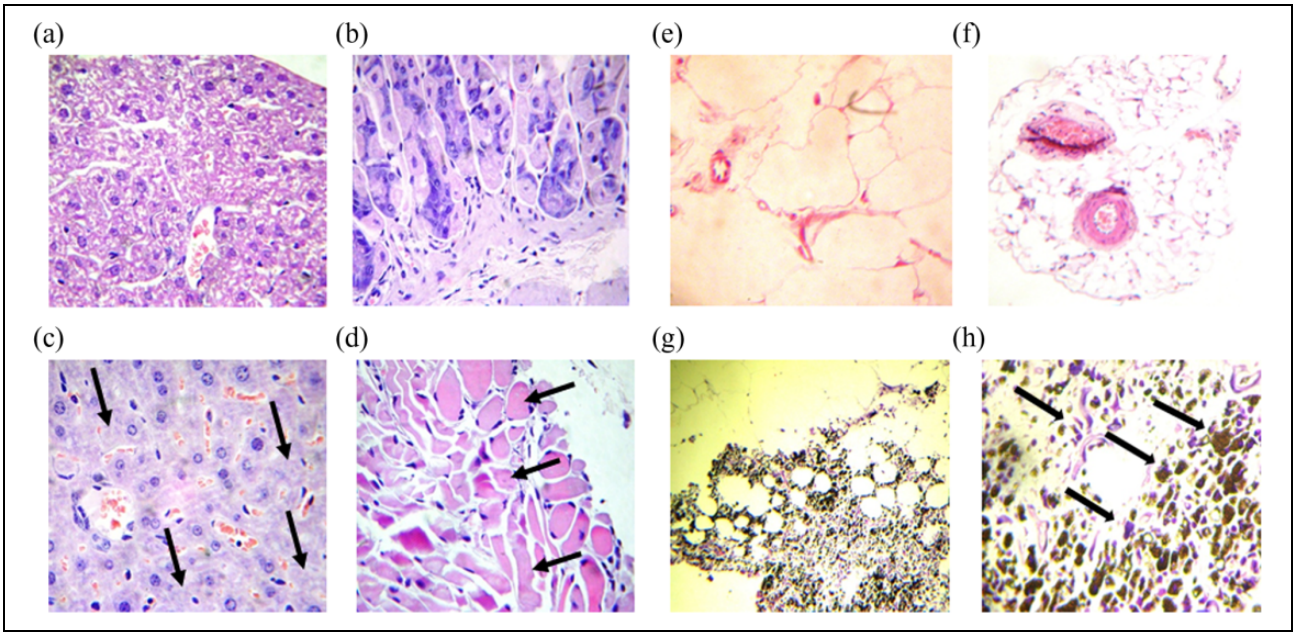

Figure 3. Histological analysis of liver, stomach, and visceral fat in mice after TiO_2 NP exposure. Tissues were stained as indicated in materials and methods. (a/b) Image (×400) shows the microscopic appearance of the hepatocytes and stomach of control animals and (c/d) the vacuolar degeneration of the hepatocytes and hyaline degeneration of the stomach smooth muscle, respectively. Images (e) (×100) and (f) (×400) show the microscopic appearance of the visceral fat of animals from control group and (g/h) the mononuclear inflammatory infiltration and change in the color of TiO_2 NP-treated tissue. TiO_2 NPs: titanium dioxide nanoparticles.

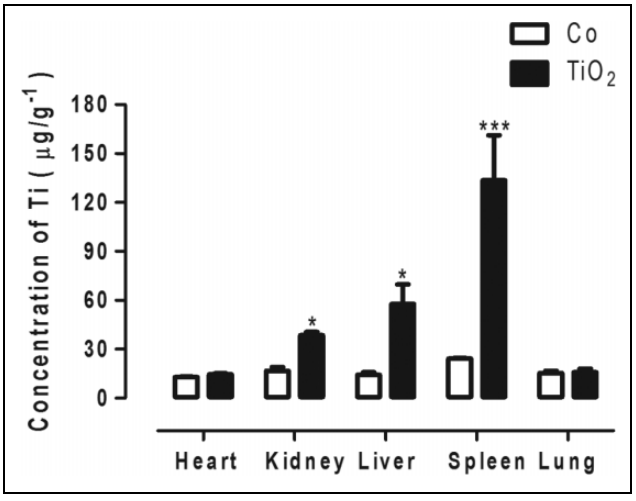

Figure 4. Determined concentration of titanium in body tissues. Animals were injected intraperitoneally with 2 mg kg^{-1} day^{-1} of TiO_2 NPs or saline (control animals–Co) for 10 consecutive days. On the last day of treatment, animals were killed and selected organs were analysed by ICP-MS to determine the titanium accumulation. *$p < 0.05$ and ***$p < 0.001$. TiO_2 NPs: titanium dioxide nanoparticles; ICP-MS: Inductively coupled plasma mass spectrometry.

yet with NPs or, the intraperitoneal administration route (Bartelt and Heeren, 2014; Young et al., 1984). An interesting aspect to further study.

Several studies have demonstrated the ability of metallic NPs, including the TiO_2 to induce inflammation, characterized by the increase of monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8) *in vitro* and leukocyte infiltration in lung and air pouch model *in vivo* (Alinovi et al., 2015; Monteiller et al., 2007; Umbreit et al., 2012). Metal oxide NPs were also identified in welders exposed to welding-related NPs (iron, manganese, and chromium oxide) leading to expression of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) in lung tissue sections (Andujar et al., 2014). However, in this study, intraperitoneal administration of metallic NPs also generated fat accumulation, and to the best of our knowledge, there are no reports addressing it after treatment with metallic NPs.

Furthermore, the inflammation can result not only in mononuclear recruitment in tissue, but also hepatocyte injury and fibrosis (Byrne, 2010). Liver damage was further confirmed by the histopathological analysis, whereas vacuolar degeneration of hepatocytes in TiO_2 NP-treated mice was observed. Despite the confirmed liver damage, a slight increase in AST levels was observed after treatment with TiO_2 NPs. Previous studies have demonstrated that AST levels, even 2–5-fold above normal values, may be predictive of

liver cell damage but not the functional capability of liver, which could be predicted by albumin and total protein serum concentrations (Peters, 2005). These facts suggest that even with the existence of hepatocyte degeneration, liver function remained preserved. Although it is most likely that this impairment in hepatotoxicity biomarkers is associated with TiO_2 NP toxicity, further experiments are needed to elucidate the biochemical mechanisms involved.

Although inflammation is known to be related with serum albumin decrease (Don and Kaysen, 2004; Kaysen et al., 2004), no alterations were observed in this protein level after TiO_2 NP treatment, perhaps because the half-lifetime of albumin is between 14 and 21 days (Kaysen et al., 1986; Kaysen and Schoenfeld, 1984) and the period evaluated by us was 10 days. Because albumin makes up more than half of the total protein present in serum (Busher, 1990), the constancy in the albumin level can be related with the constancy in total protein, even in the presence of liver damage. A study using a long-term exposition to TiO_2 NPs is necessary to better correlate the albumin and total protein changes with inflammation and liver damage.

Whereas elevated urea is considered a biomarker of weight gain, the balance between its production and urinary excretion is relevant and well documented (Hollister et al., 1967; Matsuura et al., 1998; Schimke, 1962). Besides, fat distribution may also have a relation between urea levels, as pointed by Matsuura and collaborators. The authors showed that urinary excretion of urea is significantly higher in visceral fat obesity group compared to subcutaneous fat obesity group. Although the treated animals showed increased abdominal fat, there was no significant difference in urea levels, which can be related with the increase in urinary urea excretion (Matsuura et al., 1998). A study of urea clearance should be done to address this issue.

Liver damage and splenomegaly observed in TiO_2 NP-treated mice might also be related to the high accumulation of Ti in these organs. Many studies reported that the difficulties in excretion of TiO_2 NP culminate in Ti accumulation in different organs, as the liver, which is the main detoxification organ (Oberdörster et al., 1994; Wang et al., 2007). Likewise, opsonized NPs accumulate in macrophages by the fact that lysosomal enzymes cannot digest inorganic material (Ivanov et al., 2012). Previous studies with fish also suggested that long-term exposure to TiO_2 NP favors their accumulation in important organs such as liver and kidney, representing an important toxic exposure (Scown et al., 2009). However, as creatinine levels and kidney histopathological evaluation showed similar profile between control and treated groups, TiO_2 NP does not seem to be toxic to the kidneys.

The splenomegaly could explain the changes caused by the NPs in several hematologic parameter values, since the spleen plays a key role in hematopoiesis, participating in the processes of production, development, and maturation of blood elements (Lloyd and Strickland, 2010).

The intraperitoneal route showed to be a valuable tool to study the *in vivo* toxicological effects of TiO_2 NPs, because it mimics the intravenous route (Silva et al., 2013), since the innumerous possibilities of TiO_2 NPs reach the individual bloodstream. According to Hext et al., (2005), occupational exposition can happen during production and manufacturing products containing TiO_2 and TiO_2 NPs, and while present in the pulmonary alveoli, they can reach the blood circulation (Kapp et al., 2007; van Ravenzwaay et al., 2009) and move to the organs (Donaldson et al., 2001; Nemmar et al., 2001). Exposure can also happen by body implants, as described by Skocaj and colaborators (2011). Orthopedic implants, especially the wear-exposed implants, after mechanical stress can release relevant amounts of debris in nanometer ranges into the body (Skocaj et al., 2011). The same idea must be taken into account with the additives used in food. TiO_2 is well accepted and approved by US Food and Drug Administration (FDA) in food products and can be used up to 1% of the weight food, however, no information is available about the size and structure of particles, which is mandatory in the hazard of such material (Skocaj et al., 2011). Once ingested, TiO_2 NPs can be distributed to the liver, spleen, and lung, as already showed by some authors (Cho et al., 2013; Jani et al., 1994; Wang et al., 2007). Both FDA as OECD and other regulatory agencies have been working in the development of methodologies to access the toxicological effects of TiO_2 NPs and other NPs, and the dose–response data from animal studies, as we and other researchers have been working, are mandatory to estimate risk in humans (OECD, 2012) and, subsequently, improve the regulation of nanotechnology use.

Recent studies revealed that toxicity of metal-based NPs such as silver, zinc, and quantum dots are attributed to ion release (Derfus et al., 2004; Franklin et al., 2007; Lok et al., 2007), rather than from NPs

themselves. As a limitation of this study, the Ti ion dissolution and the consequences *in vivo* was not carried out and additionally, references about it are very scarce (Eliades et al., 2004). As far as we know, there are no reports comparing the *in vivo* effects between TiO_2 NPs and metal ions released from them.

Additionally, according to Trouiller and collaborators, although TiO_2 NPs easily agglomerate in solution, they are apparently not stable and seem to dissociate in bodily fluids and tissues, and their primary particle size remains important in terms of toxicity even if NPs aggregate into larger sized agglomerates (Trouiller et al., 2009). Furthermore, the small size leads to an increased reactivity capacity with biological components, as well as uptake into certain tissues. Since the results presented here showed accumulation of TiO_2 NPs in tissues such as liver and spleen, we can suggest that some of the adverse effects triggered by TiO_2 NPs can be related with size, more pronounced than by aggregation. However, it is important to mention that there are other important NPs physicochemical properties such as degradation, shape, charge and ion release, which can also be related with the toxicological effects. Further studies should be performed to better characterize it.

Our results agree with those found by other researchers as Wang et al. (2007) and Fabian et al. (2008), assuming that a single oral gavage exposure of a very high dose (5 g kg^{-1}) of TiO_2 NPs (80 nm) in mice could elevate the ALT/AST enzyme ratio and LDH level in serum. This implies that TiO_2 NPs may induce hepatic injury and promote the accumulation of these NPs in the liver, spleen, lung, and kidneys after intravenous administration in rat (Fabian et al., 2008; Wang et al., 2007). However, our findings are novel because they show that a low dose of TiO_2 NPs (2 mg kg^{-1} day^{-1}) was capable of causing significant changes in hematologic and hepatic systems. These changes might be associated with bioaccumulation of TiO_2 NPs, since most studies had shown similar changes *in vivo* at doses 100 times higher than those used in the present study. Therefore, further toxicological studies *in vivo* have to be carried out for evaluating hazards of occupational or environmental exposure to TiO_2 NPs, since bioaccumulation over time can bring harm to health.

Conclusions

In this study, we reported the effects of mice exposure to TiO_2 NPs after 10 consecutive days of intraperitoneal administration. Several biological parameters related to toxicity, especially the induction of inflammation and liver damage, were observed. Despite the fact the exact mechanism of inflammation caused by TiO_2 NPs was not completely elucidated, the data and the discussion presented here are important given the fact that visceral fat accumulation was surprisingly found after 10 days of treatment. These results provide a basis for further *in vivo* long-term exposure studies in order to characterize the mechanism of inflammatory response as well as Ti accumulation.

Acknowledgments

The authors thank LAMEB/UFSC for providing its facilities, Prof. AA Pasa and Dr JJS Acuña for the TEM image obtained in the LCME/UFSC, and Prof. Fabíola BF Monteiro (Clinical Analysis Department/UFSC) for helpful discussion.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa de Santa Catarina (FAPESC). The grant numbers are 304229/2009-1 CNPq, 1301/2010-3 Fapesc, and 41001010044D8 PPGFAR/Capes.

References

Alinovi R, Goldoni M, Pinelli S, et al. (2015) Oxidative and pro-inflammatory effects of cobalt and titanium oxide nanoparticles on aortic and venous endothelial cells. *Toxicology in Vitro* 29(3): 426–437.

Andujar P, Simon-Deckers A, Galateau-Salle F, et al. (2014) Role of metal oxide nanoparticles in histopathological changes observed in the lung of welders. *Particle and Fibre Toxicology* 11(1): 23.

Asare N, Instanes C, Sandberg WJ, et al. (2012) Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells. *Toxicology* 291(1–3): 65–72.

Bartelt A and Heeren J (2014) Adipose tissue browning and metabolic health. *Nature Reviews Endocrinology* 10(1): 24–36.

Busher JT (1990) Serum albumin and Globulin. In: Walker HK, Hall WD and Hurst JW (eds) *Clinical Methods: The*

History, Physical, and Laboratory Examination. 3rd ed. Boston: Butterworths, pp. 497–499.

Byrne CD (2010) Fatty liver: role of inflammation and fatty acid nutrition. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 82(4–6): 265–271.

Chen HW, Su SF, Chien CT, et al. (2006) Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. *The Journal of the Federation of American Societies for Experimental Biology (FASEB)* 20(13): 2393–2395.

Cho WS, Kang BC, Lee JK, et al. (2013) Comparative absorption, distribution, and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. *Particle and Fibre Toxicology* 10: 9.

Daughton CG and Ternes TA (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environmental Health Perspectives* 107(Suppl 6): 907–938.

Derfus A, Chan W and Bhatia S (2004) Probing the cytotoxicity of semiconductor quantum dots. *Nano Letters* 4: 11–18.

Devanand Venkatasubbu G, Ramasamy S, Ramakrishnan V, et al. (2013) Folate targeted PEGylated titanium dioxide nanoparticles as a nanocarrier for targeted paclitaxel drug delivery. *Advanced Powder Technology* 24(6): 947–954.

Don BR and Kaysen G (2004) Serum albumin: relationship to inflammation and nutrition. *Seminars in Dialisys* 17(6): 432–437.

Donaldson K, Stone V, Seaton A, et al. (2001) Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environmental Health Perspectives* 109(Suppl 4): 523–527.

Eliades T, Pratsinis H, Kletsas D, et al. (2004) Characterization and cytotoxicity of ions released from stainless steel and nickel-titanium orthodontic alloys. *American Journal of Orthodontics and Dentofacial Orthopedics* 125(1): 24–29.

Fabian E, Landsiedel R, Ma-Hock L, et al. (2008) Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. *Archives of Toxicology* 82(3): 151–157.

Fadeel B and Garcia-Bennett AE (2010) Better safe than sorry: understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. *Advanced Drug Delivery Review* 62(3): 362–374.

Franklin NM, Rogers NJ, Apte SC, et al. (2007) Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl2 to a freshwater microalga (Pseudokirchneriella subcapitata): the importance of particle solubility. *Environmental Science & Technology* 41(24): 8484–8490.

Garg DK and Goyal RN (1992) Haematological and hepatotoxic effects of silken styles of corn in albino rats. *Journal of Applied Toxicology* 12(5): 359–363.

Han X, Gelein R, Corson N, et al. (2011) Validation of an LDH assay for assessing nanoparticle toxicity. *Toxicology* 287(1–3): 99–104.

Hext PM, Tomenson JA and Thompson P (2005) Titanium dioxide: inhalation toxicology and epidemiology. *The Annals of Occupational Hygiene* 49(6): 461–472.

Hollister LE, Overall JE and Snow HL (1967) Relationship of obesity to serum triglyceride, cholesterol, and uric acid, and to plasma-glucose levels. *The Amercian Journal of Clinical Nutrition* 20(7): 777–782.

Huang S, Chueh PJ, Lin YW, et al. (2009) Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO_2 long-term exposure. *Toxicology Applied Pharmacology* 241(2): 182–194.

Ivanov S, Zhuravsky S, Yukina G, et al. (2012) In vivo toxicity of intravenously administered silica and silicon nanoparticles. *Materials* 5(10): 16.

Janer G, Mas del Molino E, Fernandez-Rosas E, et al. (2014) Cell uptake and oral absorption of titanium dioxide nanoparticles. *Toxicology Letters* 228(2): 103–110.

Jani PU, McCarthy DE and Florence AT (1994) Titanium dioxide (rutile) particle uptake from the rat GI tract and translocation to systemic organs after oral administration. *International Journal of Pharmaceutics* 105(2): 157–168.

Jin C-Y, Zhu B-S, Wang X-F, et al. (2008) Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. *Chemical Research in Toxicology* 21(9): 1871–1877.

Kapp N, Studer D, Gehr P, et al. (2007) Electron energy-loss spectroscopy as a tool for elemental analysis in biological specimens. *Methods in Molecular Biology* 369: 431–447.

Kaysen GA, Dubin JA, Muller HG, et al. (2004) Inflammation and reduced albumin synthesis associated with stable decline in serum albumin in hemodialysis patients. *Kidney International* 65(4): 1408–1415.

Kaysen GA, Gambertoglio J, Jimenez I, et al. (1986) Effect of dietary protein intake on albumin homeostasis in nephrotic patients. *Kidney International* 29(2): 572–577.

Kaysen GA and Schoenfeld PY (1984) Albumin homeostasis in patients undergoing continuous ambulatory peritoneal dialysis. *Kidney International* 25(1): 107–114.

Klement G, Baruchel S, Rak J, et al. (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-

2 antibody induces sustained tumor regression without overt toxicity. *The Journal of Clinical Investigation* 105(8): R15–R24.

Lasagna-Reeves C, Gonzalez-Romero D, Barria MA, et al. (2010) Bioaccumulation and toxicity of gold nanoparticles after repeated administration in mice. *Biochemical and Biophysical Research Communications* 393(4): 649–655.

Liu W-T (2006) Nanoparticles and their biological and environmental applications. *Journal of Bioscience and Bioengineering* 102(1): 1–7.

Liu R, Yin L, Pu Y, et al. (2009) Pulmonary toxicity induced by three forms of titanium dioxide nanoparticles via intra-tracheal instillation in rats. *Progress in Natural Science* 19(5): 573–579.

Lloyd DM and Strickland AD (2010) Surgery of the spleen. *Surgery* 28(5): 229–233.

Lok CN, Ho CM, Chen R, et al. (2007) Silver nanoparticles: partial oxidation and antibacterial activities. *The Journal of Biological Inorganic Chemistry* 12(4): 527–534.

Martin-Camean A, Jos A, Calleja A, et al. (2014) Validation of a method to quantify titanium, vanadium and zirconium in oral mucosa cells by inductively coupled plasma-mass spectrometry (ICP-MS). *Talanta* 118: 238–244.

Matsuura F, Yamashita S, Nakamura T, et al. (1998) Effect of visceral fat accumulation on uric acid metabolism in male obese subjects: visceral fat obesity is linked more closely to overproduction of uric acid than subcutaneous fat obesity. *Metabolism* 47(8): 929–933.

Monteiller C, Tran L, MacNee W, et al. (2007) The pro-inflammatory effects of low-toxicity low-solubility particles, nanoparticles and fine particles, on epithelial cells in vitro: the role of surface area. *Occupational and Environmental Medicine* 64(9): 609–615.

Moore MN (2006) Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment International* 32(8): 967–976.

Mueller NC and Nowack B (2008) Exposure modeling of engineered nanoparticles in the environment. *Environmental Science & Technology* 42(12): 4447–4453.

Nemmar A, Vanbilloen H, Hoylaerts M, et al. (2001) Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *American Journal of Respiratory and Critical Care Medicine* 164: 1665–1668.

Oberdörster G, Ferin J and Lehnert BE (1994) Correlation between particle size, in vivo particle persistence, and lung injury. *Environmental Health Perspectives* 102(Suppl 5): 173–179.

OECD (2001) OECD guideline for testing of chemicals. Available at: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm (accessed 16 September 2015).

OECD (2012) *Important Issues on Risk Assessment of Manufactured Nanomaterials. Series on the Safety of Manufactured Nanomaterials*. Paris: OECD, p. 57.

Pari L and Murugavel P (2005) Role of diallyl tetrasulfide in ameliorating the cadmium induced biochemical changes in rats. *Environmental Toxicology and Pharmacology* 20(3): 493–500.

Park EJ, Yi J, Chung KH, et al. (2008) Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicology Letters* 180(3): 222–229.

Peters TS (2005) Do preclinical testing strategies help predict human hepatotoxic potentials? *Toxicology Pathology* 33(1): 146–154.

Pujalté I, Passagne I, Brouillaud B, et al. (2011) Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. *Particle and Fibre Toxicology* 8: 10.

Rico CM, Majumdar S, Duarte-Gardea M, et al. (2011) Interaction of nanoparticles with edible plants and their possible implications in the food chain. *Journal of Agricultural and Food Chemistry* 59(8): 3485–3498.

Schimke RT (1962) Differential effects of fasting and protein-free diets on levels of urea cycle enzymes in rat liver. *The Journal of Biological Chemistry* 237: 1921–1924.

Scown TM, van Aerle R, Johnston BD, et al. (2009) High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicology Science* 109(2): 372–380.

Silva AH, Locatelli C, Filippin-Monteiro FB, et al. (2013) Solid lipid nanoparticles induced hematological changes and inflammatory response in mice. *Nanotoxicology* 8(2): 7.

Skocaj M, Filipic M, Petkovic J, et al. (2011) Titanium dioxide in our everyday life; is it safe? *Radiology and Oncology* 45(4): 227–247.

Soto-Alvaredo J, Blanco E, Bettmer J, et al. (2014) Evaluation of the biological effect of Ti generated debris from metal implants: ions and nanoparticles. *Metallomics* 6(9): 1702–1708.

Thiagarajan G, Greish K and Ghandehari H (2013) Charge affects the oral toxicity of poly(amidoamine) dendrimers. *European Journal of Pharmaceuticals and Biopharmaceuticals* 84(2): 330–334.

Trouiller B, Reliene R, Westbrook A, et al. (2009) Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer Research* 69(22): 8784–8789.

Turkez H (2011) The role of ascorbic acid on titanium dioxide-induced genetic damage assessed by the comet assay and cytogenetic tests. *Experimental and Toxicology Pathology* 63(5): 453–457.

Umbreit TH, Francke-Carroll S, Weaver JL, et al. (2012) Tissue distribution and histopathological effects of titanium dioxide nanoparticles after intravenous or subcutaneous injection in mice. *Journal of Applied Toxicology* 32(5): 350–357.

van Ravenzwaay B, Landsiedel R, Fabian E, et al. (2009) Comparing fate and effects of three particles of different surface properties: nano-TiO_2, pigmentary TiO_2 and quartz. *Toxicology Letters* 186(3): 152–159.

Wang J, Zhou G, Chen C, et al. (2007) Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology Letters* 168(2): 176–185.

Wang S, Yu H and Wickliffe JK (2011) Limitation of the MTT and XTT assays for measuring cell viability due to superoxide formation induced by nano-scale TiO_2. *Toxicology in Vitro* 25(8): 2147–2151.

West AR (1984) *Solid State Chemistry and its Applications*. Chichester, NY: Wiley.

Young P, Arch JRS and Ashwell M (1984) Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Letters* 167(1): 10–14.