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Biokinetics and tissue response to ultrananocrystalline diamond nanoparticles employed as coating for biomedical devices

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Abstract: Although Ultrananocrystalline diamond (UNCD) has been proposed as a coating material for titanium biomedical implants, the biological effects and toxicity of UNCD particles that could eventually detach have not been studied to date. The biokinetics and biological effects of UNCD compared to titanium dioxide (TiO_2) nanoparticles was evaluated *in vivo* using Wistar rats ($n = 30$) i.p. injected with TiO_2, UNCD or saline solution. After 6 months, blood, lung, liver, and kidney samples were histologically analyzed. Oxidative damage by membrane lipidperoxidation (thiobarbituric acid reactive substances-TBARS), generation of reactive oxygen species (superoxide anion-O_2^-), and antioxidant enzymes (superoxide dismutase-SOD, catalase-CAT) was evaluated in lung and liver. Histologic observation showed agglomerates of TiO_2 or UNCD in the parenchyma of the studied organs, though there were fewer UNCD than TiO_2 deposits. In addition, TiO_2 caused areas compatibles with foci of necrosis in the liver and renal hyaline cylinders. Regarding UNCD, no membrane damage (TBARS) or mobilization of enzymatic antioxidants was observed either in lung or liver samples. No variations in O_2^- generation were observed in lung (Co: 35.1 ± 4.02 vs. UNCD: 48 ± 9.1, $p > 0.05$). Conversely, TiO_2 exposure caused production of O_2^- in alveolar macrophages and consumption of catalase ($p < 0.05$). The studied parameters suggest that UNCD caused neither biochemical nor histological alterations, and therefore may prove useful as a surface coating for biomedical implants. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 105B: 2408–2415, 2017.

Key Words: ultrananocrystalline diamond nanoparticles, titanium dioxide, biokinetics, oxidative metabolism

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INTRODUCTION

The clinical success of an implant depends not only on cellular response at the tissue/implant interface and osseointegration of the implant, but also on the reliability of the surface coating. Hence, evaluating the physicochemical properties of the surface of coated and noncoated metallic biomedical implants and their interaction with the biological milieu is key to biomaterials research, and poses a challenge to implantology.

Titanium is one of the most frequently used metallic biomaterials in the manufacture of biomedical implants. Because the metallic implant is in contact with body tissues and fluids, ions/particles can be released from the implant surface into the biological milieu as a result of electrochemical processes (corrosion).[1] These ions/particles can also result from wear or friction processes (tribology). When corrosion combines with wear (tribology), the process is known as tribocorrosion, and when the latter process occurs in a biological system, it is termed biotribocorrosion.[2]

Specifically, metallic titanium implants are a potential source of release of titanium dioxide (TiO_2) microparticles (MPs) and nanoparticles (NPs) into the bioenvironment.[3–7]

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It is well known that NPs have a greater surface-to-volume ratio than MPs, and are therefore biologically more reactive and potentially more harmful to human health.[6,8]

With the aims to optimize implant biocompatibility, improving osseointegration and reducing the likelihood of biotribocorrosion by preventing or limiting the release of MPs and NPs into the biological milieu, a number of coatings and micro- and nanostructured surfaces have been developed, and their properties are evaluated using different biological parameters.[9–12] As carbon is the basis of any biological system, carbon-based materials are ideal candidates for these purposes.[13] Among such coatings, Ultrananocrystalline diamond (UNCD) represents a new paradigm in multifunctional materials. UNCD presents a unique synergistic combination of outstanding mechanical (greater hardness as compared to any other material and high fracture resistance/coefficient), tribological (one of the lowest coefficients of friction ($\sim 0.02 - 0.04$) of any coating developed today, and wear),[14,15] chemical (extreme resistance to corrosive attack by acids or body fluids), electrical (highly conductive with nitrogen atoms in the grain boundaries of films or boron atoms substituting carbon atoms in the diamond lattice of the film),[16] and biocompatibility properties.[15,17–20]

Diamond films are of great interest and are being explored on account of their many current and potential applications, with the aims to obtain a new generation of multifunctional devices.[18] In this regard, there are a number of research groups investigating the advantages of UNCD and its potential use in biomedical and biotechnological applications. In comparison with other kinds of diamond, the nanotopography of UNCD facilitates adhesion, proliferation, and metabolism of different cellular types, in line with the nanoscale extracellular matrix of tissue.[20] In this regard, studies assessing the interaction between UNCD films and osteoblasts,[21] fibroblasts,[22] cortical neurons,[23] and cortical stem cells,[24] have shown the suitability and non-cytotoxicity of UNCD films as a support surface for cell growth and proliferation.[25,26] The usefulness of UNCD for biosensing post-surface functionalization has also been demonstrated.[27,28] Moreover, Garret et al.[29] recently demonstrated excellent performance of N-UNCD-coated metallic electrodes for neural stimulation. In line with this finding, other authors have evaluated the use of UNCD for ophthalmological applications, aiming to restore sight to patients with retinitis pigmentosa, a retinal disease.[30–32]

Hence, UNCD has been proposed as a coating for biomedical devices, such as coxofemoral prostheses, dental implants, cardiac valves, and ocular devices, among others.[19]

Adhesion of the coating to the substrate is a critical issue from the perspective of the mechanical performance and biocompatibility of the coated-implant.[17,18,33] Although UNCD shows strong adhesion[17] to substrates, the potential release of particulate material into the biological milieu cannot be ruled out. It is therefore important to evaluate its possible biological effects and degree of toxicity as compared to TiO_2 particles that could be released from titanium implants. It must be pointed out that metallic implants employed in the field of orthopedics and dentistry usually remain in place over long periods of time. It is therefore clinically relevant to investigate the possible short and long-term cellular, tissue, and functional consequences of exposure to UNCD. Hence, in order to evaluate biocompatibility of UNCD nanoparticles, the aim of the present work was to study the biokinetics (distribution, destination, deposition, and biopersistence) and biological effects of UNCD nanoparticles compared to response to TiO_2 nanoparticles.

MATERIALS AND METHODS

Nanoparticles characterization

Ultrananocrystalline diamond nanoparticles (UNCD), with an average particle size (APS) of 4 nm, manufactured by ITC (International Technology Center, NC, USA) and TiO_2 nanoparticles (NPs) (anatase - Nanostructured and Amorphous Materials, Los Alamos, NM), APS 5 nm, were used. APS of both particles was confirmed by SEM using a Supra 40 Zeiss microscope (Oberkochen, Germany), equipped with a field emission filament. Particles were chemically identified by EDS using an Oxford Instrument detector Oxford (Bucks, England).

Animal treatment protocol

Male Wistar rats, 100 g body weight, were used. The animals were housed under standard conditions, receiving water and food *ad libitum* and under 12:12 light-dark cycles and controlled temperature (22–24°C) conditions. Biological evaluation was performed following a protocol previously developed and published by our research group.[34] The animals were randomly divided into three groups and intraperitoneally (i.p.) injected as follows: (1) TiO_2 Group: a TiO_2 suspension (1.6 g/100 g of body weight); (2) UNCD Group: a suspension of UNCD particles (1.6 g/100 g of body weight); and (3) Control Group: an equivalent volume of saline solution (0.9% NaCl vehicle). All animals were euthanized at 6 months post-injection. Blood samples (2 ml) were obtained by puncture of the tail vein, prior to euthanasia. Immediately following euthanasia, alveolar macrophages (AM) were obtained by bronchoalveolar lavage,[35] and the lungs, liver, and kidneys were excised. Adequate measures were taken to minimize animal pain and discomfort. All procedures were performed in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication - Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011) and the guidelines of the School of Dentistry of the University of Buenos Aires (Res. (CD) 352/02 and Res. (CD) 694/02). The protocol was approved by the institutional experimentation committee (School of Dentistry of the University of Buenos Aires, Resolution Number 28/11/2012-37).

Histological study of blood and organ tissues

Blood smears were stained with safranin in order to determine the presence of particles in blood cells. The excised organs (lungs, liver, and kidneys) were fixed in 10% formalin and embedded in paraffin; histological sections were obtained and stained with H-E or Grenacher's carmine. To rule out the presence of formalin pigment artifacts, the sections were treated with a saturated solution of picric acid.

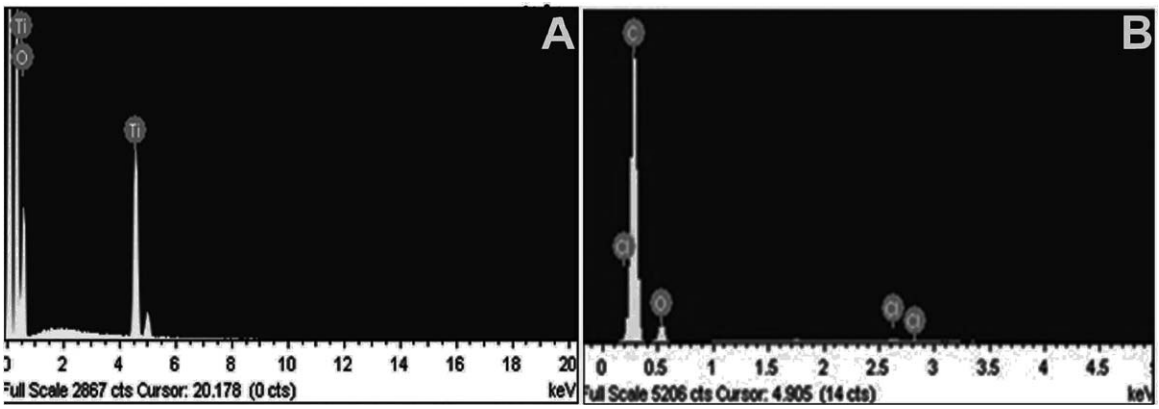

FIGURE 1. EDS. Spectra showing peaks corresponding to titanium (A) and carbon (B) in the analyzed TiO_2 and UNCD nanoparticles samples, respectively.

Body organ tissue sections were examined under a light microscope.

Analysis of oxidative metabolism

Determination of superoxide anion (O_2^-) in alveolar macrophages. Superoxide anion (O_2^-), a main reactive oxygen species generated during the respiratory burst, was evaluated in alveolar macrophages (AM) obtained by bronchoalveolar lavage, using the Nitro Blue Tetrazolium (NBT) reduction test, in all three groups.[36] Cells showing a blue formazan precipitate were considered reactive, whereas those without precipitate were scored as non-reactive. Reactive and non-reactive cells were counted using light microscopy, and the percentage of each was calculated.[37]

Determination of oxidative damage to lipids. Oxidative damage to lipids was determined in lung and liver by measuring thiobarbituric acid reactive substances (TBARS) levels using a fluorometric assay. Tissue homogenates (0.5 mL) were added to a medium consisting of 0.1 N HCl, 10% (w/v) phosphotungstic acid and 0.7% (w/v) 2-thiobarbituric acid. After incubation in boiling water for 60 min, TBARS were extracted with n-butanol. The fluorescence of the butanolic layer was measured in a Perkin Elmer LS 55 flourometer at 515 nm (excitation) and 553 nm (emission). A calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard. Results were expressed as nmol TBARS/mg protein.

Determination of enzymatic antioxidants. Antioxidant species superoxide dismutase (SOD) and catalase (CAT) were evaluated in lung and liver homogenates, as described by Llesuy et al.[38] SOD activity was determined spectrophotometrically by following the inhibition of the rate of adrenochrome formation at 480 nm, in a reaction medium containing 1 m*M* epinephrine and 50 m*M* glycine/NaOH (pH 10.5).[39] Enzymatic activity was expressed as SOD units/mg protein. One unit was defined as the amount of sample able to inhibit the rate of adrenochrome formation by 50%. Catalase activity was evaluated by following the decrease in absorbance at 240 nm in a reaction medium consisting of 100 m*M* phosphate buffer (pH 7.4) and 20 m*M* hydrogen peroxide.[40] Results were expressed as pmol catalase/mg protein.

Protein content

Protein concentration was measured using bovine serum albumin as standard, following the method described by Lowry et al.[41]

Statistical analysis

The results were compared using one-way ANOVA and Newman-Keuls or Bonferroni *post-hoc* test, accordingly. Statistical significance was set at $p < 0.05$.

RESULTS

None of the animals showed alterations in body weight, behavior, or general health throughout the study.

Physicochemical characterization of the particles

Morphological analysis by SEM confirmed TiO_2 NPs APS was 5 nm and UNCD APS was 4 nm. Elemental analysis by EDS confirmed the presence of Ti in TiO_2 NPs and of carbon in UNCD [Figure 1(A,B)].

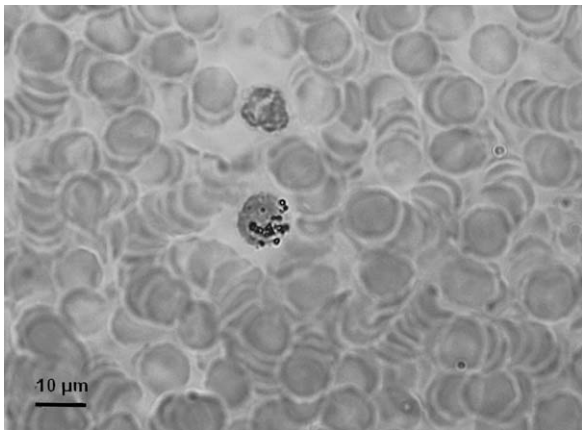

FIGURE 2. Blood smear. A monocyte containing intracytoplasmic TiO_2 nanoparticles can be observed. Safranin Stain. Orig. Mag. ×1000.

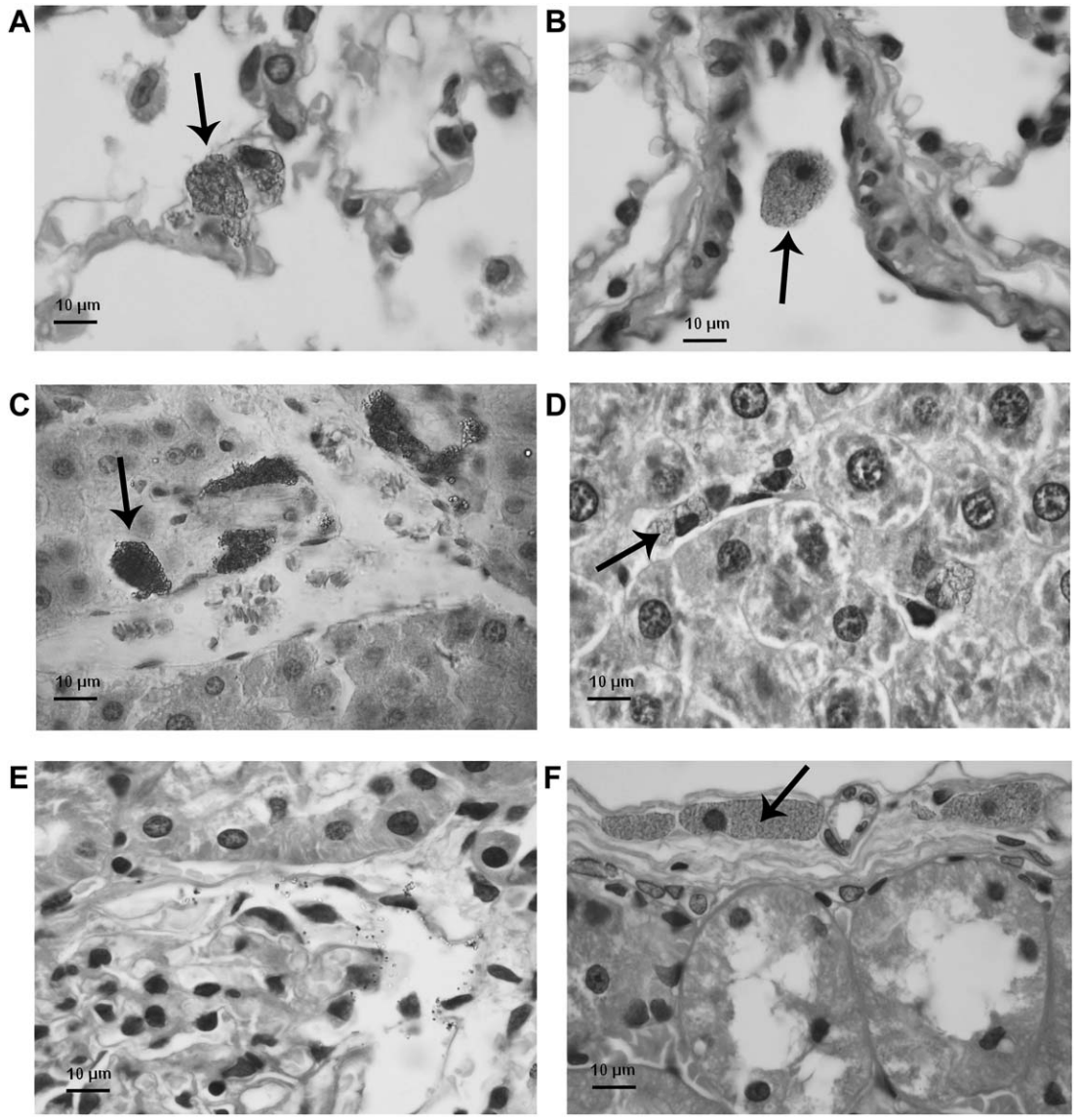

FIGURE 3. Histological Study. Lung (A and B): Deposits of TiO_2 (A) and UNCD (B) nanoparticles in alveolar macrophages (→). Liver (C and D): Deposits of TiO_2 (C) and UNCD (D) nanoparticles in Kupffer cells (→). Kidney (E and F): Scattered deposits of TiO_2 (E) in renal glomerulus and of UNCD (F) nanoparticles in macrophages in the capsule only (→). Note that TiO_2 deposits are consistently more abundant than UNCD deposits. H-E stain. Orig. Mag. ×1000.

Histological analysis of blood smears and organ tissue samples

Histological analysis of blood smears showed the presence of TiO_2 or UNCD in the cytoplasm of mononuclear phagocytic cells in particle-injected animals. A case from the TiO_2 Group is shown as an example (Figure 2). It is of note that identifying phagocytic cells loaded with UNCD was more difficult than identifying TiO_2 loaded cells. No loaded cells were found in blood smears of control animals.

The histological study of lung, liver, and kidney tissue samples showed the presence of TiO_2 or UNCD particles in the parenchyma of the studied organs [Figure 3(A–F)]. The particles formed agglomerates and were found in intercellular spaces, phagocytozed by lung alveolar macrophages or liver Kupffer cells, and in the cytoplasm of cells of the convoluted tubules and glomeruli of kidney samples. It is noteworthy that UNCD deposits [Figure 3(B,D,F)] were scant as compared to TiO_2 deposits [Figure 3(A,C,E)].

From a structural viewpoint; UNCD particles caused no alterations in any of the studied organs [Figure 3(B,D,F)]. Conversely, TiO_2 NPs caused morphological changes in the liver and kidneys [Figure 4(A,B)]. Areas compatible with foci of necrosis and hemorrhagic exudate with infiltration of mononuclear cells and polymorphonuclear neutrophils were observed in liver tissue samples [Figure 4(A)]. As regards the kidneys, although hyaline cylinders were observed in the proximal and distal tubules of control animals, they were more abundant in the TiO_2 Group [Figure 4(B)]. It must be pointed out that though TiO_2 NPs caused

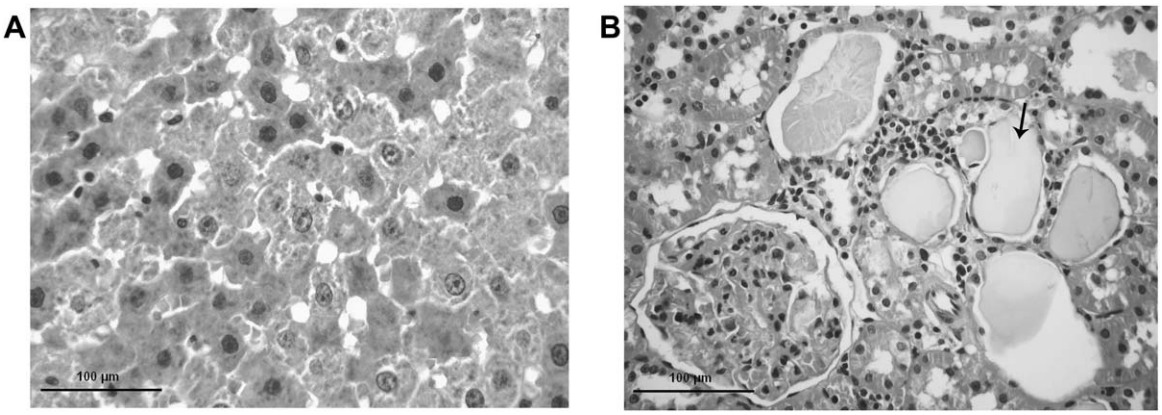

FIGURE 4. TiO_2. A: Liver; An area of necrosis can be observed. H−E stain. Orig. Mag. X400. B: Kidney; Kidney tubules containing hyaline cylinders can be seen clearly (→). H-E stain. Orig. Mag. ×400.

alterations in the liver and kidneys, they caused no morphological changes in the lungs.

Oxidative Metabolism

Determination of superoxide anion in alveolar macrophages. The percentage of reactive cells in samples obtained by bronchoalveolar lavage (BAL) was significantly higher in the TiO_2 Group compared to controls. Conversely, no significant differences in this parameter were observed between UNCD-treated and control animals (Figure 5).

Determination of oxidative damage to lipids in lung and liver. Determination of membrane damage, as assessed by TBARS, showed no significant differences in any of the studied organs between either of the experimental groups and control animals [Figure 6(A,B)].

Determination of enzymatic antioxidants. TiO_2 NPs caused significant changes in CAT levels in lung and liver homogenates, as compared to homogenates of controls [Figure 7(A,B)]. Nevertheless, administration of TiO_2 caused no changes in SOD activity in either lung or liver samples compared to controls (data not shown). UNCD caused no differences in levels of antioxidant enzymes SOD and CAT in any of the studied organs compared to controls [Figure 7(A,B)].

DISCUSSION

The human population is exposed to different types of NPs which either accidentally or not, can enter the body via inhalation, oral, and dermal routes.[42] The surface of implanted metallic medical devices can be considered a potential source of generation of particles inside the body.[43,44] Particles/ions can be released into the microenvironment as a result of electrochemical corrosion processes, material wear, or a synergistic combination of the two. Several transport mechanisms have been described for NPs, that is, systemic dissemination by the vascular and/or lymphatic systems as free particles or as phagocytized particles within macrophages.[45] When in the blood stream NPs circulate within the body and are taken up in organs and tissues; such particles particularly tend to accumulate in the reticuloendothelial system.[46] In addition, NPs exhibit many new and unexpected metabolism kinetic features *in vivo*.[47,48]

As a rule, particle toxicity correlates with particle shape, size,[49] chemical composition, and other characteristics such as crystalline structure and chemical reactivity of the particle surface.[50] The mobility of NPs shows dynamically changing properties *in vivo*, depending on the aforementioned particle physicochemical characteristics. As a result, the agglomeration, dissolution, adsorption, and biochemical activities of NPs are altered, and consequently influence their *in vivo* behavior.[47] In an agglomerate state, NPs may behave as larger particles, depending on the size of the agglomerate. Hence, it is evident that NP agglomeration size and surface reactivity must be taken into account when considering health matters.[51] In this context, in a previous study on gingival tissue we found that the agglomerates of particles in the micro-size range did not cause an inflammatory reaction.[7] Similar results were observed in lung and kidney tissue samples in the present study.

Given that particle size is one of the variables that influences the degree of cytotoxicity, TiO_2 and UNCD particles of similar average particle size (APS) were used in the present

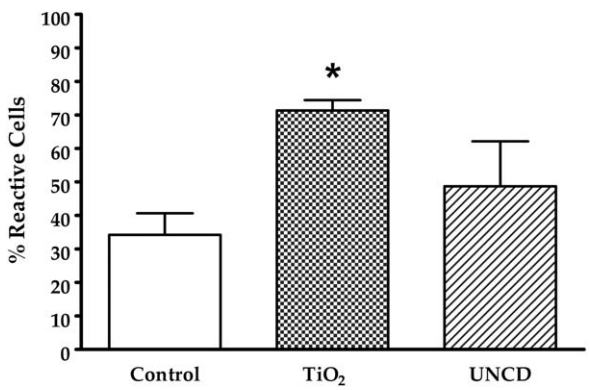

FIGURE 5. Anion superoxide generation in lung tissue samples. Percentage of reactive cells in BAL of TiO_2 and UNCD Groups. The histograms show the mean ± SD ($n \geq 6$), $^*p < 0.05$, compared to the Control Group.

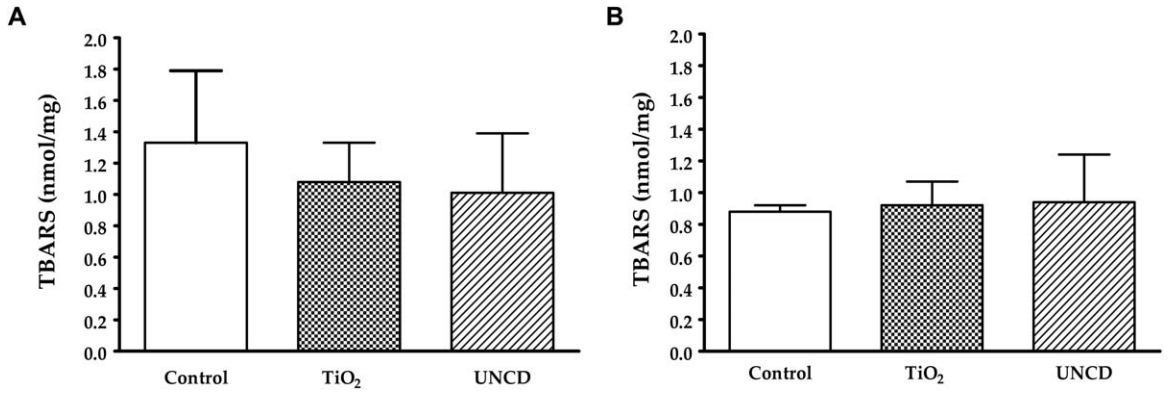

FIGURE 6. Determination of oxidative damage to lipids in lung (A) and liver (B). Thiobarbituric Acid Reactive Substances (TBARS) levels for control, TiO_2 and UNCD treated animals. No statistically significant differences were observed among groups.

study in order to control this potentially confounding variable.

Histological analysis of body organs showed the particles were agglomerated in interstitial spaces and/or phagocytozed mainly by alveolar macrophages or Kupffer cells (KCs). Qualitatively, there were more deposits in the studied organs and in blood cells in the TiO_2 injected group. Moreover, TiO_2 NPs were found to cause foci of necrosis with inflammatory infiltrate. Although it is well documented that agglomeration of particles in tissues favors giant cell recruitment,[44,52] this cell type was not observed in any of the TiO_2 or UNCD -exposed organs studied here.

As regards the liver, it has been described that NP size plays an important role in modulating target cell type and the degradation pathway. Particles larger than ~200 nm are effectively cleared by KCs because slow blood flow in liver sinusoids allows enough time for particle phagocytosis and macropinocytosis. Because the endothelium of hepatic sinusoid is discontinuous, with fenestrations approximately 100–200 nm in diameter, NPs smaller than the fenestrations can cross the endothelium into the Disse space, entering the lymphatic circulation or being taken up by hepatocytes.[46]

Despite the lung having several particle clearance mechanisms such as mucociliary transport, phagocytosis, pinocytosis and intracellular particle dissolution, NP agglomerates were found to persist in lung tissue 6 months after injection. Phagocytic uptake of NPs by macrophages may be enhanced after the formation of the protein corona which, in turn, can reduce interparticle agglomeration.[53] In comparison with the liver, the kidney is minimally involved in intracellular catabolism. Renal excretion represents a preferable clearance route for NPs from the body. Interestingly, the results of the present study showed NP deposits in the glomeruli, the first step in renal clearance.[46]

As mentioned previously, UNCD deposits were not only less abundant than TiO_2 deposits, but also caused no structural alterations in any of the studied organs. Hence, the observed biological response can be thought to be associated with the chemical composition of the studied nanoparticles.

Metallic particles can cause an increase in reactive oxygen species, which can result in oxidative stress. As pointed out by Knaapen et al.[54] ROS production could result from interactions at the particle-cell level, mainly in the lung where neutrophils and macrophages are powerful ROS generators. In agreement with their observations, our results showed that TiO_2 nanoparticles induced an increase in O_2^- generation by alveolar macrophages. Catalase consumption in lung tissue was greater in TiO_2-treated animals. This

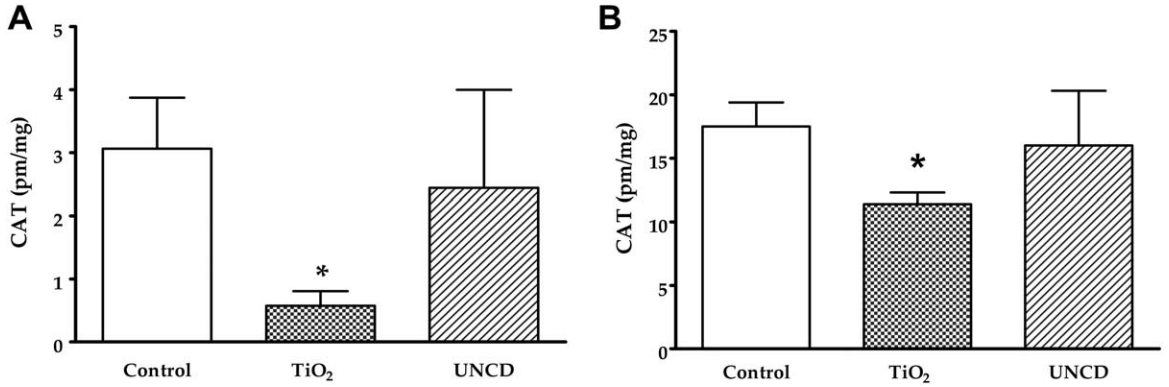

FIGURE 7. Determination of enzymatic antioxidants. Lung (A) and liver (B). Catalase (CAT) levels in control, TiO_2 and UNCD-treated animals. Note: Only TiO_2 NPs caused significant changes in CAT levels in lung and liver homogenates. The histograms show the mean ± SD ($n \geq 6$) *$p < 0.05$, compared to the Control Group.

decrease in catalase levels may be associated with the increase in superoxide anion production, indicating an adaptive mechanism that does not result in oxidative imbalance or in membrane lipoperoxidation and subsequent tissue damage. The absence of histomorphological changes in lung tissue confirms this hypothesis.

As observed in lung, CAT consumption was also observed in liver tissue samples. No significant changes in TBARS were observed in liver tissue, suggesting that, as found in the lung, the oxidative stress condition does not affect membrane phospholipids, due to the compensatory activity of CAT antioxidant enzyme in this organ. Nevertheless, it must be pointed out that foci of necrosis were found in some of the studied histological samples.

Exposure to UNCD caused no changes in oxidative metabolism in the lung, as shown by the absence of O_2^- generation, mobilization of antioxidant enzymes (SOD and CAT) and membrane damage (TBARS). Likewise, no SOD or CAT consumption or membrane damage was observed in liver samples. These biochemical parameters are in agreement with the lack of histopathological alterations in the studied organs.

The scant amount of UNCD deposits in the parenchyma of the organs studied here, the absence of morphological alterations, and the lower oxidative inductions as compared to observations in nanoparticles animals suggest that, in the event that the UNCD coating detached from the implant surface, tissue response would be less aggressive or negligible. These differences in biological response may be associated with the presence of carbon in the composition of UNCD, since it would favor biocompatibility. In this regard, Mitura et al.[55] suggested that due to the highest biocompatibility of carbon resulting from the presence of this element in the human body, it appears to be a potential biomaterial for use in biomedical implants. In addition, it was previously demonstrated that UNCD coated-Si microchips implanted inside rabbit eyes[19] could be employed to restore partial vision in blind people suffering genetically-induced degeneration of the photoreceptors. Moreover, Garret et al.[29] demonstrated excellent performance of N-UNCD-coated metallic electrodes for neural stimulation. Our findings are in agreement with Aspenberg et al.[56] who showed that diamond particles are harmless to tissues. Furthermore, the use of carbon-coated implants protects them from biological corrosion and metallosis.[57] Shi B et al.[24] studied cell culture cytotoxicity of different types of UNCDs thin films. They showed that UNCD films could support cell growth, as shown by no or little inhibition of cell proliferation, and were potentially appealing as substrate/scaffold materials.

Given the current surge in the use of nanocoatings, nanofilms, and nanostructured surfaces to enhance biocompatibility of biomedical implants, the results of the present study contribute valuable data for the manufacture of UNCD coatings as a new generation of superior medical implants.

CONCLUSION

The scant amount of UNCD deposits in the parenchyma of the organs studied here, the absence of morphological alterations, and the lower oxidative induction as compared to observations in animals exposed to TiO_2 nanoparticles suggest that, in the hypothetical case that UNCD particles detached from the surface of a UNCD-coated metallic implant, tissue response would be less aggressive. The studied parameters suggest that Ultrananocrystalline diamond (UNCD) may prove useful as a surface coating for implants exposed to the biological environment.

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