Effect of titanium dioxide on the oxidative metabolism of alveolar macrophages: An experimental study in rats

Daniel G. Olmedo,[1]* Deborah R. Tasat,[2] María B. Guglielmotti,[1] Rómulo L. Cabrini[1,3]
[1]*Department of Oral Pathology, School of Dentistry, University of Buenos Aires, Buenos Aires, Argentina*
[2]*School of Science and Technology, University of General San Martin, Buenos Aires, Argentina*
[3]*Department of Radiobiology, National Atomic Energy Commission, Buenos Aires, Argentina*

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Abstract: Metallic implants of titanium are used therapeutically in biomedicine because of its excellent biocompatibility. However, no metal or alloy is completely inert. We have previously shown that titanium oxide (TiO_2) is transported in blood by phagocytic monocytes and deposited in organs such as liver, spleen, and lung 6 months after intraperitoneal injection (ip). Furthermore, it is well known that exposure to metal traces alters the cellular redox status. Thus, the aim of the present study was to determine the presence of titanium in target organs after chronic exposure, assess the potential structural alterations, and evaluate the oxidative metabolism of alveolar macrophages (AM) in the lung. Rats were ip injected with 1.60 g/100 g body wt of TiO_2 in saline solution. Organs (liver, spleen, lung) were processed for histological evaluation. Reactive oxygen species (ROS) in AM obtained by bronchoalveolar lavage (BAL) were evaluated using the nitroblue tetrazolium test and quantitative evaluation by digital image analysis. The histological analysis of organs revealed the presence of titanium in the parenchyma of these organs with no associated tissue damage. Although in lung alveolar macrophages TiO_2 induced a significant rise in ROS generation, it failed to cause tissue alteration. This finding may be attributed to an adaptive response. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 73A: 142–149, 2005

Key words: titanium dioxide; lungs; macrophages; oxidative metablism; rats

INTRODUCTION

Titanium, both pure and in alloys, is widely used in the manufacture of dental and orthopedic osseointegrated implants.

Experimental[1–7] and clinical[8–13] studies have evidenced that intraosseous implants made from commercially pure (cp) titanium and titanium alloys are well tolerated by different tissues, including bone.

Despite the fact that osseointegration may initially be excellent, factors intrinsic to the implant materials or to the environment may lead to partial or complete loss of osseointegration.[14–17] Metal corrosion and the ensuing release of ions[18–21] may also lead to implant failure after initial success. This serious problem must be evaluated for the metallic biomaterials used in implantology. As previously shown,[22] even after an initial successful osseointegration, corrosion may lead to implant failure.

Previous findings reveal that titanium ions are released into surrounding tissues and reach the internal milieu to be excreted in the urine.[23] In a study on coxofemoral prostheses made of 90% titanium–6% aluminum–4% vanadium, Galante[23] showed that ions of these metals migrate into the plasma and are excreted in the urine. Recently, in a study of 27 autopsies, Urban et al.[24] detected the presence of metallic and plastic particles from coxofemoral prostheses and knee replacements in organs such as liver, spleen, and lymph nodes.

Since the 1960s, Ferguson et al.[25,26] and Laing et al.[27] have studied the systemic distribution of metals in different organs of the body.

We previously showed that titanium dioxide (TiO_2) is deposited in organs with macrophagic activity such as the liver, spleen, and lung,[20] 6 months after intraperitoneal injection.

We also demonstrated that TiO_2 is not only transported bound to plasma proteins in the blood but is also transported associated to cells of the phagocytic-mononuclear lineage.[28]

*Postdoctoral fellow of the National Research Council, Argentina.
Correspondence to: D. G. Olmedo; e-mail: dolmedo@argentina.com

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The toxicology of titanium has not been well studied to date. Epidemiological studies[29,30] showed that the inhalation of environmental dust containing titanium fails to exert a clear-cut deleterious effect on living organisms. However, Garabrant et al.[31] and Lee et al.[32] suggested an association between titanium particles and pathologies of the pleura, granulomatous diseases, and malignant neoplasia of the lung. Recently, Bermudez et al.[33] reported that the response of experimental animals to exposure to high concentrations of TiO_2 particles involved a persistent inflammatory response, progressive fibroproliferative lesions, and epithelial metaplasia in lung alveoli. Furthermore, it is well known that exposure to metal traces alters the redox status of the cell. This oxidative metabolism imbalance induces programmed cell death, tissue necrosis, or chronic alterations in different tissues.[34–37]

In the lung, alveolar macrophages remove the metal ions by phagocytosis. Alveolar macrophages are the main cells involved in the first line of defense and are responsible for engulfing and eliminating any element identified as "non-self." A known and distinctive feature of phagocytes is their ability to respond to appropriate stimuli by activation of a respiratory burst.[38,39] The respiratory burst involves the increased production of reactive oxygen species (ROS), mainly the superoxide anion, from the reduction of oxygen by NADPH oxidase. Detection and quantification of ROS species is of great interest to determine the association between elevated oxidative stress and pathological conditions.

Thus, the aim of the present study was to determine the presence of titanium particles in target organs after chronic exposure in rats, assess potential structural alterations in these organs, and evaluate the oxidative metabolism of alveolar macrophages.

MATERIALS AND METHODS

Animals

Male Wistar rats ($n = 10$), approximately 100 g body wt, were injected intraperitoneally with a solution of TiO_2 (anatase; Sigma Chemical Co., St. Louis, MO) at a dose of 1.60 g/100 g body wt (experimental group). Another group (n = 10) was injected with saline solution (control group). National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) were observed.

Chemicals

NBT (nitroblue tetrazolium), PBS (phosphate-buffered ssaline), PVP (polyvinylpyrrolidone), RPMI-1640 medium, TPA (tetradecanoil phorbol acetate) were purchased from Sigma Chemical Co.

Bronchoalveolar lavage

After 18 months, animals were killed and their lungs were immediately lavaged as described by Brain et al.[40] and modified by Tasat et al.[41] The thoracic cavity was partly dissected, and the trachea was cannulated with an 18-gauge needle and infused 12 times with 1 mL cold sterile PBS, pH 7.2–7.4. The bronchoalveolar lavage (BAL) fluid was immediately centrifuged at 800*g* for 10 min at 4°C and resuspended in 5 mL PBS. The samples were submitted to the Trypan Blue exclusion test and evaluation in a hemocytometer under phase contrast microscopy to determine total cell number and cell viability.

Histological analysis

Samples of liver, spleen, and lung were processed for histological evaluation. The lung samples were taken from the lobes that had not been submitted to BAL. The samples were fixed in 10% formalin and routinely processed for paraffin embedding and staining with hematoxylin-eosin. The samples were treated with picric acid to avoid the possible presence of formalin pigments.

The samples were submitted to enzymatic digestion with tripsin. A X-ray diffraction analysis of the resulting sediment was used to evaluate the presence of titanium in the tissues and perform its crystallographic characterization.

Cell viability

Macrophage viability was determined by the Trypan Blue exclusion test under an inverted phase-contrast microscope for all the experimental conditions assayed.

Generation of ROS

Alveolar macrophages (AM) from the BAL were evaluated for intracellular ROS. During the respiratory burst of AM, ROS were evaluated using the NBT reduction test as previously described.[42] The intracellular release of ROS is evidenced by the amount of a blue formazan precipitate in the cell after NBT reduction. BAL cells were treated with NBT in the presence or absence of TPA, a known inductor of superoxide anion ($O_2^{\cdot -}$) generation. BAL suspension obtained from each animal was separated into three fractions as described elsewhere.[43] Briefly, the three fractions obtained were as follows:

Fraction A was used to analyze macrophage percentage in the BAL suspension by differential staining using May-Grunwald-Giemsa.

Fraction B was used to evaluate non-TPA-stimulated macrophages. Only NBT (1 mL, 0.1% in PBS) was added for 45 min at 37°C under mild agitation to estimate the basal reaction in the cell suspension.

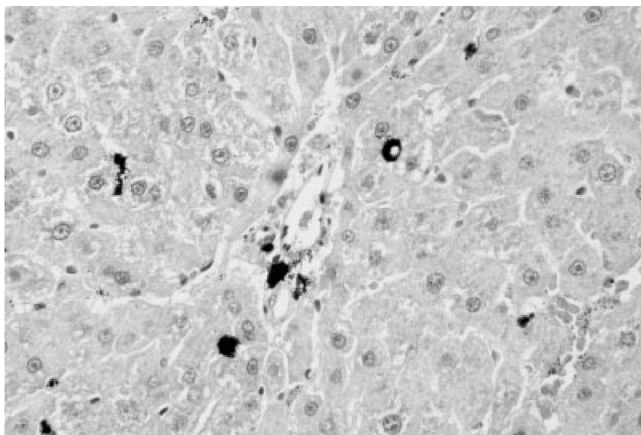

Figure 1. Liver. The presence of clusters of titanium particles is clearly observable both in the parenchyma and in the portal space. Original magnification, ×40, hematoxylin-eosin stain.

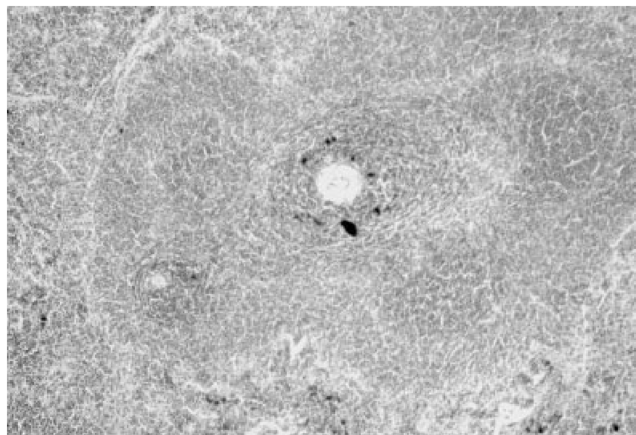

Figure 2. Spleen. Note the deposit of material in the parenchyma, particularly surrounding the artery. Original magnification, ×10, hematoxylin-eosin stain.

Fraction C was used to evaluate TPA-stimulated macrophages. NBT (1 mL, 0.1% in PBS) and TPA (10 μL, 100 μg/mL acetone) were added to the cell suspension for 45 min at 37°C under mild agitation.

No less than 2×10^5 cells were incubated for each fraction. All samples were run in duplicates.

Total optical density

The total optical density (TOD) from BAL cells was scored by cell digital image analysis as described previously.[44] Briefly, fixed cells were observed under an MPM 800 Carl Zeiss microscope (Jena, Germany) using an interferential filter (570 nm) and a plan achromat 40:1/0.75 objective coupled to a DK 7700 SXK camera (Hitachi, Hamburg, Germany). Images were digitized in 8 bites and analyzed with an Image Analysis System (IBAS-Kontron). Ten fields of approximately 20 cells each were selected at random to evaluate at least 200 macrophages per slide. The light and background intensity was determined for each sample. The software used allows for the scanning and evaluation of single cells. TOD value per cell corresponds to the sum of all the optical densities (OD) values obtained by scanning the whole cell.

Statistical analysis

The results were compared using ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

In all cases, both control and experimental groups failed to show changes in body weight or behavior.

Histological analysis

The histological analysis of liver, spleen, and lung of control animals revealed the absence of alterations. The experimental animals exhibited titanium particles in the parenchyma of these organs (Figs. 1–3). The number of macrophages in the alveolar lumen of experimental lungs was markedly higher than in controls (Fig. 4).

Crystallographic characterization

The X-ray diffraction analysis of the sediment obtained by enzymatic digestion of liver, spleen, and lung revealed the presence of TiO_2 (anatase).

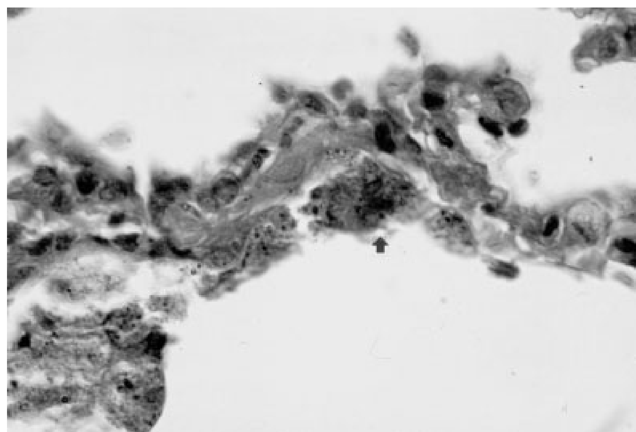

Figure 3. Lung. Macrophages loaded with particles can be observed on the alveolar wall (→). Original magnification, ×100, hematoxylin-eosin stain.

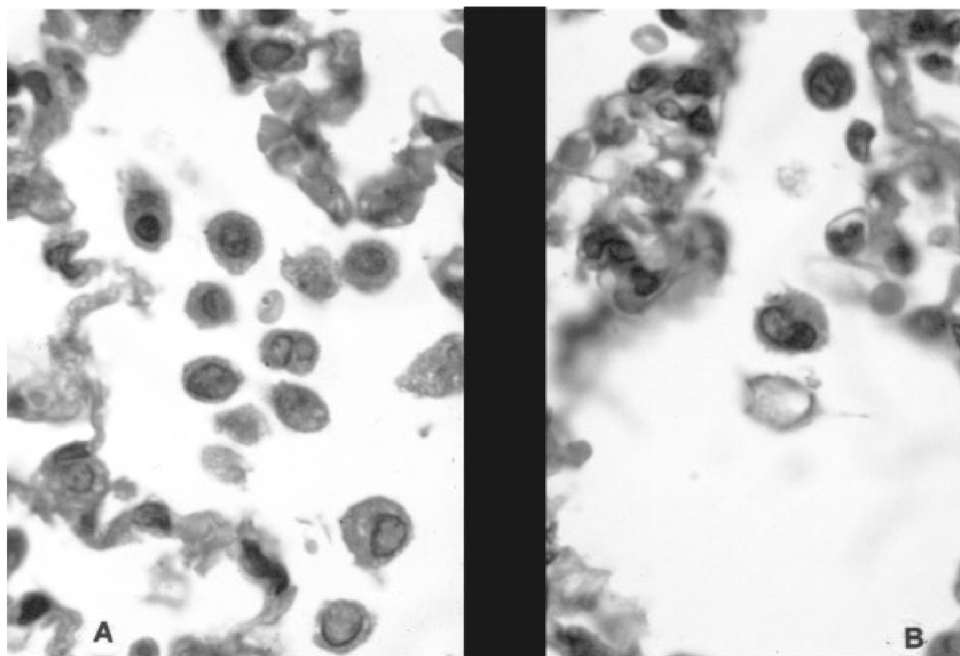

Figure 4. Lung. (A) experimental; (B) control. Note that the number of macrophages present in the alveolar lumen in (A) is larger than in (B). Original magnification, ×40, hematoxylin-eosin stain.

Cell viability

The proportion of AM in the lung lavage cells based on morphological criteria was >95%. More than 98% of AM were viable according to the Trypan Blue exclusion test.

Light microscopy and digital image analysis quantitation

Light microscopy observations of BAL cells obtained from the above samples revealed that the generation of ROS per cell varies among macrophages of the same fraction (Fig. 5). Photomicrographs of stimulated and nonstimulated BAL cells from control and experimental rats are shown in Figure 6.

The response of the control TPA-nonstimulated fraction is clearly shown in Figure 6(I). A few cells did exhibit scattered light blue granules of formazan precipitate, representing the basal reaction in the AM population of the lung. Control TPA-stimulated cells [Fig. 6(III)] showed a larger number of more intensely positive reactive cells compared to control nonstimulated cells [Fig. 6(I, III)].

The response of the experimental TPA-nonstimulated fraction [Fig. 6(II)] showed mainly reactive cells with a varying degree of reaction (slight to strong intensities), similar to those observed when experimental cells were TPA-stimulated [Fig. 6(IV)]. Very few nonreactive cells were found in these samples.

To objectively evaluate these observations, we assessed the amount of blue formazan precipitate per cell using digital image analysis.

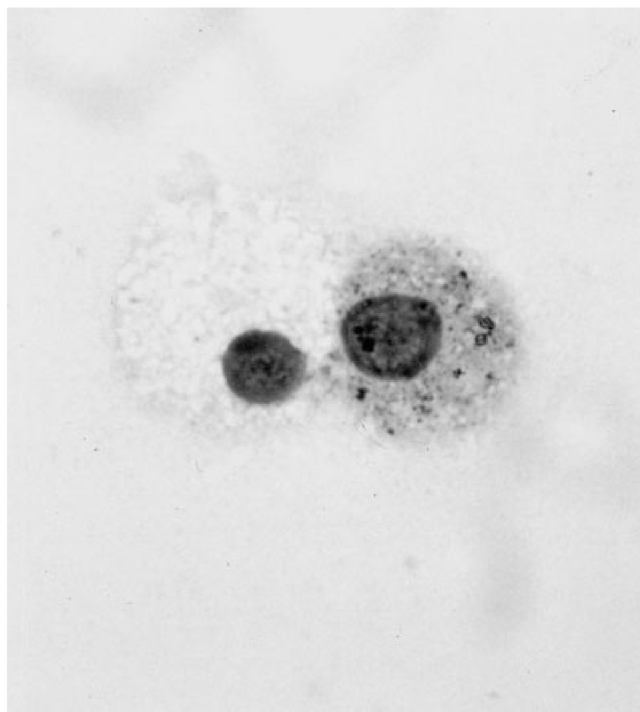

Figure 5. Macrophages from bronchoalveolar lavage submitted to the NBT test. Note the presence of a nonreactive cell and a highly NBT reactive macrophage loaded with titanium particles. Original magnification, ×100.

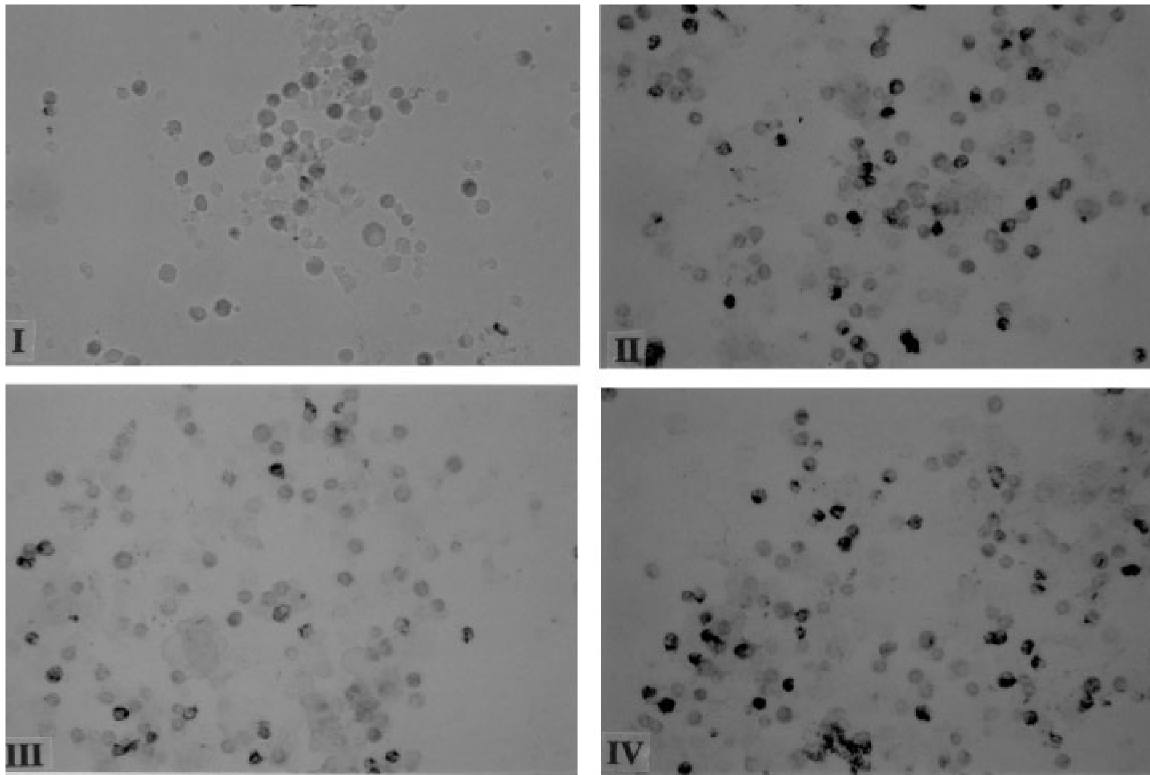

Figure 6. Light microphotographs of NBT test of BAL cells from control (I and III) and experimental (II and IV) rats. (I and II) Non-TPA-stimulated BAL cells from control and experimental animals showing basal superoxide anion generation, respectively. (III and IV) TPA-stimulated BAL cells from control and experimental animals, respectively.

TOD values were displayed and distributed in intervals of 50 arbitrary units as shown in Figure 7. In the non-TPA-stimulated fraction from control BAL cells almost all the macrophages (>80% of the population, 85.88 ± 11.3) were nonreactive, falling into the 0–50 TOD range. When these cells where TPA-stimulated they clearly responded to the stimulus. The percentage of nonreactive cells fell to around 45%

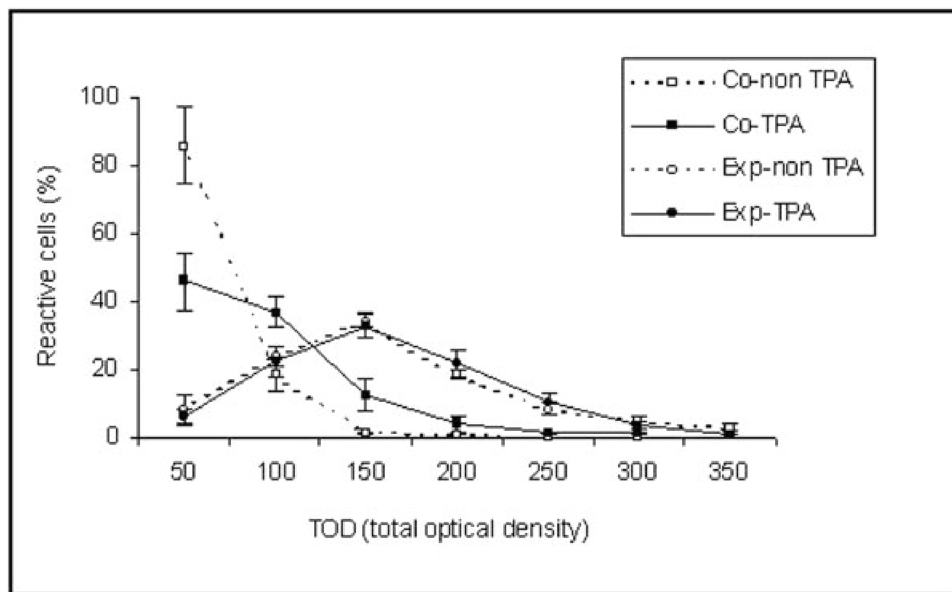

Figure 7. Generation of ROS in control and experimental rats. ROS generation was assayed by the NBT test and evaluated by Digital Image Analysis of BAL cells from control and experimental animals. Values are mean ± SE from 5 animals per group.

(45.98 ± 8.2). The 55% of the remaining population depicted higher TOD values in the range of 100–300. The difference between the number of nonreactive cells in nonstimulated BAL fractions and stimulated fractions obtained from control animals was statistically significant ($p < 0.05$).

Conversely, both TPA-nonstimulated and TPA-stimulated BAL cells fractions obtained from experimental rats showed a similar pattern with no significant differences for any of the intervals assayed. In these fractions the nonreactive subpopulation (TOD values < 50) reached only 10% of the total population (8.19 ± 4.33) and the remaining 90% depicted values > 100 and < 350 with a clear peak at 150. It is evident that in BAL cells from experimental animals TPA was not able to reduce the number of cells with 0–50 TOD values (5.82 ± 2.39).

DISCUSSION

The effect of some metals on tissues has been studied.[41,45] However, the action of metallic biomaterials used in implantology remains to be evaluated.

In a previous study we showed that 6 months after intraperitoneal injection, TiO_2 is deposited in organs such as liver, spleen, and lung.[20] The presence of titanium in lung was demonstrated by X-ray diffraction analysis and confirmed our previous findings using the microincineration technique.[21]

In the present study we showed that at longer exposure times, 18 months after injection, the presence of TiO_2 in liver and spleen did not cause observable damage. However, in lung, we observed a significant increase in the number of alveolar macrophages.

It is well known that ionizing and ultraviolet radiation, certain toxic drugs, some chemical agents such as carcinogens and metal traces can increase the physiological production of ROS. The increase in ROS may lead to an imbalance in the oxidative metabolism of the cell and cause tissue damage.[46–48] Macrophage production of ROS is linked to activation of a NADPH oxidase, which can be activated by a variety of stimuli associated with phagocyte function.[49] Using digital image analysis it is possible to quantitatively estimate cell-to-cell variations produced during the respiratory burst at the cellular level as previously described by Fernandez et al.[43]

As is clearly shown in Figure 6, TiO_2 is capable of activating a respiratory burst in macrophages to the same extent as the known carcinogen TPA. Both stimulated and nonstimulated TPA fractions from experimental rats exhibited higher TOD values when compared to stimulated and nonstimulated TPA fractions from control animals.

In 1999, Fernandez et al.[43] proposed a mechanism of macrophage autoregulation to explain the response of a population after stimulation. They suggested that the process of autoregulation guaranteed host defense capacity with a minimum damage to tissue and that impaired autoregulation would result in unregulated ROS. They also demonstrated that autoregulation is directly associated to variations in cell density. Although the cell number in BAL suspensions obtained from experimental animals was always higher than the number of cells obtained from control animals (mean ± S.E.: $4.26 \times 10^6 \pm 0.29$ and $2.16 \times 10^6 \pm 0.04$, respectively), to avoid inaccuracies in the evaluation of ROS generation we incubated BAL cells from both control and experimental rats strictly under the same conditions of cell density. In our experiments and regardless of the addition of TPA, a potent ROS inductor, the fraction of reactive cells obtained from experimental rats remained almost constant. The data presented herein show that the presence of TiO_2 induces a rise in the generation of ROS in lung alveolar macrophages. These findings are in keeping with previous studies by other authors who showed that TiO_2 induces ROS generation and lung cytotoxicity.[50]

Despite the fact that titanium particles in alveolar macrophages induced a significant increase in ROS generation, we failed to observe tissue damage at 18 months after injection. The absence of tissue damage in lung parenchyma at the experimental time assayed may be attributed to an adaptive response. However, we must point out that direct incorporation of TiO_2 particles to the lung by inhalation or instillation has been associated to lung pathologies.[31–33]

The presence of TiO_2 in the lung is significant in terms of its potential clinical value as a diagnostic indicator of corrosion processes.

The data presented herein are relevant to the fact that orthopedic and dental implants are not only a local issue. These data show that titanium dioxide may migrate and be deposited in different organs. Titanium deposits are found not only in organs that are known to metabolize and filter blood, i.e., liver and spleen, respectively, but also in lung. These findings are relevant to the biomedical field and warrant future studies.

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