Surface modification by argon plasma treatment improves antioxidant defense ability of CHO-k1 cells on titanium surfaces

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ABSTRACT

Titanium is one of the most used materials in implants and changes in its surface can modify the cellular functional response to better implant fixation. An argon plasma treatment generates a surface with improved mechanical proprieties without modifying its chemical composition. Oxidative stress induced by biomaterials is considered one of the major causes of implant failure and studies in this field are fundamental to evaluate the biocompatibility of a new material. Therefore, in this work, induction of oxidative stress by titanium surfaces subjected to plasma treatment (PTTS) was evaluated. The viability of CHO-k1 cells was higher on PTTS discs. Cells grown on titanium surfaces are subjected to intracellular oxidative stress. Titanium discs subjected to the plasma treatment induced less oxidative stress than the untreated ones, which resulted in improved cellular survival. These were associated with improved cellular antioxidant response in Plasma Treated Titanium Surface (PTTS). Furthermore, a decrease in protein and DNA oxidative damage was observed on cells grown on the roughed surface when compared to the smooth one. In conclusion, our data suggest that the treatment of titanium with argon plasma may improve its biocompatible, thus improving its performance as implants or as a scaffold in tissue engineering.

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1. Introduction

Biomaterial is any material, substance or combination of substances, of natural or synthetic sources that interacts with biological systems to stimulate the growth or replace any tissue or organ for any period of time (Binyamin et al., 2006). Several kinds of materials may be used as biomaterials, but metals are certainly the most used. Among these, titanium is the most widely used

material in the manufacture of implants for several uses, especially as bone replacement (Lefaix et al., 2008; Ratner, 2001).

The successful use of titanium-based implants for numerous applications is attributed to its excellent mechanical properties, corrosion resistance, and the biocompatibility of commercially pure titanium and its alloys (Tsaryk et al., 2007). The metal-tissue interaction is very important to cellular adaptation and seems to be dependent on the surface characteristics (Jiang et al., 2009). Apparently, the number and quality of the cells that adhere to the implant is controlled by surface properties (Rosales-Leal et al., 2010). Therefore, biomaterial's surface engineering is oriented to modify their texture and/or chemistry to improve biocompatibility and cellular colonization.

Several methods were described to change the surface properties in order to improve the osseointegration process and the mechanical resistance, and thus the biocompatibility of the implant, such as plasma treatment (Chiesa et al., 2007; Layrolle et al., 2007; Simon and Watson, 2002). In laboratory conditions, plasma is obtained through an electric gas discharge, applying a potential difference between two electrodes inserted into a

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chamber at pressures below 100 Pa. The ions produced are accelerated to the cathodically polarized electrode doing several effects such as the creation of defects on the surface. Argon plasma treatment generates a surface that has good mechanical proprieties without modified its chemical composition. (Alves et al., 2005)

In this context, biocompatibility evaluation of surface treatments ought to include assays of cell survival, metabolic activity and potential genotoxicity. The most used genotoxicity assays in mammalian cells are the micronuclei test and comet assay (Jiang et al., 2008; Shukla et al., 2011; Tavares et al., 2009). Despite the importance of genotoxic response to understand potential long term risks of biomaterials, so far only few studies have assessed the genotoxic potential of different titanium-disc surfaces (Tavares et al., 2009; Velasco-Ortega et al., 2010; Wang and Li, 1998).

The evaluation of oxidative stress induction by biomaterials has only recently received attention in tissue engineering research. Some results suggest a possible relationship between oxidative stress and cell adhesion (Parrish et al., 1999; Aikawa et al., 2002). It is well known that crucial cellular signaling pathways are regulated by the extra and intracellular redox status and that reactive oxygen species (ROS) function as intercellular signaling molecules (Nygren et al., 2001). Despite these physiological roles, ROS can also damage several biomolecules and all aerobic organisms have evolved mechanisms to defend themselves against ROS. These include important antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase, which determine the cells response to the oxidative stress (Tsaryk et al., 2007). Unbalanced ROS and reactive nitrogen species (RNS) generation can induce lipid peroxidation, protein oxidation, and DNA damage (Gurr et al., 2005; Shukla et al., 2011; Tsaryk et al., 2007), which could potentially lead to genotoxicity (Jiang et al., 2009; Tavares et al., 2009).

In a previous work, we showed that different titanium surfaces (treated and untreated by plasma energy source) present different genotoxic effects, with the Plasma Treated Titanium Surface showing lower genotoxicity than the untreated one. Regarding this, our hypothesis was the non-treated titanium surface induced higher levels of oxidants leading to the elevated genotoxic response (Tavares et al., 2009). In this context, the aim of this study was to assess oxidative stress status generated by titanium surfaces treated and non-treated (UTS) by argon plasma in the same cellular model.

2. Material and methods

2.1. Preparation and characterization of titanium discs

The titanium discs (diameter = 9 mm and thickness = 3 mm), obtained from pure commercial titanium (grade II-Sigma), were kindly provided by Prof. Dr. Clodomiro Alves Jr. of the Laboratory for Processing Material by Plasma at the Universidade Federal do Rio Grande do Norte. The discs were initially polished; and half of the samples were then bombarded with pure argon atmosphere, using a plasma energy source, generating a Plasma Treated Titanium Surface (PTTS). The Untreated Titanium Surface (UTS) was also used in this study to compare the cell response. This argon bombardment created unevenness on the polished surface, resulting in a mean roughness (Ra) of 0.11 μm against Ra of 0.027 μm on untreated surface. The treated surface was also found to be more hydrophilic. Details of the plasma treatment and the characterization procedure can be found in Sá et al. (2009).

2.2. Cell culture conditions

Chinese hamster ovarian cell line (CHO-k1) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) high glucose, supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin (Gibco), and 100 μg/ml of streptomycin sulfate (Gibco) (complete medium). The cell culture was incubated at 37 °C in a humidified atmosphere and 5% CO_2.

In this work, cells were seeded on the two kinds of titanium discs (PTTS and UTS) placed on the wells of 24-well culture plates (Costar). Cells grown on the polystyrene surface of the culture plate were used as negative control (NC). Cells (1×10^5) were plated, in each well, in 1 ml of complete medium. The plates were then incubated until the disc surface was covered with cells, for a total of 72 h. After incubation, cells were released from the surface using trypsin (IX-Gibco) and centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in PBS and collected for the bioassays described below.

2.3. Bioassays

2.3.1. Viability assays

Cell viability was measured using Trypan blue dye exclusion assay and MTT assay. Cells were seeded onto titanium discs and plastic surface of culture plate. After the growth period, the cells were incubated at 37 °C with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) for 4 h. After the supernatant removal, 1 ml of ethanol was added to each well and mixed until the formazan crystals were completely dissolved. The optical density was measured at 570 nm.

2.3.2. Analysis of cell cycle

To analyze the cell cycle, CHO-k1 cells grown on titanium surfaces were collected by tripsinzation, fixed and permeabilized with 70% ice-cold ethanol, and stored at −20 °C. The cells were resuspended in 400 μL of PI solution (0.1% Triton X-100, 20 μg/mL PI, and 200 μg/mL RNase A, in PBS) and after incubation (1 h at 37 °C, protected from light), cells were analyzed by flow cytometry (Guava® PCA-96 System, Millipore, EUA). The data was analyzed using the ModFit software.

2.3.3. Measurement of H_2O_2

The content of H_2O_2 was determined using Amplex Red (Sigma–Aldrich, St. Louis, MO). When this probe reacts with H_2O_2, in the presence of peroxidase, a fluorescent product (Resorufin) is formed. After treatment, cells were washed twice with PBS and trypsinized. The cells (1×10^6) were then centrifuged (2000 rpm, 5 min), and cells pellets were resuspended in 2 mL of reaction medium containing Amplex Red and 1 U/mL of peroxidase for fluorescence detection. The fluorescence was continuously monitored in spectrophotometer (Hitachi F-4000) with excitation and emission at 563 nm and 587 nm, respectively.

2.3.4. Antioxidant assays

Cells grown on the different surfaces for 72 h were collected and the activity of the antioxidant enzymes, catalase (CAT) and superoxide dismutase intracellular (SOD1 and SOD2), were measured using the Catalase and Superoxide Dismutase Assays Kits (Cayman Chemicals, USA), following the manufacturer's instructions. The extracellular SOD (EC-SOD or SOD3) was measured in culture medium, which was collected after the titanium exposure, using Superoxide Dismutase Assay Kit (Cayman Chemicals, USA). The protein content was determined by Bradford method.

2.3.5. Lipid peroxidation

To evaluate the lipoperoxidation on CHO-k1 cells after exposure to titanium disks, the amount of isoprostanes were measured with the 8-isoprostane EIA kit (Cayman Chemicals, USA), following the manufacturer's instructions. Isoprostanes are biomarkers for lipid oxidation produced by non-enzymatic phospholipids oxidation.

2.3.6. Protein carbonyl content

Protein carbonyl content was determined using the protein carbonyl assay kit (Cayman Chemicals, USA). This is based on the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls, which produces 2,4-dinitrophenylhydrazones that can be quantified spectrophotometrically at 370 nm. The amount of protein carbonyls was calculated on the basis of molar extinction coefficient of DNPH ($0.022 \, M^{-1} \, cm^{-1}$) and expressed as nmol carbonyl/mg protein.

2.3.7. Comet assay with FPG

The formamidopyrimidine DNA glycosylase (Fpg) enzyme recognizes and cleaves oxidized purines, in particular, 8-OH guanine, being a reliable biomarker to evaluate DNA oxidative damage. Therefore, Fpg-modified Comet assay was applied, in this study, to identify DNA oxidative damage after titanium exposure. Briefly, 10 μL of cell suspension (approximately 1×10^4 cells) of each growth condition was added to 75 μL of low-melting point agarose at 37 °C, layered onto a slide precoated with 1% regular agarose. A coverslip was added and, after agarose solidification (at 4 °C), it was gently removed and the slide immersed in freshly made lysing solution (NaCl 2,5 M; Tris 10 mM; EDTA 100 mM (pH 10); with 10% DMSO, 1% Triton X-100 added just before use) at 4 °C for 16 h. Two couples slides were prepared from each test condition. At the end of the lysing period, slides were washed three times in enzyme buffer (40 mM HEPES, 0.1 M KCl, and 0.5 mM EDTA, pH 8; 0.2 mg/mL BSA). One couple's slide was incubated with a 1:3000 Fpg solution (New England Biolabs, Arundel, Queensland, Australia), while the other was incubated with enzyme buffer. Both slides remained for 30 min at 37 °C. After that, the slides were transferred to an electrophoresis chamber, containing a high pH (>13.0) buffer (300 mM NaOH, 1 mM EDTA), and incubated at 4 °C for 20 min, and electrophoresed for another 30 min at 25 V (0.86 V/cm) and 300 mA. Finally, the slides were submerged in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min, and each slide was stained with 50 μL ethidium bromide (20 μg/mL). All the steps were conducted under dimmed light. The slides were immediately evaluated at 400X magnification using fluorescence microscope (Nikon Eclipse E200). At least 100 randomly selected images were analyzed from each sample and the DNA damage was analyzed with the CASP software package and performed in a blinded manner. Ghost cells were not counted. The tail moment comet parameter measured was used as DNA damage index in our study.

2.4. Statistical test

All the biological tests were performed in at least three independent experiments with no less than two technical replicates. The GraphPad Prism (GraphPad, San Diego, CA) was used to perform the statistical analyses. Data were analyzed using one way ANOVA. When significance was demonstrated ($p < 0.05$), comparisons were made using Tukey test.

3. Results

3.1. Viability assays

The viability of CHO-k1 cells, measured by trypan blue exclusion assay, after 72 h of growth on UTS and PTTS was ⩾80%, however the viability was higher on PTTS discs. A significant decrease ($p < 0.001$) in MTT reduction rate, measured by MTT assay, was observed in CHO-k1 cells grown on the untreated titanium discs when compared to the plasma treated ones (Fig. 1). The results demonstrate a 30% reduction to UTS against 4% reduction to PTTS,
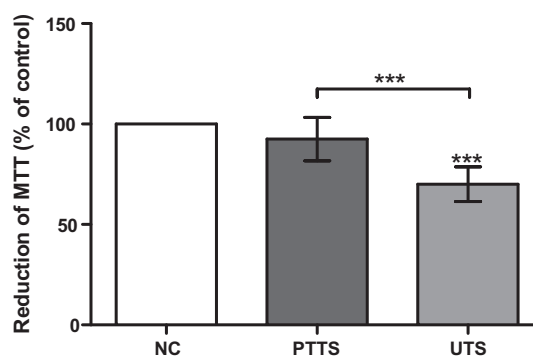

Fig. 1. Cellular toxicity of Plasma Treated Titanium Surface (PTTS) and Untreated Titanium Surface (UTS) through MTT assay. Negative control (NC) corresponds to cells grown on plastic cell culture plate. Data represent the means of three independent experiments. ***$p < 0.001$.

when both conditions were compared to cells grown over the normal plastic surface.

3.2. Analysis of cell cycle

Cell cycle progression of CHO-k1 cells grown on UTS and PTTS was studied by flow cytometry. As is reported in Table 1, after 3 days, cells growing on the titanium discs showed a cell cycle distribution quite distinct from the cultures maintained on the plastic surface (negative control). However, no significant difference in proliferation rates was observed.

3.3. Measurement of H_2O_2

The H_2O_2 content of CHO-k1, measured using Amplex Red, showed that both Ti surfaces increased H_2O_2 production when compared to the NC (Fig. 2A). These results suggest that cells growing on titanium surfaces are under oxidative stress conditions.

3.4. Antioxidant assays

The direct measurement of two antioxidant enzymes, SOD and CAT, demonstrated that changes in antioxidant response only occurred on cells grown on PTTS (Fig. 2B–C), where a significant increase in extracellular SOD ($p < 0.01$) and a significant decrease in intracellular SOD ($p < 0.01$), were observed. No change in antioxidants enzymes activity was observed for UTS. Comparing the results obtained with titanium surface, an increase of extracellular SOD and CAT and a reduction of intracellular SOD activity, were observed, when PTTS surfaces were used. When the comparison was performed between the titanium surfaces, it was observed that PTTS leads to an increase in the extracellular SOD and CAT and a reduction in the intracellular SOD activity.

Table 1
Effect of titanium surfaces on cell cycle progression (%).

| | Cell cycle phase (%) | | |
	G0/G1	S	G2/M
NC	61.75 ± 3.9	21.35 ± 4	16.09 ± 1.2
PTTS	53.84 ± 7	24.7 ± 4	21.46 ± 8
UTS	53.27 ± 8	27.2 ± 9	19.53 ± 2

NC (Negative control), PTTS (Plasma Treated Titanium Surface) and UTS (Untreated Titanium Surface). Data represents the mean ± SD of three independent experiments with two technical replicates.
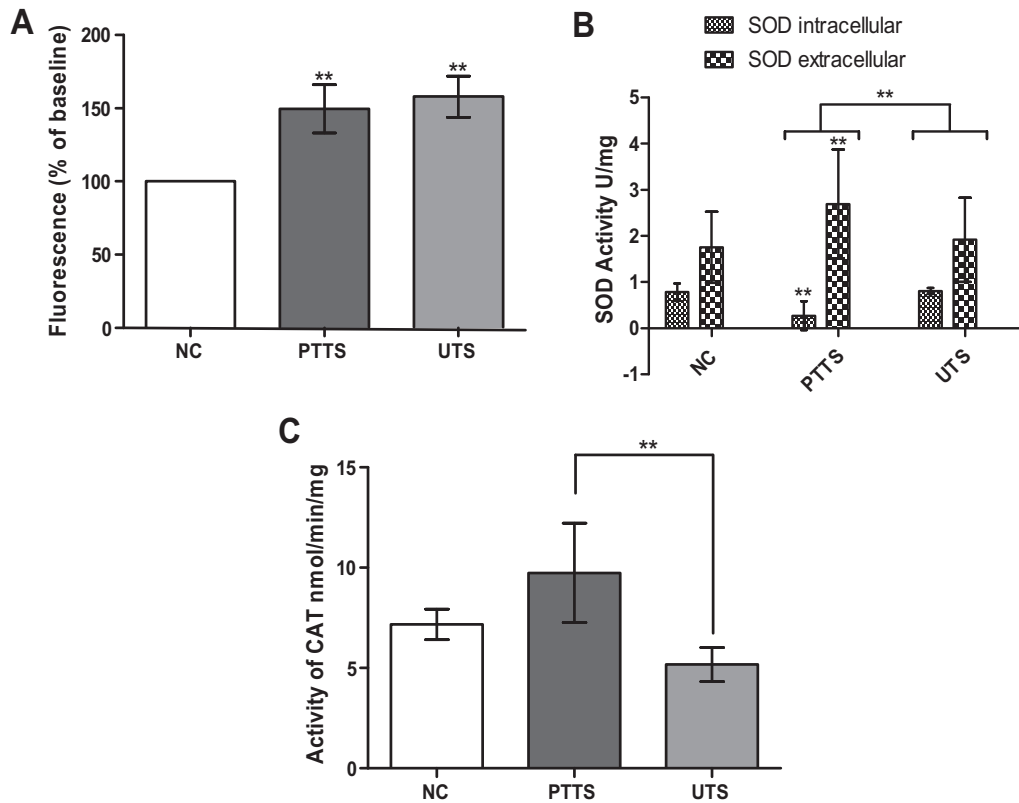
Fig. 2. Assessment of reactive species generation and antioxidant response: (A) Measurement of H_2O_2 with amplex red. (B) SOD activity. (C) CAT activity. All the analyses were done using CHO-k1 cells after 3 days of growth on titanium surfaces: Plasma Treated Titanium Surface (PTTS) and Untreated Titanium Surface (UTS), except for the measurement of extracellular SOD (SOD3) activity, which were done with the culture medium collected after the treatment period. Cells grown on plastic cell culture were used as negative control (NC). Data represent means of three experiments in triplicate except to b and c that were performed in five independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$.
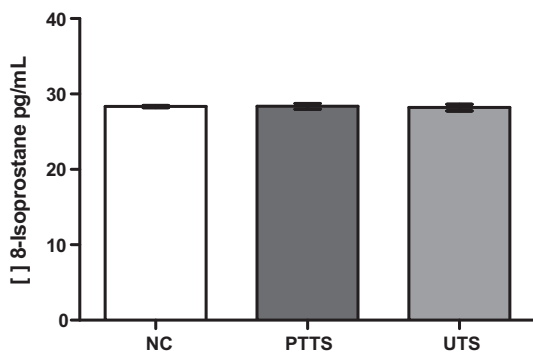

Fig. 3. Measurement of 8-Isoprostane in culture medium after 3 days of growth cellular on culture plate (NC), Plasma Treated Titanium Surface (PTTS) and Untreated Titanium Surface (UTS). Data represent the means of three independent experiments.

Fig. 4. Carbonyl content of CHO-k1 cells after 3 days of growth on culture plate (NC), Plasma Treated Titanium Surface (PTTS) and Untreated Titanium Surface (UTS). Means founded for each treatment were done in triplicate thrice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.5. Lipid peroxidation

Lipid oxidative damage was evaluated by quantification of 8-isoprostane. The amounts detected were similar, irrespective the test conditions used (Fig. 3), suggesting that titanium surfaces promote no lipid peroxidation.

3.6. Protein carbonyl content

We also ascertained whether growth on Ti surfaces leads to protein oxidation, by measuring the carbonyl content. The amount of
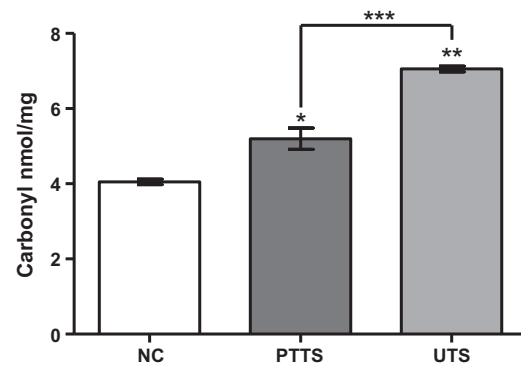
carbonyl increased in both Ti surfaces, when compared to the NC. However, the content was significantly higher on UTS ($p < 0.001$) than PTTS, indicating lower intracellular oxidative stress in cells grown on the Plasma Treated Titanium Surface (Fig. 4).

3.7. Comet assay with Fpg

DNA damage in cells grown over the three types of surfaces was assessed using the standard comet assay. To introduce strand breaks at sites containing oxidized purines, the comet assay was also performed in combination with Fpg. In both conditions, cells
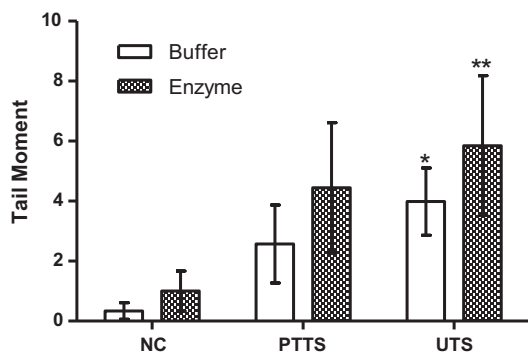
Fig. 5. Effect of an untreated surface (UTS) and Plasma-Treated Titanium Surface (PTTS) on DNA by FPG- comet assay experiment. Tail moment values represented the mean ± S.E.M. obtained from three independent evaluations. $p < 0.05$. The negative control (NC) used was cells grown on plastic cell culture.$^*p < 0.05$ compared with buffer negative control. $^{**}p < 0.05$ compared with enzyme negative control.

grown on titanium discs showed more DNA damage than the controls, although only the untreated surface induced levels of damage that were significantly higher than the controls. With the standard comet assay, tail moment (mean ± S.D) increased from 0.34 ± 0.3 in NC to 3.98 ± 1.1 in UTS and to 2.5 ± 1.3 in PTTS (Fig. 5). In the presence of Fpg, the levels of tail moment increased in all conditions used (5.85 ± 2.3 in case of UTS, 4.4 ± 2.1 in case of PTTS and 1 ± 0.7 in the case of NC). Although higher than the NC cells, DNA damage increase in cells grown on PTTS was not statistically significant. These results indicate that UTS induces more DNA breaks and oxidized base damage than the PTTS. Images of the comets could be found at Supplementary material (Fig. S1).

4. Discussion

The evaluation of oxidative stress to materials is a new field in tissue engineering. Nevertheless, there are only a few studies addressing this subject, and this work, as far as we know, is the first to directly evaluate the oxidative stress in CHO-k1 cells cultivated on pure titanium discs with (PTTS) or without (UTS) treated surface by plasma argon energy.

As expected, the PTTS showed a better cellular adhesion than UTS (Fig. S2), due to increase of hydrophilicity by plasma treatment (Tavares et al., 2009). According to the cell cycle (Table 1) and trypan blue exclusion assay results the differences obtained in the MTT (Fig. 1) could be related to the higher number of initial cell adhesion instead of material's toxicity, since it was not observed cell cycle arrest.

It is already described that a thicker oxide layer could make the metal surface more resistant to corrosion avoiding the release of particles, ions, and unstable ions during the electrochemical process of corrosion and oxidative stress enhancing the biocompatibility (Tavares et al., 2009; Jo et al., 2011; Xin et al., 2012). We found that cells grown over both the titanium surfaces are under oxidative stress (Fig. 2A). These findings are in agreement with earlier literature data, showing an increase in H_2O_2 content associated with Ti exposure (Kalbacova et al., 2007; Rahman et al., 1997; Schapira et al., 1995). However, the different behaviors observed on the surfaces is this work cannot be attributed only to H_2O_2 since others ROS were not evaluated.

The oxidant/antioxidant balance is vital to the cells function (Fujii et al., 2003). Antioxidant defense system includes several enzymes, among them superoxide dismutase (SOD), glutathione peroxidase (GPx), and CAT play an important role. The analyses of antioxidant response showed that different behaviors to titanium

surfaces (Fig. 2B–C), suggesting that the plasma treated titanium induces an up regulation of the cellular response to oxidative stress due titanium exposure.

SODs are the first and the most important line of antioxidant enzyme defense systems against ROS damage, particularly superoxide anion radicals, by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Yang et al., 2013). The extracellular superoxide dismutase (SOD 3 or EC-SOD), found in the extracellular matrix of tissues, is ideally situated to prevent cell and tissue damage initiated by extracellular produced ROS (Fattman et al., 2003). The plasma treatment seemingly led to decrease corrosion resistance (Fig. S3) and this behavior suggests that the oxide layer on PTTS is very thin film and showed propensity to oxygen evolution reaction, causing the increase of the amount of superoxide anion radicals at extracellular medium and the stimulation of SOD3 release. This behavior could be owing to a higher permeability of the passive film and favored charge transport through this film comparatively to UTS due to the higher roughness of PTTS. The higher levels of SOD3 on PTTS confirm this hypothesis: once released to extracellular medium, SOD3 converts the superoxide anion radical into H_2O_2, which easily cross the cellular membrane, increasing the ROS levels. This is not expecting to happen in UTS, therefore, it remains unclear how this surface induces oxidative stress. A probable explanation is that the stress generated during cell adhesion on UTS, due its higher hydrophobicity, impairs the adsorption of proteins, which hinders the cellular adaptation to titanium surface (Tavares et al., 2009; Kasemo, 2002).

The literature is still controversial over the effect of titanium into the antioxidants enzymes. Titanium exposure has been reported to decrease (Ozmen et al., 2006), increase (Ge et al., 2011) or not affect (Freyre-Fonseca et al., 2011) the activity of antioxidant enzymes. This shows the importance of this kind of research, not only to improve the knowledge in this field, but also to elucidate the role of oxidative stress in biomaterials rejection. It is well known that the activity of antioxidant enzymes can be regulated by ROS. Superoxide anion decreases catalase activity, while H_2O_2 inhibits SOD activity and stimulates catalase activity (Barrilet et al., 2010; Gutteridge, 1985; Pereira et al., 2008). In several cellular models, oxidative stress increases the activity of antioxidants enzymes (Husain et al., 2003; Matés, 2000) via direct modulation of the activity of existing enzymes or by induction of *de novo* synthesis (Meilhac et al., 2000).

The increase of the levels of DNA damage may indicate a higher oxidative stress on UTS. Thus, it is likely that these cells are under persistent oxidative stress (Davies et al., 1987) leading to exhaustion of antioxidant defenses, resulting in ROS intracellular accumulation (Barrilet et al., 2010). Lino-dos-Santos et al. (2011) observed that, under higher oxidative stress, the activity of antioxidant enzymes was not affected or had a slight decrease. On the other hand, an increase on gene expression of these enzymes was reported, which may reflect an excessive consumption or degradation of these enzymes. Therefore, UTS could be reducing the capacity of cells to cope with ROS, and they are accumulating oxidative damage. This is supported by the results showing higher levels of protein carbonyls and DNA oxidative damage in the UTS grown cells when compared to the PTTS. Oxidative DNA damage mediated by ROS after Ti nanoparticles exposure had been reported earlier (Shukla et al., 2011; Tsaryk et al., 2007), indicating that titanium surfaces with different topographies can lead to diverse antioxidant responses.

Altogether the results presented here indicate that despite the induction of oxidative stress by both surfaces; the PTTS seems to be less harmful to cells when compared to UTS. Serrano et al. (2005) proposed that higher hydrophilicity on polycaprolactone (PCL) films improves cell ability to response to the transitory oxidative stress generated by polycaprolactone. In assessing the
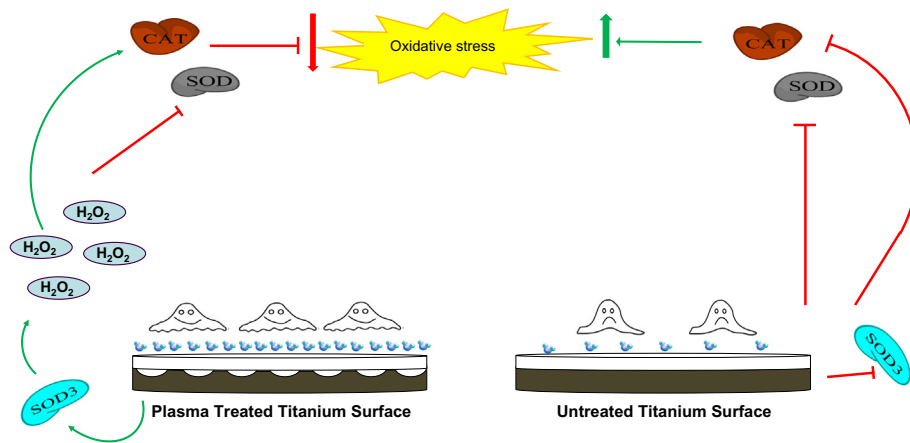
Fig. 6. Schematic model for the difference found on cellular response to oxidative stress generated by exposure to titanium surfaces.

genotoxicity of titanium surfaces Tavares et al. (2009) also attributed the lower genotoxic effect of PTTS to its higher hydrophilicity. Therefore, a model based on the hydrophilicity of these surfaces is proposed to explain the differences between PTTS and UTS found in this study (Fig. 6). Accordingly, plasma treatment could be an important tool for improving biocompatibility of implant materials on guide bone regeneration. Meanwhile, more research is warranted to examine the effects of surface modification on cellular behavior.

5. Conclusion

In conclusion, oxidative stress is a negative issue for cellular adhesion and thus osseointegration. As our results indicate, a more efficient antioxidant response is obtained with a simple modification in surface topography, which will change the cellular functional response in a significant way, decreasing oxidative damage caused by exposure to titanium. Considering this, the modification of titanium surfaces by plasma treatment can be an alternative for obtaining a safer material with respect to the oxidative stress.

Conflict of Interest

No conflict.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.11.012.

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