Dental implant surfaces treated with phosphoric acid can modulate cytokine production by blood MN cells

Abstract: The study characterizes dental implant surfaces treated with phosphoric acid to assess the effects of acid treatment on blood cells and correlate them with cytokine levels. The implant surfaces examined were divided into untreated metal surface (US; n = 50), metal surface treated with phosphoric acid (ATS; n = 50) and cement surface (CS; n = 50) groups. The samples were characterized by scanning electron microscopy (SEM) and rheometry. The implants were incubated with human blood mononuclear cells for 24 h, with surface rinsing in the ATS treatment. Cell viability was determined by colorimetric methods and cytokines in the culture supernatant were quantified using flow cytometry. In the ATS group, the surface porosity and contact surface were increased and plaques were observed on the surface. The blood flow and viscosity curves were similar among the treatments, and the high cell viability rates indicate the biocompatibility of the materials used. An increase in the levels of IL-2, IL-4, IL-6, IL-10 and TNF-α was observed in the ATS and CS groups. There were positive correlations between IL-10 and IL-2 levels and between IL-10 and IL-4 levels in the culture supernatant of the ATS group. The results suggest that implant surface treatment with phosphoric acid activates the production of inflammatory cytokines. The increased cytokine levels can modulate the immune response, thereby improving biofunctional processes and promoting the success of dental implants.

Keywords: Dental Implantation; Phosphoric Acids; Cytokines; Rheology.

Introduction

In dental implants, the prosthesis design, implant surface condition, host condition, surgical technique used and the loading applied after implant installation are key factors determining their biocompatibility and success. Thus, several studies compare methods of implant surface treatment and their effects on implant acceptance or rejection. Increased host bone-to-implant contact can be achieved by changing the implant topography or increasing the surface roughness. The relationship between the success of the implants and the cements used to fix the prostheses is only partially understood. A few studies investigated...
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Surface treatment can be an alternative procedure to promote implant success. It can be performed by additive methods, that is, the input of different substances on the implant surface, or by subtractive methods, which remove part of the surface layer. Acid etching is a subtractive method for surface treatment. It consists of the immersion of machined metal implants in a pure acid or in solution to produce a slight roughness and enhance the surface retention capacity.

The response of host cells can depend on the type of surface treatment applied to the implant. Chemical treatments can produce a nanotopography that promotes the deposition of specific ions and bone formation. Clinical studies show the high success rate of implants that receive surface treatment, even when the bone quality is not good.

Implant success also depends on cell adsorption, that is, the intimate interaction between the cell and the biological substrate, solely and exclusively promoted by the action of interfacial electrostatic and Brownian forces. The process of cell attachment to the substrate involves the participation of extracellular matrix proteins, cytoskeleton proteins, cadherins, integrins and other components, and the binding of specific proteins as well as cell proliferation and differentiation can be mediated by growth factors and cytokines.

Cytokines can be generally classified as pro-inflammatory or anti-inflammatory. They allow for the body to respond quickly to a challenge by coordinating an adequate immune response. Blood mononuclear (MN) cells, particularly T-cells, are the main cytokine producers. Based on the type of cytokine they produce, T-cells are classified into two populations, T-helper 1 (Th1) and T-helper 2 (Th2). Th1 cells participate in cell-mediated immunity, promote inflammation and cytotoxicity and are responsible for late hypersensitivity whereas Th2 cells act on the humoral immunity and regulate the inflammatory activity of Th1. Another important cytokine is IL-17, which exhibits a proinflammatory action Th17-type response. In addition, Th17 was found to induce inflammation in autoimmune diseases and acute rejection of transplants.

Inflammatory processes are common in dental implants. They are regulated and signaled locally and systemically by several biochemical agents including cytokines. Therefore, adequate interaction between these biological factors and the substances such as acids used to treat implant surfaces may determine dental implant success. Thus, the present study analyzed dental implant surfaces treated with phosphoric acid to assess the effects of this treatment on blood cells and correlate them with cytokine levels.

**Methodology**

**Samples**

The tests were performed using 150 dentures, which were divided into 3 groups (n = 50 each) according to the implant treatment. Group US were untreated (acid-free) nickel-chromium metal implants, for dental prostheses, group ATS were metal implants that underwent phosphoric acid treatment of the implant surface and group CS were standard implants with a cement surface.

**Treatment of the samples with phosphoric acid**

The acid treatment was applied to the inner and outer surfaces of the ATS group and consisted of immersion in 37% phosphoric acid for 60 s. The control treatments (US and CS) did not undergo the acid phosphoric bath. All three treatments were cemented with SSWYTE™ zinc phosphate cement.

**Cementation**

The cementation procedures followed the manufacturer’s protocols (SS Whyte, Rio de Janeiro, Brazil).

**Characterization of Implant Surfaces by Scanning Electron Microscopy (SEM)**

Scanning electron microscopy (SEM) was used to map the inner surface of the implants with and without cementation in the presence or absence of treatment with phosphoric acid. The observations
were performed using a Scanning Electron Microscope (TESCAN Kohoutovice, Czech Republic) and the surface alterations were examined using a SKYSCAN 1272 micrograph.

**Blood Sampling and Blood Cell Separation**

Blood samples (10 mL) were collected from six volunteer donors in tubes containing anticoagulant. This study was approved by the institutional Research Ethics Committee, and all of the subjects provided written informed consent before entering the experimental protocol. Total blood was used for the rheological analysis. A portion of the samples was centrifuged at 160 x g for 15 min to separate plasma from the cells. The cells were separated over a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden) to produce preparations with 95% pure mononuclear cells as analyzed by light microscopy. The purified mononuclear cells were resuspended independently in serum-free 199 medium at a final concentration of 2 x 10⁶ cells.mL⁻¹.

**Incubation of blood cells with the surface of the implant**

The surfaces of the implants with and without cementation in the presence or absence of treatment with phosphoric acid were placed in polymer molds (7 x 3 x 3mm). These dimensions resulted in a surface area of 21 mm in contact with the cells. The surfaces were separated over a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden) to produce preparations with 95% pure mononuclear cells as analyzed by light microscopy. The purified mononuclear cells were resuspended independently in serum-free 199 medium at a final concentration of 2 x 10⁶ cells.mL⁻¹.

**Rheological characterization of the implant surfaces**

The rheological parameters were measured using a Modular Compact Rheometer – MCR 102 (Anton Paar® Germany GmbH, Ostfildern, Germany) according to França et al.¹⁴ In all of the experiments, the implant surfaces treated or untreated with phosphoric acid were placed in 600 µL human blood and put on the reading plate surface, and the excess sample was removed. Readings were taken with continuous control of the gap measurement with the supported TruGap™ of 0.099 mm. The measuring cell was a Toolmaster™ CP 50, and precise temperature control was achieved with T-Ready™. The data were compiled using Rheoplus V3.61 software. Graphics were also obtained using Rheoplus software. The flow and viscosity curves were based on the established parameters for the control of shear stress (τ) of 0–5 Pa for the upsweep and 5–0 Pa for the downward curve. These tests were conducted under isothermal conditions at 25°C, and 75 readings were taken for analysis.

The viscosity curve was generated using established parameters that were relative to temperature and based on the fixed control shear stress (τ) to 1 Pa with variation in temperature from 5 ± 0.1 to 45 ± 0.1°C at a heating rate of 1°C min⁻¹. Parameters were recorded every 0.5 ± 0.1°C, and 41 readings were taken for analysis.

**Cell cytotoxicity**

Cell viability was determined using the colorimetric method with tetrazolium bromide 3- (4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT, Sigma, St Louis, USA), which is an indirect procedure to assess the mitochondrial enzymatic activity of living cells. Mononuclear (MN) cells were distributed into plate wells and incubated with their respective treatments in a humidified chamber with 95% air and 5% CO₂ at 37°C for 24h. After incubation, the supernatant was removed and 40 µL 5 mg.mL⁻¹ MTT and 360 µL complete culture medium were added to each well. The plate was incubated for 3h in the same humidified chamber. The supernatant was then discarded and 150 µL DMSO (dimethylsulfoxide) was
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Quantification of cytokines

The cellular supernatant was collected and stored at \(-80^\circ\text{C}\) prior to the analyses. The samples were thawed and the cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-\(\alpha\), and IFN-\(\gamma\)) were measured by cytometric bead array (CBA, BD Biosciences, USA) according to the manufacturer’s procedures. A flow cytometer was used for these analyses (FACSCalibur, BD Biosciences, USA). The data analyses used FCAP Array 1.0 software (CBA, BD Biosciences, USA).

Statistical analysis

The data were expressed as the mean \(\pm\) standard deviation (SD). The statistically significant differences were evaluated using the Analysis of variance (ANOVA) for the rheological parameters, cell cytotoxicity and cytokines. The correlation of cytokines was evaluated using the Pearson’s linear correlation test. Statistical significance was considered a p-value lower than 0.05 (p < 0.05).

Results

The SEM images of the ATS group (Figure 1A) show changes in porosity, with enlargement of the...
contact area and the presence of plaques covering the surface (Figure 1B).

The blood flow (Figure 2A) and viscosity (Figure 2B) curves were similar among the treatments (p > 0.05). The high rates of MN cell viability, indicated by the feasibility index (%) (Figure 3), indicate the nontoxicity of the treatments.

Table 1 shows the cytokine levels in the culture supernatant of MN cells incubated with the different surfaces. The IL-2, IL-4, IL-6, IL-10 and TNF-α levels increased in the ATS and CS groups, but their levels were not affected in the US group. The IL-17 levels in the MN cell cultures under the different treatments were high, and higher in the ATS group.

Figure 2. Blood flow (A) and viscosity curves represented by the shear rates (B) under normal conditions and the different treatments. The results are expressed as the median of 6 experiments with different individuals. p > 0.05.
supernatant cell culture, the IFN-γ levels were similar among the treatments.

Table 2 shows the correlation tests between IL-10 and other cytokine levels determined in the supernatant of MN cell culture in the ATS and CS treatments. In the ATS group, IL-10 was positively correlated with IL-2 and with IL-4. In the CS group, the IL-10 and IL-2 cytokines were correlated. The levels of the other cytokines showed no correlation with IL-10 levels (Table 2).

**Discussion**

Dental treatments aimed at alleviating tooth absence have raised the need for studies on prosthesis and the development of retention systems that provide adhesion and stability of the prosthetic element, allowing for the patient to use it with functionality and aesthetics.

In dental implants, treatment of the inner surface of the denture can directly affect the physical and mechanical properties of the prosthesis or implant assembly, reducing the cement line and improving denture fixation. The present study showed that the treatment of implant surfaces with phosphoric acid causes structural modifications that increase the chances of implant success and promote cytokine production in the presence of blood cells.

Different pretreatments (chemical, mechanical or both) for implant surfaces in contact with the cement line have been proposed in other studies. In dental implants, treatment of the inner surface of the denture can directly affect the physical and mechanical properties of the prosthesis or implant assembly, reducing the cement line and improving denture fixation. The present study showed that the treatment of implant surfaces with phosphoric acid causes structural modifications that increase the chances of implant success and promote cytokine production in the presence of blood cells.

Clinical complications associated with cement prostheses are not uncommon in dental implants, and strict clinical controls are required to verify changes in the peri-implant tissue. Inflammatory

**Table 1.** Cytokine concentrations (pg/mL) in the culture supernatant of human blood MN cells. Cells on an untreated (acid-free) surface (US), cells on an acid-treated surface (ATS) and cells on cement-treated surfaces (CS). The results are expressed as the mean and standard deviation of 6 replicates with human blood cells from different individuals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cells</th>
<th>US</th>
<th>ATS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>33.6 ± 2.7</td>
<td>39.6 ± 13.6</td>
<td>146.8 ± 50.0*</td>
<td>38.81 ± 16.8</td>
</tr>
<tr>
<td>IL-4</td>
<td>10.2 ± 2.6</td>
<td>28.5 ± 8.6</td>
<td>158.1 ± 43.0*</td>
<td>33.6 ± 6.3*</td>
</tr>
<tr>
<td>IL-6</td>
<td>9.1 ± 1.4</td>
<td>21.6 ± 4.4</td>
<td>117.4 ± 35.4*</td>
<td>31.7 ± 6.3*</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.6 ± 0.2</td>
<td>26.0 ± 10.1</td>
<td>124.2 ± 45.95*</td>
<td>23.9 ± 9.8*</td>
</tr>
<tr>
<td>IL-17</td>
<td>84.9 ± 24.6</td>
<td>168.0 ± 26.3</td>
<td>353.5 ± 127.4*</td>
<td>238.5 ± 38.6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.72 ± 0.5</td>
<td>41.5 ± 23.4</td>
<td>115.3 ± 32.5*</td>
<td>59.7 ± 25.8*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>15.4 ± 5.3</td>
<td>26.3 ± 14.4</td>
<td>14.1 ± 1.1</td>
<td>18.1 ± 0.8</td>
</tr>
</tbody>
</table>

*p < 0.05 indicates intergroup differences.
processes and changes in blood flow and in blood cells may compromise implant success. Inflammation affects the total blood viscosity, which is associated with the macro and microvascular blood flow. In the present study, characterization of the rheological and structural changes in the surface of implants of the ATC and CS groups was performed by rheometry, and the treatments showed a similar response for the variables tested (flow curve). Adequate blood flow and viscosity is fundamental to maintain the structural integrity and non deformation of cells.

Rheological alterations play an important role in implant success, and changes in the cell and micro and macrocirculation vary according to the shear rates. In the present study, characterization of the rheological and structural changes in the surface of implants of the ATC and CS groups was performed by rheometry, and the treatments showed a similar response for the variables tested (flow curve). Adequate blood flow and viscosity is fundamental to maintain the structural integrity and non deformation of cells.

Given that the blood flow and viscosity were not changed in the ATS group, the acid treatment did not affect blood components. Moreover, in the presence of blood, the acid treatment exhibited biocompatible rheological behavior and the functional activity of the cell types is homogeneous. However, the success of an implant depends on the biological interaction with tissues adjacent to the implant. The use of in vitro tests, through cell viability assays, is an alternative to assess the biological compatibility of a substance or material to provide important elements for the analysis of implant biocompatibility.

In the present study, we chose to test the viability of the cells in contact with implant surfaces treated with acid and in the presence of cement, and the viability index was similar to that of untreated surfaces. Other studies relate cell viability to its functional activity. Several candidate functional materials have been evaluated by assessing the biocompatibility and interaction with human cells. The data suggest that the acid and cement treatments applied to the metal surfaces resulted in a material with low cellular toxicity, which can be used to improve the application of cemented implant prostheses.

In different dental implant methods, proper adhesion also depends on cell adsorption, which is the close interaction between the cells and the biological substrate. The implant surface interacts with blood cells, maintains a migratory pathway of osteogenic cells and stimulates angiogenesis likely by extracellular matrix and growth factor release. Specific proteins participate in this process, promoting cell adhesion, proliferation and differentiation. Cytokines are proteins that can stimulate cell proliferation and differentiation, in addition to exerting pro- or anti-inflammatory effects.

In dental implants the initial inflammatory response is important for cellular recruitment, which can be modulated by the immune system during implant recognition. During the first 24 h, inflammation is characterized by high levels of proinflammatory cytokines IL-6, IL-8, TNF-α, IFN-γ and recruitment of lymphocytes and macrophages. In the present study, the interaction of blood MN cells with implant surfaces treated with phosphoric acid and cement resulted in the increase of cytokines levels in the culture supernatant (24-h culture).

Cytokines are produced by several cell populations; mainly MN cells such macrophages and T-helper lymphocytes. According to the cytokine type they produce, T-helper (Th) lymphocytes are classified into classes, such as Th1, Th2, Th17 and Treg cells. T cells (Th1 and Th2) secrete inflammation mediators,
proinflammatory cytokines such as interleukins-2 (IL-2), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and Th2 cells secrete anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. 34

In the present study, the IL-2 levels increased in the supernatant of MN cell culture in the ATS treatment. Considering that IL-2 is a main promoter of cell proliferation, its increase suggests that, in addition to the roughness and improved fixation due to the acid treatment, it may also be useful in the biological process of implant acceptance.

Cytokines IL-4 and IL-6 also increased in the culture of MN cells in the ATS group. IL-4 is the dominant factor promoting growth and differentiation of Th2 cells and directly inhibits the development of Th135 whereas IL-6 is involved in the control of differentiation, proliferation and migration of cells, 36 which is important for prostheses fixation.

Patients with peri-implantitis show higher levels of IL-6 and IL-17, which suggests that these cytokines intensify local inflammation.37 IL-17 is proinflammatory and is involved in inflammation and autoimmune diseases.38 Interestingly, IL-6 and IL-17 can play a dual role, i.e., protective or destructive,40 to the tissues. In the present study, irrespective of the treatment, similar levels of IL-17 were observed in the MN cell cultures of implant surfaces treated with acid, suggesting that an initial inflammation in response to the implant is important for healing.

Other studies on peri-implantitis report increased TNF-α levels41 and low levels of other cytokines, suggesting slight inflammation in the tissues surrounding the implant42. In the acid treatment group, we observed high levels of this cytokine in MN cell culture, reinforcing the intensification of the inflammatory response.

IL-10 is known to inhibit proinflammatory cytokines. It suppresses Th17 and macrophage responses by inhibiting the inflammatory cytokines IL-6 and TNF-α.33 Because of the proinflammatory activity of Th17 CD4+ T cells and the possible damages they cause, their response needs strong regulation.44 IL-10 can downregulate the expression of cytokines by Th17 cells and increase Treg cells.43,44 IL-10 is a multifunctional cytokine, with diverse effects on hematopoietic cell types. Its main function appears to be the limitation and elimination of inflammatory responses.40 Thus, this cytokine is a key immunomodulator of the immune system,49 and plays an important role in vivo regulation, producing a negative feedback loop that reduces the release of inflammatory mediators and dampens acute inflammatory responses. For instance, the production of tolerogenic IL-10 was found to increase in titanium implants.47 In the present study, a significant increase in IL-10 and positive correlations between IL-10 and IL-2 and between IL-10 and IL-4 were detected, suggesting an important regulatory role of this cytokine in cell proliferation, differentiation and interaction with dental implant surfaces.

**Conclusion**

The results suggest that treatment of implant surfaces with phosphoric acid modulates cytokine production by blood MN cells, establishing a balance between proteins with anti- and proinflammatory activity. The findings support the development of a novel treatment based on application of phosphoric acid to the implant surface to improve biofunctional processes and enhance the chances of success of dental implants.

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