Oxidative Stress Evaluation During Perimplantar Bone Resorption in Immediate Post-Extractive Implant

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Abstract

Background and purpose: The pathological events, caries, periodontitis and trauma, leading to the destruction and loss of tooth and periodontium are representing complex interactions involving an excessive osteoclastic activity. The osteoclasts can especially produce reactive oxygen species (ROS) that may be involved in the pathogenesis of periodontal disease and development of several inflammatory oral pathologies. The polydatin, a natural precursor of resveratrol, could be used as adjuncts to counteract plaque-associated bacteria and inflammatory diseases of oral cavity. Aim: 1) to relate the perimplantar bone resorption to lipid peroxidation and systemic inflammation in patients subjected to implant integration, 2) to value the future prospect of an antioxidant polydatin therapy that may decreases the bone perimplantar resorption due to oxidative stress. Methods: Fifteen patients (implant-group) receiving treatment with immediate single post-extractive implants. In relation to the sockets forms, periodontal biotype, the titanium implants was selected to obtain immediate tooth loss restoration. The bone implant integration was monitored by radiographic examinations and the saliva samples were drawn in different times during the entire process and performed to NO, MDA assay and inflammatory enzyme. Results: we have observed an increase, during implant integration between the first and third week, of both oxidative stress markers and cyclooxygenase-2 expression. At sixteen week the parameters evaluated returned to basal values. The oxidative stress and inflammatory markers could be used to evaluate the implant bone-integration. Furthermore our data showed that polydatin has both antibacterial and antioxidant activity and it could represent a adjuvants for bone perimplantar resorption.

Key Words: Reactive oxygen species, Peroxide, Lipid peroxidation, Implant

Introduction

The adult skeleton regenerates by temporary cellular structures that comprise teams of juxtaposed osteoclast and osteoblast and replace periodically old bone with new [1]. Osteoclasts are multinuclear cells derived from hematopoietic stem cells [2]. The development and differentiation of osteoblasts and osteoclasts are controlled by growth factors and cytokines produced in the bone marrow as well as adhesion molecules that mediate cell-cell and cell-matrix interactions [3,4]. The balance between osteoblasts and osteoclasts is also critical in case of dental implants [5,6]. Indeed, the bone peri-implant resorption is the results of complex interactions between the host and pathogen agents. Many mechanisms to explain this type of resorption has been proposed [7]. This mechanism includes the action of enzymes released by pathogen bacteria and by immunitary system reactions. Cell like neutrophilis, leukocytes and mainly osteoblast and osteoclast are involved in the bone resorption process [8,9]. The action of these cells is mediated by cytokines binding the information required to start the bone resorption [10,11].

The pathological mechanisms which induce the periodontal disruption during the inflammatory processes of the oral maxillo-facial cavity are mainly represented by an alteration of enzymatic and non-enzymatic degradative mechanisms [12-15]. The osteoclasts can especially produce reactive oxygen species (ROS) such as peroxide ($O^{\bullet 2}$) and it is been supposed that ROS may be involved in the pathogenesis of periodontal tissue destruction and play a pivotal role in the activation of NF-kB factor that enhances the transcription of genes encoding defence and signalling proteins [16-20]. The ROS, the free radicals, some lipid and protein catabolities, such as malondialdehyde (MDA), N-epsilon-2propenal-lysine can: 1) to modify proteins and lipid structures 2) to start oxidative degradation mechanisms 3) to modulate the inflammatory molecules expression 4) to induce the accumulation of toxic catabolities [21,22]. Studies conducted by Hall et al. and Bax et al. suggest that ROS such as O^{2-} and H_2O_2 play an important role in osteoclast activation rather than in the direct degradation of bone matrix [23-25]. Besides osteoblasts, ROS can be released by some pathogen bacteria [26] and could regulate the cytokines release [27]. The balance oxidize-reductive cellular and cytokine network are the cross-road of the responsible phenomena for a clinically significant osteopenias [28,29].

The process of mastication promotes a variety of such reactions, including oxidative stress and lipid peroxidation [30]. The saliva represents a mixture of the salivary glands secretions together with the gingival fluid [31] with antioxidant and antibacterial properties [32]. Recently, it has been claimed that the imbalances in levels of free radicals and reactive oxygen species with antioxidants [33-36] may play an important role in the onset and development of several inflammatory oral pathologies. In this context it is also important the use of supplements as polydatin, a precursor of

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the resveratrol that exhibits more than antioxidant and antibacterial activity also antiosteoporotic activity via regulating osteoprotegerin, RANKL [37].

The aim of this study is: 1) to relate the perimplantar bone resorption in patients subjected to implant integration to lipid peroxidation and systemic inflammation 2) to value the future prospect of an antioxidant therapy that may decreases the bone perimplantar resorption due to increases free radical-mediated oxidative stress.

Materials and Methods

Patient selection

All Italian subjects, sexes, aged between 18-40 years, without systemic pathologies, no smoker were enrolled in Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties, between December 2013 and December 2015. All the patients were informed of the research and gave permission for the use of their saliva and serum samples.

We enrolled: 1) fifteen patients receiving implant treatment (implant-group); 2) ten volunteers were orally treated with Polydatin (polidatin-group); 3) ten volunteers healthy subject (control group).

Implant group

The single tooth extracted from implant-group was placed immediately with a single titanium conical connection implants (V3 MIS® implat technologies Ltd). Seven patients were extracted lateral incisors (5 right and 2 left) and eight patients central incisors (4 right and 4 left). The saliva samples were collected in different times, in particular before (0 h) and 1-3-5-8-16 weeks after implant apposition, on saliva samples were measured the lipid peroxidation by TBARS assay, nitric oxide by Griess methods, and cyclooxygenase-2 (COX2) expression for asses the oxidative stress and inflammation during the entire process of bone implant integration. The entire process of bone implant integration was monitored by radiographic examinations and plasma aliquots were used to investigate cytokinic network.

Polydatin-group

The subjects were orally administered doses of 20 mg a day Polydatin (Polidal®; GHIMAS under license by GLURES srl-academic spin off Ca' Foscari University-Venice, Italy). The saliva samples were withdrawn before (control) and after one week from Polydatin treatment. In the saliva samples were evaluated the bacteria counting, Nitric Oxide levels, and lipid peroxidation by TBARS assay.

Control group

In the saliva samples were evaluated the bacteria counting, Nitric Oxide levels, and lipid peroxidation by TBARS assay.

Collection of whole saliva

Unstimulated whole saliva samples were collected by 200 µl pipettes drawing. Subjects had no food intake, drink or oral rinsing 2 h prior to sample collection. The collected saliva samples were centrifuged (4000 g for 5 min). The supernatants were stored at 70°C until further analysis.

TBARs assay

Thiobarbituric acid reactive substances (TBARs) were measured on saliva samples of control-group, implant group and Polydatin-group. The TBA-TCA solution was prepared dissolving 0.2 g of TBA in 100 mL of 15% tricloroacetic acid (TCA) and butilated hidrossitoluene BHT (7.2%) was dissolved in ethanol 98%. Salivary samples were diluited in water containing an antioxidant agent like BHT 7.2%. The salivary samples were centrifuged at 4°C for 10 min at 13000 g and the oxidation state was valuated using an analytical-quantitative methodology. It predicts the formation of colored adduct produced by the stechiometric reaction of malondialdheyde (MDA) with thiobarbituric acid (TBA): the assay was conducted incubating equal volume of sample and TBA 0.2%-TCA 15% at 100°C for 30 min. The reaction was stopped setting the sample in cold water and after a centrifugation at 15000 g for 10 min the cromogen (TBARS) was quantified by Jenway Genova spectrofotometric reading at a wavelength of 532 nm [38].

Nitrite assay

Nitrite assay was conducted on saliva samples of control-group, implant group and Polydatin-group. It predicts a centrifugation at 1600 g for 5 min of salivary samples to remove possible existing cells and the addition of Griess reactive (sulphanilamide 1% naphtilendiamine 0.1% in phosphoric acid 5%). After incubation the absorbance at 550 nm was measured by Jenway Genova spectrophotometer. The amounts of nitrite were calculated using the triple test media referred to a standard nitrite curve [39].

Immunoenzymatic assay for interleukin

Plasma samples for the interleukin valuations were taken by implant-group at 0 h, 1-8-16 weeks of implant integration process, before meal at the same time of the morning. The interleukin cellular expression was valued by the use of immunoenzymatic colorimetrical kits (ELISA) in accordance with manufacturer's instructions (Diaclone).

Cicloxygenase-2 (COX-2) immunoprecipitation

Saliva samples (20 µL) of implant group at 0 h, 1-8-16 weeks of implant integration process, were incubated at 4°C overnight with rat monoclonal antibody anti-COX-2 (1:1000). Moreover protein A Sepharose was added for 3 h at 4°C and the solution was centrifuged for 3 min at 13000 g. Pellet was resuspended with 20 µL 3X SDS sample buffer. COX-2 were separated by vertical electrophoresis poliacrilamide gel in 12% SDS (SDS-PAGE) and identified by Western Blotting with rat monoclonal primary antibody anti-COX-2. Immunoreactivity was detected using conjugated antibody anti-rabbit IgG alkaline phosphatase (diluted 1:1000) by a Western-Light1 immunodetection system (Tropix Company).

Counting bacteria evaluation

Aerobic bacteria samples were drawn through sterile tampons on both dental arch of Polydatin-group (one week of Polydatin

administration) and control group and grown with LB (Bacto-tryptone, Bacto-yeast extract, NaCl pH 7.0) medium in tube on a shaker for 24 h at 37°C. Counting bacteria were evaluated after 24 h of incubation and quantified (1 $O.D._{.600\ nm}$ = 8 × 10^8 bacteria) by Jenway Genova spectrofotometric.

Statistical analysis

All data analysis is presented as means ± standard deviations (SD). An unpaired Student's t test and one way Anova were applied to determine the significance of various biochemical changes between different groups of patients and their respective controls. A P-value of <0.05 was considered to indicate a statistically significant difference. All graph analysis was made with GraphPad Prism 4.01 software.

Results

Lipid peroxidation status and NO^{2-} production in bone remodelling associated to implant integration

At the patients of implant-group were removed compromised teeth and the resultant fresh sockets were immediately placed with implants. The patient's tooth crown has been used for the restoration before harvest, and autologous bone block has been used for filling the gap between the implant and the alveolar bone (*Figure 1A*). The implant integration was monitoring by radiographic examination (*Figure 1B*). At the 3-year follow-up, no patient dropped out and no implant failures or complications occurred after loading.

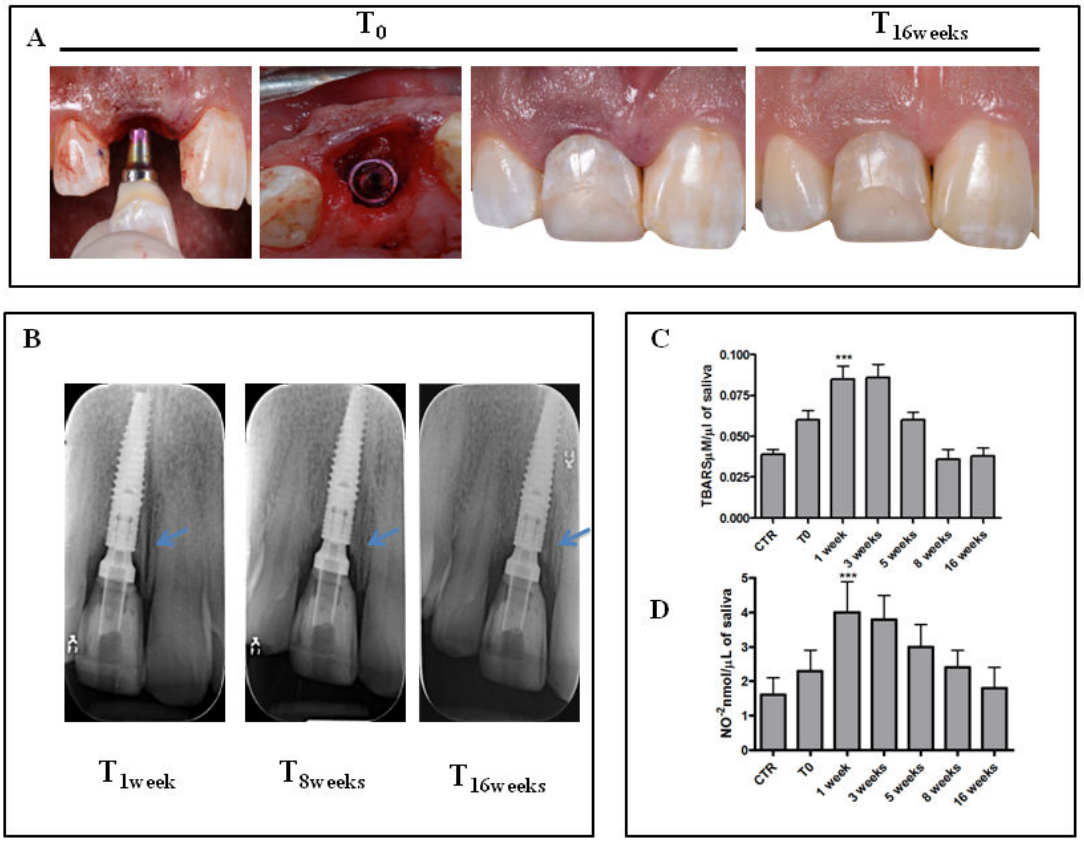

Figure 1. *(A) Patient after single tooth extraction and placement of implants in the upper central incisor regions. (B) radiographic examinations during implant bone-integration. (C) TBARs level (D) NO2- concentration. Thiobarbituric acid reactive substances (TBARs) and nitrite oxide (NO^{2-}) were measured on saliva samples drawn of implant-group at different times. The TBARS was quantified at 532 nm. Results are expressed as μM TBARs/μl of saliva. The absorbance of NO^{2-} at was measured at 550 nm. The amounts of nitrite were calculated using the triple test media referred to a standard nitrite curve. Results are expressed as nmol NO^{2-}/μl of saliva. All data analysis is presented as means ± standard deviations (SD) (***P<0.001, **P<0.02 *P<0.05).*

In *Figure 1C* showed the levels of lipid peroxidation marker (TBARs) in saliva samples of implant-group in relation to different clinical stages (0 h before of the implant, 1-3-5-8-16 weeks after implant). These values significantly increased (P=0,0007) about two-fold compared to the control group until 6 weeks as consequence of the bone remodelling during implant integration process and decreased after 8 weeks. Saliva NO^{2-} values (implant-group) are showed in *Figure 1B* and increased of about two fold compared to control group after 1-3 weeks of the implant apposition, returning at basal levels after 5 week until the complete integration of implant.

COX-2 expression and serum cytokinic network in bone remodelling associated to implant integration

We next analyzed the COX-2 expression in saliva sample and serum IL-3-5-6 cytokines of implant-group examined in the previous experiments at 0 h, 1 week, 8-16 weeks. Cyclooxygenase (COX, EC 1.14.99.1) is the rate-limiting enzyme in PG synthesis. Two isoforms of COX have been identified. COX-1 is constitutively produced in most tissues to maintain homeostasis, whereas COX-2, which is normally undetectable, is induced by endotoxin or cytokines during inflammation. In *Figure 2* panel A reported the COX-2 expression by western blotting analysis. The COX-2 increase at 1 week while decreased at 8 weeks and 16 weeks of implant integration process (*Figure 2B*) compared with healthy control subjects. The increase expression of COX-2 was correlated only to the IL-6 serum variation (*Figure 2B*), but not to IL-3 and -5 (data not shown). These data showed that at 1 week, bone remodelling induced by implant integration may produce systemic inflammatory. All inflammatory markers (COX2 and IL-6) returned at basal values when complete implant integration occurred.

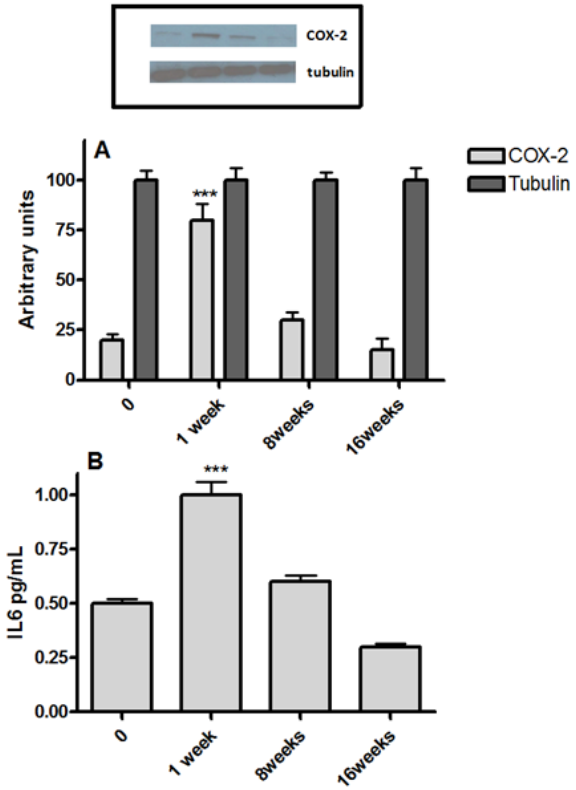

Figure 2*. COX-2 immunoblotting (panel A), variation of serum IL-6 (panel B) and COX-2 level (panel C) in saliva during implant bone-integration. Saliva samples (20 µL) of patient affected by a partial edentulia referred to 1 or 2 adjoining dental proof at 0h,1 week, 8 weeks and 16 weeks of implant integration process, were incubated at 4°C overnight with rat monoclonal antibody Anti-COX-2. Moreover protein A Sepharose was added for 3 h at 4°C. COX-2 was separated by vertical electrophoresis poliacrilamide gel in 12% SDS (SDS-PAGE) and identified by Western Blotting with rat monoclonal primary antibody Anti-COX-2. The concentration data of bar graph were referred to densiometric measurements. The interleukin-6 cellular expression in plasma sample was valued by the use of immunoenzymatic colorimetrical kits (ELISA) in accordance with manufacturer's instructions (Diaclone). All data analysis is presented as means ± standard deviations (SD). (***P<0.001, **P<0.02 *P<0.05).*

Evaluation of oxidative stress and growth bacteria in the saliva of Polydatin-group

We was tested the antioxidant and antibacterial effect on saliva of healthy subjects before (control subjects) and after one week of the Polydatin oral administration. Experimental data (*Figure 3*) showed that one week of oral Polydatin administration decreased 44% the aerobic bacterial growth 30% the TBARS values and reduced saliva NO^{2-} concentration of about 12% compared to the baseline value of same subjects before the Polydatin treatment (CTR).

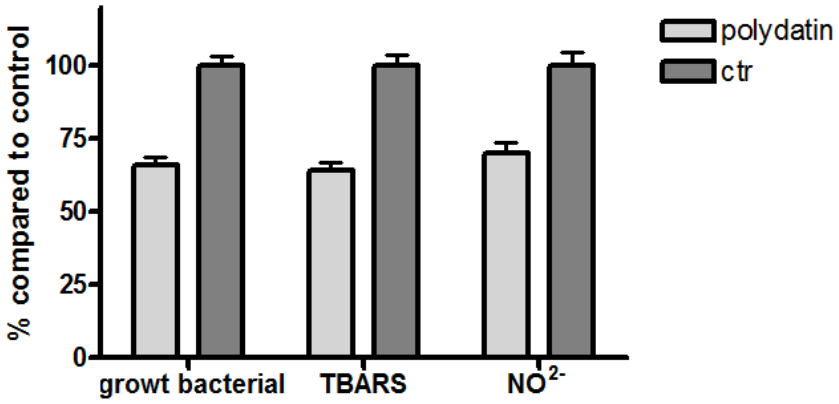

Figure 3. *Counting bacteria, TBARS and Nitric oxide (NO^{2-}) in saliva of healthy subjects before and after one week of Polidatin administration. Bacteria samples were drawn through sterile tampons on both dental archs of healthy subjects. Counting bacteria was evaluated after 24 h of incubation at 37°C and quantified (1 $O.D._{600nm}$ = 8 × 10^8 bacteria) at 600 nm. Thiobarbituric acid reactive substances (TBARs) were measured on saliva sample the cromogen (TBARS) was quantified at 532 nm. Nitrite was measured by the Griess reaction and the cromogen was assayed at 550 nm. Result are expressed as percent of the control (subjects before Polydatin treatment). All data analysis is presented as means ± standard deviations (SD).*

Discussion and Conclusion

In the present study were evaluated the oxidative status, (by lipid peroxidation and nitric oxide evaluation), and inflammation (by COX2 protein expression) in patients subjected to immediate post-extractive implant integration. Malondialdehyde (MDA) and other aldehydes are physiological compounds produced by peroxidative decomposition of unsaturated lipids (lipid peroxidation) as a by-product of arachidonic metabolism. Excessive production of aldehydes, as a result of tissue injury damages DNA and alters biological properties of proteins [21,22]. Nitric oxide (NO) is a free radical, produced by nitric oxide synthases (NOS) a group of enzymes characterized by three isoforms [40]. Inducible NOS (iNOS) in bone occurs during inflammation, and its expression induces growth inhibition, differentiation of osteoblasts, and at high concentrations apoptosis. In particular the interactions of NO with free radicals, generated by cytokines and/or LPS-activated macrophages may also be significant for osteoclast function, as superoxide and hydrogen peroxide stimulate osteoclastic bone resorption. In this study we have observed an increase of saliva lipid peroxidation markers (TBARS) and NO level between the 1-3 weeks after immediate post extractive implant and returned at basal levels after 8 weeks when the implant integration process was completed. Lipid peroxidation products could be involved in COX-2 over-expression Cyclooxygenase-2 (COX-2) a major mediator of inflammation also known as prostaglandin endoperoxide synthase 2, it converts arachidonic acid to prostaglandin. Deregulated expression of COX-2 is reported in various proliferative and inflammatory diseases such as cancer, rheumatoid arthritis, diabetes, myocardial infarction, and Alzheimer's disease [41,42]. Some studies also show that it was involved in the pathogenesis of chronic periodontitis, in fact the expression of COX-2 is increased in a rat model of periodontitis, leading to alveolar bone destruction as well as gingival inflammation. In saliva of implant-group patient we showed that COX-2 had high values at 1 week while decreased between 8-16 weeks (*Figure 3*) compared with healthy control patients.

Recent medical and dental researches in this area are geared towards the prevention of free radical-mediated diseases by using herbal medicines. Indeed, Ramesh et al. reviewed a number of studies that describe different types of herbs such

as: green tea, triphala, rubia cordifolia, ginkbo biloba etc. which possess biological properties such as antimicrobial, antioxidant and anti-inflammatory and ability in the treating periodontal disease [43]. Polydatin, is the natural precursor of resveratrol, presents a glucose molecule linked to resveratrol that modifies its pharmacodynamic and pharmacokinetic properties. We have tested if the polydatin could represent a valid adjuvant for bone perimplantar resorption diseases treatment. Our data showed that one week of polydatin treatment induced both antioxidant and antibacterial effects in the saliva of healthy subjects. Our study evidenced the therapeutic efficacy of polydatin in vivo not only by reducing saliva TBARS, markers of lipid peroxidation and nitric oxide, but also reducing aerobic bacterial growth. This study suggests the use of a therapeutic strategy in immediate post-extractive implant based on the administration of oral polydatin.

Conflict of interest

The authors declare that they do not have conflict of interest regarding the present study.

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