Int. J. Oral Maxillofac. Surg. 2019; 48: 962–970
https://doi.org/10.1016/j.ijom.2018.12.012, available online at https://www.sciencedirect.com

International Journal of
Oral & Maxillofacial Surgery

Randomised Controlled Trial
Dental Implants

The impact of implant abutment surface treatment with TiO_2 on peri-implant levels of angiogenesis and bone-related markers: a randomized clinical trial

A. L. S. Óbice, M. G. Correa, H. S. Feng, F. V. Ribeiro, F. R. Cirano, M. Z. Casati, S. P. Pimentel
Dental Research Division, School of Dentistry, Paulista University (UNIP), São Paulo, Brazil

A. L. S. Óbice, M. G. Correa, H. S. Feng, F. V. Ribeiro, F. R. Cirano, M. Z. Casati, S. P. Pimentel: The impact of implant abutment surface treatment with TiO_2 on peri-implant levels of angiogenesis and bone-related markers: a randomized clinical trial. Int. J. Oral Maxillofac. Surg. 2019; 48: 962–970.

Abstract. The goal of this randomized, blinded, split-mouth controlled clinical trial was to assess the influence of abutment surface treatment on tissue healing. Fifteen patients received two implants distributed randomly to two groups: test (TiO_2 abutment surface), control (standard abutment surface). Levels of epidermal growth factor (EGF), bone morphogenetic protein 9 (BMP-9), endothelin 1 (ET-1), fibroblast growth factor (FGF), placental growth factor (PlGF), and vascular endothelial growth factor (VEGF) were quantified in the peri-implant fluid after 3, 14, 30, and 60 days. Inter-group comparisons indicated higher levels of EGF, BMP-9, ET-1, FGF, and PlGF in the test group after 30 days ($P < 0.05$). PlGF levels were also higher in the test group after 60 days. In the test group, intra-group analysis revealed different levels of ET-1 and FGF between days 3 and 30, and days 3 and 60 ($P < 0.05$); furthermore, VEGF levels were significantly higher on day 60 than on day 3 ($P < 0.05$). In the control group, intra-group analysis demonstrated significantly different levels of ET-1, FGF, and PlGF between days 3 and 60 and of PlGF between days 14 and 60 ($P < 0.05$). In conclusion, abutment surfaces treated with TiO_2 influenced the levels of angiogenesis and bone-related markers.

Key words: dental implants; dental abutment; surface treatment; bone; biological markers; protein array.

Accepted for publication 19 December 2018
Available online 17 January 2019

After prosthetic loading, peri-implant bone loss of up to 2 mm is considered physiological, and its mechanism is defined as multifactorial (micro-gap, biological space, surgical trauma, implant position with respect to the bone crest, type of connection between implant and abutment, and cervical area design, among others)[1–3]. The success of long-term implants depends on preservation of the alveolar bone crest height, and loss of this height in the region of the first implant thread should not exceed 0.1 mm per year[4–6].

Although an annual loss of 0.1 mm is considered acceptable, methods to avoid this have been studied. Implant surface treatment has been proposed to reduce peri-implant bone loss, and positive outcomes have been observed regarding bone integration[7,8]. In addition, platform-switched abutments have proven to be effective in maintaining bone and soft tissue levels in the cervical region of implants[9]. Additionally, preclinical and clinical studies have begun to investigate the role of surface treatment in the cervical region of implants, and have reported conjunctive tissue attachment, a reduction of microbial colonization, and a reduction of peri-implant bone loss[10–12].

Recently, Gehrke and Neto assessed a different approach[13]. These authors assessed the effect of surface treatment with titanium dioxide (TiO_2) of a 1-mm collar on the intermediate abutment of implants inserted in rabbits in comparison with smooth-surface abutments. The histological results revealed greater bone volume in the region of the bone crest adjacent to the abutment that had received the surface treatment, showing the great potential for biological sealing in this critical region of the implant–abutment interface.

With respect to molecular and cellular aspects, the literature indicates that surface treatment influences the processes of protein adsorption, platelet attachment and homeostasis, activation of the complement system, inflammation, and osteogenesis[14–19]. The roughness of the abutment surface could help in the chemo-attachment of platelets and the formation of a denser fibrin clot, resulting in a greater velocity of bone integration[20].

Previous studies have reported the effect of micro−textured implant surfaces treated with growth factors on the attachment, distribution, orientation, and growth of osteoblasts and fibroblasts[21]. It was observed that the micro−texture controlled the direction of bone growth, suggesting that this type of texture could favour the insertion of soft tissue and bone tissue through the control of cell attachment, proliferation, and differentiation[21]. In addition, Simmons et al. observed that the presence of these textures stimulated tyrosine phosphorylation and the expression of messenger RNA for fibronectin, which is important in the adhesion between non−epithelial cells and the extracellular matrix[22]. In vivo and in vitro studies have found that treated surfaces promote less formation of fibrous tissue and greater integration of bone tissue with metal surfaces[22], and prevent apical migration of epithelial cells[23].

Taking into consideration the positive effects of surface treatment at the molecular and cellular levels, as described above, as well as the promising histological results obtained in the study conducted by Gehrke and Neto[13], it would be interesting to determine the effect of surface treatment of intermediate abutments at the molecular level clinically. Such an investigation could be performed by analysing the local levels of important markers of angiogenesis and osteoblastogenesis in the peri-implant fluid during the initial phases of peri-implant bone regeneration. These molecules are important markers in the tissue regeneration process. The analysis of their release pattern could significantly elucidate the possible benefits of treated abutments and indicate whether abutment surface treatment could lead to a greater potential for bone formation and biological sealing in the area of the abutment–implant interface.

Thus, the goal of this clinical laboratory study was to evaluate the impact of TiO2 treatment at the intermediate abutment surface of Morse cone connection implants on initial bone and peri−implant tissue regeneration, by means of enzyme immunoassay analyses.

Materials and methods

Population

The population of this prospective, randomized, blinded, split−mouth controlled clinical trial was recruited from patients who sought dental treatment at Paulista University, São Paulo, Brazil, between August 2015 and January 2017. The clinical procedures and assessments were conducted between November 2015 and March 2017. The statistical analysis of the data was performed in June 2017. Fifteen patients were selected, 11 female and four male; they were aged between 23 and 63 years. This study was approved by the Research Ethics Committee of Paulista University and was designed based on the CONSORT statement.

Inclusion and exclusion criteria

The inclusion criteria were patients with bilateral absence of lower posterior teeth, extractions performed at least 4 months before the treatment, minimum bone height of 9 mm, minimum thickness of 6 mm, healthy periodontal status, and age between 18 and 65 years.

The presence of any systemic disease that could interfere with bone regeneration (diabetes, arthritis, hypothyroidism, hyperparathyroidism, and osteoporosis) and the use of medications that contraindicated the performance of surgical procedures or that could alter bone regeneration around implants (e.g., anti−inflammatory and bisphosphonate drugs) were considered exclusion criteria.

All participants were informed about the nature of the study and the potential risks and benefits of their participation, and all signed an informed consent agreement.

Experimental groups

All patients received two implants, one on each side of the mandible. After insertion of the implants, they were distributed to the following groups by means of a computer−generated list (managed by S.P.P.): control group, in which a conventional intermediate abutment (smooth surface) was used (Fig. 1A); test group, in which a 1.5−mm collar of the intermediate abutment was surface−treated with TiO2 (Fig. 1B).

The treatment of implant abutment surfaces blasted with TiO2 particles (50–100 μm) was conducted by the manufacturer (Implacil de Bortoli, São Paulo, Brazil); this was followed by ultrasonic cleaning with alkaline solution Riozyme IV-E Neutro Gold (Indústria Farmacêutica Rioquímica, São José do Rio Preto, Brazil), washing with distilled water, and scouring using maleic acid. Each abutment was prepared, packaged, and sterilized according to the same procedure, as required for the packaging of implants[24].

Gehrke et al. characterized the surfaces using a scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS)[24]. For the smooth surface, the SEM images revealed a surface with smooth grooves caused by the cutting tool during machining. For the treated surface, topographical uniformity was observed, with the presence of deep grooves and a

Fig. 1. Photographs of the abutments used in this study. (A) Control group: conventional intermediate abutment with a smooth surface. (B) Test group: intermediate abutment with a 1.5-mm collar surface-treated with TiO2.

regular microrough surface. The treated surface also presented rounded edges due to acid conditioning, and residues of the blasting particles were observed in the high-magnification images. Concerning the EDS evaluation, a surface with a high concentration of titanium was observed in both groups, and other metal ions were not identified. Regarding the analysis of surface roughness, the roughness data point mean (standard deviation) values were 0.159 (0.033) μm for the smooth surface and 0.699 (0.056) μm for the treated surface. Another characteristic, the wettability (mean value of the contact angle over different times of water drop), was also evaluated. The authors reported that on the smooth surface, the contact angle showed a continuous, almost linear decrease at different times. On the treated surface, the contact angle did not change, remaining stable. The wetting area was greater (27.2%) on the smooth surface.

Installation of implants

All surgeries and postoperative follow-up examinations were performed by the same surgeon (A.L.S.O.) at the Dentistry Clinic of Paulista University, São Paulo, Brazil. All patients received two Morse cone connection implants in one stage (Implacil de Bortoli), which had their prosthetic platform positioned 2 mm below the bone crest after the installation of the implants with insertion torque varying from 30 to 45 N. Hexagonal conical abutments and conical abutment protectors (Implacil de Bortoli) were installed.

For the procedure, the area was anaesthetized and then a mucoperiosteal flap was raised after incision in the centre of the bone crest. The preparation of the bed was conducted according to the protocol recommended by the manufacturer. The intermediate implants, with and without surface treatment, and abutment protectors were subsequently installed. Uninterrupted sutures were performed using 4–0 nylon (Nylon, Ethicon, Somerville, NJ, USA). Amoxicillin (2 g administered 1 hour before the procedure), sodium dipyrone (500 mg every 6 hours for 2 days postoperative), and mouthwash with 0.12% chlorhexidine digluconate (every 12 hours for 7 days postoperative) were prescribed.

Assessment of the profile of markers related to osteoblastogenesis and angiogenesis

A blinded examiner performed the collection of peri-implant fluid in each group, using filter paper strips (PerioPaper; Oraflow, Plainview, NY, USA), on days 3, 14, 30, and 60 after installation of the implants, as described above[25]. The levels of mediators of osteoblastogenesis (epidermal growth factor (EGF), bone morphogenetic protein 9 (BMP-9), and endothelin 1 (ET-1)) and angiogenic factors (fibroblast growth factor (FGF), placental growth factor (PlGF), and vascular endothelial growth factor (VEGF)) were determined using a specific kit (HAGP1MAG–12K; Millipore Corporation, Billerica, MA, USA) and measured with a multiplex instrument (MAGpix; MiraiBio, Alameda, CA, USA), following the manufacturers' instructions.

Briefly, a 96-well plate was pre-wetted with washing buffer. After discarding the wash buffer, microsphere magnetic beads coated with monoclonal antibodies against the different target analytes were added to the wells. Samples and standards were added to the wells and incubated overnight at 4 °C. The wells were washed using a magnetic manifold, and a mixture of biotinylated secondary antibodies was added. After incubation for 1 h, streptavidin conjugated to the fluorescent protein R-phycoerythrin (streptavidin–RPE), was added to the beads and incubated for 30 min. After washing to remove the unbound reagents, sheath fluid was added to the wells, and the beads (minimum of 50 per analyte) were analysed in the MAGpix instrument. Samples were diluted with the kit diluents. The dilution was taken into consideration when calculating the concentration of each substance with a standard curve, prepared using the standard proteins in the kit. The standard curve range used was 2.7–2000 pg/ml for EGF, BMP-9, and ET-1; 13.7–10,000 pg/ml for FGF and VEGF; and 1.4–1000 pg/ml for PlGF. The samples were analysed individually, and the levels were estimated using a polynomial curve (xPONENT software, Millipore Corporation). The results were adjusted for the volume of peri-implant crevicular fluid for each implant and the values were expressed in picograms per millilitre (pg/ml).

Statistical analysis

The number of patients included in the study was based on previous studies that had found significant differences in the levels of bone, angiogenic, and inflammatory markers in peri-implant fluid[25–27]. Initially, the data were submitted to tests for normality (Kolmogorov–Smirnov test). Subsequently, the data were submitted to two-way analysis of variance (ANOVA)/Tukey test. The analyses were performed using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA), considering each implant as an experimental unit; α was set at 5%.

Results

Initially, 62 patients were selected; however, 47 were then excluded because they

did not meet the inclusion criteria. Hence, 15 individuals were included in the study. No patient withdrew from the study during the experimental stage (Fig. 2). Among the 15 patients included, 11 were female (73.3%) and four were male (26.7%). The average age of the patients was 40 ± 10.41 years (range 23–63 years).

Levels of osteoblastogenic and angiogenic markers

Analysis of the results indicated a significant difference between the groups, with higher levels of EGF, BMP-9, ET-1, FGF, and PlGF in the test group at 30 days following implant installation ($P < 0.05$). In addition, PlGF levels were also higher in the test group at 60 days following the procedure ($P < 0.05$). The intra-group analysis indicated significant differences in ET-1 and FGF levels between days 3 and 30, and between days 3 and 60, in the test group ($P < 0.05$); furthermore, there was also a difference in VEGF levels in the test group between days 3 and 60 ($P < 0.05$). With respect to the control group, there was a significant difference in ET-1, FGFand PlGF, levels between days 3 and 60 ($P < 0.05$). Additionally, significant differences in PlGF concentration were observed between days 14 and 60 in the control group ($P < 0.05$). The results are illustrated in Fig. 3.

Discussion

A greater amount of bone tissue and bone integration of the prosthetic abutment could favour biological sealing of the implant–tissue interface, thus promoting bone crest stability. A previous study used an animal model to compare the effect of an abutment with a treated surface and the effect of an abutment with a smooth surface, and showed that surface treatment favoured the stability of the peri-implant crestal bone[13]. However, to date, there appears to be no evidence in humans that treatment of the prosthetic abutment surface could influence bone regeneration and optimize bone integration of the prosthetic implant abutment.

This study investigated the effect of surface treatment of the prosthetic abutment on osteoblastogenic and angiogenic markers. In general terms, the results indicated that the treatment of the abutment surface influenced the levels of vascular and bone mediators released during the initial stages of peri−implant bone regeneration.

Interestingly, the levels of EGF, BMP-9, ET-1, FGF, and PlGF were higher in the presence of the abutment collar treated with TiO2 at 30 days following the insertion of the implants and abutments. These growth factors are expressed during the regeneration and development phases, and their local availability could contribute to the acceleration of the regeneration process, given that these markers have an effect on regeneration, chemical attraction, proliferation, and cell differentiation into osteoblasts[28]. Bhardwaj and Webster observed increased proliferation of osteoblasts and reduced bacterial proliferation on a surface treated with TiO2[29]. Thus, the presence of the collar treated with TiO_2 seems to have a positive effect on the release of these factors, producing a favourable environment for bone regeneration and vascular neoformation.

Higher EGF concentrations were observed in the test group than in the control group at 30 days following implant installation. EGF is related to osteoblast differentiation and mineralization, as well as DNA synthesis, and is directly associated with rapid tissue regeneration after injuries[30]. It is a marker found in osteoblasts, osteoclasts, and endothelial cells[31]. An in vitro study found covalent immobilization of EGF in TiO_2 nanotubes, demonstrating that the number and activity of mesenchymal cells increased significantly in the presence of this molecule[32]. The results of the present study showed that TiO_2 promoted greater release of EGF, indicating osteoblastic activity in the initial regeneration stages.

On the other hand, EGF is also related to the induction of growth and proliferation of mesenchymal and epithelial cells, with stimulation of granulation tissue formation and angiogenesis. When this marker invades the blood vessels, it plays an important role in tissue regeneration, since it stimulates vascular permeability and tissue proliferation. This phenomenon suggests that EGF is important in the regeneration process, because its actions on angiogenesis and osteoblastogenesis appear to be linked during the initial peri-implant bone regeneration phases[33,34]. In this way, the treatment of surfaces with TiO_2 seems to stimulate angiogenesis through the release of EGF and could thus favour bone regeneration and integration.

In addition to the increased EGF levels, a greater concentration of ET-1 was also observed at 30 days after the procedure. EGF is among the factors that regulate the function of ET-1[35]. ET-1 is a vasoactive mediator with important function for cellular proliferation, angiogenesis, apoptosis, and migration of diverse cell lines[36,37]. This mediator binds to cellular receptors on surfaces and activates signalling pathways that regulate mesenchymal cell behaviour, such

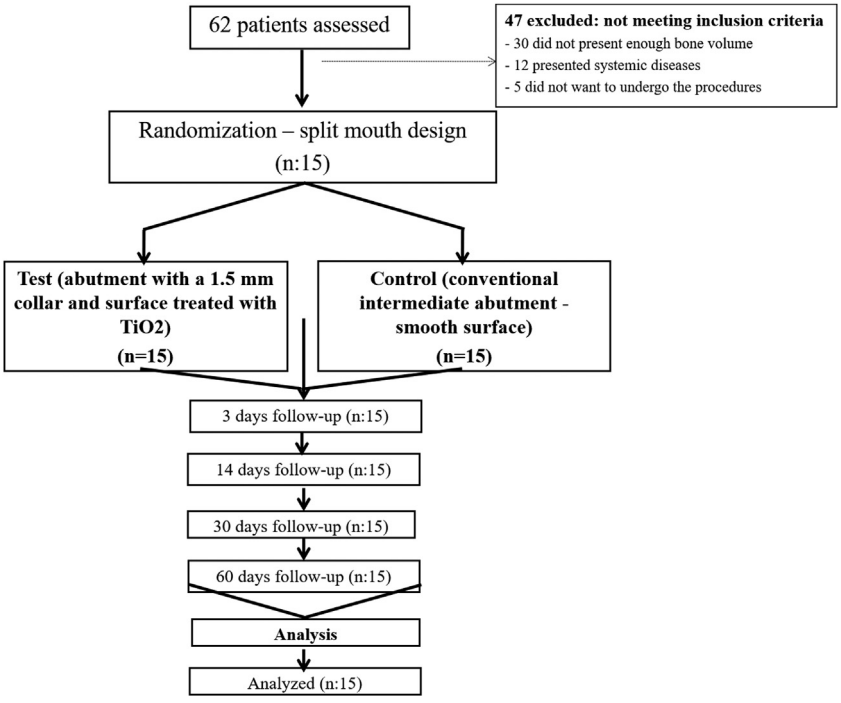

Fig. 2. Flowchart of the study showing the patients enrolled in the pre-study phase and the selection of individuals for the study phase.

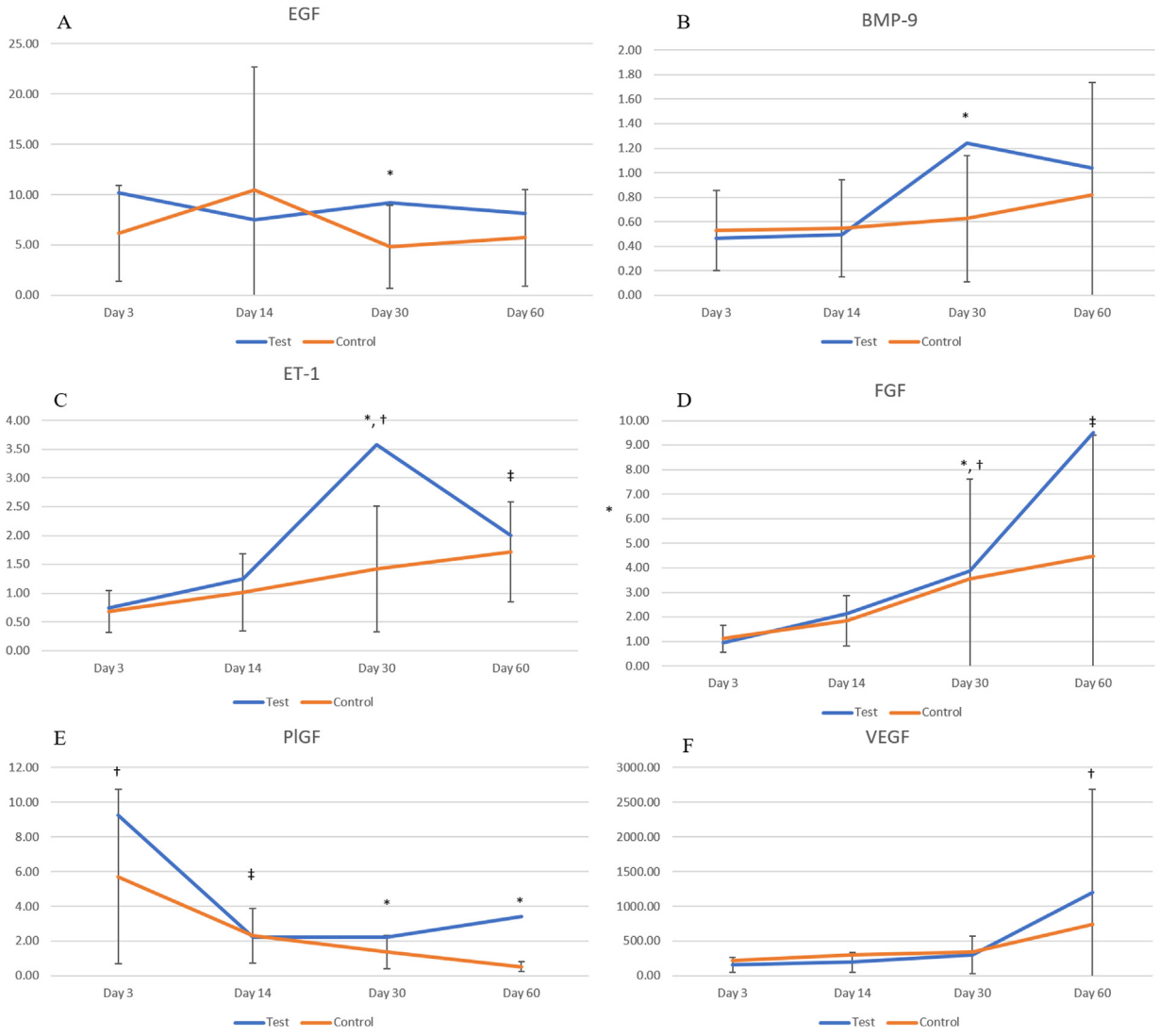

Fig. 3. Levels of epidermal growth factor (EGF), bone morphogenetic protein 9 (BMP-9), endothelin 1 (ET-1), fibroblast growth factor (FGF), placental growth factor (PlGF), and vascular endothelial growth factor (VEFG) (pg/ml) in the peri-implant fluid in the test and control groups. (A) EGF: *significant difference between the groups (two-way ANOVA/Tukey, $P < 0.05$); there was no difference in the intra-group analysis (two-way ANOVA/Tukey, $P > 0.05$). (B) BMP-9: *significant difference between the groups (two-way ANOVA/Tukey, $P < 0.05$); there was no difference in the intra-group analysis (two-way ANOVA/Tukey, $P > 0.05$). (C) ET-1: *significant difference between the groups (two-way ANOVA/Tukey, $P < 0.05$); †significant difference between day 3 and day 60 for the test group intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$); ‡significant difference between day 14 and day 60in the control group intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$). (D) FGF: *significant difference between the groups (two-way ANOVA/Tukey, $P < 0.05$); †significant difference between day 3 and day 30 in the test group intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$); ‡significant difference between day 3 and day 60 for both groups in the intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$). (E) PlGF: *significant difference between the groups (two-way ANOVA/Tukey, $P < 0.05$); †significant difference between day 14 and day 60 in the control group intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$); ‡significant difference between day 3 and day 60 in the control group intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$). (F) VEGF: †significant difference between day 3 and day 30 for the test group intra-group analysis (two-way ANOVA/Tukey, $P < 0.05$); there was no difference in the inter-group analysis (two-way ANOVA/Tukey, $P > 0.05$).

as Ca^{2+} calmodulin-dependent protein kinase, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC)[38].

Mesenchymal cells treated with ET-1 exhibited increased expression of VEGF and angiopoietins 2 and 4, and increased migration and formation of blood vessels of endothelial cells[39]. ET-1 is also known as a growth factor that stimulates hypertrophy, as well as tissue and cell proliferation, including epithelial cells[40] and fibroblasts[41–43]. Therefore, TiO2 treatment was found to stimulate angiogenic activity and it can be thesized that this treatment can also stimulate mesenchymal cell proliferation and differentiation during the peri-implant regeneration process.

ET-1 also has positive effects on bone regeneration. Mice with an ET-1 deficiency exhibited craniofacial abnormalities, with poor development of the mandible, low bone mass, and cardiovascular abnormalities[44,45]. ET-1 plays a role in regulating bone metabolism through the stimulation of bone formation[44], increases the proliferation of osteoprogenitor cells, and promotes their differentiation into mature osteoblasts[46–48]. In addition, ET-1 has been shown to promote greater bone

formation in animal models, with higher density and bone trabeculae[47]. Therefore, it is suggested that the high levels of ET-1 in the test group were related to higher osteogenesis activity and, possibly, higher density of the neoformed bone.

Among the markers assessed, only VEGF did not exhibit higher levels in the presence of the treated abutment collars. VEGF is an important growth factor in the angiogenesis process, related to differentiation into osteoblasts, chondroblasts, and osteoclasts[49–51]. Interestingly, this lack of higher VEGF levels was correlated with the higher levels of ET-1, which is the only cytokine that has an inhibiting effect on VEGF, reducing both the expression of messenger RNA and protein secretion in a dose-dependent and time-dependent manner[48,51].

On the other hand, it has also been found that ET-1 induces VEGF synthesis in immortalized pre-osteoblasts[52], suggesting that this molecule could play a key role in angiogenesis during the bone regeneration and remodelling process. The mechanisms by which this regulation occurs are not yet known. However, it is believed that the reduction of VEGF levels due to ET-1 is important to avoid uncontrolled angiogenesis (which might result in vascular tumours or hemangiomas[53,54]) and promote a spatial and temporal link between angiogenesis and bone formation[55].

BMPs are members of the transforming growth factor beta (TGF-β) family and play an important role in bone formation and the differentiation of undifferentiated mesenchymal cells[56]. BMP-9 is one of the bone morphogenetic proteins with the highest osteogenic activity, and promotes the differentiation of mesenchymal cells into osteoblasts[57,58]. The results of the present study indicate that TiO_2 treatment promoted the release of BMP-9. This demonstrates the occurrence of osteoblastic activity in the early phase of peri-implant regeneration, favouring biological sealing in the region of the abutment through bone formation.

Another important result is related to the FGF level. This growth factor has a pleiotropic effect on the development of different cells and organs, as evidenced via in vitro and in vivo studies[59,60]. It is a protein produced by fibroblasts, endothelial cells, and osteoblasts[61,62], with a mitogenic effect on the same cell types. It induces angiogenesis and stimulates the proliferation and migration of endothelial cells and the expression of proteases, growth factors, and integrins involved in angiogenesis[63]. FGF also influences bone development, remodelling, and regeneration[64,65]. In addition, FGF plays a role in the proliferation of precursor cells, promoting osteoblast proliferation[66,67], and induces recruitment, formation, differentiation, and resorption activity[68]. This growth factor appears to play an important role in bone turnover, regulating bone formation and resorption. Therefore, the higher level of this marker in the test group at 30 days demonstrates that the treatment of the intermediate abutment surface stimulated the release of this grow factor, favouring peri-implant bone remodelling.

Another relevant result found in the present study concerns the levels of PlGF, which is an angiogenic factor related to bone formation and regeneration[69]. This marker increases the proliferation, migration, and survival of endothelial cells[70,71], and stimulates fibroblast proliferation[72,73]. PlGF has been regarded as an important growth factor for bone regeneration, functioning in the initial phase of the inflammatory and post-trauma angiogenesis process, and in the proliferation and differentiation of mesenchymal cells[74]. In addition, an in vivo and in vitro study found toxicity of TiO_2 nanoparticles in lung epithelial cells, which showed increased PlGF levels (amount of mRNA and protein)[75]. Hence, higher levels of PlGF in the presence of the abutment collars treated with TiO_2 indicate greater angiogenic and bone regeneration activity in this group.

Histological studies have demonstrated that connective tissue around titanium or zirconia abutments exhibits a parallel orientation of the fibres and that epithelium adhesion to the polished surface of the implants is only promoted by hemidesmosomes[76]. Clinical studies conducted in animal models, using implants with a 1-mm laser-microtextured collar, demonstrated a perpendicular orientation of the fibres towards the abutment, with fibre insertion mimicking Sharpey's fibres[10,77]. Interestingly, some authors have suggested that laser treatment of surfaces inhibits apical migration of the epithelium[10,77–79].

In vitro studies have found a stimulatory effect of TiO_2 particles, including the induction of human fibroblasts and undifferentiated mesenchymal cells, suggesting that TiO_2 can increase cell integration[80,81]. In addition, an increase in type 1 collagen secretion and fibronectin synthesis by gingival fibroblasts has been observed, which promotes initial adhesion and consequently leads to improved soft tissue adhesion[81]. Although no histological analysis was performed in the present study, it could be hypothesized that the behaviour of fibres and epithelial tissue around the abutment collars treated with TiO_2 would be similar to the behaviour described above, given that TiO_2 exhibits this stimulatory effect on important molecules and cells related to regeneration.

Some studies have attempted to find methods to promote greater stability of the peri-implant bone crest and to ensure a greater bone tissue height and greater and faster bone integration. A study conducted on animals found higher bone tissue height (70% higher than for machined collars) and greater stability of bone crest in the presence of treated collars[13]. Similarly, from a molecular perspective, the data obtained by analysing the peri-implant fluid in the present study demonstrated that the abutments treated with TiO_2 had additional benefits in comparison to machined abutment surfaces due to the release of vascular and bone mediators.

The results of this study suggest that osteoblastic and angiogenic activities are predominantly observed in the initial phases of peri-implant regeneration, taking into account the higher levels of EGF, BMP-9, ET-1, FGF, and PlGF in the peri-implant fluid during this period. Of note, these osteoblastic and angiogenic activities were observed in the early phase (30 days postoperative), which may suggest an acceleration in the bone formation process around the abutment and consequently faster biological sealing of the implant–abutment interface.

A limitation of this study is the small number of patients included in the trial. Twenty patients were considered eligible for the study. However, five did not wish to undergo the procedures. However, the split-mouth design represents a powerful tool for the comparison of treatments, due to the increased efficiency of the statistical tests and statistical power. This is because most of the variability among patients is excluded from the intervention effect and each subject is its own control[82,83]. Besides, as well as the present study, previous studies performed by this research group using the same methodology and similar number of patients were able to determine statistical significance[25–27].

Although the molecular findings of this study may help elucidate the mechanisms involved in peri-implant tissue regeneration in the early phases of bone integration in the presence of treated intermediate abutment surfaces, further studies are required to determine the behaviour of these tissues after the establishment of function (prosthetic loading), considering the levels of bone tissue (radiographic stability), the

patterns of pro- and anti-inflammatory cytokine release, and the microbiological profile over time. In addition, other types of treatments should be assessed and compared to determine the best effect and the best clinical management.

The results of this study suggest some biological mechanisms that could help explain the positive effects promoted by the treatment of intermediate abutment surfaces with TiO_2. These findings may also improve our understanding of the vascular–bone modulation that occurs around implants through surface treatment, and thus enable better clinical strategies that could help preserve the peri-implant bone crest. In conclusion, the treatment of intermediate abutment surfaces with TiO_2 modulated the release of bone, angiogenesis, and tissue regeneration markers.

Funding

This research received no specific grant from any funding agency.

Competing interests

The authors declare that they have no conflict of interest. All authors have viewed and agreed to the submission.

Ethical approval

This study was approved by the Research Ethics Committee of Paulista University (58754416.2.0000.5512).

Patient consent

All participants were informed about the nature of the study and the potential risks and benefits of their participation; all signed an informed consent agreement.

References

1. Albrektsson T, Zarb G, Worthington P, Eriksson AR. The long-term efficacy of currently used dental implants: a review and proposed criteria of success. *Int J Oral Maxillofac Implants* 1986;**1**:11–25.
2. Albrektsson T, Donos N, Working Group 1. Implant survival and complications. The Third EAO Consensus Conference 2012. *Clin Oral Implants Res* 2012;**23**(Suppl 6):63—65.
3. Albrektsson T, Buser D, Sennerby L. Crestal bone loss and oral implants. *Clin Implant Dent Relat Res* 2012;**14**:783–91.
4. Berglundh T, Lindhe J. Dimension of the periimplant mucosa: Biological width revisited. *J Clin Periodontol* 1996;**23**:971–3.
5. Hermann JS, Cochran DL, Nummikoski PV, Buser D. Crestal bone changes around titanium implants: A radiographic evaluation of unloaded nonsubmerged and submerged implants in the canine mandible. *J Periodontol* 1997;**68**:1117–30.
6. Hermann JS, Buser D, Schenk RK, Higginbottom FL, Cochran DL. Biologic width around titanium implants: A physiologically formed and stable dimension over time. *Clin Oral Implants Res* 2000;**11**:1–11.
7. Albrektsson T, Wennerberg A. Oral implant surfaces: part 1—review focusing on topographic and chemical properties of different surfaces and in vivo responses to them. *Int J Prosthodont* 2004;**17**:536–43.
8. Albrektsson T, Wennerberg A. Oral implant surfaces: part 2—review focusing on clinical knowledge of different surfaces. *Int J Prosthodont* 2004;**17**:544–56.
9. Atieh MA, Ibrahim HM, Atieh AH. Platform switching for marginal bone preservation around dental implants: a systematic review and meta-analysis. *J Periodontol* 2010;**81**:1350–66.
10. Nevins M, Kim DM, Jun SH, Guze K, Schupbach P, Nevins ML. Histologic evidence of a connective tissue attachment to laser microgrooved abutments: a canine study. *Int J Periodontics Restorative Dent* 2010;**30**:245–55.
11. Val JE, Gómez-Moreno G, Ruiz-Linares M, Prados-Frutos JC, Gehrke SA, Calvo-Guirado JL. Effects of surface treatment modification and implant design in implants placed crestal and subcrestally applying delayed loading protocol. *J Craniofac Surg* 2017;**28**:552–8.
12. Weiner S, Simon J, Ehrenberg OS, Zweig B, Ricci JL. The effects of laser microtextured collars upon crestal bone levels of dental implants. *Implant Dent* 2008;**17**:217–28.
13. Gehrke SA, Neto UTS. Evaluation of the surface treatment on bone healing in a transmucosal 1-mm area of implant abutment: an experimental study in the rabbit tibia. *Clin Implant Dent Relat Res* 2016;**18**:489–97.
14. Boyan BD, Batzer R, Kieswetter K, Liu Y, Cochran DL, Szmuckler-Moncler S, Dean DD, Schwartz Z. Titanium surface roughness alters responsiveness of MG63 osteoblast-like cells to 1a,25-$(OH)_2D_3$. *J Biomed Mater Res* 1998;**39**:77–85.
15. Linderback P, Harmankaya N, Askendal A, Areva S, Lausmaa J, Tengvall P. The effect of heat- or ultraviolet ozone-treatment of titanium on complement deposition from human blood plasma. *Biomaterials* 2010;**31**:4795–801.
16. Masaki C, Schneider GB, Zaharias R, Seabold D, Stanford C. Effects of implant surface microtopography on osteoblast gene expression. *Clin Oral Implants Res* 2005;**16**:650–6.
17. Nygren H, Tengvall P, Lundstrom I. The initial reactions of TiO_2 with blood. *J Biomed Mater Res* 1997;**34**:487–92.
18. Schneider GB, Perinpanayagam H, Clegg M, Zaharias R, Seabold D, Keller J, Stanford C. Implant surface roughness affects osteoblast gene expression. *J Dent Res* 2003;**82**:372–6.
19. Tan KS, Qian L, Rosado R, Flood PM, Cooper LF. The role of titanium surface topography on J774A.1 macrophage inflammatory cytokines and nitric oxide production. *Biomaterials* 2006;**27**:5170–7.
20. Cooper L. A role for surface topography in creating and maintaining bone at titanium endosseous implants. *J Prosthet Dent* 2000;**84**:522–34.
21. Frenkel SR, Simon J, Alexander H, Dennis M, Ricci JL. Osseointegration on metallic implant surfaces: effects of microgeometry and growth factor treatment. *J Biomed Mater Res* 2002;**63**:706–13.
22. Simmons CA, Meguid SA, Pilliar RM. Mechanical regulation of localized and appositional bone formation around bone-interfacing implants. *J Biomed Mater Res* 2001;**55**:63–71.
23. Clark P, Connolly P, Curtis AS, Dow JA, Wilkinson CD. Cell guidance by ultrafine topography in vitro. *J Cell Sci* 1991;**99**(Pt 1):73–7.
24. Gehrke SA, Zizzari VL, Iaculli F, Mortellaro C, Tetè S, Piattelli A. Relationship between the surface energy and the histologic results of different titanium surfaces. *J Craniofac Surg* 2014;**25**:863–7.
25. Ghiraldini B, Conte A, Casarin RC, Casati MZ, Pimentel SP, Cirano FR, Ribeiro FV. Influence of glycemic control on peri-implant bone healing: 12-month outcomes of local release of bone-related factors and implant stabilization in type 2 diabetics. *Clin Implant Dent Relat Res* 2016;**18**:801–9.
26. Prati AJ, Casati MZ, Ribeiro FV, Cirano FR, Pastore GP, Pimentel SP, Casarin RC. Release of bone markers in immediately loaded and nonloaded dental implants: a randomized clinical trial. *J Dent Res* 2013;**92**(12 Suppl):161S–7S.
27. Verrastro Neto A, Andrade R, Corrêa MG, Casarin RCV, Casati MZ, Pimentel SP, Ribeiro FV, Cirano FR. The impact of different torques for the insertion of immediately loaded implants on the peri-implant levels of angiogenesis- and bone-related markers. *Int J Oral Maxillofac Surg* 2018;**47**:651–7.
28. Luginbuehl V, Meinel L, Merkle HP, Gander B. Localized delivery of growth factors for bone repair. *Eur J Pharm Biopharm* 2004;**58**:197–208.
29. Bhardwaj G, Webster TJ. Reduced bacterial growth and increased osteoblast proliferation on titanium with a nanophase TiO_2 surface treatment. *Int J Nanomedicine* 2017;**6**:363–9.
30. Savani RC, Cao G, Pooler PM, Zaman A, Zhou Z, DeLisser HM. Differential involvement of the hyaluronan (HA) receptors CD44 and receptor for HA-mediated motili-

ty in endothelial cell function and angiogenesis. *J Biol Chem* 2001;**276**:36770–8.
31. Adam AB, Özdamar MY, Esen HH, Günel E. Local effects of epidermal growth factor on the wound healing in esophageal anastomosis: an experimental study. *Int J Pediatr Otorhinolaryngol* 2017;**99**:8–12.
32. Bauer S, Park J, Pittrof A, Song YY, von der Mark K, Schmuki P. Covalent functionalization of TiO_2 nanotube arrays with EGF and BMP-2 for modified behavior towards mesenchymal stem cells. *Integr Biol (Camb)* 2011;**3**:927–36.
33. Alves JB, Ferreira CL, Martins AF, Silva GA, Alves GD, Paulino TP, Ciancaglini P, Thedei Jr G, Napimoga MH. Local delivery of EGF-liposome mediated bone modeling in orthodontic tooth movement by increasing RANKL expression. *Life Sci* 2009;**4**:693–9.
34. Sezer O, Niemöller K, Jakob C, Zavrski I, Heider U, Eucker J, Kaufmann O, Possinger K. Relationship between bone marrow angiogenesis and plasma cell infiltration and serum beta2-microglobulin levels in patients with multiple myeloma. *Ann Hematol* 2001;**80**:598–601.
35. Ahnstedt H, Stenman E, Cao L, Henriksson M, Edvinsson L. Cytokines and growth factors modify the upregulation of contractile endothelin ET(A) and ET(B) receptors in rat cerebral arteries after organ culture. *Acta Physiol (Oxf)* 2012;**205**:266–78.
36. Dawas K, Loizidou M, Shankar A, Ali H, Taylor I. Angiogenesis in cancer: the role of endothelin-1. *Ann R Coll Surg Engl* 1999;**81**:306–10.
37. Shichiri M, Hirata Y, Nakajima T, Ando K, Imai T, Yanagisawa M, Masaki T, Marumo F. Endothelin-1 is an autocrine/paracrine growth factor for human cancer cell lines. *J Clin Invest* 1991;**87**:1867–71.
38. Tsai TL, Wang B, Squire MW, Guo LW, Li WJ. Endothelial cells direct human mesenchymal stem cells for osteo- and chondrolineage differentiation through endothelin-1 and AKT signaling. *Stem Cell Res Ther* 2015;**6**:1–14.
39. Pourjafar M, Saidijam M, Mansouri K, Malih S, Ranjbar Nejad T, Shabab N, Najafi R. Cytoprotective effects of endothelin-1 on mesenchymal stem cells: an in vitro study. *Clin Exp Pharmacol Physiol* 2016;**43**:769–76.
40. Murlas CG, Gulati A, Singh G, Najmabadi F. Endothelin-1 stimulates proliferation of normal airway epithelial cells. *Biochem Biophys Res Commun* 1995;**212**:953–9.
41. Gray MO, Long CS, Kalinyak JE, Li HT, Karliner JS. Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts. *Cardiovasc Res* 1998;**40**:352–63.
42. Villarreal FJ, Kim NN, Ungab GD, Printz MP, Dillmann WH. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation* 1993;**88**:2849–61.
43. Yamamoto E, Awano S, Koseki T, Ansai T, Takehara T. Expression of endothelin-1 in gingival epithelial cells. *J Periodontal Res* 2003;**38**:417–21.
44. Kitano Y, Kurihara H, Kurihara Y, Maemura K, Ryo Y, Yazaki Y, et al. Gene expression of bone matrix proteins and endothelin receptors in endothelin-1-deficient mice revealed by in situ hybridization. *J Bone Miner Res* 1998;**13**:237–44.
45. Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, Oda H, Kuwaki T, Cao WH, Kamada N, Jishage K, Ouchi Y, Azuma Y, Toyoda Y, Ishikawa T, Kumada M, Yazaki Y. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* 1994;**368**:703–10.
46. Clines GA, Mohammad KS, Grunda JM, Clines KL, Niewolna M, McKenna CR, McKibbin CR, Yanagisawa M, Suva LJ, Chirgwin JM, Guise TA. Regulation of postnatal trabecular bone formation by the osteoblast endothelin A receptor. *J Bone Miner Res* 2011;**26**:2523–36.
47. Hu LW, Wang X, Jiang XQ, Xu LQ, Pan HY. In vivo and in vitro study of osteogenic potency of endothelin-1 on bone marrow-derived mesenchymal stem cells. *Exp Cell Res* 2017;**357**:25–32.
48. Veillette CJ, von Schroeder HP. Endothelin-1 down-regulates the expression of vascular endothelial growth factor-A associated with osteoprogenitor proliferation and differentiation. *Bone* 2004;**34**:288–96.
49. Li R, Stewart DJ, von Schroeder HP, Mackinnon ES, Schemitsch EH. Effect of cell-based VEGF gene therapy on healing of a segmental bone defect. *J Orthop Res* 2009;**27**:8–14.
50. Zelzer E, McLean W, Ng YS, Fukai N, Reginato AM, Lovejoy S, D'Amore PA, Olsen BR. Skeletal defects in VEGF (120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development* 2002;**129**:1893–904.
51. Iglarz M, Silvestre JS, Duriez M, Henrion D, Levy BI. Chronic blockade of endothelin receptors improves ischemia-induced angiogenesis in rat hindlimbs through activation of vascular endothelial growth factor—NO pathway. *Arterioscler Thromb Vasc Biol* 2001;**21**:1598–603.
52. Kozawa O, Kawamura H, Hatakeyama D, Matsuno H, Uematsu T. Endothelin-1 induces vascular endothelial growth factor synthesis in osteoblasts: involvement of p38 mitogen-activated protein kinase. *Cell Signal* 2000;**12**:375–80.
53. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 2000;**6**:389–95.
54. Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest* 2004;**113**:516–27.
55. Qu G, von Schroeder HP. Role of osterix in endothelin-1-induced downregulation of vascular endothelial growth factor in osteoblastic cells. *Bone* 2006;**38**:21–9.
56. Luther G, Wagner ER, Zhu G, Kang Q, Luo Q, Lamplot J, Bi Y, Luo X, Luo J, Teven C, Shi Q, Kim SH, Gao JL, Huang E, Yang K, Rames R, Liu X, Li M, Hu N, Liu H, Su Y, Chen L, He BC, Zuo GW, Deng ZL, Reid RR, Luu HH, Haydon RC, He TC. BMP-9 induced osteogenic differentiation of mesenchymal stem cells: molecular mechanism and therapeutic potential. *Curr Gene Ther* 2011;**11**:229–40.
57. Peng Y, Kang Q, Cheng H, Li X, Sun MH, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC. Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. *J Cell Biochem* 2003;**90**:1149–65.
58. Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, Luu HH, Park JK, Li X, Luo J, Montag AG, Haydon RC, He TC. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. *J Biol Chem* 2004;**279**:32941–9.
59. Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. *Endocr Rev* 1997;**18**:26–45.
60. Collin-Osdoby P, Rothe L, Bekker S, Anderson F, Huang Y, Osdoby P. Basic fibroblast growth factor stimulates osteoclast recruitment, development, and bone pit resorption in association with angiogenesis in vivo on the chick chorioallantoic membrane and activates isolated avian osteoclast resorption in vitro. *J Bone Miner Res* 2002;**17**:1859–71.
61. Montero A, Okada Y, Tomita M, Ito M, Tsurukami H, Nakamura T, Doetschman T, Coffin JD, Hurley MM. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *J Clin Invest* 2000;**105**:1085–93.
62. Sato Y, Shimada T, Takaki R. Autocrinological role of basic fibroblast growth factor on tube formation of vascular endothelial cells in vitro. *Biochem Biophys Res Commun* 1991;**180**:1098–102.
63. Chim SM, Tickner J, Chow ST, Kuek V, Guo B, Zhang G, Rosen V, Erber W, Xu J. Angiogenic factors in bone local environment. *Cytokine Growth Factor Rev* 2013;**24**:297–310.
64. Arman E, Haffner-Krausz R, Gorivodsky M, Lonai P. Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proc Natl Acad Sci U S A* 1999;**96**:11895–9.
65. Marie P, Debiais F, Cohen-Solal M, de Vernejoul MC. New factors controlling bone remodeling. *Joint Bone Spine* 2000;**67**:150–6.
66. Debiais F, Hott M, Graulet AM, Marie PJ. The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific. *J Bone Miner Res* 1998;**13**:645–54.
67. Hanada K, Dennis JE, Caplan AI. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteo-

genic differentiation of rat bone marrow-derived mesenchymal stem cells. *J Bone Miner Res* 1997;**12**:1606–14.
68. Chikazu D, Hakeda Y, Ogata N, Nemoto K, Itabashi A, Takato T, Kumegawa M, Nakamura K, Kawaguchi H. Fibroblast growth factor (FGF)-2 directly stimulates mature osteoclast function through activation of FGF receptor 1 and p42/p44 MAP kinase. *J Biol Chem* 2000;**275**:31444–50.
69. Jacobsen KA, Al-Aql ZS, Wan C, Fitch JL, Stapleton SN, Mason ZD, Cole RM, Gilbert SR, Clemens TL, Morgan EF, Einhorn TA, Gerstenfeld LC. Bone formation during distraction osteogenesis is dependent on both VEGFR1 and VEGFR2 signaling. *J Bone Miner Res* 2008;**23**:596–609.
70. Adini A, Kornaga T, Firoozbakht F, Benjamin LE. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res* 2002;**62**:2749–52.
71. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, Chorianopoulos E, Liesenborghs L, Koch M, De Mol M, Autiero M, Wyns S, Plaisance S, Moons L, van Rooijen N, Giacca M, Stassen JM, Dewerchin M, Collen D, Carmeliet P. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 2007;**131**:463–75.
72. Bellik L, Vinci MC, Filippi S, Ledda F, Parenti A. Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. *Br J Pharmacol* 2005;**146**:568–75.
73. Yonekura H, Sakurai S, Liu X, Migita H, Wang H, Yamagishi S, Nomura M, Abedin MJ, Unoki H, Yamamoto Y, Yamamoto H. Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. *J Biol Chem* 2016;**1999**(274):35172–8.
74. Maes C, Coenegrachts L, Stockmans I, Daci E, Luttun A, Petryk A, Gopalakrishnan R, Moermans K, Smets N, Verfaillie CM, Carmeliet P, Bouillon R, Carmeliet G. Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. *J Clin Invest* 2006;**116**:1230–42.
75. Chen HW, Su SF, Chien CT, Lin WH, Yu SL, Chou CC, Chen JJ, Yang PC. Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. *FASEB J* 2006;**20**:2393–5.
76. Berglundh T, Lindhe J, Ericsson I, Marinello CP, Liljenberg B, Thomsen P. The soft tissue barrier at implants and teeth. *Clin Oral Implants Res* 1991;**2**:81–90.
77. Nevins M, Camelo M, Nevins ML, Schupbach P, Kim DM. Reattachment of connective tissue fibers to a laser-microgrooved abutment surface. *Int J Periodontics Restorative Dent* 2012;**32**:e131–4.
78. Nevins M, Camelo M, Nevins ML, Schupbach P, Kim DM. Connective tissue attachment to laser-microgrooved abutments: a human histologic case report. *Int J Periodontics Restorative Dent* 2012;**32**:385–92.
79. Ricci JL, Grew JC, Alexander H. Connective-tissue responses to defined biomaterial surfaces: I. Growth of rat fibroblast and bone marrow cell colonies on microgrooved substrates. *J Biomed Mater Res A* 2008;**85**:313–25.
80. Guida L, Annunziata M, Rocci A, Contaldo M, Rullo R, Oliva A. Biological response of human bone marrow mesenchymal stem cells to fluoride-modified titanium surfaces. *Clin Oral Implants Res* 2010;**21**:1234–41.
81. Wang X, Lu T, Wen J, Xu L, Zeng D, Wu Q, Cao L, Lin S, Liu X, Jiang X. Selective responses of human gingival fibroblasts and bacteria on carbon fiber reinforced polyetheretherketone with multilevel nanostructured TiO_2. *Biomaterials* 2016;**83**:207–18.
82. Hujoel PP, DeRouen TA. Validity issues in split-mouth trials. *J Clin Periodontol* 1992;**19**(9 Pt 1):625–7.
83. Hujoel PP, Loesche WJ. Efficiency of split-mouth designs. *J Clin Periodontol* 1990;**17**:722–8.

Address:
Departamento de Odontologia
Universidade Paulista (UNIP)
Av. Dr. Bacelar
1212
Vila Clementino
São Paulo
04026-002
Brazil
Tel./Fax: +55 11 5586 4000
E-mails: suppimentel@yahoo.com, suzana.pimentel@docente.unip.br