Original Article

Propolis extract and bovine bone graft combination in the expression of VEGF and FGF2 on the preservation of post extraction socket

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Abstract

Aim: To determine the potential of propolis extract and BBG combination on the quantity of fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), and osteoblasts in the preservation of tooth extraction socket on days 3 and 7.

Settings and Design: Laboratory in vivo reseach using animal model.

Materials and Methods: Fifty-six Cavia cobaya were divided into eight groups containing seven animals in each group. The extraction socket on the lower left incisor was filled with polyethylene glycol (PEG) at a concentration of 2% (Groups I and II) as a control; active materials consisted of propolis extract and PEG (Groups III and IV); active materials consisted of BBG and PEG (Groups V and VI); and active materials consisted of propolis extract, BBG, and PEG (Groups VII and VIII). Then, an examination was done using immunohistochemistry to perform an expression of VEGF, FGF2, as well as histology of osteoblasts.

Statistical Analysis Used: The statistical analysis performed using a one-way ANOVA and Tukey's honestly significant difference test.

Results: Propolis extract, BBG and PEG had the most significant result related to the formation of FGF2, VEGF, and osteoblasts.

Conclusion: The combination of propolis extract with BBG and PEG in socket preservation is effective in increasing the expression of FGF2, VEGF, and osteoblasts.

Keywords: Bovine bone graft, fibroblast growth factor-2, osteoblast, propolis, vascular endothelial growth factor

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INTRODUCTION

Tooth extraction is a common procedure performed in the field of dentistry. A normal tooth extraction process will always be followed by bone resorption and regeneration. Tooth extraction followed by socket-healing process usually leads to alveolar bone deformities, including the reduction of residual ridge height and width.[1]

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After tooth extraction, bleeding occurs in the tooth socket followed by the initiation of the inflammatory mediator and the production of blood clots that will cover the extraction socket. Inflammation leads to the increasing activity of osteoclast, facilitated by the pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β. Therefore, the inflammatory cytokines induces increasing of the Receptor Activator of Nuclear-κB (RANK) and RANK Ligand (RANKL). In other words, if the osteoclast cells increase, the alveolar bone resorption will occur.[2]

Three to five days after the tooth extraction, fibrin degeneration and early granulation tissue formation containing blood vessels, fibroblasts, and chronic inflammatory cells will occur. In the fibroblast inflammation process, the tissue stimulates osteoprogenitor (OPG) to inhibit RANKL binding to RANK and trigger the growth of fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) that play a role in osteoblast cell proliferation and differentiation. On day 7 after the tooth extraction, the immature bone begins to form through differentiation of osteoblasts and bone matrix with high osteocyte proportions at the edge of the socket wall and extends to the central area of the socket with a centripetal pattern, leading to the trabecular bone.[2]

One year after tooth extraction, a decrease in the alveolar bone as much as 40% will perform, and the bone resorption continues for the next three years. Large resorption without rapid bone regeneration can alter the structure of the mandibular and maxillary bones, triggering problems in the use of dentures, in terms of retention, stability, and comfort of the dentures.[3] A research conducted by Kresnoadi *et al*. showed that the potential of *Garcinia mangostana* peel extract combined with demineralized freeze-dried bovine bone xenograft leads to reduced ridge resorption and alveolar bone regeneration in preserving the tooth extraction socket.[4] Therefore, it is important to perform socket preservation procedures to maintain alveolar bone after tooth extraction and minimize alveolar bone resorption.

One of the bone graft materials often used is the socket preservation procedure is bovine bone graft (BBG). BBG is often used due to its osteoconductive inorganic matrix components, which serve to provide a scaffold for bone regeneration without getting involved in bone formation itself.[5] The innovative material is needed to induce osteogenetic activity to accelerate bone formation.

Natural ingredients, one of which is propolis, are extensively employed in traditional medicine. Propolis, which had previously been obtained from Lawang, East Java, possesses certain bioactive components, such as artepyline, apigenin, flavonoid, cinnamic acid, saponin, quercetin, terpenoid, and caffeic acid phenethyl ester (CAPE), which have many beneficial effects, including antibacterial, antifungal, anti-inflammatory, antiviral, antioxidant, immunostimulator, and anticancer. Propolis extract is also known to contain polyphenolic compounds, such as flavonoids and CAPE, which can improve the number of osteoblasts and possess anti-inflammatory activity.[6]

A study conducted by Kresnoadi *et al*. proved that the combination of propolis extract and BBG can increase the intracellular HSP70 and osteocalcin expression.[7] In prosthodontics, prominent residual ridges are needed in the success of removable denture, fixed denture, and dental implant treatment. The increasing osteoblast growth activity is needed to prevent ridge resorption, which leads to a prominent residual ridge.

Therefore, this research aimed to reveal the effects of propolis extract and BBG combination on the quantity of FGF2, VEGF, and osteoblasts in the preservation of tooth extraction socket on days 3 and 7.

SUBJECTS AND METHODS

Research procedure and animal model

This research was of experimental laboratory design. Ethical clearance for this research was issued by the Committee of Health Research Ethics, Faculty of Dental Medicine, Universitas Airlangga, No. 014/HRECCIODM/III/2018.

The materials used were propolis extract from Lawang, BBG (from Biomaterial Center Dr. Soetomo Tissue Bank, 150–355 μ/500 mg), polythylene glycol (Merck®) 400 and 4000, 100 mg of ketamine, absolute alcohol, 10% formaldehyde buffer (Brataco®), ethanol 96%, paraffin solution, xylol, ethylenediaminetetraacetic acid (Merck®), hematoxylin and eosin staining material®, monoclonal antibodies VEGF and FGF, and immune staining kit reagent (Novocastra-Leica®, Buffalo Grove,Illinois, USA). Indirect immunohistochemistry examination was conducted using primary antibodies as well as secondary antibodies. The experimental animals used were healthy and active male *Cavia cobaya*, weighing around 300–350 g, aged 3–3.5 months.

The most appropriate dosage of the mixture of propolis extract, BBG, and PEG was calculated, followed by a

histopathologic examination of osteoblasts, along with a staining and immunohistochemistry examination using monoclonal antibodies FGF-2 and VEGF.

Animal preparation

Fifty-six *C. cobaya* were divided into eight groups containing seven animals in each group. The mandibular left incisor tooth was extracted. Afterward, the *C. cobaya* was taken from their treatment facility and anesthetized intravenously with 0.2 cc/300 g ketamine. The mandibular left incisor extraction was conducted with specialized pliers (needle holder). The extraction socket was then filled with PEG, propolis extract + PEG, BBG + PEG, and a combination of propolis extract + BBG + PEG. Each mixture was allowed as much as 0.1 cc, corresponding with the volume of the tooth extraction socket, which was then sutured with polyamide monofilament sewing thread DS 12 3 / 8c, 12 mm, 6/10 met, 0.7 (Braun VetCare SA, Rubi, Spain). The groups were sorted as follows: Groups I and II: tooth extraction was performed, followed by filling in the socket with PEG only; Groups III and IV: tooth extraction was performed followed by filling in the socket using propolis extract + PEG; Groups V and VI: tooth extraction was performed, followed by filling in the socket with BBG and PEG; and Groups VII and VIII: tooth extraction was performed followed by filling in the socket with a combination of propolis extract + BBG + PEG. Groups I, III, V, and VII were evaluated for 3 days, whereas Groups II, IV, VI, and VIII were evaluated for 7 days.

Histopathologic specimen preparation

After 3 and 7 days after animal preparation, all the *C. cobaya* were euthanized and their jaws were removed with a surgical incision. The paraffin block was then prepared. The paraffin block was sliced, using a rotary microtome (Novocastra-Leica®, Buffalo Grove,Illinois, USA) with a thickness of approximately 4 μ. The observation was subsequently carried out under a light microscope, with each slide being examined at ×400 magnification and a maximum of 10 fields of view. The calculation results were recorded in a worksheet with a mean value per field of view. At this point, the quantity of FGF-2, VEGF, and osteoblasts was calculated.

For statistical analysis, a Kolmogorov–Smirnov statistical test was performed on the data obtained. To identify differences between the groups, a one-way ANOVA test was conducted, followed by a multifactorial comparison test involving a Tukey's honestly significant difference (HSD) test.

RESULTS

The results of the average calculation and the standard deviation of the quantity of FGF2 and VEGF at the tooth extraction socket on the 3rd and 7th days are shown in Figure 1. Group VIII had the highest quantity of FGF2, at 28.71 ± 6.370, and the highest quantity of VEGF, at 14.86 ± 4.180. Meanwhile, the lowest quantity of FGF2 and VEGF was found in Group I.

A normality test was carried out in each group using the Kolmogorov–Smirnov test. The results obtained $P > 0.05$, indicating that the data were normally distributed. The homogeneity test showed that all research groups had $P > 0.05$, which showed that all groups have the same variant (homogeneous). With the prerequisites of normal and homogeneous distribution, one-way ANOVA test was then performed to observe the significance between groups.

ANOVA statistical calculations on FGF2 expression on the 3rd and 7th days showed significant differences in the FGF2 results among the group, as indicated by statistically significant values of $P = 0.00$ ($P < 0.05$). This was then followed by a Tukey's HSD statistical test on the FGF2 expression results, which showed significant differences in the FGF2 expression for 3rd and 7th days. The results of the Tukey's HSD test, as stated in Tables 1 and 2, showed that there was a significant increase in FGF2 expression in Group VII compared to Groups I, III, and V and Group VIII compared to Groups II, IV, and VI with statistical significance ($P < 0.05$). The microscopic expression of FGF2 is shown in Figure 2.

ANOVA statistical calculations on VEGF expression on the 3rd and 7th days showed significant differences in the VEGF results among the group, as indicated by statistically significant values of $P = 0.00$ ($P < 0.05$). This was then

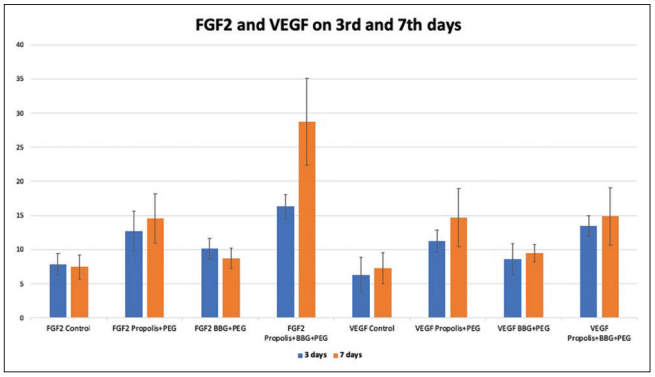

Figure 1: Mean value of fibroblast growth factor-2 and vascular endothelial growth factor, Y-axis: Expression number of fibroblast growth factor-2 and vascular endothelial growth factor. X-axis: Treatment groups at 3rd and 7th days

followed by a Tukey's HSD statistical test on the VEGF expression results, which showed significant differences in the VEGF expression on the 3rd and 7th days. The results of the Tukey's HSD test, as stated in Tables 3 and 4, showed that there was a significant increase in VEGF expression in Group VII compared to Groups I and V; Group I compared to Group III; Group VIII compared to Groups II and VI; and Group IV compared to Group II and VI with statistical significance ($P < 0.05$). The microscopic expression of VEGF is shown in Figure 3.

The results of the average calculation and the standard deviation of the quantity of osteoblast cell at the tooth extraction socket on the 3rd and 7th days are shown in Figure 4. Group VIII had the highest quantity of osteoblast cell, at 18.86 ± 2.61. Meanwhile, the lowest quantity of osteoblasts was found in Group I.

ANOVA statistical calculations on osteoblasts on the 3rd and 7th days showed significant differences among the groups, as indicated by statistically significant values of $P = 0.00$ ($P < 0.05$). This was then followed by a Tukey's HSD statistical test on the number of osteoblasts, which showed significant differences in the osteoblasts on the 3rd and 7th days. The results of the Tukey's HSD test, as stated in Tables 5 and 6, showed that there was a significant increase in the number of osteoblasts in Group VII compared to Groups I, III, and V and Group VIII compared to Groups II, IV, and VI with

Table 1: The results of Tukey's honestly significant difference test on the quantity of fibroblast growth factor-2 in each treatment group at 3 days

Group	Group I	Group III	Group V	Group VII
Group I		*		*
Group III				*
Group V				*

*A significant difference between group

Table 2: The results of Tukey's honestly significant difference test on the quantity of fibroblast growth factor-2 in each treatment group at 7 days

Group	Group II	Group IV	Group VI	Group VIII
Group II		*		*
Group IV			*	*
Group VI				*

*A significant difference between group

Table 3: The results of Tukey's honestly significant difference test on the quantity of vascular endothelial growth factor in each treatment group at 3 days

Group	Group I	Group III	Group V	Group VII
Group I		*		*
Group III				
Group V				*

*A significant difference between group

Table 4: The results of Tukey's honestly significant difference test on the quantity of vascular endothelial growth factor in each treatment group at 7 days

Group	Group II	Group IV	Group VI	Group VIII
Group II		*		*
Group IV			*	
Group VI				*

*A significant difference between group

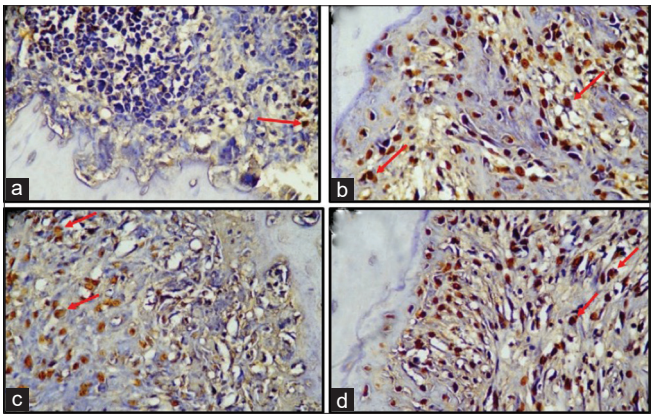

Figure 2: Microscopic expression of fibroblast growth factor-2 at 7 days. (a) Group II; (b) Group IV; (c). Group VI; (d) Group VIII. The red arrow shows fibroblast growth factor-2 expression

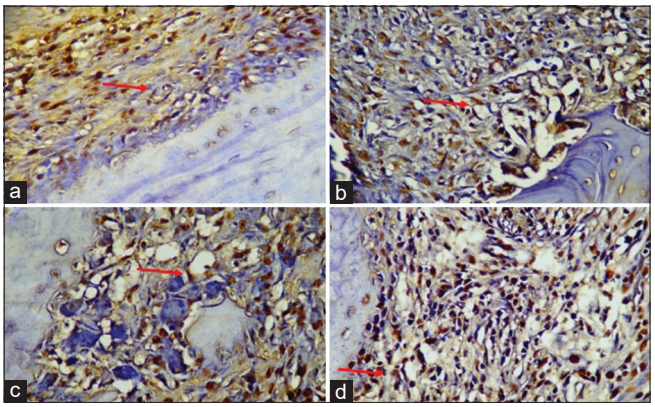

Figure 3: Microscopic expression of vascular endothelial growth factor at 7 days. (a) Group II; (b) Group IV; (c). Group VI; (d) Group VIII. The red arrow shows vascular endothelial growth factor expression

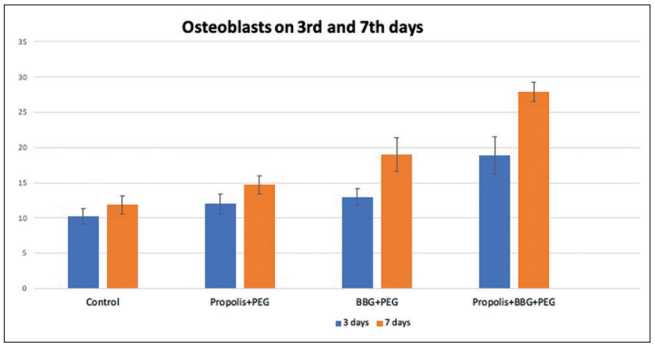

Figure 4: Mean value of the osteoblast number. Y-axis: The number of osteoblasts. X-axis: Treatment groups at 3rd and 7th days

Table 5: The results of Tukey's honestly significant difference test on the quantity of osteoblasts in each treatment group at 3 days

Group	Group I	Group III	Group V	Group VII
Group I			*	*
Group III				*
Group V				*

*A significant difference between group

Table 6: The results of Tukey's honestly significant difference test on the quantity of osteoblasts in each treatment group at 7 days

Group	Group II	Group IV	Group VI	Group VIII
Group II		*	*	*
Group IV			*	*
Group VI				*

*A significant difference between group

statistical significance ($P < 0.05$). The microscopic view of osteoblasts is shown in Figure 5.

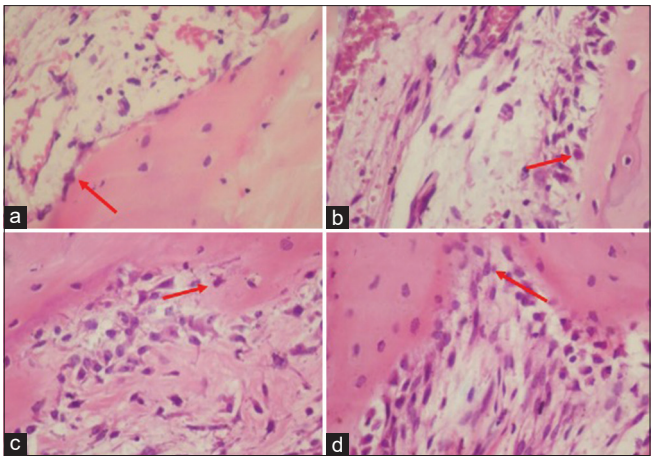

Figure 5: Histological view of osteoblasts at 7 days. (a) Group II; (b) Group IV; (c). Group VI; (d) Group VIII. The red arrow shows vascular endothelial growth factor expression

DISCUSSION

A tooth extraction will create a socket in the alveolar bone because the trauma of extraction can cause cell reactions during the wound healing process. One of the stages in the wound-healing process is the inflammatory phase. Inflammation is the body's defense reaction due to trauma.[6] This inflammatory process triggers the infiltration of inflammatory cells, one of which is the macrophage. Subsequently, macrophages will stimulate the activation of the nuclear factor-kappa beta (NF-κB). NF-κB is a negative regulator of the osteoblast differentiation process of mesenchymal cells. Next, the increased NF-κB stimulates pro-inflammatory cytokine activities, such as TNF-α, IL-6, and IL-1β, triggering the differentiation of preosteoclasts into osteoclasts, resulting in bone resorption.[6]

On day 3 after the tooth extraction, fibrin degeneration and early formation of granulation tissue containing blood vessels, fibroblasts, and chronic inflammatory cells will occur. In the fibroblast inflammation process, the tissue stimulates OPG to inhibit RANKL binding to RANK and triggers the growth of FGF-2 and VEGF, which play a role in osteoblast proliferation and differentiation. On day 7 after the tooth extraction, the immature bone begins to form through differentiation of osteoblasts and bone matrix with high osteocyte proportions at the edge of the socket wall and extends to the central area of the socket with a centripetal pattern, leading to the trabecular bone.[5]

The results indicate that propolis extract and BBG stimulate the growth of alveolar bone in the socket of tooth extraction. BBG is one of the graft materials in the form of xenograft used in this study. Xenograft is used to stimulate the proliferation of fibroblasts, osteoblasts, and endothelial cells. BBG contains a component of osteoconduction inorganic matrix which acts as a scaffold for bone regeneration without being involved in bone formation itself. The active components in BBG are more osteoconductive than osteoinductive so that in this study, there was an increase in the quantity of FGF 2, VEGF, and osteoblasts in the extraction socket, which were given BBG.[8]

BBG is a type of xenograft derived from bovine. Bovine xenograft is the most commonly used graft because it has hydroxyapatite which is almost the same as human bone which allows the graft to revascularize and be replaced with the new bone. Inorganic materials from BBG can support osteoblast cell attachment and proliferation, which is the first step in the process of osteogenesis. The material supports bone matrix for regulation through three mechanisms, namely (1) forming strong spaces for fillers; (2) forming osteoblast attachment and proliferation; and (3) acting as a means to stimulate bone formation. The function of osteoblasts is not only limited to bone formation but is also responsible for the initiation of bone resorption.[8]

The quantity of FGF-2, VEGF, and osteoblasts in the group induced with propolis extract was higher than those in the group induced with BBG. It indicates that propolis extract that obtained from The Research and Consultation Laboratory of Surabaya Industrial Research and Consulting Center at Lawang, East Java, Indonesia, contains cinnamic acid (2.56%), apigenin (1.05%), flavonoid (1.28%), saponin (0.82%), quercetin (1.03%), and terpenoid (1.15%), which have some beneficial effects as anti-inflammation, antibacterial, antiviral, immunostimulatory, antifungal, and anticancer.[4]

The flavonoids present in propolis promote wound healing by increasing the formation of FGF-2 and VEGF A. FGF-2 constitutes a pleiotropic growth factor that can stimulate fibroblast cells and progenitor osteoblasts. FGF-2 expression in osteoblasts is detected during fracture repair. Therefore, it is recognized that FGF-2 may play a crucial role in fracture healing, bone remodeling, and osteogenesis.[9]

VEGF constitutes an angiogenic growth factor highly specific for vascular endothelial cells. As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion, concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate.[10] Evidence is accumulating that osteoblasts among bone cells produce and secrete VEGF in response to various physiological agents. We have previously shown that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells, and that among the mitogen-activated protein kinase superfamily, p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase act as positive regulators in the VEGF release. These findings led us to speculate that VEGF secreted from osteoblasts may play a pivotal role in the regulation of bone metabolism.[11]

Caffeic acid phenethyl ester (CAPE) represents a group of flavonoids in propolis that has an antioxidant that inhibits excessive oxidative reactions as a result of inflammatory reactions and metabolic processes followed by cell injury. As an anti-inflammatory agent, CAPE acts to inhibit phospholipase in arachidonic acid cascade, so it does not release prostaglandin and leukotriene. CAPE can also inhibit lipoxygenase and cyclooxygenase, which play a role in the metabolic pathway. CAPE is lipophilic and facilitates cell infiltration, freeing anti-inflammatory cytokines (transforming growth factor-beta [TGF-β], IL-10, and IL-4); inhibits inflammatory mediators; inhibits T cell proliferation and lymphokine production; inhibits NF-κB activity which acts to regulate genes that encode pro-inflammatory cytokines (TNF-α, IL-1, and IL-6): adhesin, chemokine, and enzyme molecules; induces nitric oxide synthase; and increases the proliferation of FGF-2.[12]

Propolis extract containing flavonoids plays a role in increasing osterix and Run×2 with the result that osteoblasts mature more rapidly. Saponin in propolis plays an active role in increasing alkaline phosphate (ALP), increasing mineralization, and promoting the expression of osteogenic ALP, RUNX2 gene.[13] Besides, quercetin in propolis promotes an increase in the formation of osterix and RunX2 which plays a role in stimulating osteoblast differentiation and bone formation by suppressing lipopolysaccharide.[14] Cinnamic acid in propolis acts as an immunomodulator. It can also increase ALP activity and calcium that can stimulate bone formation while also inhibiting the production of NF-κB and TNF-α.[15]

This result is accordance with some researches that showed that the combination of mangosteen peel extract and xenograft can increase the number of osteoblast cells and reduce the number of osteoclasts. Besides, it can potentially increase osteocalcin and collagen 1 expressions, thereby accelerating alveolar bone regeneration.[16,17] Another study showed that a combination of xenograft and *Moringa* leaf extract can effectively generate TGF-β1 and osteocalcin expressions during the preservation of tooth extraction sockets.[18] The increase in osteoblast cells in this study was strongly induced by the combination of propolis extract and BBG.

CONCLUSION

The combination of propolis extract with BBG and PEG in socket preservation increases FGF-2 and VEGF expression and the number of osteoblasts. This study suggests that the combination of propolis and BBG for socket preservation and maintaining the alveolar bone dimension is to support the success of prosthetic treatment.

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Conflicts of interest
There are no conflicts of interest.

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