Evaluation of the Possible Synergic Regenerative Effects of Platelet-Rich Plasma and Hydroxyapatite/Zirconia in the Rabbit Mandible Defect Model

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Received: 15 April 2017
Revised: 6 May 2017
Accepted: 14 May 2017

What's Known
- Platelet-rich plasma and hydroxyapatite exert beneficial effects on bone repair.
- Zirconia also has beneficial effects on bone repair and improves the mechanical strength of the scaffold for bone tissue engineering.

What's New
- Combination of platelet-rich plasma/hydroxyapatite/zirconia provides a superior osteoconductive environment for bone repair in the short term.
- In the long term, the platelet-rich plasma/hydroxyapatite/zirconia scaffold showed no significant synergic regenerative effects in the rabbit mandible defect.

Abstract

**Background:** Platelet-rich plasma (PRP) and bioceramics such as hydroxyapatite (HA) and zirconium oxide (ZrO₂) are used to reconstruct mandibular defects. We sought to determine the synergistic effects of HA/ZrO₂ and PRP and compare their osteogenic activity.

**Methods:** ZrO₂ scaffolds were constructed by the slurry method and were then coated with HA and impregnated by PRP/heparan sulfate (HS). Bilateral mandibular defects were created in 26 male rabbits. In 20 rabbits, the left defects were treated with HA/ZrO₂/PRP (Group 1) and the corresponding right defects were filled with HA/ZrO₂ (Group 2). The 6 remaining models were treated with PRP gels at both sides (Group 3). The osteoconductivity of HA/ZrO₂/PRP was compared with that of HA/ZrO₂ or PRP by radiological and histological methods after the follow-up period, at weeks 2, 6 and 8. The statistical analyses were performed by ANOVA and LSD using SPSS, version 16.0, for Windows (P<0.05).

**Results:** After 2 weeks, the percentage of the surface occupied by bone was significantly higher in the HA/ZrO₂/PRP-treated defects than in the PRP-treated defects (P=0.007). Osteoblast and osteocyte counts were higher significantly in the PRP-treated group (P=0.032); however, the cells had not started matrix formation on a large scale and just small islands of osteoid with trapped osteocytes were observed. In the long term, the regenerative potential of all the scaffolds was the same.

**Conclusion:** HA/ZrO₂ showed a superior osteoconductive capacity over PRP in the short term; however, they showed no long-term synergic effects.

Please cite this article as: Shahsavari-Pour S, Aliabadi E, Latifi M, Zareifard N, Namavar MR, Talaei-Khozani T. Evaluation of the Possible Synergic Regenerative Effects of Platelet-Rich Plasma and Hydroxyapatite/Zirconia in the Rabbit Mandible Defect Model. Iran J Med Sci. 2018;43(6):633-644.

Keywords: Durapatite ● Hydroxyapatite ● Zirconium oxide ● Platelet-Rich plasma ● Heparan sulfate proteoglycans ● Osteogenesis

Introduction

Ablative surgery of the mandible due to maxillofacial disorders leads to large defects, which can be reconstructed by autogenous grafts and alloplastic materials; however, each one has particular advantages and disadvantages.1 Autologous graft surgery...
has some disadvantages such as hematoma, residual pain, and esthetic problems. Also, the mandible reconstructed with autogenous grafts is not esthetically satisfying; hence the need for the application of a better reconstruction method such as engineered bone replacement.

Biomimetic bone scaffolds recapitulate an appropriate base for cell attachment, proliferation, and differentiation. Bioactive ceramics such as hydroxyapatite (HA) are helpful in the restoration of the bone with satisfying osteoinduction, osteocompatibility, and osteoconduction properties without triggering a considerable immune response. The biologic similarity of these ceramics with the body’s mineral tissues has made them a useful bone replacement biomaterial in orthopedics and dentistry. Nonetheless, porous HA is weak and is not concordant with the mechanical properties of the cortical bone in load-bearing situations; it is, therefore, feasible to combine the bioactivity of HA with the mechanical properties of bio-inert ceramics such as zirconium oxide (ZrO2). ZrO2 with adequate bioactivity, biocompatibility, mechanical strength, and toughness can be widely used in orthopedic grafts. Despite such advantages, however, ZrO2 usually generates a flat surface not suitable for cell attachment, which affects the osteointegration of the graft.

Platelet-rich plasma (PRP) contains fibrin and growth factors (GFs) and exerts beneficial effects on bone growth. PRP provides a gel with bioactivity, integrity, and osteogenic potential. Since osteoblasts are immobile cells, there is a tendency to use osteoconductive biomaterials such as fibrin to increase their motility. On the other hand, some GFs present in PRP motivate proliferation, migration, and osteoblast differentiation among mesenchymal cells. The beneficial effects of PRP on the migration of human osteoblasts have been reported previously.

Bone formation is a lengthy process that requires the constant presence of GFs at its site. Most GFs such as transforming growth factor-β (TGF-β), with the ability to bond heparan sulfate (HS), play a crucial role in osteogenesis. HS along with HA is used to sequester GFs and protect them from local protease; therefore, GFs permeate gradually and persist longer in the composite. In addition, HS has some interactions with bone morphogenetic protein antagonists, which blocks the inhibitory effects of these agents and leads to an even higher rate of bone formation.

Various studies have examined different types of materials as bone replacement such as the powder derived from demineralized bone, HA, and other forms of allografts in combination with PRP. Nonetheless, the addition of PRP to HA/ZrO2 composites has not been investigated yet. Moreover, there are contradictory results regarding the synergistic effects of a combination of bioceramics and PRP.

The objectives of the present study were to determine whether the combination of HA/ZrO2 with PRP and HS could exert a synergistic impact on bone regeneration and to compare the in vivo bone regeneration capacity of the HA/ZrO2 scaffold with or without PRP.

**Materials and Methods**

**Ethical Statement**

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences and conducted according to the ethical principles of animal handling and treatment.

**Fabricating the HA/ZrO2 Composite**

The HA/ZrO2 composite was prepared by adding 100 g of ZrO2 (US Research Nanomaterial, USA) and 6 g of triethyl phosphate (Sigma, USA) to distilled water and mixing them for 24 hours. Subsequently, 15 g of polyvinyl buty1 (Sigma Aldrich, USA) was added as a binder. Polyurethane foam was immersed into the slurry and desiccated at 80 °C for 10 minutes. After 4 cycles of immersion/desiccation, the foam was heated again at 800 °C for 5 hours at a heating rate of 2 °C/min. It was ultimately heated at 1400 °C for 3 hours.

HA and ZrO2 interaction was prevented by dispersing 15 g of homemade fluorapatite, 1 g of triethyl phosphate, and 6 g of polyvinyl buty1 in distilled water and mixing for 24 hours. The ZrO2 scaffold was immersed in the fluorapatite slurry twice and dried at 90 °C for 20 minutes. The scaffold was heated at 800 °C for 5 hours, followed by another 3 hours at 1250 °C at the rate of 2 °C/min. Thereafter, a layer of HA was applied on the fluorapatite-coated ZrO2 latticework through the same method. The scaffolds prepared were 9 mm in length, 5 mm in width, and 4 mm in thickness.

**Loading PRP/HS**

Ten mL of the rabbit blood was transferred to a tube containing citric acid and centrifuged at 5000 rpm for 15 minutes. The plasma and the buffy coat were centrifuged again at 2000 rpm for 10 minutes, and the bottom one-third was preserved in the freezer as PRP. The PRP gel was fortified with 2.5% CaCl2 and immediately mixed with HS just prior to impregnation within the pores of the HA/ZrO2 scaffold in a vacuum oven at 37 °C.
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Scanning Electron Microscopy (SEM)
The scaffolds were fixed with 4% paraformaldehyde, dehydrated with gradually increasing concentrations of ethanol, and then dried by immersion in hexamethyldisilazane. The hexamethyldisilazane was allowed to evaporate and the samples were exposed to gold with a sputter coating machine (Ted Pella, Inc., USA).

Study Design and Surgical Procedure
Twenty-six male rabbits in normal health condition were selected randomly and anesthetized with an intramuscular injection of 10 mg/kg of ketamine (Alfasan, Holland) and 2 mg/kg of xylazine (Alfasan, Holland). The rabbits’ mandibles were shaved and prepped. Next, via a submandibular incision, the myocutaneous flap was reflected. After muscle and periosteal retraction, a rectangular defect was applied in the body of the mandible with a fissure burr on each side (figure 1A). The left side was filled with HA/ZrO₂/PRP and the right side with HA/ZrO₂ in 20 rabbits (figure 1B). For the 6 remaining models, PRP gels were placed in both sides. The periosteum and skin were sutured with VICRYL 4/0 and silk 3/0, respectively, and coated with oxytetracycline.

After the follow-up period, X-ray radiography was done with an X-ray machine (Planmeca Intra, Finland) in the second, sixth, and eighth postoperative weeks. Scaffold absorption was compared through an analysis of the radiographs with respect to pixel intensity by ImageJ. (http://imagej.nih.gov/ij/index.html).

Histologic Preparation
After the muscle was stripped, the specimens were fixed in 10% formaldehyde (Sina Chemical Industrial, Iran), decalcified by 10% EDTA (pH 7.4) for about 3 months, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with the hematoxylin and eosin method. Morphometric analyses were performed by ImageJ, and the percentages of the area occupied by bone and connective tissue and also the number of osteoblasts and osteocytes were estimated.

Statistical Analysis
The data were analyzed using the analysis of variance (ANOVA) to compare the difference in the mean of the percentage of the area occupied by bone and connective tissue and the number of bone cells between the 3 groups. Further analysis was done using the least significant difference (LSD) test to compare the mean value of each 2 groups. A P value less than 0.05 was considered significant. All the analyses were performed by SPSS, version 16.0, for Windows and the graph was depicted by GraphPad 5.

Results
All the rabbits tolerated the surgery and were able to eat normal diet postoperatively, but 1 rabbit was eliminated from the analysis because of the surgical site infection.

SEM
The examination of the scaffolds with SEM revealed good porosity in the HA/ZrO₂ scaffold. The pores were filled well with the PRP gel in the HA/ZrO₂/PRP scaffold. The higher magnification of the PRP scaffolds exhibited nanoscale porosity. Some platelets were found within the scaffolds (figure 2).
**Gross Examination**

At the second week, light new bone formation at the peripheries of the scaffold started in the HA/ZrO\(_2\)/PRP-treated group and, to a lesser extent, in the HA/ZrO\(_2\)-treated group. In the PRP-treated animals, the initiation of bone formation was seen at the defect margins.

At the sixth week, a reduction in the scaffold size was observed in the HA/ZrO\(_2\)/PRP-treated animals; however, the scaffold materials could be seen easily. In the PRP-treated animals, bone defects could be detected easily with new bone formation.

At the eighth week, scaffold resorption increased and bone formation improved in both HA/ZrO\(_2\)/PRP- and HA/ZrO\(_2\)-treated groups with a slightly higher absorption in the HA/ZrO\(_2\)/PRP-treated rabbits (figure 3). The PRP-treated defects were partially filled with the new bone formation.

**Radiologic Findings**

The brightness of the scaffold area was maximum in the HA/ZrO\(_2\) group at the second week, which may indicate scaffold absorption, although there was no significant difference in the pixel-intensity evaluation between HA/ZrO\(_2\)/PRP and HA/ZrO\(_2\). Pixel-intensity evaluation also showed a nonsignificant reduction in both groups as time progressed (figure 4).

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**Figure 2:** Scanning electron micrographs at low magnification show good porosity in the HA/ZrO\(_2\) scaffold (A). Pores are also filled with PRP/HS (B). PRP scaffold contains randomly oriented fibrin fibers and platelets. It also contains nano-sized pores (C). Scale bar is 2.5 mm for A and B and 5 µm for C.

**Figure 3:** Gross view of the specimens at the sixth and eighth weeks shows better healing in HA/ZrO\(_2\)/PRP than in HA/ZrO\(_2\).
Histological Findings
At the second week, osteoid and woven bone appeared in both HA/ZrO₂/PRP- and HA/ZrO₂-treated groups, while just small islands of osteoid along with larger islands of fibrin remnant were found in the PRP-treated defects. After the specimens were decalcified, the scaffold remnant (ZrO₂) was observed as a black area. Osteoid was found in most of the cases. The estimation of the percentage of the area occupied by bone revealed a nonsignificant increase in the HA/ZrO₂/PRP-treated group compared with the HA/ZrO₂-treated group. According to the LSD test, the estimation of the percentage of the area occupied by bone revealed a nonsignificant increase in the HA/ZrO₂/PRP-treated group compared with the HA/ZrO₂-treated group and a significant increase compared with the PRP-treated defects (P=0.007). Fibrous connective tissue also predominantly filled the space between HA/ZrO₂ and newly formed woven bone in both HA/ZrO₂/PRP- and HA/ZrO₂-treated animals; however, a small area filled with adipose tissue was found in 50% of the cases treated with HA/ZrO₂/PRP and 25% of the cases treated with HA/ZrO₂. In the PRP-treated group, 84.33%±0.2 of the total area was occupied by connective tissue containing a large number of blood vessels (figure 5).

The estimation of cell count/mm² revealed that the defects treated with HA/ZrO₂/PRP contained a nonsignificantly higher number of osteocytes but a lower number of osteoblasts than those treated with HA/ZrO₂ after 2 weeks. The PRP-treated defects contained cell aggregates composed of newly differentiated osteoblasts. The newly formed osteocytes were also trapped in the osteoid. Comparing the 3 groups showed that the highest number of osteoblasts and osteocytes was present in the PRP-treated group (both Ps=0.001). No sign of inflammation was present in the histological sections of the defected bones.

At the sixth week, the percentage of the area occupied by bone increased significantly in all the groups compared with their counterparts at the second week (P=0.001 for all). Both HA/ZrO₂/PRP and HA/ZrO₂ scaffolds induced woven bone along with lamellar bone and immature Haversian canal formation. A significant increase in the percentage of the area occupied by bone was also shown in the PRP-treated defects compared with the HA/ZrO₂-treated defects at this week (P=0.028). The amount of fibrous tissue also exhibited a significant reduction in both HA/ZrO₂/PRP- and HA/ZrO₂-treated defects compared with their counterparts at the second week (P=0.001). In the PRP-treated defects, bone cells produced extracellular matrix and, as a result, the percentage of the surface occupied by connective tissue decreased significantly compared with its counterpart at the second week (P=0.014). A large area filled with connective tissue was also estimated in the HA/ZrO₂/PRP-treated defects compared with the
HA/ZrO₂-treated defects (P=0.023). The amount of connective tissue was also significantly larger in the PRP-treated defects than in the HA/ZrO₂-treated defects (P=0.008). Angiogenesis was reduced in all the groups by comparison with the second week, and osteoid was hardly ever found in the cases (figure 6). The estimation of the osteoblast and osteocyte counts was the same in all the groups.

At the eighth week, a significant increase in the ossified area was detected in the HA/ZrO₂/PRP- and HA/ZrO₂-treated defects compared with their counterpart defects at the second week (P=0.0001 and P=0.025, respectively). The defects treated with HA/ZrO₂/PRP showed the best repair area. Connective tissue was estimated to occupy a significantly lower area in the HA/ZrO₂/PRP-treated defects than in the HA/ZrO₂- or PRP-treated defects (P=0.027 and P=0.012, respectively). Lamellar bone and Haversian canals were detected in both HA/ZrO₂/PRP- and HA/ZrO₂-treated defects but not in the PRP-treated defects (figure 7). The number of osteocytes and osteoblasts was the same in all the groups. The percentage of the area occupied by bone and connective tissue and also the number of osteoblasts and osteocytes/mm² are summarized in figures 8 and 9 and table 1.

**Discussion**

Our data showed that PRP incorporation within a prefabricated 3D bioceramic scaffold had no significant synergic impact on bone regeneration; however, the percentage of the area occupied by connective tissue decreased in the HA/ZrO₂/PRP-treated defects. Although both bioceramic and PRP have osteogenic properties, there are contradictory reports regarding the effectiveness of such a combination in bone regeneration. Soaking HA with PRP enhances cell activity involved in bone regeneration. Also, PRP provides a bridge that connects the scaffold with the defective bone and facilitates endothelial and osteoprogenitor cell migration. HA has the ability to absorb proteins temporarily and release them gradually. The HA/PRP composite has been reported to increase the vertebral union in the rat lumbar interbody fusion model. Further, it has been suggested that autologous bone with PRP can enhance bone formation in the rabbit defective calvaria. In contrast, lack of efficiency...
Figure 7: Micrographs show the histological sections of the defects treated with PRP (A), HA/ZrO₂ (B), and HA/ZrO₂/PRP (C) at the eighth week after surgery. Percentage of the area occupied by osseous zone also shows a significant increase in all the defect areas compared with the corresponding defects at the second week. Higher magnification of the PRP-treated defects shows that the area occupied by osteoid has decreased (a). Scale bar is 200 µm for A, B, and C and 30 µm for a.

Figure 8: Comparison of the surface occupied by bone (A) and connective tissue (B) between the HA/ZrO₂/PRP-treated defects and the HA/ZrO₂- and PRP-treated defects.
*Significant difference with the counterpart at the sixth and eighth weeks (all Ps=0.001).
γ Significant difference with PRP-treated defects at the second week (P=0.007, P=0.034).
ψ Significant difference with PRP-treated at the sixth week (P=0.028).
α Significant difference with the counterpart at the sixth week (P=0.015).
€ Significant difference with both HA/ZrO₂- and PRP-treated defects at the eighth week (P=0.027 and P=0.012, respectively).
Ψ Significant difference with the HA/ZrO₂-treated defects at the sixth week (P=0.023).
ϧ Significant difference with PRP-treated defects at the eighth week (P=0.008).
× Significant difference with the counterpart at the sixth and eighth weeks (P=0.014 and P=0.046).

Figure 9: Comparison of the number of osteoblasts (A) and osteocytes (B) between the HA/ZrO₂/PRP-treated defects and the HA/ZrO₂- and PRP-treated defects.
† Significant difference with PRP-treated defects at the second week (all Ps=0.001).
‖ Significant difference with counterparts at the sixth week (P=0.009, P=0.017, respectively).
§ Significant difference with all other groups (all Ps=0.0001).
¶ Significant difference with the counterparts at the sixth and eighth weeks (Ps=0.003, P=0.007, respectively).
has been reported in rabbit defective maxilla regeneration with the same combination, and no synergic effect has been shown by treating the rabbit vertebra with HA granules and PRP. Still, there is other evidence hinting at the formation of fibrous tissue rather than bone in chitosan/gelatin/platelet/HA-treated defects in rats. Our data also suggested no synergic effect of such a combination.

In the current study, both HA/ZrO$_2$-based scaffolds were more effective than PRP in early bone regeneration. The early stage of the healing period of the goat tibia can be improved by covering the PRP-coated titanium cylinder with calcium phosphate. This finding along with covering the PRP-coated titanium cylinder with HA/ZrO$_2$ has been in the goat tibia can be improved

| Groups       | Weeks  | Bone Area (%)±SEM | Connective Tissue Area (%)±SEM | Number of Osteoblasts/mm$^2$±SEM | Number of Osteocytes/mm$^2$±SEM |
|--------------|--------|------------------|-------------------------------|----------------------------------|----------------------------------|
| HA/ZrO$_2$/PRP | Second wk | 38.388±2.868γ | 62.496±2.785Δ | 1.787±0.133¥ | 1.352±0.152¥  |
|              |       | 59.999±6.769ζ | 40.001±6.769η | 1.749±0.51γ  | 0.349±0.129ν  |
|              | Eighth wk | 61.717±4.952ζ | 37.545±5.29μ | 1.815±0.452μ | 1.218±0.376ν  |
| HA/ZrO$_2$   | Second wk | 30.111±2.908γ | 69.889±2.908η | 1.173±0.111λ | 1.495±0.267ν  |
|              | Sixth wk | 72.409±6.127ψ | 23.947±4.635μ | 1.613±0.428ζ | 0.550±0.267μ  |
|              | Eighth wk | 46.849±6.7ζ | 53.151±6.7ο | 3.361±1.116δ | 0.649±0.105μ  |
| PRP          | Second wk | 0.00±0.00α | 84.336±0.148β | 6.208±0.365μ | 4.503±0.997ν  |
|              | Sixth wk | 51.894±6.673ζ | 48.106±6.673ζ | 1.208±0.139ζ | 0.352±0.123μ  |
|              | Eighth wk | 43.323±3.579ζ | 56.677±3.579ζ | 2.429±1.203ζ | 1.380±0.471μ  |

*Significant difference with the counterpart at the sixth and eighth weeks (all Ps=0.001).
γ Significant difference with the PRP-treated defects at the second week (P=0.007 and P=0.034).
ψ Significant difference with the PRP-treated defects at the sixth week (P=0.026).
α Significant difference with the counterpart at the sixth week (P=0.015).
λ Significant difference with the counterpart at the sixth and eighth weeks (P=0.022 and P=0.046, respectively).
€ Significant difference with the HA/ZrO$_2$-treated defects at the sixth week (P=0.023).
ό Significant difference with both HA/ZrO$_2$- and PRP-treated defects at the eighth week (P=0.027 and P=0.012, respectively).
ξ Significant difference with the HA/ZrO$_2$-treated defects at the seventh week (P=0.02).
× Significant difference with the PRP-treated defects at the sixth week (P=0.008).
† Significant difference with the counterpart at the sixth and eighth weeks (P=0.014 and P=0.046).
ς Significant difference with the HA/ZrO$_2$-treated defects at the second week (all Ps=0.001).
ύ Significant difference with the counterparts at the sixth and eighth weeks (P=0.001 and P=0.007, respectively).
ϕ Significant difference with the HA/ZrO$_2$-treated effects at the second week (all Ps=0.0001).
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influenced by its combination with HA-based scaffolds.\(^{37}\) Our data showed that neither HA nor HS exerted a significant effect on PRP osteogenic capacity. PRP is also a rich source of angiogenic factors.\(^{32}\) According to our findings, PRP induced more angiogenesis in the short term; nevertheless, in the long term, there was no difference in angiogenesis between the defects treated with or without PRP.

The data obtained from the present study showed that the highest number of osteoblasts and osteocytes/mm\(^2\) was present in the PRP-treated defects, where osteoid and osteoblast condensations were observed after 2 weeks. A deceleration in cell migration in the PRP-treated defects may explain the presence of the higher number of bone cells in the defective area. The lag in osteogenesis may be explained by the nanoscale porosity in PRP scaffolds. In the HA/ZrO\(_2\)/PRP- and HA/ZrO\(_2\)-treated groups, osteoblast differentiation was accelerated and the production of bone-specific extracellular matrix was promoted, which was detectable with scattered cells. As a result, there was a drop in the cell count/mm\(^2\).

In vitro study showed that the HA/ZrO\(_2\) scaffold provided an appropriate environment for cell attachment, proliferation, and differentiation. ZrO\(_2\) also improved the mechanical strength of the scaffold, which is a necessary feature for the application of engineered bone replacement in the load-bearing area.\(^{38}\) Radiologic and histologic evidence from the current study showed that PRP had no influence on ZrO\(_2\) degradation.

Both PRP and platelet-rich fibrin have antimicrobial effects, making them appropriate autologous additives for the regeneration of infected bone injuries.\(^ {39}\) The data from the present study indicated the lack of any sign of inflammation in the defect site.

The current study has several limitations. First, the amount and the content of GFs in the rabbit PRP differ from those in the humans and the osteogenesis property of PRP is species-dependent.\(^ {30}\) Second, our findings demonstrated that HA/ZrO\(_2\) and PRP had no synergic impact in the gel form; be that as it may, we might have observed the possible synergic effects of PRP and HA/ZrO\(_2\) had we examined different PRP preparation procedures or different PRP sources. Third, the presence of leukocytes in PRP and the way of administration have been suggested as critical issues,\(^ {30}\) which may explain the contradictory results.

Conclusion

HA/ZrO\(_2\) with or without PRP provided a better environment for the regeneration of the rabbit mandible defect in the short term. However, in the long term, all the scaffolds acted in the same manner apropos bone regeneration. In addition, the application of PRP may have beneficial effects vis-à-vis short-term osteoinduction and antimicrobial properties. Although this study showed that PRP and ZrO\(_2\)/HA had no synergistic effect, this finding may be attributed to the species, gel versus liquid administration, and the method of HA preparation rather than the ineffectiveness of such a combination.

Acknowledgment

This manuscript is based on a thesis by Dr. S. Shahsavari for a degree in maxillofacial surgery. The project was financially supported by the Research Deputyship of Shiraz University of Medical Sciences (94-01-03-10533). We wish to thank E. Noori and M. Dehghani for their excellent technical support and Dr. Nasrin Shokrpour for her editorial assistance. Many thanks are also due to Dr. Vosooghi in the Center for Research Improvement, School of Dentistry, for the statistical analyses. We appreciate the collaboration of Shiraz University of Medical Sciences, Shiraz, Iran, and also the Center for the Development of Clinical Research of Nemazee Hospital.

Conflict of Interest: None declared.

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