ARTICLE DOI: 10.5504/BBEQ.2011.0070 MB

COMPARISON OF THE EFFECTS OF LOCAL APPLICATION OF HYDROXYAPATITE GRAFT SOAKED WITH ALENDRONATE SOLUTION AND PURE HYDROXYAPATITE GRAFT IN THE MANDIBLE OF OVARIECTOMIZED RATS

Ahmet Arslan[1], Hatice Altundal[2], Ozge Cevik[3], Vakur Olgac[4]
[1] Yeditepe University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Istanbul, Turkey
[2] Private Oral and Maxillofacial Surgeon, Istanbul, Turkey
[3] Marmara University, Faculty of Pharmacy, Department of Biochemistry, Istanbul, Turkey
[4] Istanbul University, Faculty of Medicine, Department of Pathology, Istanbul, Turkey
Correspondence to: Ahmet Arslan
E-mail: ahmetarslan@doctor.com

ABSTRACT

This study aimed to investigate whether local delivery of alendronate could improve bone formation and decrease bone resorption after hydroxyapatite grafting in ovariectomised rat mandibular defects. Thirty-six female Wistar rats were divided into control and experimental groups. Both groups were ovariectomised. After two months a surgical defect was created on the vestibular side of their mandible. In the experimental group, hydroxyapatite graft soaked with alendronate solution was placed in the bone defect, and in the control group hydroxyapatite graft soaked with physiological saline was used. Both groups were divided into 3 subgroups: 2-, 4-, and 8-week follow-up groups. Each of the groups consisted of 6 rats. The animals were killed at the end of the designated periods. The number of osteoclasts and the amount of new bone formation were evaluated and compared. Eight weeks after surgery, the experimental group had more bone formation than the control group but it was not statistically significant. The number of osteclasts was increased significantly in the 4th week compared to the 2nd week and decreased significantly in the 8th week compared to 4th week in both groups. Local application of a single dose of alendronate did not have a decreasing effect on the number of osteoclasts between the second and fourth week. Although hydroxyapatite graft soaked with alendronate solution showed a trend towards better performance for bone formation at the 4th week, no statistically significant intergroup difference was found.

Biotechnol. & Biotechnol. Eq. 2011, **25**(3), 2513-2521

Keywords: alendronate, local application, ovariectomized rat

Introduction

Bone augmentation to provide skeletal healing has become one of the most common surgical techniques in recent years. The regeneration of injured bone tissue begins with recruitment, attachment and proliferation of progenitor cells. This process continues with cell differentiation into appropriate phenotypes which can restore the damaged tissue (7). To improve the quantity and quality of the regenerated bone different biological mediators are used (6). An example of such bone metabolism mediators are the bisphosphonates, a group of carbon-substituted pyrophosphate analogs, which are effective bone resorption inhibitors. They have been effectively applied in the control of osteolysis or reduction of systemic bone loss in Paget's disease, metastatic bone disease, hypercalcemia of malignancy (1), and osteoporosis (25). Bisphosphonates bind strongly to apatite crystals, mainly on remodeling surfaces and inhibit their growth, aggregation and dissolution (19, 24, 26, 34). They restrain osteoclast activity without ruining cellular effectiveness (30). This prevention of the resorptive activity causes an alteration in the bone metabolism in favor of osteoblastic activity. It has been shown that bisphosphonates diminish bone resorption when medicated systemically or locally (31, 36, 37). Bisphosphonates have a high affinity to the bone mineral and it has been reported that a single dose of locally applied bisphophonate can be sufficiently diffused to the bone (37). After topical application of alendronate, reduced alveolar bone resorption has also been observed following mucoperiostal surgery in rats (35).

Data indicate that alendronate is one of the most potent bisphosphonates in inhibiting bone resorption both *in vitro* and *in vivo* (9, 11, 29). Besides its systemic effects, alendronate has also been shown to reduce tooth resorption, graft resorption and alveolar bone loss in the dental fields (5, 10, 24).

Although anti-resorptive effects of bisphosphonates are well-known, little research has been conducted on their effects on bone formation (21, 27, 33). Therefore the aim of this study was to investigate whether local application of alendronate could improve bone formation after hydroxyapatite (HA) grafting in osteoporotic rat mandible.

Materials and Methods

Animals

Thirty-six female Wistar rats, weighing 240-250 g, obtained from the animal laboratory of the Faculty of Medicine,

Marmara University, were used. All rats were 12 weeks old, kept 6/cage, and provided ground laboratory food and water *ad libitum*. The animals were divided into 2 groups (18 rats/group): control group (hydroxyapatite graft soaked with physiological saline) and experimental group (hydroxyapatite graft soaked with alendronate solution). Both groups were divided into 3 subgroups: 2-, 4-, and 8-week follow-up groups. Each of the groups contained 6 rats. The handling of the animals was supervised by the Animal Ethics Research Committee, Faculty of Medicine, Marmara University.

Surgical procedures

All surgeries were carried out by one investigator using aseptic techniques. Prior to surgery, the animals were anesthetized by intramuscular injections of a mixture of ketamine hydrochloride (100 mg/kg body weight) and chlorpromazine (25 mg/kg body weight). Surgery was performed under sterile conditions. In order to develop osteoporotic bone, all rats were ovariectomized. Eight weeks after the ovariectomization a defect was created on the vestibular side of the mandible in all rats. In order to access this region, a linear incision was made through the skin, subcutaneous tissues and masseter muscle parallel to the inferior border of the mandible, and by elevating mucoperiosteal flaps, the lateral aspect of the bone surface around the angle of mandible was exposed. A standardized round bur through-and-through bone defect (5 mm in diameter) (33) was created in the middle region of the mandible body using a round carbide bur. In the experimental group, hydroxyapatite soaked with alendronate solution was placed in the bone defect. In the control group, hydoxyapatite graft soaked with physiologic saline was placed in the bone defect. Then the flaps were carefully repositioned and sutured with resorbable sutures. Cefazolin was given to the animals as intramuscular injections intraoperatively and for 3 days postoperatively. Healing progressed uneventfully in all animals and no postoperative complications were noticed during the 2, 4, and 8 weeks' observation periods.

Histological procedures

The rats in the control and experimental groups were killed at the end of the designated periods. They were killed by overdose injection of anaesthetic solution. Mandibles of all rats were dissected and prepared for the histomorphometric investigation. All specimens were fixed in 10% buffered formalin for one week. After the fixation procedure, all specimens were decalcified in the solution prepared from formic acid of 50% and 20% of sodium citrate. Paraffin blocks prepared from routinely processed specimens were cut into 5-7 μm slices. They were stained with hematoxylin and eosin (H&E) and examined with light microscope (Olympus Bx60, Japan).

Histomorphometric analysis

Olympus Soft imaging system analysis FIVE, Japan, was used for the histomorphometric examination. Digital images magnified ×400 were examined. Inflammation, necrosis, fibrosis, number of osteoclasts and new bone formation were examined. According to the staining percentage which they cover on a digital image, these findings were scored ranging from 1 to 3: 1 (1-30%), 2 (30-60%) and 3 (> 60%). Three different parts of one and the same block section (0.14 mm^2 at ×400 magnification) were determined and examined to calculate the average number of osteoclasts. The most central stained section that represented the maximum diameter of the defect was selected in each block. It was not possible to capture the entire defect in one image at the level of magnification that was used, therefore it was obtained from three different parts of the block.

Statistical analyses

Data analyses and frequency tables were calculated with SPSS 16 (SPSS Inc. Chicago, Illinois-USA). The differences of the values at 2, 4 and 8 weeks of osteoclastic activity, new bone formation, inflammation, fibrosis and necrosis were compared with Mann-Whitney U-test. Probability less than 0.05 was considered statistically significant.

Results and Discussion

In this study, we used hydroxyapatite (HA) as bone graft substitute because *in vitro/vivo* studies have reported favorable clinical results following surgical treatment of intrabony defects with hydroxyapatite graft materials (14, 18, 23, 38). Our results showed that the use of HA granules did not cause any undesirable reaction. All animals recovered without complication. Hydoxyapatite grafts were well adapted in critical bone defects. No foreign body reactions were observed. New bone formation was observed along the borders of the surgical defect both in the control and experimental group. Although there was no statistically significant intergroup difference, the experimental group showed a trend towards better performance with a slight increase in bone formation ($P > 0.05$) at the 2nd, 4th and 8th week (**Table 1**). In the control group a nonsignificant increase in the bone formation between the 2nd and the 4th week was also observed. But after the 4th week, stable bone formation was visible in the control group (**Table 2** and **Table 3**). The replacement of HA granules by new bone in our study was consistent with the previous study of Jain et al. (16), indicating that the healing of the grafted area occured by the osteoconductive property.

TABLE 1

Difference between the 2nd and 8th week in the experimental group

Experimental group	**2 weeks**	**8 weeks**	**P**
Inflammation	2.33±0.52	1.33±0.52	0.26
Necrosis	0.5±0.55	0	0.18
Fibrosis	1.67±0.52	1.5±0.55	0.69
New Bone Formation	1.67±0.52	2.5±0.55	0.06
Osteoclasts	3±0.63	2.5±0.55	0.24

Alendronate sodium is a bisphosphonate which is known as a potent inhibitor of bone resorption. It is responsible for

TABLE 2

Comparison of the experimental and the control group between the 2nd and 4th week

	Experimental group		P	Control group		P
	2 weeks	4 weeks		2 weeks	4 weeks	
Inflammation	2.33±0.52	1.5±0.55	0.65	2.17±0.41	1.33±0.52	**0.04**
Necrosis	0.5±0.55	0.33±0.52	0.69	0.5±0.55	0.33±0.52	0.69
Fibrosis	1.67±0.52	2	0.39	2	2	1
New Bone Formation	1.67±0.52	2.33±0.52	0.13	1.5±0.55	2.17±0.41	0.09
Osteoclasts	3±0.63	5.33±0.82	**0.002**	2.33±0.52	4±0.89	**0.009**

TABLE 3

Comparison of the experimental and the control group between the 4th and 8th week

	Experimental group		P	Control group		P
	4 weeks	8 weeks		4 weeks	8 weeks	
Inflammation	1.5±0.55	1.33±0.52	0.69	1.33±0.52	1.5±0.84	0.93
Necrosis	0.33±0.52	0	0.39	0.33±0.52	0.5±0.55	0.69
Fibrosis	2	1.5±0.55	0.18	2	2	1
New Bone Formation	2.33±0.52	2.5±0.55	0.69	2.17±0.41	2.17±0.41	1
Osteoclasts	5.33±0.82	2.5±0.55	**0.002**	4±0.89	1.67±0.52	**0.002**

the inhibition of osteoclast recruitment, inhibition of osteclast adhesion, reduction of osteoclast lifespan and inhibition of osteoclastic activity (12). In addition to inhibition of bone resorption, it also stimulates the formation of osteoblast precursors and mineralised nodules and thus boosts early osteoblastogenesis (15). It is known that subcutaneous application of alendronate results in improved bone formation around the autogenous free bone graft in rats (4). Srisubut et al. (33) reported that a single dose of local delivery of alendronate improved bone formation. It can be hypothesized that the topical application of alendronate will modify the local osteclastic activity and thereby slow down the bone resorption during initial modeling (22), leading to better bone formation in the defect area, although in our study, topically applied alendronate did not enhance significantly the local bone conditions. In both groups, a notable increase in the number of osteoclasts was observed between the 2-week and 4-week periods ($P < 0.05$). But the number of osteoclasts was significantly lower at the 8th week in each group (**Table 3**). Unlike Srisubut et al. (33), Bodde et al. (8) have obtained similar to our results and showed no increased bone formation around alendronate loaded bioactive bone cements in femoral defects. Jakobsen et al. (17) found decreased biomechanical fixation of all the implants soaked in alendronate and also reported that local alendronate treatment blocked formation of new bone and inhibited resorption of the graft material.

Regarding the differences between the experimental and control groups, the only statistically significant difference was observed in the 4th week. The number of osteoclasts was significantly lower in the control group at the end of the 4th week ($P < 0.05$) (**Table 4**). No differences were determined in the 2nd and 8th week between the experimental and control groups. In the experimental group, the number of osteoclasts was always higher than that in the control group. The local delivery of pure alendronate did not have a damaging effect on the osteoclasts. It is possible that the concentration of the alendronate placed in the defect was not large enough to cause apoptosis. Alakangas et al. (2) stated that bone resorption was not related to a decrease in osteoclast number, but inactivates the osteoclasts by mechanisms that impair their intracellular vesicle transport. Local application of alendronate may have inhibited the function of osteoclasts but at the same time it increased new bone formation in the experimental group. Osteoblastic activity in the experimental group is shown in **Fig. 1** and **Fig. 2**. **Fig. 3** and **Fig. 4** show new bone formation in the control group. We think that the statistically nonsignificant result for osteoblastic activity in our study might be due to the indirect inhibition of the resorptive mechanism via osteoblastic activity. The chief advantage of topical application is the possibility to administer a single dose to stimulate new bone formation. Garcia-Moreno et al. (13) revealed that alendronate *in vitro* did not affect the viability, proliferation, and mineral deposit capacity of human osteoblasts at the concentration at which it inhibited the resorptive capacity of osteoclasts by 50%. Meraw and Reeve (23) reported that locally applied alendronate resulted in significant increases in the amounts of bone in the peripheral area with both hyroxyapatite and titanium machine-polished implants. Our results suggested that a single dose of local application of alendronate combined with HA was able to induce only a slight increase in the bone regeneration which might be useful in alveolar bone defects. The significant increase in the number of the osteoclasts in both groups at the 4th week may have been a result of increased osteoblastic activity. **Fig. 5** and **Fig. 6** show examples of the pictures we

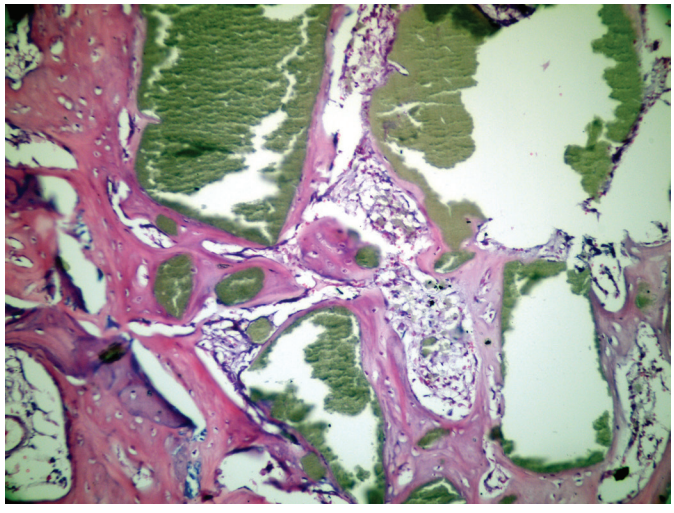

Fig. 1. New bone formation around the HA graft in the experimental group in the 4th week (H&EX200).

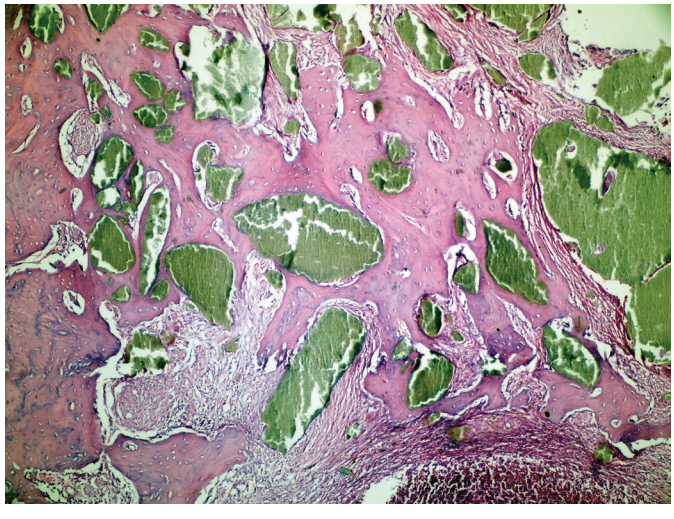

Fig. 2. Thickened new bone formation around the HA graft in the experimental group at the end of the 8th week (H&EX200).

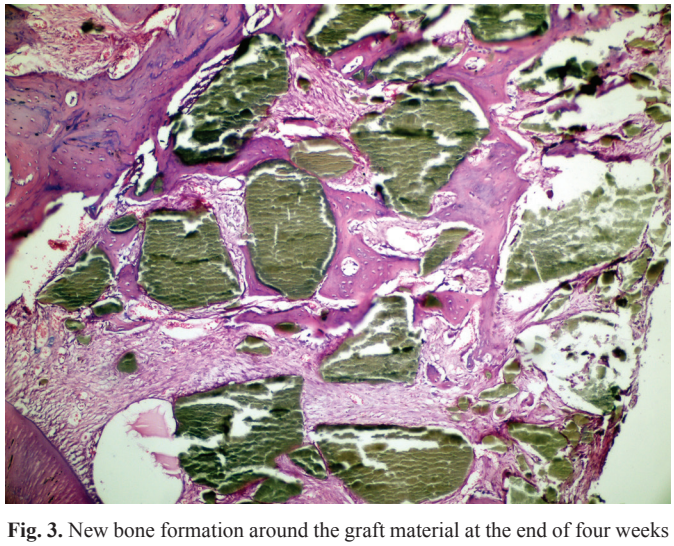

Fig. 3. New bone formation around the graft material at the end of four weeks in the control group (H&EX100).

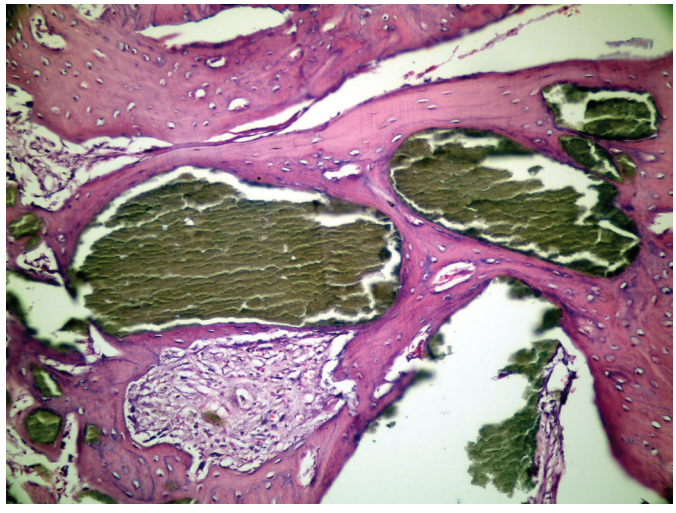

Fig. 4. New bone formation at the end of 8 weeks in the control group (H&EX100).

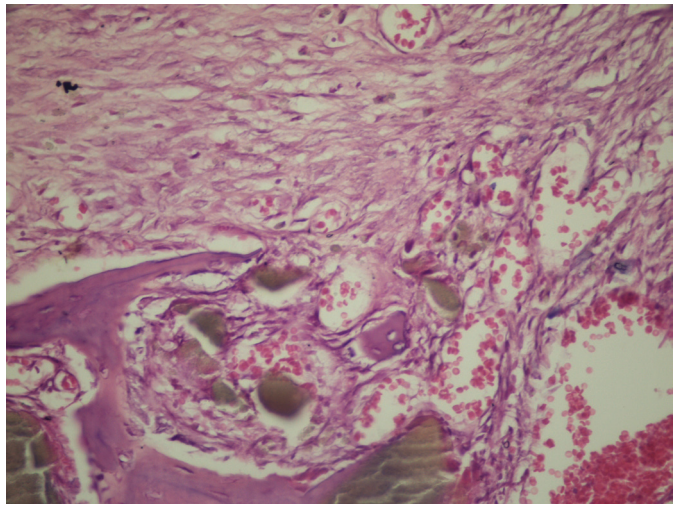

Fig. 5. Picture used to determine the number of osteoclasts in 0.14 mm^2 at ×400 magnification at the end of the second week in the experimental group.

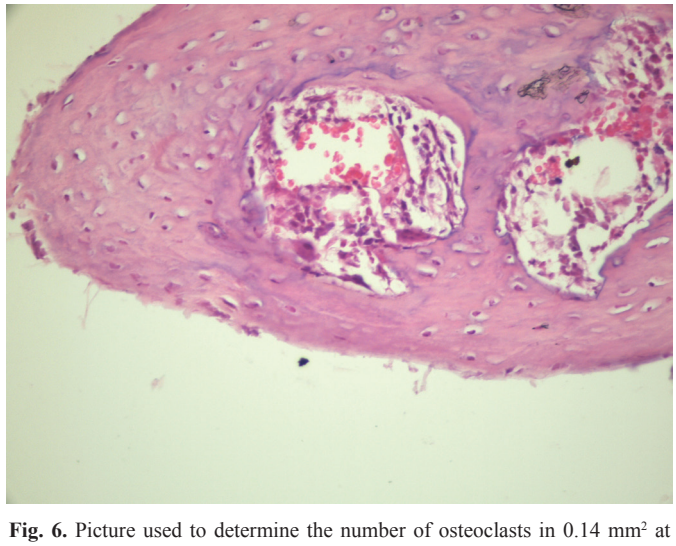

Fig. 6. Picture used to determine the number of osteoclasts in 0.14 mm^2 at ×400 magnification at the end of the eighth week in the experimental group.

used to count the osteoclasts at 400× magnification. Further studies need to be done to understand the potential mechanism before moving onto human studies.

TABLE 4

Comparison of both groups in the 4th week

4 weeks	**Experimental group**	**Control group**	**P**
Inflammation	1.5±0.55	1.33±0.52	0.69
Necrosis	0.33±0.52	0.33±0.52	1
Fibrosis	2	2	1
New Bone Formation	2.33±0.52	2.17±0.41	0.69
Osteoclasts	5.33±0.82	4±0.89	**0.04**

Experiments performed on bone cell cultures, using very low concentrations of bisphosphonates, have revealed increased parameters of bone formation (21, 28). However, as mentioned before, besides these positive effects of alendronate on bone formation, there are also conflicting results. Nobre et al. (27) stated that local application of alendronate did not contribute to bone repair but it might be responsible for the extracortical bone formation in spontaneously hypertensive rats. Altundal and Güvener (3) demonstrated that osteoblastic activity was less in the alendronate-treated group than in the saline-treated group following tooth extraction. These different results may be due to differences in compounds, duration of treatment, dosage, methods of administration and research models. The proportion of alendronate absorbed by the skeleton may vary according to bone turnover; it is highest at sites of active bone remodeling. In our study, in contrast to Srisubut et al. (33), pure alendronate was dissolved in saline and mixed with HA before application in intrabony defect area of the rat. Srisubut et al. (33) dissolved Fosamax to place it into the bone defect and reported that the ingredients of this drug, other than alendronate, like microcrystalline cellulose, anhydrous lactose, croscarmellose sodium and magnesium stearate may have contributed to the stimulation of bone growth.

In our study, comparisons of the number of osteoclasts between alendronate treated versus saline treated HA grafts provided statistically significant results only at the fourth week and the number of osteoclasts was always higher in the experimental group. Other investigators have previously reported that the number of osteclasts tended to increase with the administration of bisphosphonate (20, 32). The results from our experiments revealed that local application of a single dose of alendronate did not have an effect on the number of osteoclasts, but it improved slightly bone formation. Although hydroxyapatite graft soaked with alendronate solution showed a trend towards better performance, no statistically significant intergroup difference was found. To improve the effectiveness of alendronate, further studies needed to focus on demonstrating the effects of local application of a single dose of alendronate and finding the most effective dosage and duration of application in different research models.

Conclusions

The results from this study showed that local application of a single dose of alendronate could not prevent the increase in the number of osteoclasts between the second and fourth week. Application of hydroxyapatite graft soaked with alendronate solution showed a tendency towards better regeneration for bone formation after 4 and 8 weeks but the intergroup difference was not statistically significant.

REFERENCES

1. **Adami S., Zamberlan N., Mian M., Dorizzi R., Rossini M., Braga B, Gatti D., Bertoldo F., Locascio V.** (1994) Bone Miner., **25**, 75-82.
2. **Alakangas A., Selander K., Mulari M., Hallen J., Lehenkari P., Mönkkönnen J., Salo J., Väänänen K.** (2002) Calcif. Tissue Int., **70**, 40-47.
3. **Altundal H. and Güvener Ö.** (2004) Int. J. Oral Maxillofac. Surg., **33**, 286-293.
4. **Altundal H. and Gursoy B.** (2005) Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod., **99**, 285-291.
5. **Aspenberg P. and Astrand J.** (2002) Acta Orthop. Scand., **73**, 20-23.
6. **Baylink D.J., Finkelman R.D., Mohan S.** (1993) J. Bone Miner. Res., **8**, 565-72.
7. **Binderman I.** (1991) Curr. Opin. Dent., **1**, 836-840.
8. **Bodde E.W., Kowalski R.S., Spauwen P.H., Jansen J.A.** (2008) Tissue Eng. Part A., **14**, 29-39.
9. **Devogelaer J.P., Broll H., Correa-Rotter R., Cumming D.C., De Deuxchaisnes C.N., Geusens P., Hosking D., Jaeger P., Kaufman J.M., Leite M., Leon J., Liberman U., Menkes C.J., Meunier P.J., Reid I., Rodriguez J., Romanowicz A., Seeman E., Vermeulen A., Hirsch L.J., Lombardi A., Plezia K., Santora A.C., Yates A.J., Yuan W.** (1996) Bone, **18**, 141-150.
10. **El-Shinnawi U.M. and El-Tantawy S.I.** (2003) J. Int. Acad. Periodontol., **5**, 5-10.
11. **Fleisch H.** (1993) Osteoporos. Int., **2**, 15-22.
12. **Fleisch H.** (1998) Endocr. Rev., **19**, 80-100.
13. **Garcia-Moreno C., Serrano S., Nacher M., Farre M., Diez A., Marinozo M.L., Carbonell J., Mellibovsky L., Nogues X., Ballester J., Aubia J.** (1998) Bone, **22**, 233-239.
14. **Galgut P.N., Waite I.M., Bookshaw J.D., Kingston C.P.** (1992) J. Clin. Periodontol., **19**, 570-577.
15. **Giuliani N., Pedrazzoni M., Negri G., Passeri G., Impicciatore M., Girasole G.** (1998) Bone, **22**, 455-461.
16. **Jain A., Dixit J., Prakash D.** (2008) J. Int. Acad. Periodontol., **10**, 59-65.
17. **Jakobsen T., Baas J., Bechtold J.E., Elmengaard B., Soballe K.** (2007) Clin. Orthop. Relat Res., **463**, 195-201.

18. **John A., Varma H.K., Vijayan S., Bernhardt A., Lode A., Vogel A.,Burmeister B., Hanke T., Domaschke H., Gelinsky M.** (2009) Biomed. Mater., **4**, 15007.
19. **Kanis J.A., Gertz B.J., Sibger F., Ortolani S.** (1995) Osteoporos. Int., **5**, 1-13.
20. **Kaynak D., Meffert R., Bostancı H., Günhan Ö., Özkaya Ö.G.** (2003) J Periodontol., **74**, 1348-1354.
21. **Komatsu K., Shimada A., Shibata T., Shimoda S., Oida S., Kawasaki K., Nifuji A.** (2008) J. Periodontol. Res., **43**, 194-200.
22. **Mcleod K., Anderson G.I., Dutta N.K., Smart R.S., Voelcker N.H., Sekel R., Kumar S.** (2006) J. Biomed. Mater. Res. A., **79**, 271-281.
23. **Meraw S.J. and Reeve C.M.** (1999) J. Periodontol., **70**, 1228-1233.
24. **Levin L., Bryson E.C., Caplan D., Trope M**. (2001) Dent. Traumatol., **17**, 120-126.
25. **Liberman U.A., Weiss S.R., Broll J., Minne H.W., Quan H., Bell N.H., Rodriguez-Portales J., Downs R.W., Dequeker J., Favus M.** (1995) N. Eng. J. Med., **333**, 1437-1443.
26. **McClung M.R**. (1996) Bone, **19**, 195-198.
27. **Nobre M.D., Fernandes R.G., Chin C.M., Faig-Leite H.** (2008) Arq. Bras. Cardiol., **90**, 239-246.
28. **Reinholz G.G., Getz B., Pederson L., Sanders E.S., Subramaniam M,. Ingle J.N., Spelsberg T.C.** (2000) Cancer Res., **60**, 6001-6007.
29. **Sass D.A., Bowman A.R., Yuan Z., Ma Y., Jee W.S.S., Epstein S**. (1997) Bone, **21**, 65-70.
30. **Sato M., Grasser W., Endo N., Akins R., Simmons H., Thompson D.D., Golub E., Rodan G.A**. (1991) J. Clin. Invest., **88**, 2095-2105.
31. **Schenk R., Eggli P., Fleisch H., Rosini S.** (1986) Calcif. Tissue Int., **38**, 342-349.
32. **Shoji K., Horiuchi H., Shinoda H.J.** (1995) J. Periodont. Res., **30**, 277-284.
33. **Srisubut S., Teerakapong A., Vattraphodes T., Taweechaisupapong S.** (2007) Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod., **104**, e11-e16.
34. **Tucci J.R., Tonino R.P., Emkey R.D., Peverly C.A., Kher U., Santora A.C**. (1996) Am. J. Med., **101**, 488-501.
35. **Yaffe A., Binderman A., Breuer E., Pinto T., Golomb G.** (1999) J. Periodontol., **70**, 893-895.
36. **Yaffe A., Fine N., Alt I., Binderman I.** (1995) J. Periodontol., **66,** 999-1003.
37. **Yaffe A., Iztkovich M., Earon Y., Alt I., Lilov R., Binderman I.** (1997) J. Periodontol., **68**, 884-889.
38. **Yukna R.A., Callan D.P., Krauser J.T., Evans G.H., Aichelmann-Reidy M.E., Moore K., Cruz R., Scott J.B.** (1998) J. Periodontol., **69**, 655-663.