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Original Article

Evaluation of the effects of two different bone resorption inhibitors on osteoclast numbers and activity: An animal study

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ABSTRACT

Background: The aim of this study was to evaluate the effects of bone resorption inhibitors, doxycycline (DOX) and erythromycin (EM), on osseous wound healing in rat alveolar socket.

Materials and Methods: In this randomized controlled trial, 45 8–10-week-old male Wistar rats had their maxillary right molar extracted. They were divided into three groups of 15. In Group 1 normal saline, Group 2 DOX, and Group 3 EM were administered at the doses of 5 ml/kg/day, 5 mg/kg/day, and 2 mg/kg/day, respectively, for 7 consecutive days. The rats were sacrificed 7, 14, and 21 days after surgery. Real-time polymerase chain reaction was employed to evaluate the mRNA expression of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) and immunohistochemical staining for tartrate-resistant acid phosphatase (TRAP) to determine osteoclasts. The data were analyzed by one-way analysis of variance followed by Tukey's *post hoc* test using SPSS version 20. Significant level was set at 0.05.

Results: The results showed that when drug-treated groups compared to control groups, RANKL gene expression significantly decreased, TRAP + cells decreased on day 7. The RANKL/OPG ratios in the first two weeks in the test groups were significantly lower than the control group. There was no significant difference in the studied indices between DOX and EM groups.

Conclusion: Following administration of DOX and EM, the number of osteoclasts and RANKL/OPG ratio decreased suggesting their anti-osteoclastogenesis activity. These two drugs have no advantage over each other in increasing the bone formation.

Key Words: Immunohistochemistry, real-time polymerase chain reaction, tartrate-resistant acid phosphatase

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INTRODUCTION

Wound healing after tooth extraction or dental implantation is an abstruse process involving a series of biological events which consist of repair and remodeling of soft and hard tissues in response to injury. The focus of research in bone biology and healing is now centered on molecular events that regulate the repair of injured tissue. Identification of cellular and molecular biology and many signaling

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molecules associated with formation and repair of skeletal tissues, like members of the transforming growth factor-β superfamily (including the bone morphogenetic proteins) and several additional signaling molecules such as fibroblast growth factors, insulin-like growth factors and platelet derived growth factors have resulted in rapid progress of our knowledge of the complex process of wound healing.[1] Various intracellular and intercellular pathways are activated after an injury occurs. Many types of cells involve in the wound healing process, such as immune cells, endothelial cells, fibroblasts, progenitors, and stem cells, whose proliferation, differentiation and migration are a prerequisite for this phenomenon.[2]

One of the mechanisms which plays an important role in the remodeling of bone is the relationship between the elements osteoprotegerin (OPG), receptor activator of nuclear factor κB (RANK), and RANK ligand (RANKL). OPG is a soluble glycoprotein member of the tumor necrosis factor receptor-α superfamily. The manner in which OPG interacts with the target cells is binding to RANKL; a transmembrane cytokine expressed on the surface of the preosteoclastic/stromal cells; this binds to RANK and RANKL, triggers a series of mechanisms that result in differentiation, maturation and activation of osteoclasts. OPG inhibits osteoclastogenesis by binding to RANKL and blocks interaction with RANK.[3,4]

Tartrate-resistant acid phosphatase (TRAP or TRAPase), also called acid phosphatase 5, tartrate resistant, is a glycosylated monomeric metalloprotein enzyme expressed in mammals.[5] Under normal circumstances, TRAP is highly expressed by osteoclasts, activated macrophages, neurons, and by the porcine endometrium during pregnancy.[6,7] In osteoclasts, TRAP is localized within the ruffled border area, the lysosomes, the Golgi cisternae, and vesicles. It has been shown that osteopontin and bone sialoprotein, and bone matrix phosphoproteins, are highly efficient *in vitro* TRAP substrates, which bind to osteoclasts when phosphorylated. On partial dephosphorylation, both osteopontin and bone sialoprotein are incapable of binding to osteoclasts. From this effect, it has been hypothesized that TRAP is secreted from the ruffled border of osteoclasts dephosphorylates osteopontin and allows osteoclast migration, and further resorption to occur.[8,9]

A number of drugs such as bisphosphontes,[10,11] esteriods,[12] nonesteroidal anti-inflammatory drugs,[13,14] and chemically modified teracyclines[15] are recognized for interaction with these complicated mechanisms in different stages, although the exact mechanisms of these agents have not been exactly described.

Chemically modified tetracyclines (CMTs) are tetracycline compounds which have substantially no antibacterial activity but have been found to possess a number of interesting properties, such as the inhibition of excessive collagenolytic activity *in vivo*. They have been used for their anticancer potential in a variety of cancers: melanoma, lung, breast, and prostate cancers.[16] Bone resorption is also suppressed due to their combined antiproteinase and apoptotic effects on osteoblasts and osteoclasts, respectively. Development of resistant bacteria and gastrointestinal toxicity seen with parent tetracyclines is not produced by CMTs.[17]

In 1984, Gomes *et al*. compared four antibiotics; penicillin, streptomycin, ampicillin, and tetracycline for their inhibitory effect on bone resorption. They concluded that only tetracycline could prevent bone resorption.[18] After the introduction of nonantibacterial tetracycline formulations in dentistry, a low-dose of minocycline was initially tested but was soon replaced by low-dose doxycycline (DOX).[19-21]

The recent literature described the inhibitory effect of DOX on bone resorption. Chaturvedi *et al*. showed more linear bone fill using a membrane loaded with 25% DOX paste in the treatment of human periodontal infrabony defects.[22]

Macrolides such as erythromycin (EM) are reported to reduce exacerbation of chronic inflammatory respiratory disease and chronic obstructive pulmonary disease; their anti-inflammatory effects *in vitro* and *in vivo* are shown.[23,24] Ren *et al*. concluded that EM can inhibit wear debris-induced osteoclastogenesis by modulation of murine macrophage NF-κB activity. They also reported that when EM was applied to the Peri-Apatite™ layer of the titanium pins in the tibial bone of rats, the bone volume percentage increased significantly around the pin area.[25]

To the best of our knowledge, no *in vivo* study has yet been conducted to report the effect of DOX on the expression of OPG and RANKL genes following tooth extraction. The objective of this research is to evaluate the RANKL and OPG gene expressions using real-time polymerase chain reaction (RT-PCR) and the number of osteoclasts by TRAP staining, in the presence of subantimicrobial concentrations of DOX and EM following tooth extraction in rats.

MATERIALS AND METHODS

In this animal study, forty five (8–10) week-old male Wistar rats had their maxillary right molar extracted after immobilization and using general anesthesia with ketamine 10% (Alfasan International, Woerden, Holland, 80 mg/kg) and xylazine (Neurotranq, Alfasan, Woerden, Holand, 8 mg/kg). Teeth were loosed using a hemostat with modified beaks (two cavities were made in each beak) (day 1). Finally, they were extracted by a cotton plier. The percedures of this study were approved by the Animal Research Ethics Committee of the Isfahan University of Medical Sciences, Isfahan, Iran. Postoperative bleeding was insignificant. The animals were observed till fully recovered. Then animals were divided into three groups of fifteen. In Group 1 (control), Groups 2 and 3 the rats received normal saline (5 ml/kg/day), DOX (subantimicrobial dose, 5 mg/kg/day by gavage) and EM (subantimicrobial dose, 2 mg/kg/day intraperitoneally [i.p.]), respectively at day 1 and daily for 1 week. Each group was evaluated at three different times: In 7, 14, and 21 days following tooth extraction. Five animals were used for each reading time. The animals were kept in an artificially controlled environment with temperature ranging from 20°C to 24°C, on a 12:12 h light/dark cycle and were fed food and water. Then samples were euthanized in 7, 14, and 21 days after surgery in a chamber saturated with halothane vapor.

After the administration of subantimicrobial dose of DOX, immobilization of the rats, an aqueous solution of DOX hyclate 10% was released into their stomachs using a gavage tube. In Group 3, EM dissolved in distilled water and was injected i.p. from the day of tooth extraction and maintained daily injection until the 7th day. The right sections of upper jaws of animals were cut and maintained in a 4% paraformaldehyde solution. The study was approved by the Ethics Committee of the by the Ethical Committee for Animal Experiment in Isfahan University of Medical Sciences.

Real-time quantitative polymerase chain reaction

First strand cDNA was synthesized using 1 μl of total RNA and random hexamers. Real-time quantitative polymerase chain reaction (TaqMan PCR) using an ABI Step One Real-Time Sequence Detection System and a TaqMan PCR Core Reagent Kit (Perkin–Elmer Corp.) was performed according to the manufacturer's protocol. One microliter of the first strand cDNA was used in the following assay. The copy number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The following primers and TaqMan probes were used. GAPDH: forward 5'-GCATTGATGGTGAGGTGAGCAAA-3', reverse 5'-TCGCTCCTGGAAGATGGTGA-3', TaqMan probe 5' (FAM)-CCACGGCAAGTTCAACGGCACAGT-(TAMRA) 3'; OPG: forward, 5'-AGAGGGCGCATAGTCAGTAGACA-3', reverse 5'-ATATTGCCCCCAACGTTCAAC-3', TaqMan probe 5' (FAM)-TGTGCACTCCTGGTGTTCTTGGACA-(TAMRA) 3'; RANKL: forward 5'-CTTGGCCCAGCCTCGAT-3', reverse 5'-ACCATCAATGCTGCCGACAT-3', TaqMan probe 5' (FAM)-AAGGTTCGTGGCTCGATGTGGCC-(TAMRA) 3'. The copy number of each cDNA was measured using a separate plate. The conditions for the OPG gene were as follows: 95°C for 5 min, followed by 48 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 28 s. For RANKL gene the setting was 95°C for 5 min, followed by 28 cycles of 95°C for 25 s, 58°C for 30 s, and 72°C for 20 s and an extra cycle of 62°C for 10 s. The negative controls for each target showed an absence of carryover.

Immunohistochemistry

To detect the specific antigens of TRAP, the tissues were immunohistochemically stained by Biotin–streptavidin method. Briefly, the main procedure included serial sectioning (in 3–4 μm sections), deparaffinization, rehydration, and antigen retrieval. All specimens were placed in phosphate-buffered saline, treated with protein block (RE 1102) for 5 min to prevent any false staining. Next, the specimens were incubated for 30 min with primary antibody of TRAP (NCL-TRAP) clone 514H12 (Novocastra). (The sections were then exposed to Novolink Polymer [RE7112] or secondary antibody for 30 min and washed in phosphate buffer saline, after that they were incubated with 3,3'-diaminobenzidine for 5 min for visualization. After washing, the slides were counter-stained with hematoxylin. Sections through tonsils served as positive controls and the breast tissue were used as negative control. The slides were mounted after drying). To quantities the number of cells within the lesions positive for TRAP marker in the middle third of the rat alveolus, sections were observed by a single examiner who was also blinded to the identity of samples using a 400 magnification of Olympus light microscope (Olympus Corporation,

Tokyo, Japan). Four nonoverlapping high-power fields were selected randomly. The amount of stained cells (multinuclear TRAP-positive cells that were considered as osteoclasts) in each field was measured. The mean of all four fields was calculated in each group.

Statistical analysis

All data of three groups were presented as the mean ± standard error of the mean in Table 1 and were analyzed by SPSS version 20 software (Statistical Package for the Social Sciences, IBM SPSS, Inc. in Chicago, Illinois, USA) and one-way analysis of variance (ANOVA). If a significant difference between them was found, *post hoc* (Tukey multiple comparisons) test was used for comparison between groups. A significance level of $P < 0.05$ was utilized for all comparisons.

RESULTS

Receptor activator of nuclear factor κB ligand gene expression

One-way ANOVA showed that differences in the mean of RANKL in EM, DOX, and control groups were statistically significant with regard to evaluated time points [Table 1]. In all of the studied intervals in EM group compared to control group, a significant RANKL reduction on gene expression level was observed. In the DOX group, the expression of RANKL in three evaluated time points diminished significantly rather than in the control group ($P < 0.05$). Based on *post hoc* analysis (Tukey's multiple comparisons test), no meaningful difference in the expression of RANKL gene was observed between DOX and EM groups [$P > 0.05$, Table 1 and Chart 1a].

Table 1: The mean expression of receptor activator of nuclear factor κB ligand, osteoprotegerin, receptor activator of nuclear factor κB ligand/ osteoprotegerin ratio and the mean number of osteoclasts in dental socket of rat in test and control groups

Group	*n*	Weeks	RANKL	OPG	RANKL/ OPG	Osteoclasts ($TRAP^+$ cells) (hpf)
DOX	5	1	2.01±0.14	3±0.09	0.67±0.07	3.71±0.31
EM	5	1	1.9±0.18	3.12±0.13	0.61±0.03	3.89±0.31
Control	5	1	2.55±0.17	2.53±0.24	1.01±0.08	5.08±0.24
P			0.036	0.43	0.02	0.002
DOX	5	2	1.7±0.29	3.1±0.22	0.57±0.05	3.34±0.31
EM	5	2	2.24±0.18	3.2±0.11	0.7±0.06	4.06±0.27
Control	5	2	3.2±0.24	3.02±0.21	1.06±0.21	4.22±0.18
P			0.001	0.81	0.046	0.53
DOX	5	3	1.51±0.13	3.22±0.27	0.49±0.13	3.12±0.27
EM	5	3	2.15±0.29	3.48±0.23	0.62±0.07	3.61±0.33
Control	5	3	3.52±0.31	3.42±0.2	1.03±0.18	4.26±0.31
P			0.031	0.73	0.059	0.089

Data are mean±SEM. *P* values calculated by one-way ANOVA and $P<0.05$ considered statistically significant. RANKL: Receptor activator of nuclear factor κB ligand; OPG: Osteoprotegerin; TRAP: Tartrate-resistant acid phosphatase; DOX: Doxycycline; EM: Erythromycin; SEM: Standard error of mean; ANOVA: Analysis of variance

Osteoprotegerin gene expression

In all of the studied intervals in DOX and EM groups, the expression of OPG was higher when compared to control group in same periods [Table 1], although this enhancement was not statistically significant ($P > 0.05$). When two intervened groups were compared in similar evaluated time points, despite higher levels of OPG expression in EM group, no significant difference was observed [$P > 0.05$, Table 1 and Chart 1b].

Receptor activator of nuclear factor κB ligand/osteoprotegerin ratio

As shown in Table 1, the ratio of RANKL/OPG in EM and DOX groups in the days 7 and 14, was significantly lower than the control group ($P < 0.05$), but on day 21 it was not. In the comparison of two drugs, there was no meaningful difference between them [$P > 0.05$, Table 1, Chart 1c].

Immunohistochemical observation (tartrate-resistant acid phosphatase + cells)

Osteoclasts are characterized by a cytoplasm with a homogeneous, "foamy" appearance. This appearance is due to a high concentration of vesicles and vacuoles. These vacuoles include lysosomes filled with acid phosphatase. This permits characterization of osteoclasts by their staining for high expression of TRAP, and cathepsin K. Osteoclast rough endoplasmic reticulum is sparse, and the Golgi complex is extensive.[26,27] In osteoclasts, TRAP is localized within the ruffled border area, the lysosomes, the Golgi cisternae, and vesicles.[8]

On day 7, the number of TRAP + cells in control group was significantly lower than DOX and EM groups, without any meaningful difference between two tested groups [$P < 0.05$, Table 1 and Chart 2]. On days 14 and 21, based on one-way ANOVA, there was no significant difference in the studied groups [$P > 0.05$, Table 1 and Figure 1].

DISCUSSION

To the best of our knowledge, no *in vivo* study has yet been conducted to report the effect of DOX on

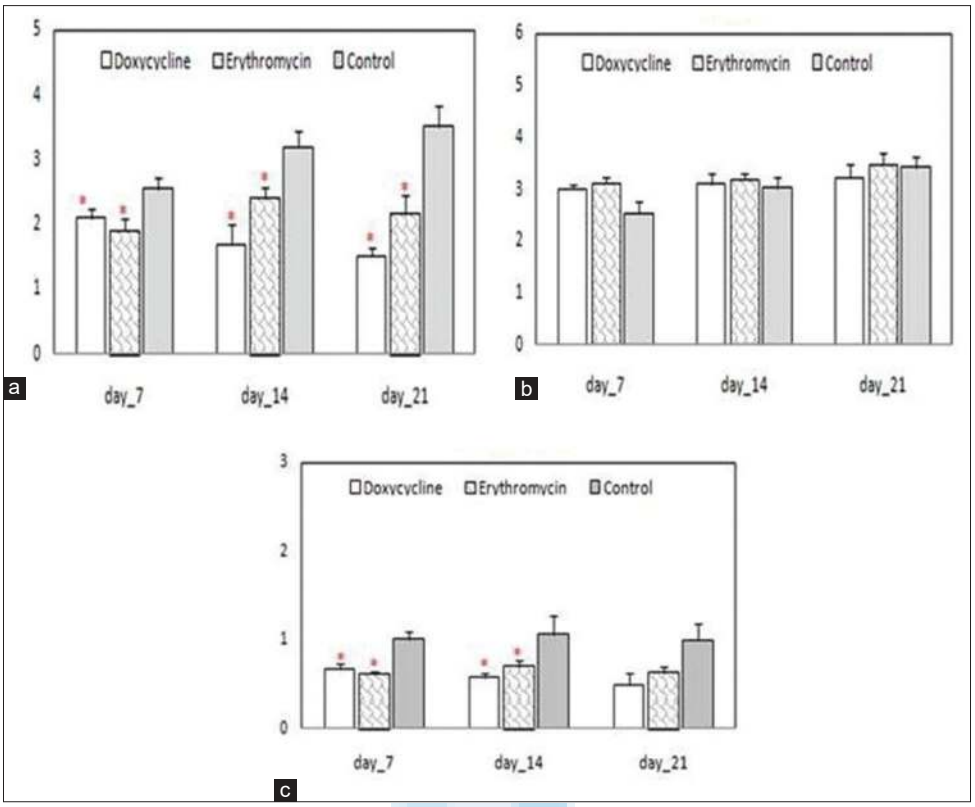

Chart 1: Comparison of variables between study groups. (a) Receptor activator of nuclear factor κB ligand expression in the dental socket of rat, (b) osteoprotegerin expression in the dental socket of rat, (c) receptor activator of nuclear factor κB ligand/osteoprotegerin ratio. *Significant difference was found between intervention groups with control group, $P < 0.05$. X-axis: Time Points. Y-axis: Mean + standard error of the mean of variables. *P* values derived from by Tukey's *post hoc* test.

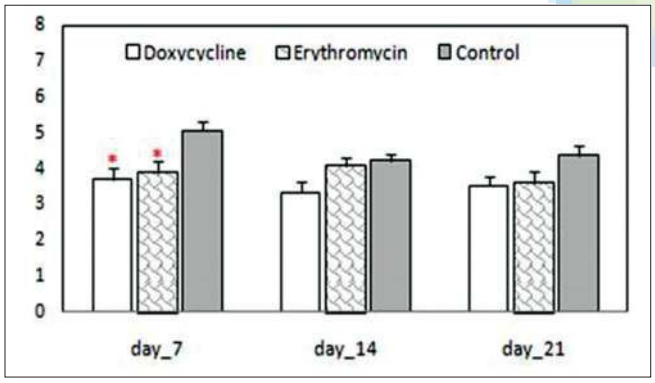

Chart 2: Comparison of tartrate-resistant acid phosphatase+ cells. *Significant difference was found between intervention groups with control group, $P < 0.05$. X-axis: Time points, Y-axis: Mean + standard error of mean of variables, *P* derived from by Tukey's *post hoc* test.

the expression of OPG and RANKL genes following tooth extraction. In fact, we reported the short-term sequential expression of RANKL and OPG from week 1 to week 3 (every week in the dental socket of rat), using the advanced method of RT-PCR to register the delicate alterations as far as possible.

The RANKL/OPG ratio illustrates the importance of the balance between these molecules in bone remodeling, in health, and in disease states.[28,29] Both studied genes, OPG and RANKL, are expressed by osteoblasts and play a major role at early stages of alveolar bone healing. OPG is the natural decoy receptor for RANKL that is known as osteoclastogenesis inhibitory factor[30,31] Since these molecules are known as key factors for osteoclastogenesis and primary regulators of bone remodeling,[32,33] we focused our attention on them.

Glucocorticoids have been demonstrated to upregulate RANKL mRNA levels and decrease OPG mRNA level in human osteoblasts. A similar pattern of these changes has been indicated following the administration of immunosuppressant (cyclosporine A, rapanyein, tacrolimus), by contrast bisphosphonates have been shown a reverse pattern in the expression of these genes.[34,35]

In control group, on days 7 and 14, the RANKL/OPG ratio was significantly higher

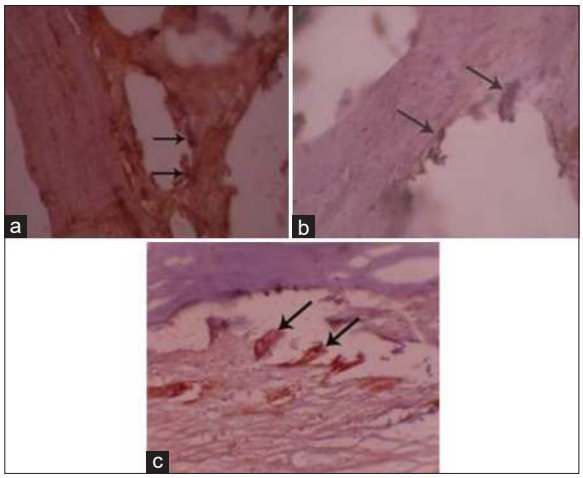

Figure 1: Rat alveolar socket 1 week after tooth extraction (TRAP, ×400). The arrows show osteoclasts in control, erythromycin, and doxycycline groups. (a) Control group, (b) Erythromycin, (c) Doxycyclin.

than other two groups that indicate the anti-osteoclastogenesis of DOX and EM, again. The increase in OPG (marker of bone formation) and reduction in RANKL (marker of bone resorption) expressions observed in both DOX and EM groups, (than the control group,) indicates more activation or increase of the osteoblast cells.

In EM-treated group, a significant down-regulation of RANKL gene expression was observed at 3 weeks, suggesting low differentiation of osteoclasts, and show the low level of bone resorption than control group. This finding is confirmed the decreased number of osteoclasts by TRAP staining (a genetic marker of osteoclast differentiation) with the first week in control group and increased with the amount of new bone formation.[36]

In the DOX-treated group, there was a gradual up-regulation of OPG gene expression over time; while the pattern of RANKL gene expression had a gradually decreasing behavior. The RANKL expression peak, which occurred during the bone resorption phase (7th day), preceded the expression of OPG that occurred during the bone formation phase (21st day). It meant that bone formation was stimulated by DOX and bone resorption was inhibited; comparing the control group in which RANKL gene expression or induction of bone resorption was enhanced gradually. The RANKL/OPG ratio peaked in the first week, rather than other periods, and then gradually decreased. This might be related to the initial increase in bone resorption that preceded normal bone formation following tooth extraction which has been noted by Guglielmotti and Cabrini[37] and Iizuka *et al*.[38]

It has been demonstrated that 14 days after tooth extraction in rats is a time in which maximal bone formation and alveolar volume occurs.[38] The effect of tetracyclines on osteoblastic cells was addressed in few previous studies, by different cell systems and situations. Gomes and Fernandes reported that therapeutic concentrations of doxycycline and minocycline can stimulate the proliferation of osteoblastic-induced bone marrow cells. They caused an increase in the cell growth, in the maintenance of alkaline phosphatase activity and higher abundance of mineral deposition.[39] Ferraz *et al*. used nanohydroxyapatite microspheres as a delivery system for EM, amoxicillin, and augmentin to evaluate their interaction with osteoblasts. He concluded that EM-induced osteoblastic cells proliferation higher than others, and also suggested the use of microsphere and EM as a good alternative carrier to enhance bone regeneration while treating periodontal defects.[40]

Reduction of resorbing activity in our investigation was not in agreement of Folwarczna *et al*.'s study which indicated administration of DOX in rats with normal process of bone remodeling, increased bone resorption, and this effect may be dose-dependent.[41] In another study with the aim of comparison of new bone formation following use of DOX and vehicle in the bony defects, no meaningful difference was observed.[42]

On the other hand, many articles showed the inhibitory effect of DOX in bone resorption. In an *in vivo* investigation, it was reported that the DOX-treated rats' (7 days, i.p., 3.0 mg/kg) skin, muscle and bone healing clearly improved compared with the saline-treated animals.[43] In another investigation by use of DOX after periradicular surgeries or following applied orthodontic forces, bone loss reduced significantly.[44,45] Büchter *et al*. reported that a bioabsorbable polymer that delivers doxycycline (Atridox) can be used for the treatment of severe peri-implant bone loss when is combined with autogenous bone.[46] In another study in 2008, Metzger *et al*. concluded that low-dose doxycycline reduced the area of bone resorption associated with apical periodontitis in the mandibular first molar teeth of rats.[47]

We know that there is a biologic balance between inflammatory cytokines and growth factors so that

upregulation of one results in the down-regulation of the other one. Both studied drugs have an anti-inflammatory effect which is independent of their antimicrobial one, so a decrease in the inflammatory cytokines caused enhancement of the growth factors and increased regeneration and rate of wound healing.[48,49]

Since the principles of osseointegration of dental implants are on the basis of bone healing, understanding its mechanism, rate and the factors which may accelerate this phenomenon is very important. Other routes of administration of these drugs such as local delivery as surface coating have not yet been investigated in conjunction with endosseous dental implants, which is strongly suggested for the future studies.

CONCLUSION

According to these findings, it seems that DOX and EM improve healing of alveolar bony socket by decreasing RANKL/OPG ratio and the number of osteoclasts that suggests their anti-osteoclastogenesis activity. It is reasonable to propose that the RANKL/OPG system plays an important role in bone healing.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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