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Original Article

Effects of diclofenac and celecoxib on osteoclastogenesis during alveolar bone healing, *in vivo*

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ABSTRACT

Background: Osteoclastogenesis is coordinated by the interaction of members of the tumor necrosis factor (TNF) superfamily: Receptor activator of nuclear factor-κB ligand (RANKL) and Osteoprotegerin (OPG). The aim of this study was to compare the effect of two different types of non-steroidal anti-inflammatory drugs (NSAIDs) on the RANKL/OPG balance during the healing of the alveolar process.

Materials and Methods: This was an experimental study, carried on 45 male Wistar rats (200 ± 25 g, 8-10 weeks old). After extraction of the right maxillary first molar, 15 rats received 5 mg/kg/day of diclofenac and 15 rats received 15 mg/kg/day of celecoxib and 15 rats received normal saline. The animals were sacrificed 7, 14 and 21 days after tooth extraction. The number of osteoclasts, OPG and RANKL messenger ribonucleic acid expression were determined by tartrate-resistant acid phosphate (TRAP) staining and polymerase chain reaction (PCR) respectively. The data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test. Values of $P < 0.05$ were considered significant.

Results: On days 7, 14 and 21 the ratio of RANKL/OPG in the control group was higher than diclofenac and celecoxib groups. TRAP immunolabeling of the control group was more than diclofenac group on day 7 and was more than celecoxib group on day 14. On day 21, no significant differences were noted among the three studied groups.

Conclusion: Both drugs affect RANKL/OPG gene expression and also osteoclastogenesis in alveolar socket during the experimental period of 21 days.

Key Words: Non-steroidal anti-inflammatory drugs, osteoclastogenesis, osteoprotegerin, receptor activator of nuclear factor-κB ligand, tartrate-resistant acid phosphatase

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INTRODUCTION

Bone is a mineralized tissue that is influenced by numerous local and systemic factors which regulate the proliferation, differentiation and function of bone cells.[1-3] Osteoclasts are known to differentiate from hematopoietic precursor cells. Recent studies have identified that the development of osteoclasts from their precursors usually requires the presence of osteoblasts through a mechanism that requires three new members of the tumor necrosis factor (TNF) and TNF receptor families including receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG) and receptor activator nuclear factor-κB (RANK).[4] RANKL is produced and secreted by osteoblasts.[5] It stimulates osteoclasts differentiation through its known receptor RANK, which is expressed on osteoclasts and their precursors. Although, the

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interaction between RANKL and RANK could be inhibited by the decoy receptor OPG that is produced by osteoblasts.[5-7] RANKL and OPG have been considered relatively specific for osteoblasts[6-9] and the balance between RANKL and OPG determines osteoclast functions. Alterations of the RANKL/OPG ratio are critical in the pathogenesis of bone diseases[10] and this ratio is a major determinant of osteoblast-regulated osteoclastogenesis.[11]

Tartrate-resistant acid phosphatase (TRAP) is a different form of acid-phosphatase enzyme that is mainly found in bone and some blood cells. This enzyme is expressed in osteoclasts and is related to bone resorption.[12,13]

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used drugs world-wide.[14] These are prescribed for analgesia following trauma, fractures and osteoarthritis.[15-17] NSAIDs act as an inhibitor of the cyclooxygenase (COX) isoenzymes and effectively reduce pain and inflammation. Prostaglandin (PG) production is controlled by COX-1 and-2. In fact, selective COX-2 inhibitors are currently used for the suppression of PG production because of their superior gastrointestinal safety compared with traditional nonselective NSAIDs.[18,19] The COX-2 specific inhibitors exhibit analgesic and anti-inflammatory effects equivalent or superior to conventional NSAIDs while decreasing systemic complications.[20] Although increased cardiovascular adverse events associated with prolonged use of COX-2 inhibitors has raised safety concerns regarding these drugs, but the short-term use in pain management does not warrant similar concerns.[20]

Numerous studies have shown traditional NSAIDs may inhibit bone metabolism and healing,[21,22] but there are numerous controversies and also little is known about the alleged inhibitory effects of the newer COX-2 inhibitors on this process.[23-25] Therefore, the role of COX-2 in bone regeneration needs to be better defined in order to further elucidate the impact of NSAIDs on bone healing.[26]

On the other hand, these experimental and clinical evidences were taken from the orthopedic area which prolonged use of both non-selective and selective NSAIDs can hinder reparational long bone formation.[27] Although NSAIDs are commonly used for swelling and pain management in clinical dentistry, very few assessments with results still controversial have been conducted to assess their deleterious effects on alveolar bone.[28]

To the best of our knowledge, there are no experimental or clinical studies in dentistry conducted to compare the effect of two different types of NSAIDs on RANKL/OPG balance during the healing of the alveolar process. Therefore, this study was designed to evaluate the effect of traditional NSAID, diclofenac sodium and a COX-2 inhibitor, celecoxib, on the RANKL and OPG gene expression in osteoblasts by means of polymerase chain reaction (PCR) over a period of 21 days. Furthermore, expression of TRAP protein in osteoclasts was evaluated.

MATERIALS AND METHODS

In this experimental study, 45 male Wistar rats (8-10 weeks old at the beginning of the study), weighing 200 ± 25 g were used. Throughout the experiment, they were housed under similar conditions (22°C room temperature, 40% humidity and 12 h light/12 h dark cycle, with free access to water and rat chow).

The principles of laboratory animal care and national laws[29] on animal use were complied with in the present study, which was authorized by the Animal Research Ethics Committee of the Isfahan University of Medical Sciences, Isfahan, Iran.

In all rats, general anesthesia was induced by intramuscular injection of ketamine 10% (Alfasan International, Woerden, Holland, 80 mg/kg) and xylasine (Neurotranq, alfasan, Woerden, Holland, 8 mg/kg) and their right upper molars were luxated with the aid of a tapered instrument and extracted by means of a hemostat with modified beaks (two cavities were made in each beak). A surgical technique was used, which allows tooth extraction without post-operative complications. The animals were then observed until fully recovered. No antibiotic or other medication was used. The animals were randomly separated in three groups (n = 15). Following extraction, a group received a daily dose (5 mg/kg) of diclofenac sodium, diluted with sterile distilled water and was injected subcutaneously. Other group received a daily dose (15 mg/kg) of celecoxib by gavage administration. Animals in extraction control group received daily normal saline (5 ml/kg) by gavage administration. All medications were administered for a period of 7 days, starting on the day of tooth extraction. Doses of all drugs were chosen based on prior studies and pharmacokinetic data to simulate the doses that could be used in human, taking into account the species-dependent differences in metabolism.[30]

On day 7, 14 and 21, five animals from each extraction group were sacrificed by over dose of ether inhalation and then, the maxilla was removed. The obtained samples (maxilla) were postfixed in 4% paraformaldehyde solution, demineralized with 10% EDTA (Merck, Darmstadt, Germany) and embedded with paraffin (Merck, Darmstadt, Germany). The samples were sectioned perpendicular to the long axis of the alveolar process with a microtome (Accu-Cut SRM, SAKURA, USA) in order to obtain slices with 5 μm thicknesses, which were mounted in previously poly-L-lysine slides.

For each specimen, one slide of H and E, staining was prepared. For the immunohistochemistry reactions, blocking with 0.03% hydrogen peroxide followed by primary antibodies anti TRAP (Goat anti trap polyclonal-Santa Cruz, CA, USA) and the biotinylated donkey anti-goat antibodies (Biotin-SP-AffiniPure donkey anti-goat IgG-Jackson Immunoresearch Laboratories, West Grove, PA, USA) was the secondary antibody; the immunohistochemistry reaction signal was amplified with the Avidin-Biotin system (Kit ABC Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA) and the reaction was revealed using diaminobenzidine (DAB-Sigma, Saint Louis, MO, USA) as the cromogen.

Sections were used for immunohistochemical staining to determine the expression of TRAP protein in the alveolar tissues during the healing process after tooth extraction and then were counterstained with Harris's hematoxylin. Immunostaining evaluated alveolar bone under light microscope. A negative control was prepared for each specimen using the same method except for the primary antibody. For immunohistochemical staining to determine the expression of TRAP, light microscope with ×1:400 magnification was used. Four non-overlapping fields were selected and the numbers of stained cells in each field were evaluated by an expert blinded Pathologist. Then, the percentage of positive (stained) cells to total cells was determined. Finally, the average of four fields was calculated as "the average of the percentages" for each specimen.

The images of the sections representative of TRAP protein in each tooth were captured by a digital camera (Canon powershot A650 IS; Tokyo, Japan) coupled to the light microscope (Olympus CX21FS, Olympus Corporation, Tokyo, Japan).

For each group, there were three time points for PCR testing: 7, 14 and 21 days. From each extraction group, 15 rats were harvested at each time point and five normal rats for a total of 50 rats. First strand complementary deoxyribonucleic acid (cDNA) was synthesized as described previously using 1 μl of total ribonucleic acid (RNA) and random hexamers. Real-time quantitative PCR (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. 1 μl of the first strand cDNA was used in the following assay. The copy number of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was used as an internal control. The following primers and TaqMan probes are listed in Table 1.

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.

All analyses were done using the statistical package for the social sciences-20. (IBM SPSS Statistics for Windows, Version 20.0., IBM Corp, Armonk, NY). The results are presented as means ± standard error of the mean. Statistical differences among groups were determined by one-way ANOVA followed by Tukey's *post-hoc* test. Values of $P < 0.05$ were considered to be significant.

Table 1: Primers and TaqMan probes used in real-time PCR

Gene	Primer sequence
GAPDH	5′-GCATTGATGGTGAGGTGAGCAAA-3′
	5′-TCGCTCCTGGAAGATGGTGA-3′
TaqMan probe	5′(FAM)-CCACGGCAAGTTCAACGGCACAGT-(TAMRA)3′ (5)
OPG	5′-AGAGGGCGCATAGTCAGTAGACA-3′
	5′-ATATTGCCCCCAACGTTCAAC-3′
TaqMan probe	5′(FAM)-TGTGCACTCCTGGTGTTCTTGGACA-(TAMRA)3′ (5)
RANKL	5′-CTTGGCCCAGCCTCGAT-3′
	5′-ACCATCAATGCTGCCGACAT-3′
Taq Man probe	5′(FAM)-AAGGTTCGTGGCTCGATGTGGCC-(TAMRA)3′ (5)

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PCR: Polymerase chain reaction; OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor kappa B ligand

RESULTS

All animals tolerated the whole experimental period. No significant differences in behavior or body weight were noted among the control, diclofenac and celecoxib groups throughout the experimental period.

Results are expressed as relative expression in comparison to GAPDH. On the day 7, RANKL mRNA expression within extraction sockets was stimulated to the largest by the control group and this augmentation was suppressed by both NSAIDs. However, the difference between diclofenac and celecoxib groups was not statistically significant. On the day 14, only the control group showed higher RANKL mRNA expression than that of the day 7. Again, the control group had the highest RANKL mRNA expression and it was significantly inhibited in rat treated with diclofenac and celecoxib. This inhibition was slightly more noticeable in celecoxib. In the control group, RANKL gene expression level of the day 21 was higher than that of the day 7 and 14. RANKL gene expression of the control group was significantly higher than other groups [Figure 1a].

OPG gene expression pattern did not follow a similar trend to that of the RANKL results. No significant difference in OPG gene expression among studied groups at each time point was found [Figure 1b].

With respect to contrary effects on OPG and RANKL, the ratio of RANKL/OPG was taken in order to evaluate the resulting effect. The findings are presented in Figure 1c. On the day 7, the RANKL/OPG ratio differed significantly among the studied groups. The control group had the maximum ratio. Results showed that the ratio of RANKL/OPG in the control group was higher than diclofenac and celecoxib groups. On the day 14, the control group increased the RANKL/OPG ratio significantly. It showed that the ratio of RANKL/OPG in the control group was higher than two other groups. Diclofenac had higher RANKL/OPG ratio in comparison with celecoxib. On the day 21, RANKL/OPG ratio within extraction sockets was stimulated to the highest in the control group and this augmentation was suppressed slightly by both NSAIDs. Furthermore, the difference between diclofenac and celecoxib effect was not statistically significant.

Comparisons in the number of TRAP-positive osteoclasts between groups are shown in Figure 1d. On the day 7, TRAP immunolabeling of the control group was higher than that of other groups. Data showed that the number of osteoclasts in the control group was more than diclofenac group. On the day 14, the difference between the control group and celecoxib group was statistically significant. The osteoclast number value in the control group was higher than diclofenac and celecoxib groups on day 21. Furthermore, no significant differences were noted among the three studied groups [Figure 2].

DISCUSSION

Understanding the circumstances that lead to the regeneration of oral hard tissues has been a major challenge for dental research. It is known that a great variety of signals are released when an injury occurs, inducing neighboring cell populations to respond with proliferation, migration, or differentiation. Current research has focused on elucidating the biomolecular mechanism of extraction-socket healing, especially by evaluating the expression of the RANKL/OPG system components.

To the best of our knowledge, this is the first description to determine the effect of diclofenac sodium (traditional NSAID) and celecoxib (COX-2 inhibitor) treatment on the RANKL/OPG ratio in the healing of the alveolar process *in vivo*. Real-time PCR analysis showed that the RANKL gene expression was reduced by test drugs. The OPG gene expression was not meaningfully changed by used drugs.[4] As a result, the RANKL/OPG ratio is declined by diclofenac sodium and celecoxib.

RANKL and OPG have a controversial impact on osteoclastogenesis and osteoclast differentiation: RANKL, a soluble paracrine-secreted protein, promotes the osteoclast differentiation while OPG prevents through a decoy receptor binding. Therefore, the ratio of RANKL/OPG gene expression should allow a sufficient assessment of osteoblast induced stimulus to osteoclasts. By a RANKL/OPG ratio >1, a stimulating influence of osteoblasts on osteoclastogenesis and differentiation could be assumed.[4,31] In the control group, this ratio was more than one and both diclofenac and celecoxib decreased this ratio to less than one. The RANKL/OPG ratio, elevated by tooth extraction, could suggest an anabolic effect on osteoclasts via osteoblasts' secretion after tooth extraction.[31] In the present study, there was no statistically significant difference in OPG mRNA expression between control and used

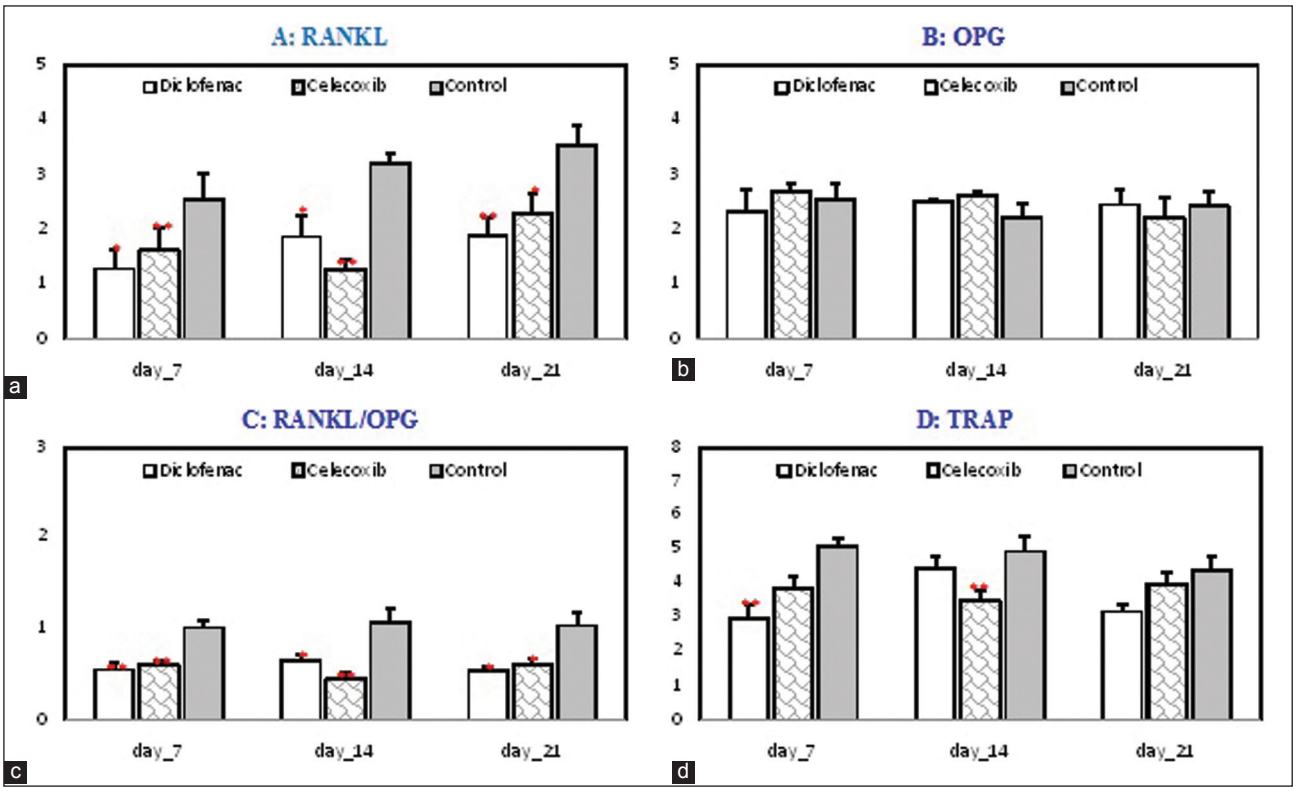

Figure 1: Comparison of variables between study groups. (a) Receptor activator of nuclear factor-κB ligand (RANKL) messenger ribonucleic acid (mRNA) expression in the bone marrow cells, (b) osteoprotegerin (OPG) mRNA expression in the bone marrow cells, (c) RANKL/OPG ratio, (d) tartrate-resistant acid phosphate. Significant difference was observed: **P* < 0.05, ***P* < 0.01. X axis: Time points. Y axis: Mean±standard error of the mean of variables *P* values derived from by Tukey's *post-hoc* test

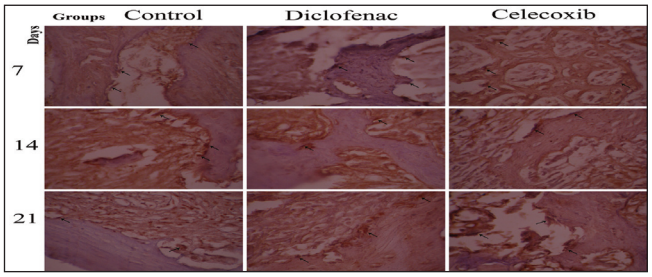

Figure 2: Expressions of tartrate-resistant acid phosphate labeling at 7, 14 and 21 post-extraction days in animals of control, diclofenac and celecoxib groups

drugs groups, which indicates that diclofenac and celecoxib had no effect on OPG mRNA expression. However, RANKL gene expression was significantly decreased in the test groups compared to control group. These results showed that used drugs with a decrease in RANKL gene expression and no major change in OPG gene expression seemed to cause an OPG gene expression in osteoblasts that exceeds the RANKL gene expression and reduced the RANKL/OPG ratio accordingly. Therefore, diclofenac and celecoxib might inhibit osteoclasts' differentiation.

The temporal effect of NSAIDs administration on expression patterns of RANKL/OPG mRNA were similar to those described in an inflamed joint model.[32] The authors in this study showed that celecoxib suppressed the ratio of RANKL/OPG mRNA expression in an inflamed joint and a rat mandibular distraction osteogenesis model. The OPG expression peaked during the bone formation.[32]

During the treatment period of the present study, diclofenac sodium and celecoxib had lower clastic activity as demonstrated by the poor TRAP expression. Osteoclasts are readily distinguished from macrophages by the presence of TRAP in their cytoplasm. This type-V isoenzyme of acid phosphatase presents an intense activity in osteoclasts, being considered a specific marker for osteoclasts. Also, test drugs led to a significant decrease in TRAP expression during 3 weeks post extraction; especially in the first 14 days. Previous studies have shown that celecoxib and dicloenac reduced osteoclastogenesis *in vitro*.[33-35]

Diclofenac and celecoxib interfere with bone remodeling processes that are controlled by mediators

such as RANKL and OPG. Our study demonstrated that administration of used drugs, suppressed osteoclastogenesis by down regulation of RANKL/OPG ratio in early stage of extraction-socket healing. An increased RANKL/OPG ratio would favor osteoclast formation and activation; as a result, bone resorption occurs. On the other hand, a decreased RANKL/OPG ratio promotes bone formation by inhibiting osteoclastic activity. In this study, an increase in the RANKL/OPG ratio was seen with tooth extraction and diclofenac and celecoxib administrations decreased this ratio. Therefore, it can be assumed that these two different types of NSAIDs can affect bone loss after tooth extraction.

CONCLUSION

Within the limitations of this study, it can be concluded that diclofenac and celecoxib affect RANKL/OPG gene expression and osteoclastogenesis in alveolar socket of rats during the experimental period of 21 days. However, further modulation of RANKL and OPG gene expression on post-transcriptional level could not be ruled out.

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