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RESEARCH ARTICLE

OPG and RANKL Signal Transduction in Osteoblast Regulation Post Application Extract Collagen in Osteogenesis

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ABSTRACT:

Background and Aim: The current goal of periodontal therapy is to achieve periodontal regeneration. Important factor for periodontal regeneration is to promote bone formation, nowadays used bone replacement. The used of xenograft materials as gold standard for periodontal tissue regeneration using type I collagen bone graft has been widely developed. The main organic component in fish scales is type I fibril collagen, which are same as with component in bones. Specific markers of bone formation are the expression of osteoblast, osteoclast, osteoprotegerin (OPG), and receptor activator of nuclear factor κB ligands (RANKL). The purpose of this study is to assess the expression of OPG and RANKL after application of extract collagen from gourami (Osphronemus gouramy) scales. Materials and Methods: Thirty-two experimental 3-month-old male Wistar albino rats (150g and 200g) were randomly divided into four groups: 7-day control group, 7-day fish collagen group, 14-day control group and 14-day fish collagen group. The sockets were filled with blood (control group), whereas 10 mg to 30 mg extract collagen was added until the sockets were fully occupied (treatment group). **Results:** The ANOVA test showed a significance level of 0.000 (p <0.05). **Conclusion:** Expression of OPG enhanced and expression of RANKL lowered after application of type I collagen from gourami scales, accelerating osteogenesis.

KEYWORDS: Collagen, Gourami fish scale, OPG, RANKL.

INTRODUCTION:

The augmentation method is a regenerative treatment for repairing damage to the alveolar bone and periodontal tissue[1,2,3]. Bone is growing tissue and mostly made of collagen[4]. Collagen is a tough, fiber-like protein that makes up about a third of body protein[5,6]. The tissue engineering process as part of the bone augmentation method relies on three important pillars to achieve successful tissue regeneration processes, namely: scaffold, cells, and growth factors.

Scaffold acts as a temporary matrix for growth and differentiation of cells and tissues. On the other hand, cells have an important role because cells together with extracellular matrix (ECM) molecules act as biological triggers that will stimulate endogenous regeneration. The goal is to develop cells in the scaffold over a period of time which will then build a network of scaffolds / cells to be implanted *in vivo*[7,8].

Collagen from aquatic animals began to be developed to avoid transmission of bovine spongiform encephalopathy from cows[9,10,11,12]. In addition, collagen extracted from pigs is not used by some people for religious reasons[13]. Type I collagen is the main organic component of fish scales, is known to be non-toxic to cells, and has good viability[14]. Fish scales are a major by-product of the fish-processing industry, causing wastage and pollution[15]. The pore size of gourami (*Osphronemus gouramy*) scales collagen extract ranges

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from 191.6µm to 385.3µm, and the optimal pore size for bone regeneration ranges from 100µm to 500µm[16]. The materials derived from collagen is used in wound healing[17]. The size of porous collagen scaffold plays a role in providing a place for cells to penetrate and develop in the scaffold, namely in the cell seeding, cell migration, matrix deposition, and vascularization processes[16,17]. Type I collagen as an organic part of bone extracellular matrix (ECM) is able to induce migration, proliferation, and differentiation of cells during osteogenesis or new bone formation[18,19,20]. This can be seen from the expression of several protein markers of bone forming both *in vivo* and *in vitro*[20].

The receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) binding pathway is a signaling system for the communication between immune and bone cells[21]. Osteoblasts regulate osteoclasts via the RANKL-RANK signaling pathway[22]. RANKL is expressed in osteoblasts and T cells, and it is an important factor for the stimulation, differentiation, and activation of osteoclasts that bind to their specific receptors, namely RANK found in osteoclast precursors and in mature osteoclasts. RANKL and RANK binding to the surface of osteoclast precursors activates nuclear factor κB and transcription genes in osteoclastogenesis. OPG when bound to RANKL inhibits the binding of RANKL and RANK, thus inhibiting osteoclastogenesis, osteoclast activity, and bone resorption. By modulating RANKL and OPG binding, osteoblasts can control osteoclast differentiation and the activity in the remodeling process[23].

MATERIALS AND METHODS:

Ethical approval:

The study was approved by the Ethical Clearance Committee of the Faculty of Dental Medicine, Airlangga University, Indonesia (process no. 653/HRECC.FODM/X/2019), and was conducted in accordance with the guidelines on animal ethics and welfare.

Extract collagen collections:

Gourami scales were obtained by washing fish scale, freezing them, and soaking 100 g in 6% acetic acid solution for 7 days. The acetic acid solution was replaced every day. After 7 days, the scales were rinse under running water until a neutral pH was obtained. During rinsing, collagen fibers appeared and collagen clots began to form. The collagen was then freeze-dried. The collagen product was sterilized with ethylene oxide gas.

Animals:

Using 3-month-old male Wistar albino rats (*Rattus norvegicus*) weighting between 150g and 200g. The sample was randomly chosen and the sample size was determined using Lemeshow formula, including 8 rats in each group. The sample was divided into four groups: control group at 7-days and 14-days and the group given collagen extracted from gourami scales at 7-days and 14-days (treatment group).

Experimental design:

This is an experimental laboratory study with a post-test-only *in vivo* design.

Procedures:

The wistar rats according to the criteria were adapted for 2 weeks. After that, the wistar rats were given 0,2 ml of ketamine anesthesia before being tooth extracted. Whereat, the extraction was conducted on the mandibular incisors, the sockets were filled with blood on the control group, whereas 10 mg to 30 mg collagen was added until the sockets were fully occupied on the treatment group. At the 7-days and 14-days, the experimental animals were sacrificed with 10% ether inhalation anesthetic. Research data were retrieved based on the number of osteoblasts that expressed OPG, RANKL, and osteoclasts at 7 and 14 days. The incisor sockets were prepared with immunohistochemical techniques using OPG and RANKL monoclonal antibodies and hematoxylin-eosin (HE) staining for visualization of osteoblasts and osteoclasts.

Statistical analysis:

Commercially available statistical software SPSS 16 were used for the statistical analyses. A Shapiro Wilk test was used to assess the normality of the data. A Levene's test was used to assess the homogeneity of the data. ANOVA test was performed to compare the differences between control group and treatment group at 7 and 14-days.

RESULTS:

ANOVA test showed significant differences in the expression of OPG, RANKL, osteoblast, and osteoclast at 7 and 14 days between the control group and the treatment group shown in **Table.1** and **Figure.1**. Levene's test had a $p>0.05$, indicating homogeneity of variance.

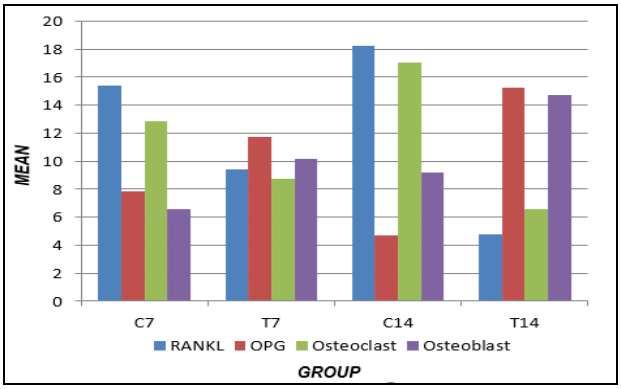

Figure 1. Average expression of RANKL, OPG, osteoclast, and osteoblast

Table 1. ANOVA results for the average expression of RANKL, OPG, osteoclast, and osteoblast in the control and treatment groups

Observation Group	Average							
	RANKL		OPG		Osteoclast		Osteoblast	
	at 7 days	at 14 days	at 7 days	at 14 days	at 7 days	at 14 days	at 7 days	at 14 days
Control group	15.375± 1.92261	18.25± 3.28416	7.8125 ± 1.16305	4.6875± 2.47758	12.875± 2.23207	17.0± 1.13389	6.5625± 1.07529	9.1875± 2.90551
Treatment group	9.375± 2.32609	4.75± 2.25198	11.75± 1.51186	15.25± 2.49285	8.75± 2.22004	6.5625± 1.56838	10.1563± 2.90608	14.750± 1.14953
p-value	0.000	0.000	0.003	0.000	0.001	0.000	0.014	0.000

Note: ANOVA results shown in the mean ± SD column indicate a significant difference (*p-value* <0.05).

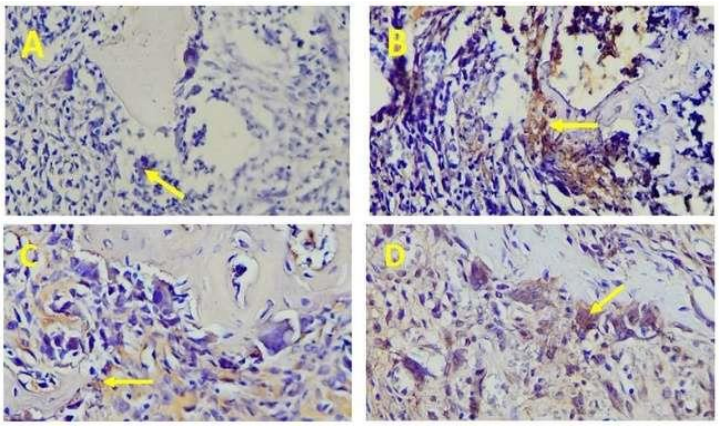

Figure 2. Immunohistochemical analysis of RANKL expression at 7 and 14 days. (A) Control group at 7 days (B) Treatment group at 7 days (C) Control group at 14 days (D) Treatment group at 14 days. The arrows indicate the RANKL expression (purplish brown).

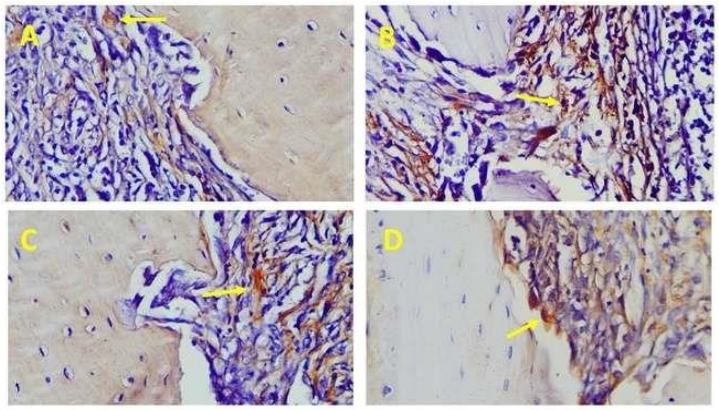

Figure 3. Immunohistochemical analysis of OPG expression at 7 and 14 days. (A) Control group at 7 days (B) Treatment group at 7 days (C) Control group at 14 days (D) Treatment group at 14 days. The arrows indicate the OPG expression (purplish brown).

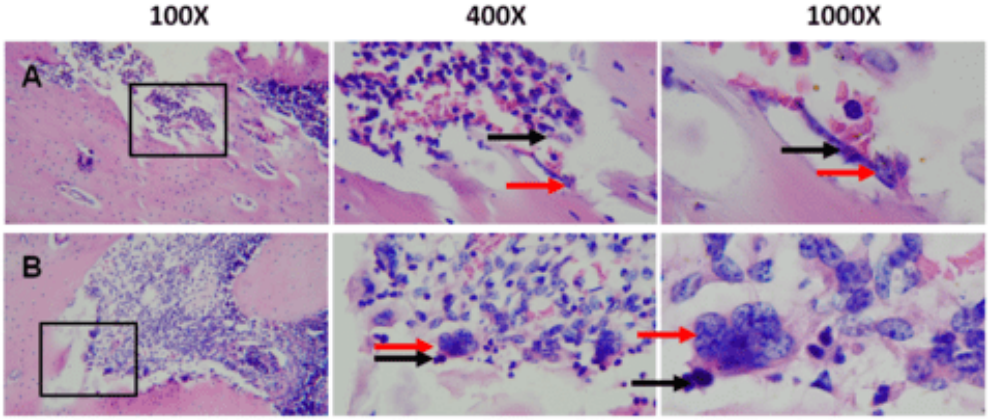

Figure 4. Immunohistochemical analysis of osteoclast (red arrow) and osteoblast (black arrow) expression at 7 days. (A) Control group (B) Treatment group

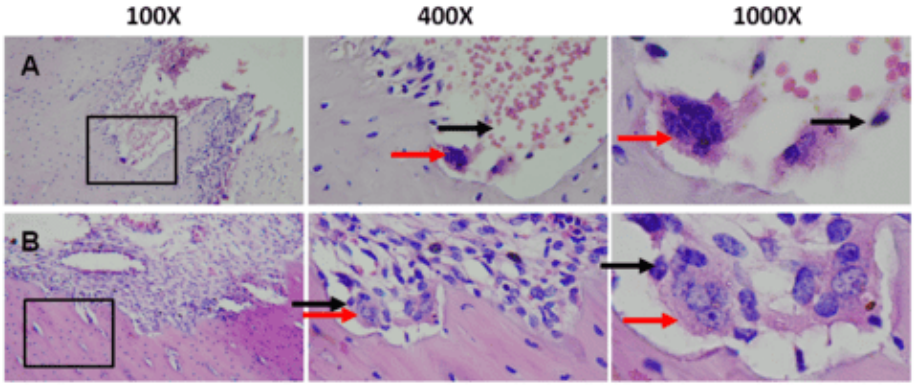

Figure 5. Immunohistochemical analysis of osteoclast (red arrow) and osteoblast (black arrow) expression at 14 days. (A) Control group (B) Treatment group

DISCUSSION:

This study was conducted on male Wistar rats (*Rattus norvegicus*) with extraction of mandibular incisors to prove the effect of type I collagen from gourami scales on OPG, RANKL, osteoblast, and osteoclast expression. Bone regeneration plays a very important role in a number of periodontal surgical procedures, such as in the remodelling bone defects of various causes. Bone regeneration need osteoprogenitor cells, signaling molecules, and scaffolds to trigger and regulate osteogenic differentiation so that new bone is eventually formed[2,24,25]. Collagen derived from mammals has been used as a biomass scaffold and various studies have shown that collagen can induce osteoblast cell proliferation[26,27,28].

In a normal bone homeostasis there is important things to maintenance the balancing between osteoblastic bone formation and osteoclastic bone resorption[22]. This concept is further clarified by the existence of molecular mechanisms regarding the identification of the RANK, RANKL, and OPG-pathway system[23]. The RANKL, RANK, and OPG binding pathway is a signaling system for communication between immune and bone cells[21].

In the present study, the use of collagen from fish scales applied to rat tooth sockets evaluated at 7 and 14 days showed a significantly higher average OPG expression at 7 and 14 days compared to mean RANKL expression at 7 and 14 days. The increase in OPG expression and the decrease in RANKL expression at 7 and 14 days are in line with research conducted by Naghsh *et al*. (2016), in which this mechanism plays an important role in bone remodeling, especially in the relationship between OPG, RANK, and RANKL. OPG as soluble glycoprotein is a tumor necrosis factor alpha that interacts with target cells by binding to RANKL and blocking RANK-RANKL interactions, thereby inhibiting osteoclastogenesis[29]. This also supports the findings by Belibasakis and Bostanci (2012), according to which OPG and RANKL are inversely proportional, i.e., if OPG expression is high, RANKL expression will decrease[30].

The application of gourami scales also decreases RANKL and osteoclast expression and increases OPG and osteoblast expression. This can be seen in the results for RANKL and osteoclast expression in control group, which increased at 14 days compared to 7 days, whereas RANKL and osteoclast expression significantly decreased in the treatment group at 14 days when compared with 7 days. This causes resorption to end and continue with bone apposition in a series of alveolar bone remodeling processes. An increase in OPG expression would theoretically reduce the possibility of interaction between RANKL and RANK, thereby preventing the possibility of osteoclastogenesis activation during bone formation and the remodeling process[31]. These findings are also supported by Boyce & Xing (2009) and Maxhimer et al. (2015), who showed that OPG expression increased and RANKL expression decreased and that bone remodeling stopped at the resorption stage and continued to the stage of bone apposition during the bone remodeling process. Bone remodeling is regulated by a system that involves a balance between resorption by osteoclasts and formation of new bone tissue by osteoblasts. The RANK/RANKL /OPG signaling pathway can explain this balance between bone resorption by osteoclasts and bone formation by osteoblasts. The binding of RANKL and RANK, produces in fusion, differentiation, and osteoclast activation in osteoclastogenesis. OPG utilities as a feed receptor for RANKL, preventing RANKL from binding to RANK. Therefore, OPG is an important factor against bone resorption[32,33]. Over the past few decades, research into the RANKL/RANK/OPG system confirms it is the primary regulator of bone resorption. Osteoblasts regulate osteoclastogenesis through the expression of OPG and RANKL. OPG expression in osteoblasts is regulated by certain hormones, cytokines, and the Wnt / β-catenin pathway[34]. The increase in OPG expression aims to inhibit the binding of RANK and RANKL, thereby causing a decrease in RANKL expression. The

expression of OPG and RANKL is modulated by proresorptive cytokines (TNF-α, TGF-β, and IL-1), parathyroid hormone (PTH), 25-dihydroxyvitamin (D3), and glucocorticoid hormones so that the effects of RANKL are blocked during osteoclastogenesis[35].

CONCLUSION:

Collagen from extract gourami scales has been shown to increase OPG and osteoblast expression and to suppress RANKL and osteoclast expression, which are important for osteoblastogenesis.

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CONFLICT OF INTERESTS:

The authors declare that they have no conflict of interests.

REFERENCES:

1. Ajay M, Negi KS, Saroj T, Kanwarjeet AS. A successfully treated case of severe periodontitis using interdisciplinary approach: Report of a case. Journal of Indian Society of Periodontology. 2016; 20(1):95-97. https://doi.org/10.4103/0972-124X.168496.
2. Keestra JAJ, Barry O, Jong LD, Wahl G. Long-term effects of vertical bone augmentation : a systematic review. J Appl Oral Sci. 2016; 24(1): 3–17. http://dx.doi.org/10.1590/1678-775720150357.
3. Kandwal A, Bhardwaj J, Sunny, Batra M. Bone Grafts In Periodontal Surgery . A Review. Journal of Dental Herald. 2014; 1(3):30–32.
4. Sakat BT, Sakhare RB, Suryvanshi UC, Kore PS, Mohite SK, Magdum CS. Osteoporosis: The Brittle Bone. Asian Journal Pharmaceutical Research.2018; 8(1):39-43. doi: 10.5958/2231-5691.2018.00008.4.
5. Shailaja KS, Saraswati P. Collagen Vascular Disease-A Review. International Journal of Advances in Nursing Management 3(4): Oct.-Dec. 2015; Page 382-384. doi: 10.5958/2454-2652.2015.00040.2
6. Shetty P, Chacko N, Alva A, Kumar V, Kandige PS, Gururaj MP, Joshi H, D'souza UP. Wound Healing Potential of Psidium guajava var, Pyrifera. Research Journal of Pharmacy and Technology. 2019; 12(12): 6067-6070. doi: 10.5958/0974-360X.2019.01053.9
7. Ostrowski MC. A New Role for OPG: Putting RANKL in Its Place. Journal of Bone Mineral Research. 2010; 25(9):1905-6. doi: 10.1002/jbmr.206.
8. Kalaivani C, Kuppusamy G, Saikamal, Karri VVSR. Simvastatin Loaded Polycaprolactone-collagen Scaffolds dor the treatment of Diabetic Foot Ulcer. . Research Journal of Pharmacy and Technology. 2019; 12(6): 2637-2644. doi: 10.5958/0974-360X.2019.00441.4.
9. Yamada S, Yoshizawa Y, Kawakubo A, Ikeda T, Yanagiguchi K, Hayashi Y. Early gene and protein expression associated with osteoblast differentiation in response to fish collagen peptides powder. Dental Materials Journal. 2013; 32(2):233–40. https://doi.org/10.4012/dmj.2012-188
10. Yamada S, Yamamoto K, Ikeda T, Yanagiguchi K, Hayashi Y. Potency of Fish Collagen as a Scaffold for Regenerative Medicine. BioMed Research International. 2014; 2014(3):1–8. http://dx.doi.org/10.1155/2014/302932.
11. Rachmasari A, Kartini RS, Alviani G, Solihah I. Healing effect of Spray gel collagen extract from Channa striata bone on burn in rats. Research Journal of Pharmaceutical Dosage Forms and Technology. 2019; 11(4):275-279. doi: 10.5958/0975-4377.2019.00046.6.
12. Zulkifeli NRAN, Zain HHM, Zainol I, Musa NHC. The properties of Hydrolysed Collagen from Oreochromis mossambicus's scale and their effect towards Cell viability. Research Journal of Pharmacy and Technology.2020; 13(12): 5855-5860. doi: 10.5958/0974-360x.2020.01020.3.
13. Zahrani RA. Extraction and Isolation of Collagen Type I from Fish Skin. New Zealand: University of Otago, Dunedin; 2010. Available from: https://www.semanticscholar.org/paper/Extraction-and-Isolation-of-Collagen-Type-I-from-Zahrani-Chen/fc1e3e67fad3aa99fb1cbe8dc35c4a92ab3f62ea
14. Nagai T, Izumi M, Ishii M. Fish scale collagen. Preparation and partial characterization. International Journal of Food Science and Technology. 2014;39(3): 239–244. doi: 10.1111/j.1365-2621.2004.00777.x.
15. Oliviya CR. Production of ecofriendly silver nanoparticle and evaluation of its potential antimicrobial activity. Research Journal of Pharmacy and Technology. 2015; 8(10):1374-1378. doi: 10.5958/0974-360X.2015.00246.2.
16. Prahasanti C, Wulandari DT, Ulfa N. Viability test of fish scale collagen (*Oshpronemus gouramy*) on baby hamster kidney fibroblasts-21 fibroblast cell culture. Veterinary World. 2018; 11(4):506–10.
17. Ridhanya RK. Skin Wound Healing: An update on the Current knowledge and Concepts. Research Journal of Pharmacy and Technology. 2019; 12(3): 1448-1452. doi: 10.5958/0974-360X.2019.00240.3
18. Arif R, Jaffat HS. Assessment of Procollagen 111N Terminal Peptide Level in Rats Undergo CCl4 and Bile Duct Ligation. Research Journal of Pharmacy and Technology. 2017; 10(7): 2132-2135. doi: 10.5958/0974-360X.2017.00374.2
19. Yani S, Soeharto S, Sumarno, Kalim H. The Effect of Eleutherine Americana Merr. Extract on Expression Changes of MMP-8 and Type 1 Collagen in Periodontitis Rat Models. Research Journal of Pharmacy and Technology. 2020; 13(5): 2407-2412. doi: 10.5958/0974-360X.2020.00432.1
20. Shi S, Kirk M, Kahn AJ. The Role of Type I Collagen in the Regulation of the Osteoblast Phenotype. Journal of Bone and Mineral Research. 2009; 11(8):1139–1145. https://doi.org/10.1002/jbmr.5650110813
21. Kasagi S, Chen W. TGF-beta1 on osteoimmunology and the bone component cells. Cell and Bioscience. 2013; 3(4):1-7. doi: 10.1186/2045-3701-3-4
22. Kim JH, Kim N. Signaling Pathways in Osteoclast Differentiation. Chonnam Medical Journal. 2016;52;12-17. http://dx.doi.org/10.4068/cmj.2016.52.1.12
23. Neve A, Corrado A, Cantatore FP. Osteoblast physiology in normal and pathological conditions. Cell and Tissue Research. 2011;343(2): 289–302. doi: 10.1007/s00441-010-1086-1
24. Newman MG, Takei HH, Klokkevold PR, Carranza FA. *Carranza's* Clinical Periodontology. 12th ed. ELSEVIER: Saunder; 2015.
25. Liu C, Sun J. Hydrolyzed Tilapia Fish Collagen Induces Osteogenic Differentiation of Human Periodontal Ligament Cell. Biomedical Materials. 2015; 10(6):1-9. doi:10.1088/1748-6041/10/6/065020
26. Bareil RP, Gauvin R, Berthod F. Collagen-Based Biomaterials for Tissue Engineering Applications. Materials. 2010; 3:1863–1887. https://doi.org/10.3390/ma3031863
27. Tang J, Saito T. Biocompatibility of Novel Type I Collagen Purified from Tilapia Fish Scale : An In Vitro Comparative Study. BioMed Research International. 2015; 2015:1–8. doi: 10.1155/2015/139476
28. Capati MLF, Nakazono A, Yamamoto K, Sugimoto K, Yanagiguchi K, Yamada S, Hayashi Y. Fish Collagen Promotes the Expression of Genes Related to Osteoblastic Activity. International Journal of Polymer Science. 2016; 2016:1–8. http://dx.doi.org/10.1155/2016/5785819
29. Teitelbaum SL, Amer YA, Ross FP. Molecular Mechanisms of Bone Resorption. J Cell Biochem. 1995; 59(1):1-10. doi: 10.1002/jcb.240590102
30. Naghsh N, Razavi SM, Minaiyan M, Shahabooei M, Birang R, Behfarnia P, Hajisadeghi S. Evaluation of the effects of two different bone resorption inhibitors on osteoclast numbers and activity: An animal study. Dental Research Journal. 2016; 13(6):500–507. https://doi.org/10.4103/1735-3327.197034
31. Delgado-Calle J, Sañudo C, Sumillera M, Garcés CM, Riancho JA. Expression of RANKL and OPG in primary osteoblasts. Rev Osteoporos Metab Miner. 2012; *4*(4):133–138.
32. Boyce BF, Li J, Xing L, Yao Z. Bone Remodeling and the Role of TRAF3 in Osteoclastic Bone Resorption. Frontiers in Immunology. 2018;8:1-12. doi: 10.3389/fimmu.2018.02263
33. Maxhimer JB, Bradley JP, Lee JC. Signaling pathways in osteogenesis and osteoclastogenesis: Lessons from cranial sutures and applications to regenerative medicine. Genes and Diseases. 2015; 2(1):57–68. https://doi.org/10.1016/j.gendis.2014.12.004
34. Liu W, Zhang X. Receptor activator of nuclear factor - κ B ligand (RANKL)/ RANK / osteoprotegerin system in bone and other tissues (Review). Molecular Medicine Reports. 2015;11:3212-3218. https://doi.org/10.3892/mmr.2015.3152
35. Khosla S. Minireview: The OPG/RANKL/RANK System. Endocrinology. 2001; 142(12):5050-5055. https://doi.org/10.1210/endo.142.12.8536