



# Osteoclast activity in osteoporosis mandibular bone based on RANKL and osteoprotegerin ratio

[Actividad de los osteoclastos en el hueso de la mandíbula con osteoporosis basada en la relación RANKL y osteoprotegerina]

Mefina Kuntjoro<sup>1,2</sup>, Nike Hendrijantini<sup>2\*</sup>, Fedik Abdul Rantam<sup>3</sup>, Guang Hong<sup>4</sup>, Sherman Salim<sup>2</sup>, Muhammad Dimas Aditya Ari<sup>2</sup>

<sup>1</sup>Doctoral Program, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

<sup>2</sup>Department of Prosthodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

<sup>3</sup>Laboratory of Virology and Immunology, Department of Microbiology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

<sup>4</sup>Liaison Center for Innovative Dentistry, Graduate School of Dentistry, Tohoku University, Aoba-Ku, Sendai, Japan.

\*E-mail: [nike-h@fg.unair.ac.id](mailto:nike-h@fg.unair.ac.id)

## Abstract

**Context:** Osteoporosis occurs not only in the lumbar bone, femur, and radius but also occurs in the mandibular bone. Therefore, osteoporosis is a disease that required attention from the dentists.

**Aims:** To determine the ratio of Receptor Activator of Nuclear Factor  $\kappa$ B-Ligand:Osteoprotegerin (RANKL:OPG) expression and the ratio of osteoclasts:osteoblasts number in the mandibular bone of the osteoporosis rat models.

**Methods:** The research subjects used were 20 *Rattus norvegicus*, which were randomly divided into two groups, namely ovariectomy (OVX) and sham surgery (SHS). Twelve weeks after surgery, the animals were terminated and mandibular bone specimens were taken for histological examination of osteoclasts, osteoblasts, RANKL, and OPG expression. An examination of estrogen levels in the blood of the research subjects was also carried out. All data obtained were tested statistically using a t-test.

**Results:** There was a decrease in the amount of estrogen in the OVX group rather than SHS ( $p = 0.005$ ). In the SHS group, the RANKL:OPG expression value ratio was  $0.51 \pm 0.15$ , while in the OVX group was  $0.86 \pm 0.22$  ( $p = 0.001$ ). In the SHS group, the ratio of osteoclasts:osteoblasts number was  $0.012 \pm 0.004$ , while in the OVX group was  $0.061 \pm 0.023$  ( $p = 0.001$ ).

**Conclusions:** A decrease in estrogen significantly induces the ratio of RANKL:OPG and osteoclast:osteoblasts in the mandibular bone.

**Keywords:** estrogen; medicine; osteoblast; osteoclast; osteoprotegerin; RANKL.

## Resumen

**Contexto:** La osteoporosis ocurre no solo en el hueso lumbar, fémur y radio, también ocurre en el hueso mandibular, por lo que la osteoporosis es una enfermedad que requiere atención por parte de los dentistas.

**Objetivos:** Determinar la proporción de expresión del Receptor Activador del Factor Nuclear  $\kappa$ B-Ligando:Osteoprotegerina (RANKL:OPG) y la proporción de osteoclastos:número de osteoblastos en el hueso mandibular de los modelos de rata con osteoporosis.

**Métodos:** Los sujetos de investigación utilizados fueron 20 *Rattus norvegicus*, que se dividieron aleatoriamente en dos grupos, a saber, ovariectomía (OVX) y cirugía simulada (SHS). Doce semanas después de la cirugía, se sacrificaron los animales y se tomaron muestras de hueso mandibular para el examen histológico de osteoclastos, osteoblastos, expresión de RANKL y OPG. También se llevó a cabo un examen de los niveles de estrógeno en la sangre de los sujetos de investigación. Todos los datos obtenidos se testaron estadísticamente mediante una prueba t.

**Resultados:** Hubo una disminución en la cantidad de estrógeno en el grupo OVX en lugar de SHS ( $p = 0,005$ ). En el grupo SHS, la relación de valor de expresión de RANKL:OPG fue de  $0,51 \pm 0,15$ , mientras que en el grupo de OVX fue de  $0,86 \pm 0,22$  ( $p = 0,001$ ). En el grupo de SHS, la relación de osteoclastos:número de osteoblastos fue de  $0,012 \pm 0,004$ , mientras que en el grupo de OVX fue de  $0,061 \pm 0,023$  ( $p = 0,001$ ).

**Conclusiones:** Una disminución de estrógenos induce significativamente la relación RANKL OPG y osteoclastos:osteoblastos en el hueso mandibular.

**Palabras Clave:** estrógeno; medicina; osteoblasto; osteoclasto; osteoprotegerina; RANKL.

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## AUTHOR INFO

ORCID: 0000-0002-9261-3626 (NH)



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## INTRODUCTION

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The occurrence of osteoporosis often complicates and causes failure in cases of dentures treatment. Mandibular bone resorption is kind of a serious problem for dentists, principally when diagnosing jawbone density, especially in postmenopausal women, before taking prosthodontic treatment because it can affect the support, retention, stability, and mastication functions of dentures users (Marcu et al., 2011).

Bone metabolism involves many factors; however, estrogen is one of the potential factors in regulating the bone mass of women as well as men (Manolagas et al., 2002). For women who experience menopause, a decrease in bone mineral density occurred and can cause the risk of losing teeth and alveolar bone (Johnson, 2003). Osteoporosis occurs not only in the lumbar bone, femur, and radius but also occurs in the mandibular because osteoporosis is a disease that required attention from dentists because osteoporosis occurs in the mandibular bone (Schorge et al., 2020). Osteoporosis also needs extra attention from dentists. Osteoporosis in the jawbone causes a reduction of bone density, increased degree of alveolar bone resorption, associated with increased risk of periodontal disease, and increased tooth loss. In patients with osteoporosis, there is a 50% decrease in trabecular bone and cortical bone up to 35% (Clowes et al., 2005).

Bone is a dynamic structure and undergoes a continuous regeneration process called the remodeling process (Robling et al., 2006). Bone remodeling depends on the balance between bone resorption by osteoclasts and bone deposition by osteoblasts (Khrisnan and Davidovitch, 2006). The imbalance of the remodeling process may cause bone disorders, one of which is osteoporosis. The ratio of osteoclasts and osteoblasts can describe the dominant number of osteoclasts as cells that play a role in the process of bone resorption against the number of osteoblasts as cells that play a role in bone formation (Hikmah et al., 2016).

The mechanism for abnormal bone mineral metabolism is still unknown, but three proteins are known to be involved in the process of regulating bone regeneration, those proteins are Receptor Activator of Nuclear *Kappa* B (RANK), Receptor Activator of Nuclear *Kappa* B ligand (RANKL) and osteoprotegerin (OPG). These three proteins are part of the superfamily of tumor necrosis factor receptors that function as a regulator of bone remodeling. RANKL is the key mediator in the process of osteoclast formation, whereas OPG is a natural inhibitor of RANKL, which leads to osteoblasts formation (Rouster-Stevens et al., 2007). The research to determine the pattern of bone turnover in osteoporosis has been widely studied (Naylor and Eastell, 2012; Shetty et al., 2016; Eastell and Szulc, 2017); however, research on the jawbone has yet to be found. Osteoporosis can induce osteoclast activity through several cell signaling regulations, including the balance between RANKL and OPG (McClung, 2007). However, in the mandible, the expression patterns of RANKL and OPG have not been fully studied. This study aims to determine the ratio of RANKL:OPG expression and osteoclasts:osteoblasts number in the osteoporosis mandibular bone of osteoporotic rat models.

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## MATERIAL AND METHODS

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### Chemicals and reagents

Reagents and kits used were the Mouse estrogen (E) ELISA Kit (CUSABIO®, CSB-E07280m, USA), anti-RANKL monoclonal antibodies (Abcam, ab45039), anti-OPG monoclonal antibodies (Abcam, ab124820).

### Animals and experimental design

The research type was experimental laboratory research, according to Hendrijantini et al. (2019). This research was conducted after being approved by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary, Universitas Airlangga (Number 397-KE). The research subjects used were 20 Wistar rats (*Rattus norvegicus*) with criteria of female, 200 g weight, 12 weeks of age, and healthy

according to the criteria of the experimental animal. All the subjects were then randomly divided into two different groups: a group with 10 ovariectomy rats (OVX) and a group with 10 sham surgery rats (SHS).

All subjects were fasted for 6-8 hours before surgery and then anesthetized using ketamine of 0.2 mL (50 mg/mL). Ovariectomy was performed by making a ventral incision from the umbilicus to the pubis, then the ovarian blood vessels and fallopian tubes were ligated separately. Bilateral retrieval of the ovary was then followed by closing the peritoneal incision with a simple interrupted suture technique. The control group was performed sham surgery, where after ventral incisions from the umbilicus to the pubis, bilateral ovaries were removed and returned to their previous positions, which were then continued by closing the peritoneal incision with a simple interrupted suture technique (Hendrijantini et al., 2019).

Twelve weeks after termination of Wistar rat ovariectomy, 0.2 mL of blood taken from the heart was taken to measure estrogen levels using ELISA (CUSABIO®, CSB-E07280m, USA). This measurement was performed as confirmation of a decrease in estrogen in the animal models. The mandibular bone dissection was performed for histological analysis. The specimens obtained were then decalcified and embedded for immunohistochemical examination. The samples were then stained using anti-RANKL monoclonal antibodies (Abcam, ab45039) and anti-OPG monoclonal antibodies (Abcam, ab124820). For osteoclasts and osteoblasts number, hematoxylin-eosin staining was performed. Examination of osteoblasts and osteoclasts number was carried out on an ordinary light microscope (Nikon H600L) with 400× magnification equipped with 300-megapixel DS camera and Nikon Image System image processing software, while RANKL and OPG examination were performed using the same microscope with 1000× magnification.

### Statistical analysis

After obtaining the calculation results of each RANKL and OPG expression and the number of osteoblasts and osteoclasts, then the ratio of

RANKL:OPG and osteoclasts:osteoblasts were calculated. All research data were performed in mean values and standard deviation. Statistical analysis was performed using SPSS version 23 (SPSS Inc., Chicago). A normality test with Shapiro-Wilk was carried out to determine the normal distribution of the research data, then proceed using the independent t-test statistical test. The data were considered as statistically significant at  $p < 0.05$ .

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## RESULTS

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### Blood estrogen levels

The results of samples of blood estrogen confirmation indicated that there was a decrease in estrogen levels of the OVX group compared to the SHS group (Table 1). The ELISA kit was used for this measurement and presented in Fig. 1. A higher level of blood estrogen was found in the SHS group with the mean value of  $84.73 \pm 16.84$  mg, while in the OVX group, the mean value of blood estrogen was  $47.07 \pm 6.35$  mg. Statistical test using Shapiro Wilk showed that the data were normally distributed ( $p > 0.05$ ). The t-test results showed a significant decrease in blood estrogen levels in the OVX group compared to the SHS group ( $p < 0.05$ ).

### The ratio of RANKL:OPG

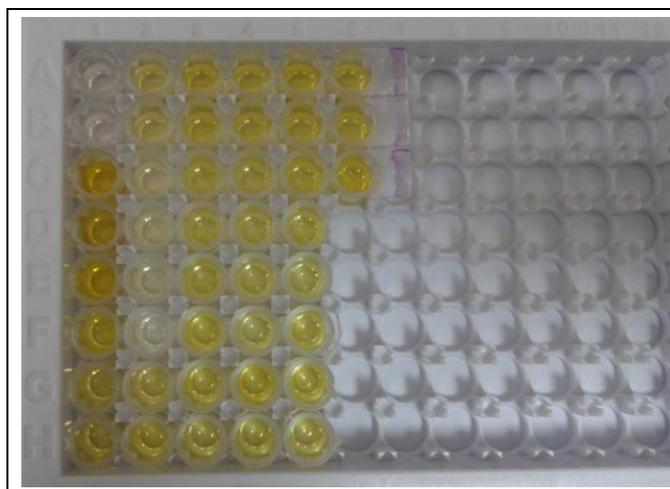
The higher RANKL:OPG expression ratio was found in the OVX group with the mean value of  $0.86 \pm 0.22$ , while in the SHS group was  $0.51 \pm 0.15$  (Table 2). Statistical test using Shapiro Wilk showed that the data were normally distributed ( $p > 0.05$ ). The test then continued using a t-test to find out differences between groups. The t-test results showed a significant increase in RANKL:OPG expression ratio in the OVX group compared to the SHS group ( $p < 0.05$ ). The microscopic view of RANKL and OPG can be seen in Figs. 2 and 3.

### The ratio of osteoclasts:osteoblasts

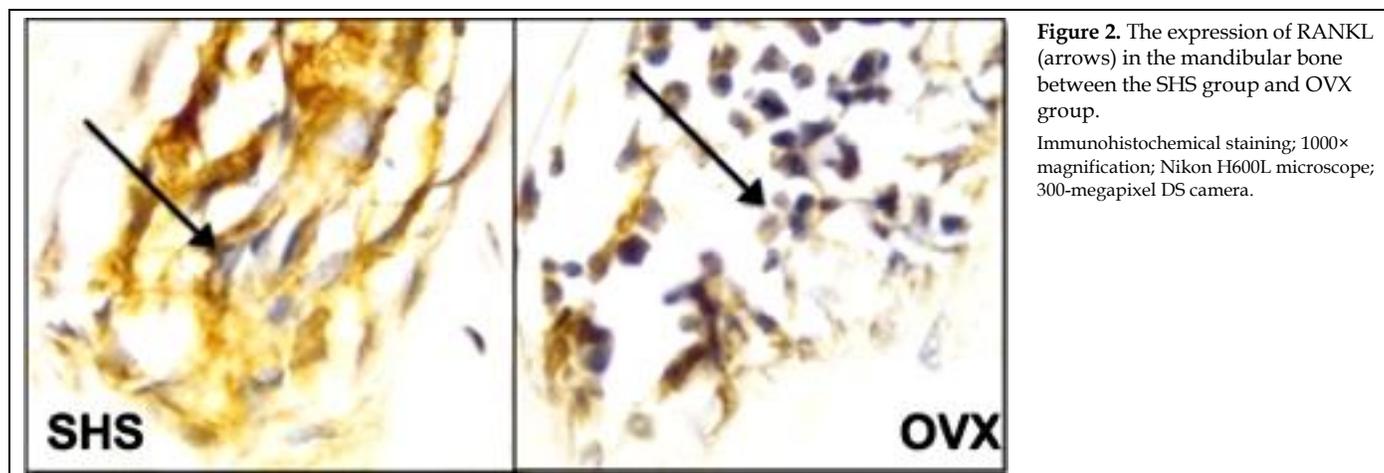
The higher number of osteoclast:osteoblast ratio was found in the OVX group with the mean value of  $0.061 \pm 0.023$ , while in the SHS group was  $0.012 \pm 0.004$  (Table 3). Based on the normality test, the data has a  $p > 0.05$ , which indicated that the data

was normally distributed. The test then continued using a t-test to find out differences between groups. The t-test results showed a significant increase in the number of osteoclast:osteoblast ratio

in the OVX group compared to the SHS group ( $p < 0.05$ ). The histological view of osteoclast and osteoblast can be seen in Fig. 4.



**Figure 1.** A 96-well plate showed a measurement for estrogen level using ELISA.



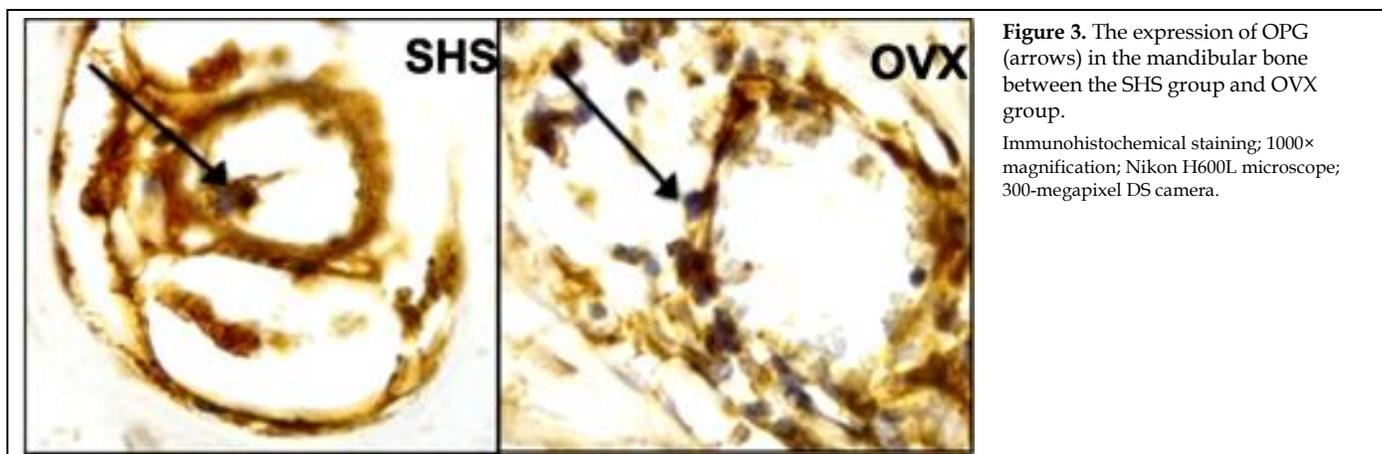
**Figure 2.** The expression of RANKL (arrows) in the mandibular bone between the SHS group and OVX group.

Immunohistochemical staining; 1000× magnification; Nikon H600L microscope; 300-megapixel DS camera.

**Table 1.** The level of estrogen in blood samples.

Group	Mean value	Standard deviation	P-value
OVX	47.07	6.35	0.005*
SHS	84.73	16.84	

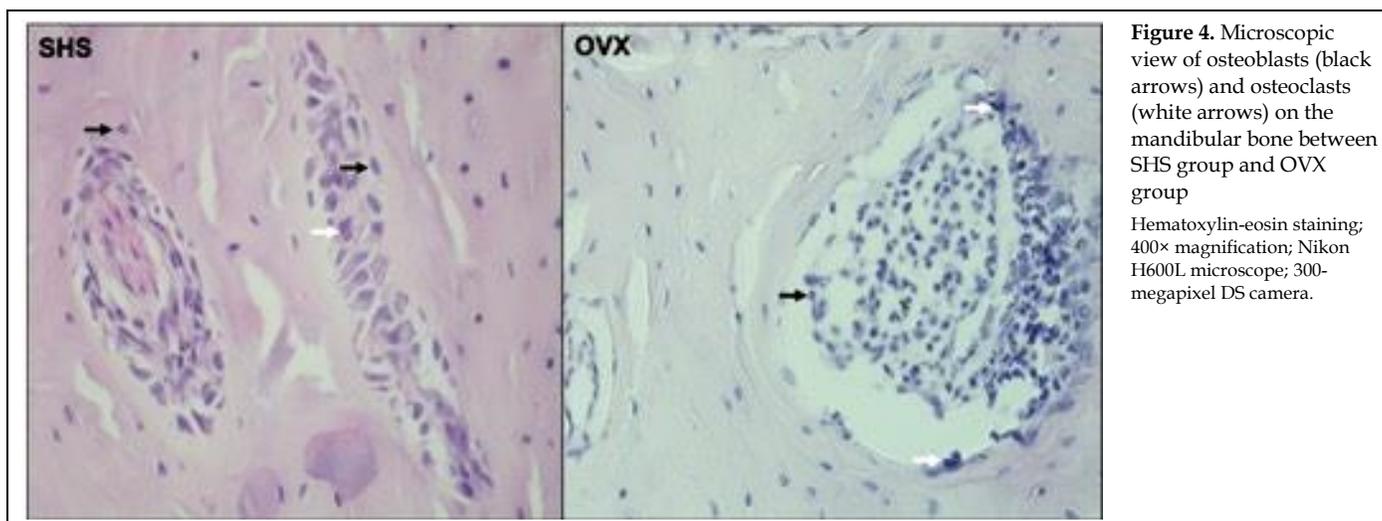
\*Indicated there was a significant difference according to the independent t-test ( $p < 0.05$ ).



**Table 2.** The mean value and standard deviation of RANKL:OPG ratio.

Group	Mean value	Standard deviation	P-value
OVX	0.86	0.22	0.001*
SHS	0.51	0.15	

\*Indicated there was a significant difference according to the independent t-test ( $p < 0.05$ ).



**Table 3.** The mean value and standard deviation of osteoclasts:osteoblasts ratio.

Group	Mean value	Standard deviation	P-value
OVX	0.061	0.023	0.001*
SHS	0.012	0.004	

\*Indicated there was a significant difference according to the independent t-test ( $p < 0.05$ ).

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## DISCUSSION

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Osteoporosis is a health problem for women when experiencing a menopause phase and affects the lives of postmenopausal women. Assessment of bone density is an important part of routine care in postmenopausal women. Osteoporosis is a bone disease characterized by a decrease in bone mass and a high risk of fracture (Susanto, 2016). Several studies have shown osteoporosis can occur after a 12-week period. This is due to the disruption of the migration capacity of Mesenchymal Stem Cell (MSC) to the place where bone damage occurs (Jee and Yao, 2001; Goergen et al., 2013). Jee and Yao (2001) study stated that there was a significant difference in the loss of vertebral trabecular bone mass and its strength after 12 weeks of ovariectomy in Wistar rats. Systemically, estrogen is a major factor that plays a role in bone metabolism by inhibiting osteoclast activity and stimulating osteoblasts. The decreasing estrogen hormone in osteoporosis conditions will affect bone metabolism (Streicher et al., 2017).

RANKL is the key mediator in the process of osteoclast formation. RANKL is expressed by osteoblasts. The binding of RANKL to the RANK on the surface of the pre-osteoblasts causes activation of jun terminal kinase and activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), which leads to osteoclast formation. OPG is the natural inhibitor of RANKL (Rouster-Stevens, 2007). A study suggests that estrogen can regulate bone metabolism primarily by targeting RANKL expression in the remodeling process. This study suggested that estrogen deficiency can selectively increase RANKL expression in bone lining cells. Bone cells can function as cellular integrators of hormonal signals from the extracellular environment and from mechanical signals originating from bone tissue through the RANKL communication mechanism (Streicher et al., 2017).

OPG is a molecule that resembles the tumor necrosis factor receptor, which acts as a binder and inhibits RANKL bond to RANK can prevent osteoclastogenesis. OPG is an inhibiting factor for osteoclastogenesis secreted by osteoblast progenitors. OPG will then bind to RANKL to inhibit osteoclas-

togenesis (Rouster-Stevens, 2007). The expression of OPG in osteoblasts is regulated by many factors, one of which is the number of hormones, cytokines, and growth factors such as estrogen and Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) (Chen et al., 2018).

Estrogen deficiency in osteoporosis caused an increase in bone remodeling with increased bone resorption over its formation against bone micro-architecture (Marie, 2010; Hendrijantini et al., 2019). Directly, estrogen deficiency can affect the quality of OPG. In detecting the occurrence of bone resorption, the molecular aspect that can be seen is the expression of RANKL and OPG. According to research conducted by Li et al. (2015), osteoporosis can increase the level of RANKL mRNA and decrease the level of OPG mRNA in the tibia. This is also in line with the results obtained in this study conducted on the mandibular bone.

The results of this study proved that estrogen deficiency could cause increased osteoclast activity in bone by expression of RANKL and OPG. The low level of estrogen induces increased production of interleukin 1, interleukin 6, and TNF- $\alpha$ , which will further cause the formation of M-CSF and RANKL. The activation of this pathway can be through various mechanisms of action, one of which is the activation of the pro-osteoclast's cytokine pathway through NF- $\kappa$ B (Khalid and Krum, 2016). Research conducted by Xiong et al. (2015) also shows the activity of osteoclastogenesis associated with estrogen. Proper estrogen regulation can regulate osteoclast activity through the activity of differentiation, apoptosis, and cell adhesion.

This study proves that there was a progressive activity of osteoclasts in the mandibular bone, which ultimately leads to poor bone quality at a low level of estrogen. It can be the rationale for conducting further therapy on the mandible so that a prominent alveolar bone can be obtained which can support prosthetic treatment.

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## CONCLUSIONS

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The decreases in estrogen levels significantly induce osteoclast activity. An increase in the ratio

of RANKL:OPG and osteoclasts:osteoblasts at low estrogen levels prove the existence of induction of the osteoclastogenesis pathway.

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## CONFLICT OF INTEREST

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The authors declare no conflicts of interests.

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**AUTHOR CONTRIBUTION:**

Contribution	Kuntjoro M	Hendrijantini N	Rantam FA	Hong G	Salim S	Ari MDA
Concepts or ideas	x	x				
Design	x	x				
Definition of intellectual content	x	x	x			x
Literature search	x	x	x	x	x	x
Experimental studies	x	x			x	x
Data acquisition	x	x	x	x	x	x
Data analysis	x	x		x	x	x
Statistical analysis	x	x			x	x
Manuscript preparation	x	x	x			x
Manuscript editing	x	x	x			x
Manuscript review	x	x	x	x	x	x

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