

EFFECTIVENESS OF LUMBRICUS RUBELLUS EARTHWORM EXTRACT AGAINST THE NUMBER OF OSTEOCLASTS IN WISTAR PERIODONTITIS RAT

IGA Ayu Dharmawati

Health Ministry Polytechnic Denpasar, Bali, Indonesia.

I Dewa Made Sukrama , I Made Bakta

Faculty of Medicine, Udayana University, Bali, Indonesia

Ida Bagus Putra Manuaba

Analitic Laboratory, Udayana University, Bali, Indonesia

Hasanuddin Thahir

Departement of Periodontology, Faculty of Dentistry, Hasanuddin University, Makasar, Indonesia

I Nyoman Mantik Astawa

Faculty of Veterinary, Udayana University, Bali, Indonesia

Ni LuhKartini

Faculty of Agrothecnology, Udayana University, Bali, Indonesia

Background/Objective(s): *Chronic periodontitis is an inflammatory tooth supporting network involving the periodontal ligament and alveolar bone by the main pathogenic bacteria Phorphyromonas gingivalis. The RANKL-RANK bond stimulates the maturation of preosteoclasts into osteoclasts, resulting in alveolar bone resorption. Lumbricus rubellus earthworm extract has activities as anti-bacterial and anti-inflammatory, which can inhibit NFkB and reduce pro-inflammatory cytokine. The aim of this study was to decide the effect of Lumbricus rubellus (EEW) earthworm extract on decreasing the number of osteoclasts.*

Materials and Methods: *Experimental study with a post test only group design. Each of the five Wistar rats in each treatment was made periodontitis by induction of P gingivalis and silk ligature bacteria. Treatment of rats: control / not given EEW (P0), oral EEW administration 200mg / kg / bb (P1), topical EEW administration of 20% (P2). Decavutaton on day 3,7,14,21 for evaluation of osteoclasts. The study was conducted at the Analytical Laboratory and the Faculty of Veterinary Medicine, Udayana University.*

Result: *Day 3 the number of osteoclasts did not differ significantly in each group ($p > 0.05$) but between the control-topical group and the oral-topical group there were significant differences ($p < 0.05$). Day 7 there were no significant differences in either group ($p > 0.05$) or between treatment groups ($p > 0.05$). Day 14 there were significant differences in each treatment group ($p < 0.05$). There were significant differences between the control-oral group and the control-topical group ($p < 0.05$), where in the oral-topical group there were no different meanings. Day 21 shows the same results as the 14th day.*

Conclusion: *Giving EEW for up to 7 days has not provided effective results for decreasing the number of osteoclasts. Giving EEW is quite effective on day 14, so also on day 21 shows the same results. The decrease in the number of osteoclasts by oral and topical administration on days 14 and 21 gave the same effect.*

Keywords: *Lumbricus rubellus extract (EEW): Oral, Topical, Osteoclast cells.*

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INTRODUCTION

Periodontal disease is the result of infection and inflammation of the gingiva and bone tissue that supports and surrounds the tooth. In the early stages, it is called gingivitis where the gingiva (gums) swells, redness and bleeds easily¹. This inflammatory process is limited to the soft tissue surrounding the teeth without bone damage. If a gingival disease is not treated as early as possible, the disease process will continue to develop affecting the alveolar bone, periodontal ligament or cementum, this condition is called periodontitis². Chronic periodontitis is an infectious disease caused by bacteria. The WHO report (2003) states that this disease has a high prevalence throughout the world. This disease is damage caused by the defense of the host (host). Pathogenic bacteria are thought to be the cause of the inflammatory response, gingival damage and periodontal tissue³. The main pathogenesis in periodontitis is *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Besides other bacteria that are often associated with the occurrence of periodontitis are *Bacteroides forsythus*, *Protella intermedia*, *Peptostreptococcus micros* and *Fusobacterium nucleatum*⁴. *Porphyromonas gingivalis* bacteria is found 85.75% in sub gingival plaque in patients with chronic periodontitis. These bacteria are non motile, asaccharolytic, Gram negative bacteria in the form of anaerobic obligate stems⁵. *Porphyromonas gingivalis* bacteria has cell wall material in the form of lipopolysaccharide (LPS). This material acts as a signal for the host to respond directly or indirectly. In the initial phase, the bacteria will release material products such as fatty acids, peptides and LPS which will diffuse into the gingival epithelial layer. Lipopolysaccharides will bind to Lipopolysaccharides Binding Proteins (LBP) and activate CD14 receptors, activate monocytes and endothelial cells through Toll like receptors (TLRs). This material will stimulate epithelial cells to produce inflammatory mediators such as interleukin-8 (IL-8), Interleukin-1 beta (IL-1 β), prostaglandin E2 (PGE2), matrix metalloproteinases (MMPs) and tumor necrosis factor alpha (TNF- α)⁶. TNF- α results in increased activity of osteoclasts which causes bone resorption. Lymphocytes release antibodies which are the host's defense mechanism but also activate osteoclasts which result in bone resorption. T lymphocytes secrete the kappa- β ligand (RANKL) receptor activator of nuclear factor which activates osteoclasts. This destructive inflammatory mediator mechanism can be inhibited by the secretion of osteoprotegerin (OPG) and tissue inhibitors of metalloproteinases (TIMPs)⁶.

The medical value of earthworms has been known for centuries. This is evident from the history of ancient southeast Asia. Earthworms are a source of protein, enzymes and physiologically active substances. Extracts taken from earthworm soft tissue can be used as a treatment for several diseases. Several studies have shown that earthworm extracts contain different macromolecules, which show various activities such as antioxidant, antibacterial, anti-inflammatory, antitumor, etc.⁷. Research Chang et al., (2011) proved that earthworm extracts stimulated the MAPK pathway in regulating MMPs activity⁸. Similarly, Azmi et al. (2014) showed that earthworm extracts could inhibit MMP1 activity in skin inflammation and photo aging⁹. Research Wang et al., (2016) proved that the extract of the earthworm *Lumbicus rubellus* can reduce the activity of MMP9¹⁰. Matrix metalloproteinase-9 is involved in tissue damage by the activation of MMP-13, causing alveolar bone resorption¹¹. Earthworm extract lumbrokinase can suppress the expression of Cox2 and inos in myocardial injury through NF κ B cascade signal activity¹². Lumbrokinase in *Lumbicus rubellus* proves that Lumbrokinase can inhibit TLR4 activation which is a product of periodontal tissue¹³. Seeing the results of this study, this study aims to determine the ability of the *Lumbicus rubellus* soil cacaing extract in suppressing osteoclast cell formation that results in resorption of alveolar bone.

METHODS AND MATERIALS

This research was conducted at the UPT Analytical Laboratory and the Faculty of Veterinary Medicine of Udayana University, this study had received ethical clearance from the Faculty of Veterinary Medicine of Udayana University. An experimental study with a randomized posttest only control group design.

Consists of three treatment groups: control (P0) no *Lumbricus rubellus* (EEW) worm extract, oral administration of EEW 200mg / kg / bb (P1), EEW topical 20% (P2). Each group consisted of five Wistar rats 8-10 weeks old, body weight 250g - 300g, decavated on day 3,7,14,21.1.

Manufacture of Wistar Periodontitis mice:

1. The rat was anesthetized by injection of ketamine Hcl i.m in the hamstrings at a dose of 0.2 ml / 250 g bb, then periodontal silk ligature was placed on the anterior mandible in the sub gingival region. Furthermore, induction of *Porphyromonas gingivalis*. *Porphyromonas gingivalis* induction with 3×10^8 Mac Farland counts, as much as 0.25ml once in the buccal region. Installation of silk ligature for 7 days, after ligature was removed, for 3 days there was no debridement, with the aim that the bacteria in plaque persist until chronic periodontitis occurs on the 11th day¹⁴.

2. Making *Lumbricus rubellus* earthworm extract: Earthworm extract is made by the method of matseration with 1kg tepund earthworm dissolved with 3500ml ethanol, stirred and soaked for 24 hours. Then filtered with Wattman paper to separate the filtrate and residue. 1500ml filtrate was obtained, then evaporated with a rotary evaporator until 15,570g of earthworm extract was obtained¹⁴.

3. Making earthworm *Lumbricus rubellus* 20% extract gel with glycerin base mixture, 0.18% methyl paraben preservative, 0.05% propyl paraben, and CMC Na 3% introductory ingredients, additional basic ingredients as glycerin 15% and aquadest 100% . Make a mixture by dissolving methyl and propyl parabens in a number of glycerin and stirring until homogeneous (Mix I). Develop CMC Na in a stirred amount of water using an overhead stirrer for 30 minutes (Mix II). Add mixture II to mixture I while stirring using an overhead stirrer until homogeneous¹⁴.

4. Oral administration of *Lumbricus rubellus* worm extract: The extract given using a syringe dose of 200 mg / kg / day was carried out by administering as much as 0.3 ml of distilled water. at 07.00-19.00wita). Giving as much as 2x a day for 14 days.

5. Topical administration of 20% *Lumbricus rubellus* earthworm extract gel which is applied with the help of a 1ml slow speed syringe tool that can be put to the bottom of the pocket as much as 0.3ml. Gifting 3 times a day at 07.00: 13.00: 19.00 Wita, for 14 days.

RESULT

Figure 1. Osteoclast cells on Day 7

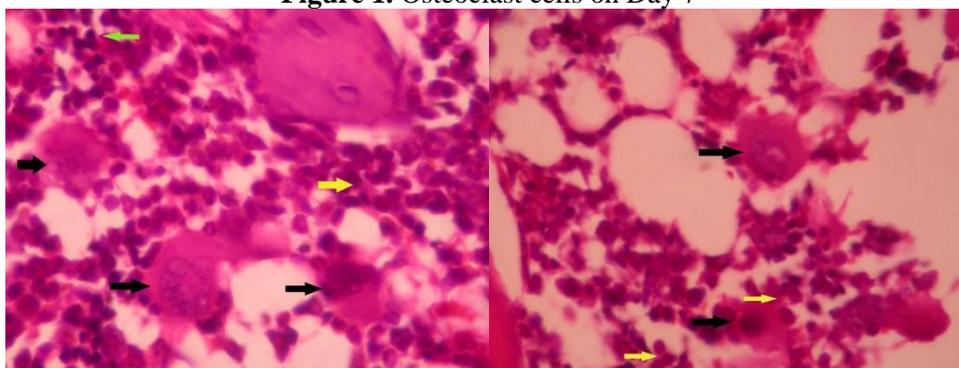


Fig.1a. Control

Fig.1b. Oral

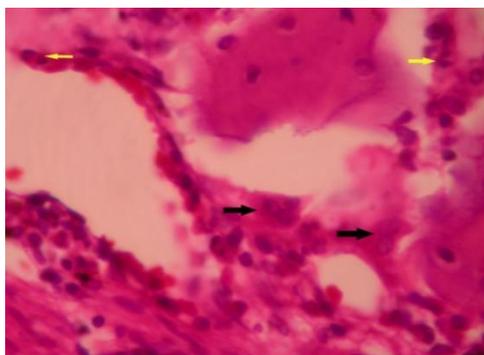


Fig. 1c Topical

Figure 2. Osteoclast cells on Day 21

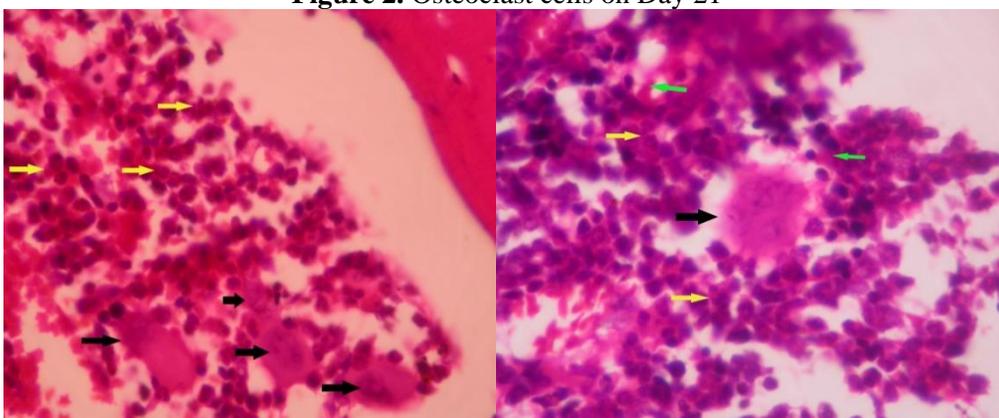


Fig. 2a Control

Fig. 2b Oral

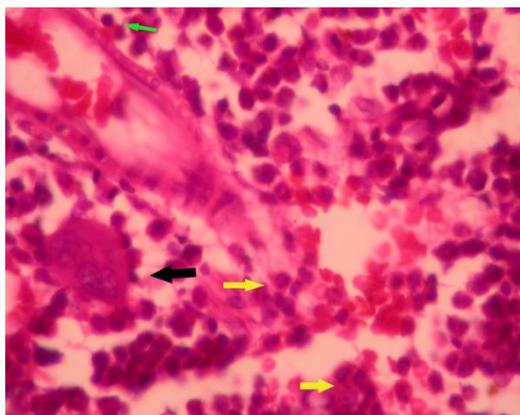


Fig. 2c Topikal

(Picture caption: Histological image with HE staining, 400x magnification. Osteoclasts are shown by black arrows.)

Table 1: The mean number of osteoclasts of the 3rd day in each treatment group.

Group	n	Mean \pm SD	p
Control	5	3 \pm 0,707	
Oral	5	3 \pm 0,707	0,071
Topikal	5	2 \pm 0,707	

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 1. The mean reduction in the number of osteoclasts of the 3rd day in each treatment group was not significant ($p > 0.05$).

Table 2: Mean differences in the number of osteoclasts of the 3rd day between treatment groups

Group	Mean diff.	<i>p</i>
Control - Oral	0,000	1,000
Control - Topical	1,000	0,045
Oral - Topical	1,000	0,045

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 2. The mean number of osteoclasts between controls and oral did not differ significantly ($p > 0.05$), whereas controls with topical and oral and topical had significant differences ($p < 0.05$).

Table 3: The mean number of osteoclasts of the 7th day in each group

Group	n	Mean±SD	<i>p</i>
Control	5	3±1,225	
Oral	5	2±0,707	0,361
Topical	5	2±1,581	

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 3. The mean number of osteoclasts of the 7th day in each treatment group was not significant ($p > 0.05$).

Table 4: Mean differences in the number of osteoclasts of the 7th day between treatment groups

Group	Mean diff.	<i>p</i>
Control - Oral	1,000	0,221
Control - Topical	1,000	0,221
Oral - Topical	0,000	1,000

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 4. Differences in mean number of osteoclasts between controls, oral and topical administration, were not significantly different ($p > 0.05$).

Table 5: The mean number of osteoclasts of the 14th day in each group

Group	n	Mean±SD	<i>p</i>
Control	5	3±1,581	
Oral	5	1±0,707	0,018
Topical	5	1±0,707	

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 5. The mean number of osteoclasts of the 14th day in each treatment group there were significant differences ($p < 0.05$).

Table 6: The difference in mean reduction in the number of osteoclasts of the 14th day between treatment groups

Group	Mean diff.	<i>p</i>
Control - Oral	2,000	0,013
Control - Topical	2,000	0,013
Oral - Topical	0,000	1,000

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 6. Difference in mean decrease in the number of osteoclasts between control and oral, control with topical there was a significant difference ($p < 0.05$), whereas between oral and topical was not significant ($p > 0.05$)

Table 7: The mean number of osteoclasts of the 21st day in each group

Group	n	Mean±SD	p
Control	5	4±0,707	
Oral	5	1±1,225	0,001
Topical	5	1±1,225	

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 7. The mean number of osteoclasts of the 21st day in each treatment group showed a significant difference ($p < 0.05$).

Table 8: Mean differences in the number of osteoclasts of the 21st day between treatment groups

Group	Mean diff.	p
Control - Oral	3,000	0,001
Control - Topical	3,000	0,001
Oral - Topical	0,000	1,000

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 8. Differences in mean number of osteoclasts of the 21st day between the control and oral groups and between controls and topicals were significant ($p < 0.05$). Between the oral and topical groups there were no significant differences ($p > 0.05$).

DISCUSSION

Induction of *P gingivalis* bacteria causes an increase in the number of osteoclast cells, through RANKL activation in osteoblasts and Matrix metalloproteinase (MMP) in fibroblasts, osteoclast formation results in bone resorption¹⁶. Table 1. The results of this study indicate that on the third day, all treatment groups had no significant difference between the control and treatment groups ($p > 0.05$). This means that the treatment given to the three groups of mice had the same effect on the number of osteoclast cells, although there were significant differences between the control group with the topical and oral with the topical (table 2) ($p > 0.05$). Table 3. The seventh day shows results that are almost the same as the third day. Where there were no significant differences in the three treatment groups, ($p > 0.05$) as well as between the control and treatment groups showed no significant results (table 4) ($p > 0.05$). The healing process is an overlapping event involving three phases: inflammation, tissue formation and tissue remodeling¹⁷. The results of this study indicate the 3rd to 7th day is an inflammatory period where platelet aggregation is followed by leukocyte infiltration to the site of inflammation resulting in an increase in proinflammatory cytokines. Cytokines involved stimulate bone resorption, including TNF- α , interleukin-1 α , interleukin-1 β , interleukin-6, interleukin-11, interleukin-15, and interleukin-17¹⁸. In the event of alveolar bone resorption osteoclastogenesis occurs, starting with the formation of a receptor activator of nuclear factor kappa beta ligand (RANKL / NF κ B Ligand) or osteoprotegerin ligand (OPGL) / osteoclast differentiation factor (ODF) or TNF related activation induced cytokine (TRANCE) which is produced by osteoblast cells or stromal cells with RANK. RANKL is expressed in two forms as an attached membrane (mRANKL) and dissolved molecule (sRANKL) which is released by TNF alpha. RANK is also known as TRANCE-R which is expressed on the surface of osteoclast progenitor cells^{19,20}. This RANKL bond - RANK will bind to the TRAF6 protein adapter (tumor necrosis factor receptor associated factor 6), and activate and translate NF κ B to the nucleus. NF κ B increases the expression of cFos and cFos interacting with NFATc 1 (Nuclear factor of activated T cells) to transcribe osteoclastogenesis genes¹⁹. Research by Yustina et al., (2012) proved that the induction of *P gingivalis* bacteria for 3 weeks showed an increase in the number of osteoclasts by 22 times compared to controls that were not induced by *P gingivalis*¹⁵. In accordance with the results of Ikeda and Takeshita's research (2016) in vivo osteoclastogenic cultures induced by M-CSF and RANKL, bone marrow macrofags (BMM) multiply and double the number of cells every 24 hours before they become pre-osteoclasts (preOCs) on day 2, which then merged with each other and eventually became multinucleated adult osteoclasts (MOC) between days 3 and 4¹⁸. This study was supported by the results of the study of Fu et al., (2014) that until day 2 extracts of earthworm no effect on the number of osteoclasts²¹.

Table 5. Showing the administration of earthworm extract (EEW) on the 14th day there were significant differences in all treatment and control groups ($p < 0.05$). Table 6. Shows that there are differences in the mean number of osteoclasts between the control group by oral administration (EEW1) and between controls and topical administration (EEW2) ($p < 0.05$). Similarly, on the 21st day there was a significant difference in the average number of osteoclasts in all groups ($p < 0.05$). This difference occurred between the control group with EEW1 and control with EEW2 ($p < 0.05$). The results of this study prove EEW can reduce the number of osteoclasts on administration until the 14th day, because of the ability of EEW to inhibit the signaling pathway of osteoclast formation through the ability of EEW that can reduce the number of osteoclasts. Research Wang et al., (2016) that the content of lumbrokinase in EEW can suppress the activity of NF κ B which is a transcriptional pathway in pro-inflammatory cytokines, the ability of lumbrokinase to suppress NF κ B activity can block the transcription of osteoclastogenesis by blocking the expression of cFos to interact with NFATc1 so osteoclastogenesis gene transcription does not occur¹¹. Research by Chauhan et al., (2018) shows that earthworm extract has an activity that can reduce the production of TNF α and increase the production of anti-inflammatory cytokines, IL-10²⁴. The ability to suppress TNF α can reduce the production of RANKL, where the formation of RANKL by activation of TNF α ²⁰. Research by Boyce and Xing (2008) shows that the RANK / RANKL / NF κ B signal is an osteoclast formation pathway¹⁷. The ability of EEW to increase Interleukin-10 as an anti-inflammatory cytokine will increase during suppression of inflammation. Research shows that IL-10 acts as a TNF α block and other pro-inflammatory cytokines, resulting in suppression of RANKL. These cytokines are secreted by monocyte, macrophage and Th2 activity^{16,24}. Another study from Fu et al., (2014) on the ability of EEW to inhibit osteoclast maturation through TRAP which is a marker of differentiation of osteoclast cells shows a decrease in TRAP activity²¹. Alveolar bone resorption in periodontitis is also influenced by MMP-9 activations by TNF α , which activates MMP-13, MMP-13 activates the preosteoclast into adult osteoclasts through the release of RANK and pro-MMP-9 secretion, inactivates galaxy 3 which is an inhibitor of osteoclastogenesis and supports the activator signal pathway into adult osteoclasts through the release of RANK and pro-MMP-9 secretion, inactivates galaxy 3 which is an inhibitor of osteoclastogenesis and supports the activator signaling pathway. RANKL is involved in alveolar bone resorption^{22,23}. The content of lumbrokinase in earthworm extracts can inhibit MMP9 expression through its antioxidant activity by inhibiting the formation of MPO, MPO is an activator to activate pro MMP9 to MMP9, so that the preosteoclast maturation pathway becomes osteoclast is inhibited^{10,11}. From the results of this study can prove that EEW can be used as a natural ingredient in periodontitis therapy which has the ability to inhibit alveolar bone resorption in chronic periodontitis.

CONCLUSION

The earthworm extract of *Lumbricus rubellus* orally administered 200mg / kg / bb or topical 20% of Wistar periodontitis rats on the 3rd and 7th day has not had an effect on the number of osteoclasts. Whereas on the 14th and 21st days it has the same effect on decreasing the number of osteoclasts. This finding could be an alternative material for periodontitis therapy both orally and topically to inhibit the formation of osteoclasts thus inhibiting alveolar bone resorption. Given the same effect on the 14th and 21st days, so that the earthworm extract of *Lumbricus rubellus* is sufficiently administered until the 14th day.

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CONFLICT OF INTEREST

All the authors hereby declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors have contributed to provide suggestions and thoughts in this research in accordance with their respective disciplines, so that this research can run smoothly and successfully obtain results in accordance with research objectives.

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