

ORIGINAL ARTICLE

The Effect of Different Tofu Liquid Waste Doses on Decreasing Alveolar Bone Resorption in Rats

Diah Diah¹, Nur Permatasari^{2,5}, Dini Rachmawati³, Jelshagryсна Jessyella Rope⁴, Tania Dewi Fitriani⁴, Saiful Anwar⁴, Andry Anthony Tanujaya⁴

¹ Department of Periodontics, Faculty of Dentistry, Universitas Brawijaya, Malang 65145, Indonesia

² Department of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, 65145, Indonesia

³ Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Brawijaya, Malang 65145, Indonesia

⁴ Bachelor of Dentistry Program, Universitas Brawijaya, Malang 65145, Indonesia

⁵ Department of Oral Biology, Faculty of Dentistry, Universitas Brawijaya, Malang, 65145, Indonesia

ABSTRACT

Introduction: *P. gingivalis* are the main bacteria that cause periodontal diseases. One of the clinical signs of periodontal disease is alveolar bone resorption. Tofu liquid waste is a typical high water-containing biomass and isoflavones. Isoflavones is a polyphenol that has an anti-inflammatory effect by reducing pro-inflammatory cytokine expression. This study aims to evaluate whether systemic administration of different tofu liquid waste doses has effects on decreasing alveolar bone resorption of the Wistar rats' periodontal tissues induced by *P. gingivalis*. **Materials and methods:** : This is true experimental research, with a *randomized posttest-only group design*. The groups are K (-) for the normal group, K (+) for the negative control group, and K-1, K-2, and K-3 for group induced with *P.gingivalis* [0.03 mL(1x 10⁹CFU/mL)] and administrated with 6, 12, and 18 mL/kgBW tofu liquid waste respectively every day for 28 days. **Results:** Freeze-dried technique result showed tofu liquid waste still contains isoflavone (daidzin and glycitin). There is no difference in mean in all parameters between K(-) and K-3. K(+) has difference mean with K-1, K-2, and K-3. Correlation test showed there was a positive correlation between the dose levels of tofu liquid waste with osteoblasts and OPG, and a negative correlation between the dose levels of tofu liquid waste with lymphocytes, neutrophils, osteoclasts, RANKL, alveolar bone resorption, and periodontal ligament space. **Conclusion:** Tofu liquid waste administration has effects on decreasing the alveolar bone resorption of Wistar rat (*Rattus norvegicus*) periodontal tissues induced by *P. gingivalis* and 18 mL/kgBW has the highest effect.

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Corresponding Author:

Diah Diah, DDS

Email: diahperio.fk@ub.ac.id

Tel : +62816553585

INTRODUCTION

Periodontal disease is a universal health problem, which globally, almost 20-50% of the population chronically suffers from (1). In Indonesia, its prevalence in all age categories reaches 74,1% (2). Periodontal disease is an inflammatory chronic and multifactorial disease involving hosts, environmental factors, and bacteria (3). Although microorganisms are the main factor of periodontal disease, their progressiveness and manifestation depend on the host's immune response. Inflammatory responses can cause periodontal tissue damage, which then leads to loss of teeth (4).

Alveolar bone resorption is one feature of periodontal disease and occurs as a result of periodontal tissue inflammation (5). Some major inflammatory cytokines are interleukin (IL)-1, tumor necrosis factor (TNF)- α , IL-6, IL-12, and interferon (IFN)- γ which if bound with receptors will activate RANKL which is the cause of alveolar bone resorption (3). Functional characterization of three novel members of the tumor necrosis factor-ligand and receptor superfamily, the receptor activator of nuclear factor- κ B (RANK), its ligand (RANK Ligand or RANKL) and the soluble decoy receptor of RANKL named osteoprotegerin, have contributed significantly to the establishment of osteoimmunology, where these molecular mediators participate as key modulators of physiological and pathological bone resorption (6).

So far, some of the reported drug administration for periodontal disease cases are (a) cyclooxygenase

inhibitor which on a clinical trial shows its ability to inhibit periodontal disease (7), and (b) TNF inhibitor (etanercept) which can fix alveolar bone damage on periodontal disease (8). The problems that come with it are its side effects and costs. Nutraceutical is a term taken from nutrition and pharmacy, which presumably can avoid or overcome some chronic diseases. Research conducted in Japan shows that women who are on nut-based diets (food high in isoflavones) have a lower risk of getting periodontal disease (9). In line with that result, in experimental research on a menopausal animal model, the administration of tofu waste is also reported as being able to fix periodontal tissue damage (10).

Tofu is an Indonesian traditional food, as a source of vegetable protein. During the tofu-making process, there are two kinds of waste: solid and liquid. Solid waste can be used to feed farm animals, whereas liquid one can be used to produce food or drinks such as nata de soya (11). Tofu waste, apart from containing organic compounds in the form of carbohydrates, protein, and fat, also contains isoflavones (12).

Isoflavones is a polyphenol that has an anti-inflammatory effect by reducing the TNF- α cellular expression in chronic inflammation animal models (13). Another research shows that flavonoids inhibit proinflammatory cytokines IL-1 β , IL-6 and TNF- α (14). The main isoflavones are daidzein, formononetin, genistein and biochanin A. Daidzein and biochanin A showed the highest affinity for RANKL (15). A study by Devitaningtyas et al showed that 12 mL/kg BW tofu liquid waste can reduce osteoclast cells in ovariectomy rat' mandible (16).

Based on the data above, this study aims to evaluate whether systemic administration of different tofu liquid waste doses has effects on Wistar rats' periodontal tissues induced by *P. gingivalis* as seen from the number of lymphocytes, neutrophils, osteoblasts, osteoclasts, OPG, RANKL, alveolar bone resorption and periodontal ligament space.

MATERIALS AND METHODS

Ethical approval

Experimental animal research in this study follows animal ethics. The research has been approved by the Research Ethics Commission (Animal Care and Use Committee) University of Brawijaya (No: 1158-KEP-UB).

Research design

This experiment is an in-vivo *true experimental design posttest control only* to establish the effect of different tofu liquid waste dose administration on lymphocytes, neutrophils, osteoblasts, osteoclasts, OPG, RANKL, alveolar bone resorption and periodontal ligament space on the alveolar bone of Wistar rat (*Rattus norvegicus*) induced by *P. gingivalis*.

Animal

The sample size is 25 male Wistar rats, with ages 2–3-month-old and weights of 250-300 g. The rats must have good health conditions. They were kept in cages for approximately 7 days, with a detailed dietary schedule, and then grouped into 5 different clusters. The groups are K (-), K (+), K-1, K-2, and K-3. K (-) for the normal group (rats without any treatment), K (+) for the negative control group (rats induced with *P. gingivalis* only), K-1, K-2, and K-3 for rats induced with *P. gingivalis* and 6 mL/kgBW, 12 mL/kgBW, and 18 mL/kgBW tofu liquid waste administrated orally (modified from a study by Devitaningtyas et al) (16). *P. gingivalis* induction by drop 0.03 mL (1x 10⁹ CFU/mL) of *P. gingivalis* and given once every 3 days (17) for 28 days with micropipette on mandibular incisor interdental gingiva. Tofu liquid waste was administrated every day for 28 days, at the same time as *P. gingivalis* induction. On day 29th, the rats were sacrificed with cervical dislocation. The mandibula decapitations were performed, and paraffin blocks and then slides for HE and IHC were created. The specimens were then observed using a light microscope with 40x and 400x dilation, and some computations were performed.

Tofu waste preparation

The steps of the tofu liquid waste production are as follows: Selection of soybeans, followed by washing and draining it, soaking the soybeans in clean water for 8 hours. Rewashing the soaked soybeans until it is completely clean. Blend the soybeans using a blender while adding some water until a paste-like consistency is achieved. Straining the mixture using a clean morii cloth to obtain the soy essence, cooking the essence until it is boiling, leaving it for several minutes and adding diluted vinegar, then leaving it until tofu lumps and clear liquid are formed, separating the lumps and the liquid using a clean morii cloth (16). The production of liquid is assumed to be 100% tofu liquid waste. We used the freeze-dried technique to determine the isoflavones content in tofu liquid waste.

Histopathological analysis

Samples of rat mandibles were put in formalin; paraffin blocks were made and processed for HE and IHC slides. Lymphocytes, neutrophils, osteoblasts, osteoclasts, alveolar bone resorption, and periodontal ligament space were examined using HE and IHC for OPG and RANKL. Viewed with a light microscope with 400x dilatation. The measurement of alveolar bone resorption is performed by drawing a horizontal line on the *cemento enamel junction* (CEJ) and calculating the distance from CEJ to the top of alveolar bone on the tooth with the worst bone resorption, conducted using the *software* Olyvia. The measurement of periodontal ligament space is done at 1/3 coronal, 1/3 centre and 1/3 apical on both distal and mesial sites, conducted using the *software* Olyvia. Statistical analysis using One Way Anova to find out the difference in the average of

each group then continued with Post Hoc to determine significant differences between groups. A correlation Test is used to determine the strength of the relationship between two or more variables.

RESULTS

Based on the results of the freeze-dried technique, we get that tofu liquid waste still contains isoflavones, namely daidzin and glycitin. (Table I.)

Table I. The two highest isoflavones in tofu liquid waste were determined by the freeze-dried technique

Isoflavone	Level
Daidzein	1.48%
Glycitin	0.85%

Observations and calculations were made regarding the mean of all parameters. The calculation results show the different mean of parameters after administering tofu liquid waste. Statistical tests showed there is no difference in mean in all parameters between K (-) and K-3. K (-) has difference mean in all parameters with K (+), K-1, and K-2; whereas K (+) has difference mean with K-1, K-2, and K-3. (Table II.) (Fig. 1.) (Fig. 2.) (Fig. 3.) (Table III.)

Table II: Calculation of cell and cytokine expression (mean ± standard deviation) in periodontal tissue

Group	Lymphocytes	Neutrophils	Osteoblasts	Osteoclasts	OPG expression	RANKL expression	Alveolar bone resorption (in µm)	Periodontal ligament space (in µm)
K (-)	5.80 ± 1.095	1.40 ± 0.55	30.48 ± 29	34.3 ± 08	2 ± 1.1	14 ± 1.14	41 ± 93,11	9 ± 6.06
K (+)	11.00 ± 2.00	5.40 ± 0.89	19.48 ± 0.769	75.2 ± 8.9	8.2 ± 0.69	29.4 ± 2.701	73 ± 6.17	12 ± 4.20
K -1	9.00 ± 1.00	4.40 ± 1.14	24.76 ± 11	62.6 ± 97	1 ± 0.5	22.6 ± 1.516	65 ± 44.87	10 ± 9.92
K -2	8.00 ± 1.00	3.00 ± 0.71	26.72 ± 23	49.8 ± 63	1 ± 0.4	19.4 ± 1.14	59 ± 81.71	10 ± 7.12
K -3	6.80 ± 0.84	2.00 ± 0.71	29.32 ± 44	37.4 ± 29	22,50 ± 99	16,2 ± 1.303	55 ± 81.05	10 ± 1.68

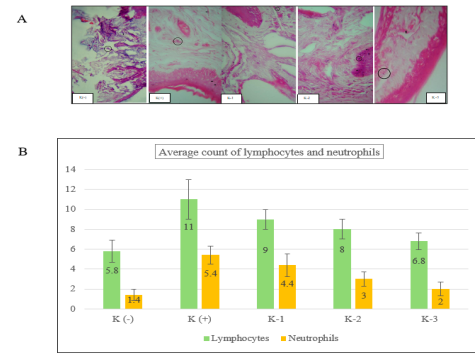


Fig. 1: The effects of tofu liquid waste on lymphocytes and neutrophils of Wistar rats' periodontal tissues induced by *P. gingivalis*. Histology of (A) Lymphocytes and neutrophils. 400 x dilatation (B) Average count of lymphocytes and neutrophils.

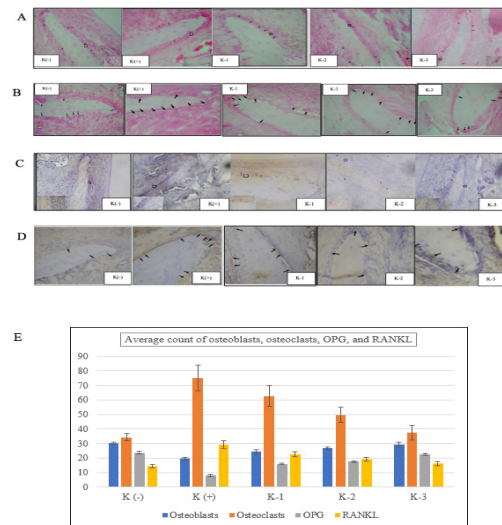


Fig. 2: The effects of tofu liquid waste osteoblasts, osteoclasts, OPG, and RANKL of Wistar rats' periodontal tissues induced by *P. gingivalis*. Histology of (A) Osteoblasts (B) Osteoclasts (C) OPG (D) RANKL. 400x dilatation (E) Average count of osteoblasts, osteoclasts, OPG and RANKL.

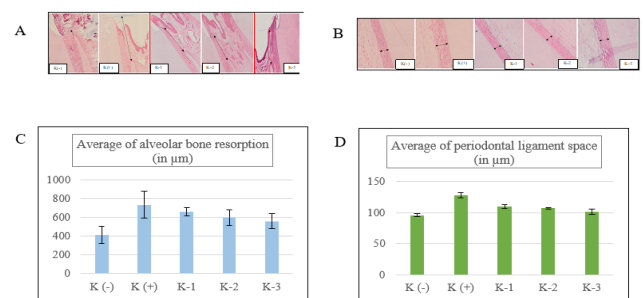


Fig. 3: The effects of tofu liquid waste on alveolar bone resorption and periodontal ligament space of Wistar rats' periodontal tissues induced by *P. gingivalis*. (A) Histology of alveolar bone resorption and (B) Histology of periodontal ligament space. 400X dilatation. The measurement is done at 1/3 coronal, 1/3 centre and 1/3 apical on both distal and mesial sites, conducted using the software Olyvia. (C) Average of alveolar bone resorption. (D) Average of periodontal ligament space.

Table III: Comparison of cell and cytokine expression in periodontal tissues

Variable	Lymphocytes	Neutrophils	Osteoblasts	Osteoclasts	OPG	RANKL	Alveolar bone resorption	Periodontal ligament space
Group	Group	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
K (-)	K (+)	.00	.07	.00	.00	.00	.00	.000
	K -1	.001	.008	.000	.000	.000	.000	.004
	K -2	.018	.013	.000	.006	.000	.002	.040
	K -3	.257*	.166*	.250*	.937*	.063*	.564*	.143* .073*
K (+)	K -1	.077*	.154*	.000	.031*	.000	.000	.716* .000
	K -2	.007	.010	.000	.000	.000	.000	.192* .000
	K -3	.000	.007	.000	.000	.000	.000	.056* .000
K -1	K -2	.257*	.049	.014	.027	.009	.046	.843* .636*
	K -3	.018	.011	.000	.000	.000	.000	.472* .004
K -2	K -3	.177*	.058*	.001	.034	.000	.046	.964* .088*

*No significant difference (p >0.05)

There was a positive correlation between osteoblasts with OPG, osteoclasts with RANKL, alveolar bone resorption with periodontal ligament, the dose levels of tofu liquid waste with osteoblasts and OPG, and a negative correlation between the dose levels of tofu liquid waste with lymphocytes, neutrophils, osteoclasts, RANKL, alveolar bone resorption, and periodontal ligament space (Table IV.)

Table IV: Correlation test between tofu liquid waste dose with all parameters and correlation test between parameters.

	Lymphocytes	Neutrophils	Osteoblasts	Osteoclasts	OPG	RANKL	Alveolar bone resorption	Periodontal ligament space
Tofu liquid waste dose	-.729	-.809	.909	-.889	.942	-.910	-.540	-.772
Sig.	.002	.000	.000	.000	.000	.000	.038	.001
Lymphocytes		.844						
Sig.		.000						
Osteoblasts					.968			
Sig.					.000			

CONTINUE

Table IV: Correlation test between tofu liquid waste dose with all parameters and correlation test between parameters.

	Lymphocytes	Neutrophils	Osteoblasts	Osteoclasts	OPG	RANKL	Alveolar bone resorption	Periodontal ligament space
Osteoclasts							.856	
Sig.							.000	
Alveolar bone resorption								.690
Sig.								.000

Correlation is significant at the 0.05 level

DISCUSSION

Daidzein is one of the main isoflavones content in soybean. Daidzein has anti-inflammatory, antimicrobial, anti-cancer, anti-oxidative, and anti-dysrhythmic activities (18) (19). Isoflavones as an anti-inflammation have an inhibition mechanism on the pro-inflammatory cytokine and chemokine production such as IL-1, IL-6, IL-12, and TNF-α (20). Soy isoflavone has benefits and effects namely on cancer, osteoporosis, learning and memory, coronary heart disease, diabetes, and anti-photoaging (21).

P. gingivalis infects hosts and ruins normal tissues by releasing virulence factors or potential toxins (22,23). One of the *P. gingivalis* virulence factors is lipopolysaccharide (LPS) which penetrates inside the junctional epithelium gingiva and starts an inflammation process. Activation of inflammation mediators and pro-inflammation cytokines like IL-1, IL-6, IL-8, TNF-α (22), and PGE₂ (20) plays an important role in osteoclast differentiation and activation as well as lowering the number of osteoblasts (24).

This study proved that the number of lymphocytes decreased with increasing doses of tofu liquid waste, and the group with the highest dose was near the normal group. This result is in line with the theory which has been put forward by Yu et al, that isoflavones have an anti-inflammation nature which influences granulocyte, monocyte, and lymphocyte (19). By making processed soybeans, Kao et al’s experiment product found that isoflavones powder and genistein standard can effectively hamper the inflammation which has been induced by LPS, reducing the number of leukocytes in rat’s blood, and lower the production of IL-1β, IL-6, NO, and PGE₂ (25). Lymphocytes are important immune cells that produce IL-1, IL-6, and IL-17, RANKL, and TNF-α. It is one of the most prominent leukocytes in the inflammatory infiltrate associated with periodontal degradation and induces bone loss (26). This result is

in line with the theory which has been put forward by Abernathy et al, that soybean isoflavones can reduce the level of inflammatory infiltration and damage in blood vessels, showing that soybean modulates the immune response which is triggered by injury. Soybean isoflavones have an anti-inflammatory mechanism through leukocyte-endothelial interaction modulation (27).

According to the research from Jannah et al, isoflavones from tofu waste, like genistein and daidzein can stimulate osteoblast production and hinder osteoclast resorption activity. Isoflavones stimulate osteoblast by making a transcript factor for mesenchymal cell differentiation and making the osteoblast progenitor cell become an active osteoblast so that the osteoblast number will increase (28). Genistein stimulated osteogenic differentiation of cultured human bone marrow mesenchymal stem cells (hBMSCs), induced the expressions of osteoblast differentiation-related genes and osteoblast mineralization in primary rat osteoblasts, and suppressed the receptor activator of RANKL-stimulated osteoclast differentiation (20).

The normal group showed the amount of OPG was almost two times the amount of RANKL, which means that a low RANKL/OPG ratio. There is no inflammation in the normal group, so the RANKL/OPG ratio is low. The RANKL/OPG ratio, known as a regulator of bone formation, is an important determinant of bone resorption. In most cases, upregulation of RANKL and downregulation of OPG lead to bone loss (29). The RANKL-OPG balance can influence osteoclast activity, thus it has been suggested that the serum RANKL/OPG ratio is an important factor in determining osteoclast activation (30). OPG works to prevent bonding between RANKL and RANK, if OPG concentration is higher than RANKL, they will bind, and the osteoclast amount will decrease. Tofu liquid waste contains isoflavones which can increase osteoblast cell proliferation and differentiation, and also stimulate osteoblasts to produce OPG (31).

All groups with *P. gingivalis* induction showed a lower OPG than RANKL, but the RANKL/OPG ratio decreased as the tofu liquid waste dose increased. The reduction of OPG is caused by *P. gingivalis*' endotoxin secretion in the form of LPS which will cause inflammation because LPS binds the CD14 receptor on monocyte surface cells and macrophage. This binding induces TLR-4 to secrete proinflammatory cytokines and inflammation mediators including TNF- α , IL-1, IL-6, and PGE₂ which will decrease OPG production as well as increase RANKL production (24,32,33).

A significant decrease in RANKL counts results after being given various doses of tofu waste. Kao et.al explain that isoflavones effectively withhold LPS-induced inflammation, reduce leukocytes amount, and

reduce the production of pro-inflammatory cytokine TNF- α , IL-1, IL-6, nitric oxide (NO), and PGE₂ (25) can lower RANKL's production and higher OPG and RANK production (32). This is in accord with the theory from Park et al (34) and Zaklos-Szyda et al (15) saying that isoflavones contained in tofu waste could increase OPG expression and decrease RANKL expression.

Bone destruction in periodontitis involves two complex processes osteoclast and osteoblast. In the inflammatory process, the osteoblast which has the function of bone formation decreases its size and activities, while the osteoclast which has the function of breaking down and resorbing bone will increase its size and activities (24). Research by Tang et al shows that isoflavones can slow down bone resorption, not by osteoblast proliferation, but by decreasing osteoclastogenesis through its inhibition on RANKL (35). The remodelling process is a bone physiology factor where osteoblast and osteoclast cells are in balance. If the quantity of osteoclast cells is higher than the osteoblast, bone resorption will happen. Excessive resorption will disturb the balance of the bone remodelling process (25).

The osteoblast's progenitor plays a role in exerting RANKL/OPG as the mediator key in the development and activity of osteoclast. The bond between RANKL and RANK is the start of the bone resorption process (36). Osteoclastogenesis happens because RANKL stimulates the activity from osteoclast to resorb the bone while OPG is part of TNF which plays a role as the resistor factor of osteoclastogenesis. The bond between RANKL and RANK causes a pre-osteoclast differentiation to become a mature osteoclast which happens because there is a transduction signal in the osteoblast precursor in the form of TRAF 6 (37). The increase in the osteoclastogenesis process causes an increase in the number of osteoclast cells and the start of bone resorption.

Isoflavones as an anti-inflammation can hinder the inflammation process which is induced by *P. gingivalis*' LPS by repressing excessive immunity response towards *P. gingivalis*. This is due to genistein and daidzein contained in isoflavones that can reduce the production of pro-inflammatory cytokines like IL-1, IL-6, TNF- α , and PGE₂ with the result of decreased RANKL expression and increased OPG expression. This is caused by decreasing pro-inflammatory cytokine production and inflammatory mediators which suppress OPG expression, so there will be an escalation of OPG expression (19). Osteoprotegerin inhibits osteoclastogenesis and induces osteopetrosis when over-expressed in transgenic mice. RANKL has been associated with diverse osteodestructive pathologies, including rheumatoid bone tumors arthritis, Paget's bone disease, osteoporosis, odontogenic lesions, osteolytic lesions of the facial skeleton, and periodontitis (6).

With the cytokine and chemokine production being

held up, the RANKL expression on the osteoblast also decreases, resulting in an imbalance between the RANKL expression level and OPG. In a balanced situation, RANKL and OPG will create more RANKL-OPG bind than RANK-RANKL bind so that the osteoclastogenesis process happens at a normal level corresponding to the physiological condition (6).

Alveolar bone resorption and periodontal ligament space decreased gradually with increasing doses of tofu liquid waste. As stated in an experiment by Intini et al., isoflavones as an anti-inflammation can inhibit the pro-inflammatory cytokine production resulting in a decrease in the pro-inflammatory cytokine production which then keeps the damage in the periodontal ligament space and alveolar bone from happening(38). This result matches the study by Devitaningtyas et al. that the isoflavones contained in the tofu liquid waste can inhibit alveolar bone resorption (16). This is also following research conducted by Bae et al, which showed that daily administration of daidzein strongly suppressed loss of alveolar bone height (39). Genistein suppresses *P. intermedia* LPS-induced inflammatory macrophages and attenuates alveolar bone loss in periodontitis rats. Genistein inhibits *P. intermedia* LPS-induced accumulation of iNOS-derived NO and IL-6 with a decrease in their mRNA expression in RAW264.7 cells, indicating that genistein inhibited these inflammatory mediators as both gene transcription and translation levels (20). Aquina and Permatasari proved that isoflavones from tofu liquid waste could decrease the periodontal ligament space. Isoflavones can inhibit increasing of periodontal ligament space, because as an anti-inflammation that decreases pro-inflammatory cytokines and MMPs production (10). Proinflammatory cytokine inhibition downregulated the inflammation response and osteoclast activity. Therefore, it decreases alveolar bone resorption and widens periodontal ligament space. A decrease in MMPs also decreases collagen degradation, so collagenase activity can play in the normal range and can keep the homeostasis of the periodontal ligament space (40).

Aquina and Permatasari's studies on female rats proved an increase of alveolar bone resorption will widen the periodontal ligament space (10). This is also in line with Lim et al., who stated that in conditions where the alveolar bone experiences decreased bone resorption, the width of the periodontal ligament space will decrease, whereas in conditions where the alveolar bone experiences increased bone resorption, the width of the periodontal ligament space will be widened (41). This study proved that the correlation between alveolar bone resorption and dose of tofu liquid waste was found to be moderate with a negative direction, the higher the dose of tofu liquid waste the resorption of the alveolar bone becomes lower. In this study, tofu liquid waste has effects on the alveolar bone resorption of Wistar rats and future studies were required.

CONCLUSION

Based on the results of this study, it can be concluded that tofu liquid waste administration has effects on reducing the alveolar bone resorption of Wistar rat (*Rattus norvegicus*) periodontal tissues induced by *P. gingivalis* and 18 mL/kgBW has the highest effect.

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