

## **The Effectiveness Of Tofu Liquid Waste As A Natural Phytoestrogen For Mandibular Bone Of Ovariectomized Rats**

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### **Abstract**

Estrogen is a steroid compound that has many benefits for the body's physiology. Hypoestrogenic women could have periodontitis in their mouth. Hormone replacement therapy (HRT) is a therapy that used to reduce the disturbance caused by estrogen deficiency. However, HRT has many side effects. Theoretical study shows that isoflavones in tofu liquid waste has an element of a steroid compound. The purpose of this study was to prove the effectiveness of isoflavones in the tofu liquid waste by examining the microscopic structure, estrogen receptor expression and MDA levels in rat mandibular bone post-ovariectomy. This research method use a true experimental laboratory with randomized post test control group design. Twenty-four female wistar rats were divided randomly into six groups, consisted of a group of normal rat that does'nt ovariectomized (C1), 4 weeks ovariectomized rats (C2), 8 weeks ovariectomized rats (C3) but no tofu liquid waste was given, and 8 weeks ovariectomized rats that given tofu liquid waste at the end of the 4<sup>th</sup> week of ovariectomy with three different doses (C4 = 1.2, C5 = 6; C6 = 12 ml / kg). Histopathological slide used to see the changes resulted in a number of osteoclasts, osteocytes, periodontal ligament width, alveolar bone height, the amount of estrogen receptor expression and MDA levels. The results of statistical tests show that there is a difference in the rats between six different groups (ANOVA,  $p < 0.05$ ) and there is a close relationship between the dose of the tofu liquid waste with all parameter. The conclusion of this study is tofu liquid waste could improve the microscopic structure, estrogen receptor expression and MDA levels in rat mandibular bone post-ovariectomy.

**Key words:** Menopause, Periodontitis, Isoflavones, Tofu Liquid Waste

### **Efektivitas Limbah Cair Tahu Sebagai Fitoestrogen Alami Terhadap Tulang Mandibula Dan Jaringan Periodontal Tikus Paska Ovariectomi.**

### **Abstrak**

Estrogen merupakan senyawa steroid yang memiliki banyak manfaat untuk kepentingan fisiologis tubuh. Pada wanita yang mengalami keadaan hipoestrogen, maka akan terjadi gangguan pada fungsi rongga mulut berupa periodontitis.

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Terapi Sulih Hormon (TSH) adalah terapi yang digunakan untuk mengurangi gangguan yang dirasakan akibat kekurangan estrogen. Namun, TSH memiliki banyak efek samping. Dari kajian teoritis menunjukkan bahwa terdapat isoflavon, suatu senyawa yang mempunyai unsur steroid pada limbah cair tahu. Tujuan dari penelitian ini adalah membuktikan efektivitas isoflavon pada limbah cair tahu terhadap struktur mikroskopik, ekspresi *estrogen receptor* dan kadar MDA pada tulang mandibula tikus paska ovariektomi. Metode penelitian ini menggunakan true experimental laboratorik yang menggunakan metode *randomized post test control group design*. Dua puluh empat tikus *wistar* betina dibagi secara random dalam enam kelompok, terdiri dari kelompok tikus normal yang tidak di ovariektomi (K1), tikus yang di ovariektomi selama 4 minggu (K2) dan tikus yang di ovariektomi selama 8 minggu (K3) tidak diberikan limbah cair tahu, dan tikus yang di ovariektomi selama 8 minggu dan diberikan limbah cair tahu pada akhir 4 minggu ovariektomi dengan tiga dosis yang berbeda (K4 = 1,2 ; K5 = 6; K6 = 12 ml/kgBB). Digunakan sediaan histopatologi untuk melihat perubahan hasil berupa jumlah osteoklas, osteosit, lebar ligament periodontal, tinggi tulang alveolar, jumlah ekspresi estrogen receptor serta kadar MDA. Hasil uji statistik menunjukkan terdapat perbedaan pada tikus antara enam kelompok yang berbeda (ANOVA,  $p < 0,05$ ) dan terdapat hubungan yang erat antara dosis limbah cair tahu dengan keenam parameter. Kesimpulan dari penelitian ini adalah secara mikroskopis limbah cair tahu dapat memperbaiki struktur mikroskopis, ekspresi *estrogen receptor* dan kadar MDA tulang mandibula pada tikus paska ovariektomi.

**Kata Kunci** : Menopause, Periodontitis, Isoflavon, Limbah Cair Tahu

## Introduction

Estrogen is a steroid hormone that is mainly produced by ovarian follicles. Estrogen production would reduce because the lower number of follicles in the ovaries at the time of menopause.<sup>1</sup>

As many as 30% of menopausal women suffer periodontitis.<sup>2</sup> Periodontitis is an oral disease that most often encountered. Facts show at the time of menopause, women lose the protective effects of estrogen that increases the risk of periodontitis.<sup>3</sup> American Society for Reproductive

Medicine says that ten million people had periodontitis, and 14 million postmenopausal women with low bone mass has a high risk of severe periodontitis.<sup>4</sup>

In 1970 scientist introduced a treatment of menopause by using (Hormonal Replacement Therapy / HRT). However, until now hormonal replacement drugs (hormonal replacement therapy / HRT) is still considered expensive, and has a various risks such as endometrial cancer and breast cancer.<sup>5</sup>

The use of natural materials that contain phytohormones has been

developed at this time. One of them is a phytoestrogen. Phytoestrogens are a substrate from a plant which has similar activity with estrogen.<sup>6</sup> Natural phytoestrogens which are now starting to be developed is from *Leguminosae* class. This class contain of *Isoflavones* compound. *Isoflavones* compound shown to have hormonal effects, especially estrogenic effect. Estrogenic effect of *Isoflavones* is associated with a structure that can be transformed into equol, which has a phenolic structure similar with estrogen. In other words, *Isoflavones* may prevent the process of periodontitis in the bone so the bone remains solid and massive.<sup>7</sup>

In the process of making tofu, there are two kinds of waste products, namely solid waste and liquid waste, solid waste used as livestock feed, while the liquid waste discharged into the environment thus contaminating the environment. Research prove that the liquid waste products still contains organic compounds such as fats, proteins, carbohydrates, and also there is a compounds of isoflavan.<sup>8</sup> Therefore, research is needed to demonstrate the use of liquid waste as an alternative therapy to reduce the risk of periodontitis in postmenopausal women.

## Methods

**Research Design.** The methodology of this study is a true experimental laboratory research that uses methods of randomized post test control group design. Subjects were divided into 6 groups. C1 (not ovariectomized and not given tofu liquid waste), C2 (ovariectomized 4 weeks and were not given tofu liquid

waste), C3 (ovariectomized 8 weeks and were not given tofu liquid waste), C4 (ovariectomy + 1, 2 ml/kg of tofu liquid waste), C5 (ovariectomized + 6 ml/kg of tofu liquid waste), C6 (ovariectomized + 12 ml/kg of tofu liquid waste). After that, the sixth group would performed mandibular bone and periodontal tissue surgery to see the microscopic structure, expression of estrogen receptors and levels of MDA.

**Ovariectomy Procedure.** Weight of rats were measured, and then the rat were fixed in a supine position. Performed anesthesia using ketamine im dose 40mg/kgBW then carried out sterilization using 70% alcohol and betadine solution. Performed transabdominal incision about the top of the uterus along the 1.5 - 2 cm layer by layer to penetrate the wall of the peritoneum. Find uterine horn-oviduct-ovary uteru. Oviduct and ovary tissue freed from surrounding fat and connective tissue. The distal of oviduct and ovary was ligated. Then the oviduct and the ovaries are removed. Give the basitrasin powder (Nebacetin). Use the same procedure for the right ovary. Then the incision given Gentamycin i.m at a dose of 60-80 mg/kgBW 1 time per day for 3 days, and Novalgin i.m at a dose of 0.3 ml for 1 day.<sup>9</sup>

**Procedure of Making Tofu Liquid Waste.** Choose a clean and washed soybeans. Soak in water for 8 hours (at least 3 liters of water to 1 kg of soy). Wash repeatedly soaked soybeans. Mash soybeans and add warm water to form slurry. Cook the pulp, not to coagulate at a temperature of 70<sup>0</sup> ~ 80<sup>0C</sup>. Strain pureed soybeans and water using 3 ml of vinegar to 1 liter of soymilk.

Pressed to remove the sediment water (tofu liquid waste).<sup>10</sup> This liquid waste is used as a research variable. After that, ovariectomized rats were given out through a sonde with a specific dose for 4 weeks.

**Slide processing.** Decalcification process for 5 days by sediment the organ in oncalek liquid. The process of fixation, dehydration, clearing and impregnation by dipping into a solution of the tissue in sequence

according to the specified time. Performed tissue embedding with microtome then painted using haematoxylin eosin procedure and immunohistochemistry. Microscopic structure and expression of estrogen receptor observation using a microscope Olympus BX51 with the Photo Slide DP71 12 Megapixel camera with 1000x magnification in each tissue / slides from each rat by 4 field of view and then on the average.

**Table 1. Result of Average Data**

Group	Osteoclast	Osteocyte	Alveolar Height	Ligament Periodontal Space	Estrogen Receptor Expression	MDA's Level
1	19.1425	93	8672.07	18.283	5.0625	0.1567
2	19.9475	39.125	7248.02	28.852	11.9375	0.2849
3	33.6875	28.0625	4547.23	42.039	16.1875	0.3328
4	16.5	45	5731.97	23.273	14.625	0.2819
5	13.25	64.4375	6679.63	14.301	10.5625	0.2281
6	10.4375	67.875	7876.87	10.437	5.6875	0.1905

**Test Lipid Peroxides (MDA) Mandible Bone Powder.** Weigh 50 mg of bone that has been powdered, then dimortal with phosphate buffer as much as 1 cc. Add TCA 100% of 250 microliters, 200 1 N HCl 200 microliters, Natio Barbiturates 1% of 200 microliters. Heat the water to 100o C for 20-25 minutes. Centrifuged at 2000 rpm then take the supernatant with 3 cc posphat aquabides or buffer. Read results using a spectrophotometer with 532 waves.

**Analysis.** Observations on the control and treated rat were

statistically analyzed using SPSS program for Windows 16:00 with a significance level of 0.05 (p = 0.05) and 95% confidence level ( $\alpha = 0.05$ ). With the method of data analysis using One-way ANOVA test, Multiple Comparison Test post hoc Tukey by Equal Variance, and Pearson Correlation Test.

**Results And Discussion**

Number of Osteoclasts. Administration of Tofu liquid waste containing *Isoflavones* have an estrogenic activity that can bind to

estrogen receptors located in the bone.<sup>11</sup> So that it can cause changes in the bone that can be seen microscopically.

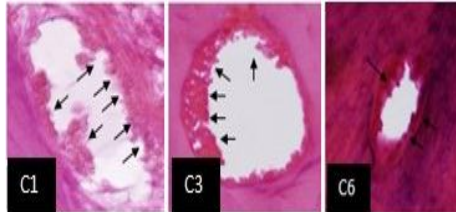


Figure 1. Histopathology of Osteoclast

One of the changes that happened was in osteoclasts. Homogeneity test showed normal distribution. ANOVA test shows significant value  $p = 0.001$ , then from post hoc test showed there were significant differences in group C2, C3, C4, C5, with a significant value  $p < 0.05$ . The pearson test showed that tofu liquid waste has a enough relationship with the number of osteoclasts on mandibular bone ( $r = -0,432$ ). This suggests that the dosage of different Tofu liquid waste know can reduce the number of osteoclasts in ovariectomized rats.

#### Number of Osteocytes.

Effect of low estrogen in the body will lead to increased expression of pro-apoptotic genes (genes *Insp3Rs*) and the activity of caspase 3/7 on the osteoblast cell apoptosis.<sup>12</sup> Increased apoptosis resulting in decreased osteoblast cell number. Mature osteoblasts was called osteocytes which is a major component of adult bone constituent on mandibular bone. If the number of osteoblast cells decreased, the osteocytes cell numbers can also be confirmed

decreased.

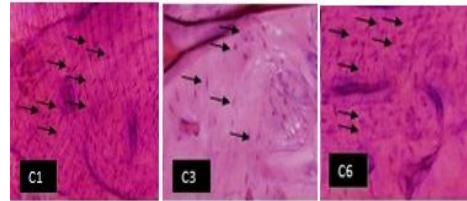


Figure 2. Histopathology of Osteocytes

Decreased of osteocytes cell numbers result in a decrease of bone regeneration. Decreased structure regeneration of bone structure can weaken bones and cause periodontitis structures. Provision of Tofu liquid waste containing in this study *Isoflavones* are expected to act as phytoestrogens that characterized by an increase in the number of osteocytes cells, so that the tofu liquid waste can prevent the occurrence of periodontitis. ANOVA test shows significant value  $p = 0.000$ , then from post hoc test showed there were significant differences in group C2, C3, C4, C5, C6 with a significant value  $p < 0.05$ . The pearson test showed that tofu liquid waste has a very strong relationship with the number of osteocytes on mandibular bone ( $r = 0,883$ ). This suggests that the dosage of different tofu liquid waste will increase the number of osteocytes in rats post-ovariectomy.

**Alveolar Bone Height.** Tofu liquid waste contain *Isoflavones* is proved to have hormonal effects, especially the estrogenic effect. *Isoflavones* are able to influence calcification process. *Isoflavones* have a role in preventing alveolar bone resorption that occurs in hipoestrogenic condition.<sup>13</sup>

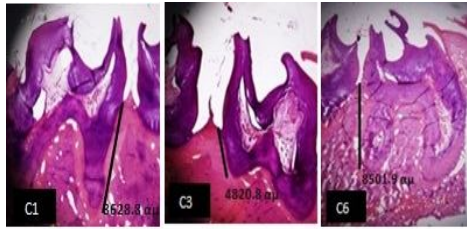


Figure 3. Histopathology of Alveolar Bone Height

ANOVA test shows that there are significant differences ( $p = 0.000$ ), then from post hoc test showed there were significant differences in group C1, C2, C3, C4, C5, C6 with a significant value  $p < 0.05$ . The pearson test showed that tofu liquid waste has a very strong relationship with the number of osteocytes on mandibular bone ( $r = 0.933$ ). This suggests that the dosage of tofu liquid waste will increase the height alveolar bone ovariectomized rats.

**Periodontal Ligament Space Width.** Decreased of estrogen levels could be a trigger in inflammation and decreased synthesis of fibroblasts that making up collagen so it can makes an increased of bone resorption of periodontal tissue. Tofu liquid waste treatment inhibit the inflammatory cytokines that inhibit the expression of inflammation that causes bone resorption, decrease of periodontal tissue fibroblasts as well as collagen formation in periodontal tissues may also be hampered.<sup>14</sup> So there is no increase in periodontal ligament space width.

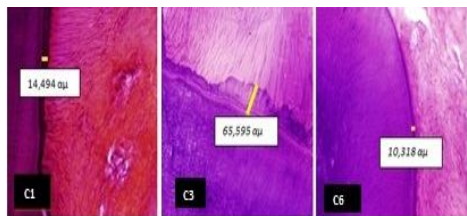


Figure 4. Histopathology of Ligament Periodontal Width

From the ANOVA test can be seen that there are significant differences ( $p = 0.001$ ), then from post hoc test showed there were significant differences in group C1, C2, C3, C4, C5, C6 with a significant value  $p < 0.05$ . The pearson test showed that tofu liquid waste has a very strong relationship with the width of ligament periodontal space on mandibular bone ( $r = -0.945$ ). This suggests that the dosage of tofu liquid waste will decrease the width of periodontal ligament space in ovariectomized rats.

**Expression of Estrogen Receptor.** Tofu liquid waste containing *Isoflavones* is known to be having estrogenic activity that can bind to estrogen receptors located in the bone. As the number of estrogen decreased, the number of free estrogen receptors are increased.<sup>1</sup>

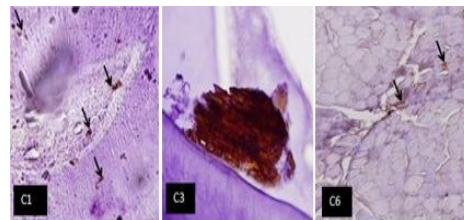


Figure 5. Histopathology of Estrogen Receptor Expression

ANOVA test shows significant value  $p = 0.000$ , then from post hoc test showed there were significant differences in group C1, C2, C3, C4, C5, C6 with a significant value  $p < 0.05$ . The pearson test showed that tofu liquid waste has a very strong relationship with the number of estrogen receptor expression ( $r = -0.940$ ). This suggests that the dosage of tofu liquid waste will decrease the number of estrogen receptor expression in ovariectomized rats.

**MDA levels of Mandible Bone.** MDA is the result of calculation by using spectrophotometer. Administration of tofu liquid waste containing *Isoflavones* works through two pathway mechanisms. The first *Isoflavones* have estrogenic activity that can bind to estrogen receptors and produces cellular endogenous antioxidant (ex, SOD) and the second through free radical scavenger antioxidant activity, so as to reduce levels of free radicals in post-menopausal.<sup>[15]</sup> From the ANOVA test can be seen that there are significant differences ( $p = 0.000$ ) in the levels of MDA (Malodialdehyd) which is one product of free radicals. In the post hoc test showed a significant difference between C1, C2, C3, C4, C5, C6.



Figure 3. MDA Tube

The pearson test showed that tofu liquid waste has a strong relationship with the expression of estrogen receptor ( $r = -0,700$ ). So that the tofu liquid waste can improve MDA levels nearly normal mandibular bone.

### Conclusion

The tofu liquid waste is a tofu byproduct. It is potential enough to be used as a therapeutic in hypoestrogen conditions. The conclusion of this research that tofu

liquid waste can improve the microscopic structure, estrogen receptor expression and MDA levels of mandibular bone post-ovariectomy in rats.

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