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Psychological Insights into the Impact of Yellowfin Fish Bone Powder and CaCO3 Therapy on Histological Features of Vertebrae Bone in an Ovariectomized Mice Model

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Abstract

Introduction: The therapy of giving yellowfin tuna bone meal to experimental rats is one of the models used to determine the effectiveness of bone meal on changes in healing in ovariectomized rats. Treatment for osteoporosis to date only use provision of calcium as an ingredient for therapy for osteoporosis sufferers. Calcium is needed by the body to help the growth of bones and teeth.

Purpose: This study aims to determine the effect of bone meal on histological features of vertebral bones in an ovariectomized rat model using yellowfin bone meal and $Caco_3$. therapy.

Methods: The method used in this study was an experimental study (experimental study) randomized post test control group design to determine the effect of surgical treatment (ovariectomy) and treatment of deproteinated, non-deproteinated yellowfin tuna bone meal and CaCO3 at a dose of (0, 400 , 800 and 1600 mg/kg BW/day) in ovariectomized rats.

Results: The results showed that the greater the dose of yellowfin bone meal at a dose of 1600 mg/kg BW/day, the faster the vertebral bone repair towards healing, this was indicated by the bone thickness with the lowest value in the treatment and CaCo3 (63 mm); non-deproteinated (72 mm) and the highest was in the deproteinated treatment at a dose of 1600 (92.24 mm).

Keywords: Histological features, yellowfin bone meal, ovariectomized rats

1. Introduction:

Marine biota is a hidden natural wealth and so far it has not been widely used because it is priceless. Various attempts and efforts have been made by humans to reveal the secrets contained in marine biota and other fishery products. The tireless effort has begun to show results with the discovery of various types of novel compounds that are not found in land biota. Empirically humans have used various types of plants both on land and in the ocean as raw materials for medicine since ancient times, although the compounds contained in them are not known with certainty (Rasyid A, 2008)[1]. Marine products that have not been widely used so far are fish bones. So far, fish bone waste has not been utilized because most people view it as waste or material that is not useful. These waste products are found in parts of the bones, heads, scales, fins and skin as well as stomach contents which are usually disposed of because they have a negative impact on the environment (Talib *et al*, 2009)[2]. Even though this waste, when processed, can provide added value economically and can be used in additional food products, as a source of minerals, fiber and iodine (Noor *et al*, 2019)[3]. Fish bone meal contains many minerals, especially macro and micro minerals and the largest amounts are calcium and phosphorus (Talib *et al, 2018*)[4]. The high mineral content in fish bones must be treated so that the bioavailability level also increases. One way to increase bioability is by deproteination treatment. Deproteination is the process of releasing proteins from their bonds which are covalently bound and can be degraded by chemical treatment, namely dissolving in a strong alkaline solution or by biological treatment.

In principle, deproteination is carried out by providing alkaline conditions followed by heating for a certain period of time. Treatment by way of diproteinasi and non-deproteinasi and increase the level of absorption before use to test animals. Dosage of deproteinated, non-deproteinated yellowfin bone meal and

calcium carbonate in the ovariectomized rat model group can be seen through *Hematoxylin-Eosin* (HE) staining. Hematoxylin is an alkaline dye that binds to acidic structures in cells and stains it to a purplish blue while Eosin is an acidic dye that has a negative charge (Pecham, 2014)[5]. In the picture using Hematoxylin-Eosin (HE) it can be seen that there were differences in the histological picture between the control rats without ovariectomy, the ovariectomized rats without treatment and the ovariectomized rat group using dose therapy (0.400, 800 and 1600 mg/g BW/day).

The ovariectomy process in white rats was carried out based on the Hartiningsih method (2012), carried out through skin incisions in the left and right flank areas[6]. Mice were first anesthetized using a dose of 1-4 mg/kg BW intravenously through the coccygeal vein. After the rats were anesthetized, the skin in the flank area was slashed with an incision length of approximately 1-1.5 cm. Furthermore, the subcutaneous tissue is exposed, then the abdominal muscles are slashed, then the fat pads are pulled so that the ovaries along with the Fallopian tubes (uterine tubes) and the uterine coruna are also carried out of the abdominal cavity. The ovary can be found, then the hanging part of the ovary is tied with cat gut thread.

2. Result

In ovariectomized control rats without therapy, the thickness of the vertebral body wall was 83 mm and the lowest was 44 mm. For the dose of 400 mg/g BW/day, the highest was 71 mm for the CaCo3 treatment and the lowest was using deproteination of 66 mm. The highest dose of 800 mg/g BW/day was the deproteinized treatment with a thickness of 84 mm and the lowest was the non-deproteinated treatment, namely 52 mm. The highest dose of 1600 mg/g BW/day for deproteination parameters resulted in a thickness of 92.24 mm in the vertebral wall; and the lowest was with the CaCo3 treatment with a thickness of the vertebrae wall of 63 mm. The histopathological results of three groups of rats were treated with deproteinated, non-deproteinated bone meal and calcium carbonate with each dose (0.400, 800 and 1600 mg/g BW/day). These results indicate that there is a correlation between the thickness of the vertebral body wall and the administration of calcium from fish bone meal and calcium carbonate.

The highest dose for the parameters of the deproteination treatment showed the thickest wall thickness compared to the other treatments. Giving deproteinated fish bone meal therapy has high calcium levels and will increase calcium levels in the body. Calcium from bone meal that has been absorbed will be used in metabolic processes and bone repair. Calcium that has been absorbed in the body will speed up the absorption process so that bone repair will run faster. The process of vertebral bone repair in ovariectomized rats is followed by thickening of the vertebral body walls. The availability of calcium in the jejunum will be transported by 1,25 dihydroxycalciferol produced by the kidneys. Doses of 1600 mg/g BW/day for deproteination can have an effect on the amount of calcium availability in the body so that absorption is more than other doses.

This absorption causes the bone remodeling process to run perfectly so that the osteocyte cells that are degraded due to ovariectomy can be replaced immediately. Mice treated with ovariectomy without deproteinated, non-deproteinated and calcium carbonate yellowfin bone meal treatment showed the lowest average bone thickness of 44 mm. Whereas in ovariectomized rats with deproteination treatment the dose of 1600 mg/g BW/day was the highest, this was evidenced by the thickening of the walls of the corpus vertebrae which was 92 mm. The same study was conducted by Eklou-Kolonji (1999) that ovariectomized rats would experience reduced bone mass and decreased bone density[7]. The addition of corpus wall thickening showed that deproteinated yellowfin bone meal therapy had an effect towards bone repair as a result of ovariectomy treatment.

These results indicate that the effect of bone meal administration and treatment of fish bone meal doses greatly influences the improvement of vertebrae wall thickening. Significant differences were found in the treatment group of rats without ovariectomy and the group of ovariectomized rats and the group of rats treated with calcium carbonate. In the control group without ovariectomy, it was clear that there was no damage to the vertebral bones, but in the ovariectomy group without therapy there was permanent damage on days 10, 20 and 30. On the 10th day after the ovariectomy there was no significant damage to the vertebral body but on the 20th and 30th day the damage was very visible with widening of the follicles. This dilation is caused by estrogen,

which normally functions properly, now begins to be disrupted by the ovariectomy treatment. Histological picture of the vertebrae of the ovariectomized rats is presented in Figure 1.

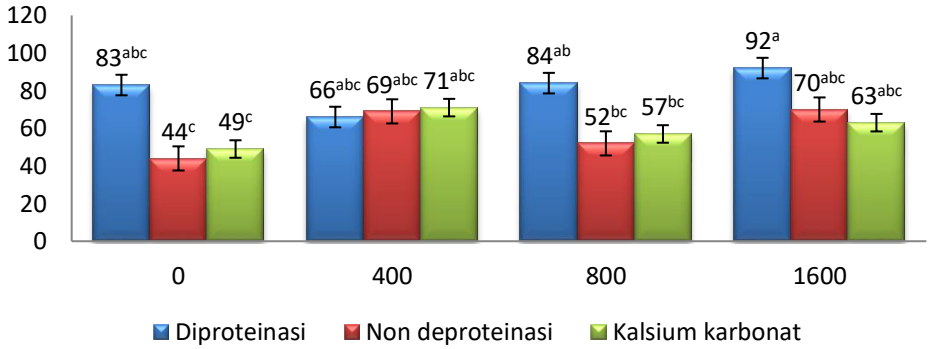


Figure 1. Histological view of the vertebrae of ovariectomized rats during observation

Note: Numbers in the same diagram followed by different superscript letters show significantly different results ($p<0.05$)

Histopathological observations of doses of 0, 400, 800 and 1600 mg/kg BW/day showed a significant difference between the thickness of the walls of the vertebral body and the treatment with yellowfin bone meal and calcium carbonate. These results were statistically significantly different ($p<0.05$) between doses of 0, 400, 800 and 1600 mg/g BW/day. The results of these observations indicate that the greater the dose given can affect the thickness of the corpus wall compared to the control without ovariectomy and the ovariectomy group of rats without therapy. To determine the thickness of the cell wall is done by measuring the bottom of the wall of the vertebral body. Bone density is determined by the amount of content in the bone matrix, especially calcium and phosphorus. The high content of calcium and phosphorus in yellowfin bone meal and calcium carbonate can affect the levels of calcium and phosphorus in rat bones. The use of high doses of calcium without being followed by phosphorus in a balanced dose is feared to disrupt the balance of calcium and phosphorus in the body. This calcium deficiency can result in the precipitation of calcium salts in various tissues, especially the kidneys (Wiwik *et al.*, 2008)[8].

This is in line with the study of Firmansyah (2005) who reported that administration of supplemental doses of calcium carbonate (450 mg/day) could affect the process of improving the histopathological picture of the femur in ovariectomized rats[9]. Ovariectomy resulted in decreased bone appearance in rats and led to osteoporosis. Adult rats aged 10-16 weeks after ovariectomy rapidly develop osteoporosis within 3 weeks due to significant loss of trabecular bone. In some cases, cortical bone loss usually takes much longer. The amount of bone loss depends on the treatment performed (femur, tibia, spine) and the method used to determine bone density and volume (Robert, 2011)[10].

Osteoporosis in humans can affect postmenopausal women and cause brittle bones or fractures (Iwamanto *et al.*, 2008). Loss of sex steroids in postmenopausal women triggers accelerated bone turnover, i.e. predominance of bone resorption over bone formation[11]. Bone formation causes a negative balance of calcium that will support bone loss, increase bone fragility and increase the risk of fracture (Karsdal *et al.*, 2007)[12]. In women affected by menopause or in men who experience castration the speed of bone remodeling will increase very rapidly. This is in line with studies conducted using mice, that loss of sex steroids will trigger regulation of osteoclast and osteoblast formation in the marrow through regulation of the production and action of cytokines which are responsible for osteoclastogenesis and osteoblastogenesis (Manolagas, 2000)[13].

The mechanism is initiated by the stimulus of differentiation of mesenchymal cells into the osteoblast lineage due to hormonal changes. The next process, osteoclastogenesis causes bone loss which is downstream (Manolagas, 2000)[13]. In postmenopausal women, the number of osteoclasts as well as the number of osteoblasts actively forming bone will increase. The increased frequency of activation in postmenopausal women triggers an increase in the number of osteoclasts and resorption lacunae in the skeleton. Osteocalcin is one of the new molecules formed by osteoblasts and incorporated into the organic matrix and bones will increase in postmenopausal women. Ovariectomy causes ovarian function to be disrupted resulting in

estrogenic effects, namely loss of the ability to produce estrogen resulting in changes in menstrual patterns. The menstrual cycle causes irregular estrogen receptors, and will eventually stop and this process is called menopause. Menopause causes a decrease in bone density and bone formation (Lerner, 2006)[14]. Comparison of normal vertebral bones without ovariectomy, ovariectomy without therapy and treatment with deproteinated, non-deproteinated yellowfin bone meal and calcium carbonate is presented in Figures 2.3, 4.5 and 6 at doses of 0, 400, 800 and 1600 mg/kg BW/day.

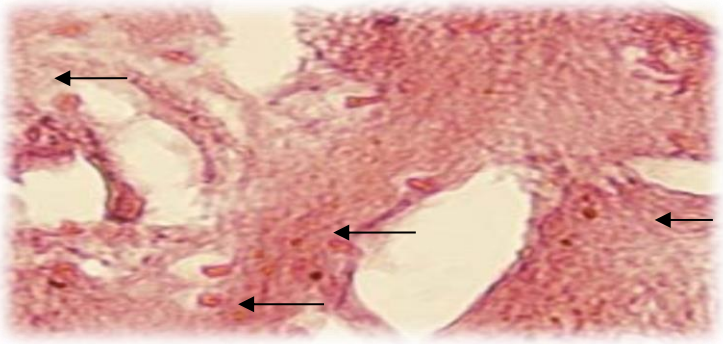


Figure 2. Normal vertebrae without ovariectomy
Note: Arrows indicate osteoblast cells

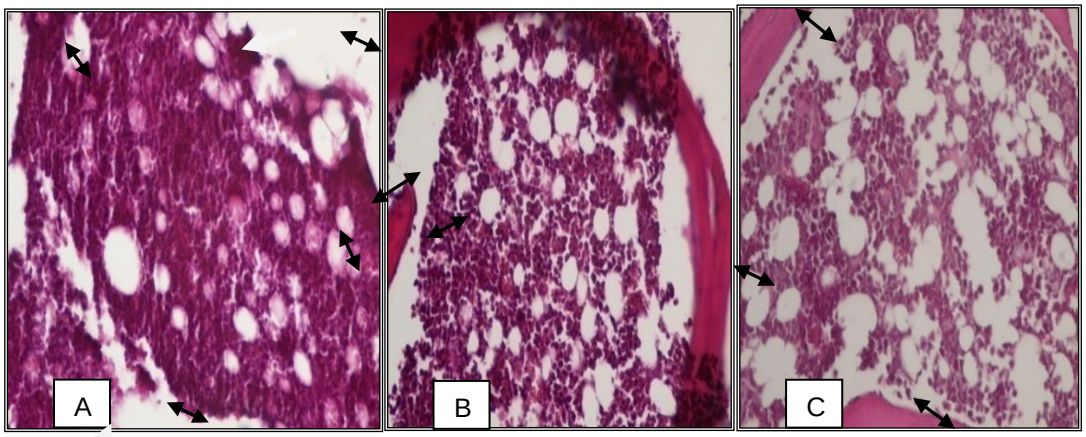
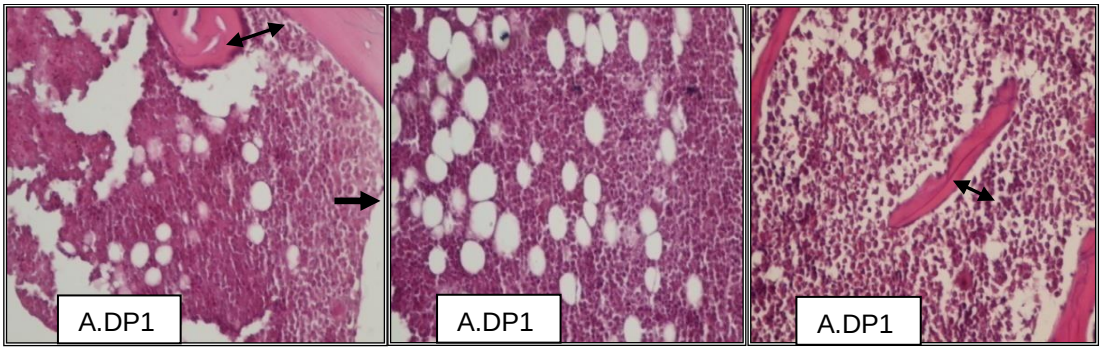


Figure 3. Normal vertebrae without ovariectomy
Note: Arrows indicate osteoblast cells

Description: (A). Histological picture of the 10th day control rat vertebrae during the study (100x magnification).
(b). Histological picture of vertebrae control rats on the 20th day during the study (100x magnification)
(C). Histological picture of control rats on the 30th day during the study (100x magnification)



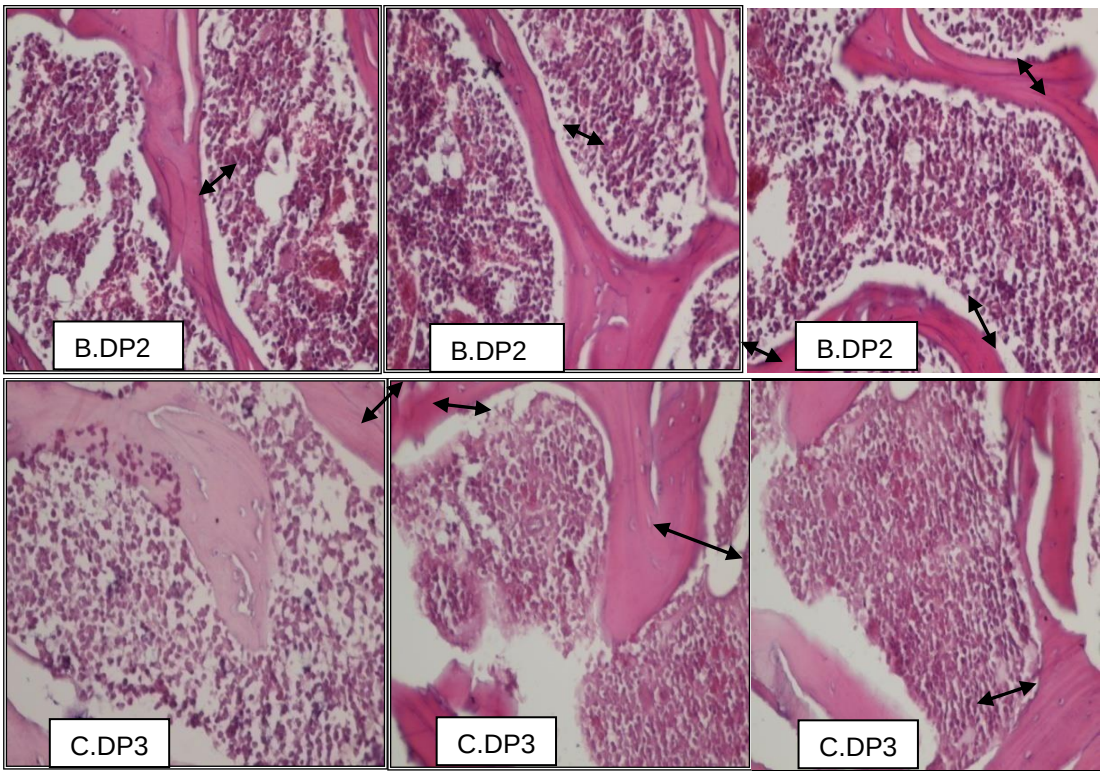


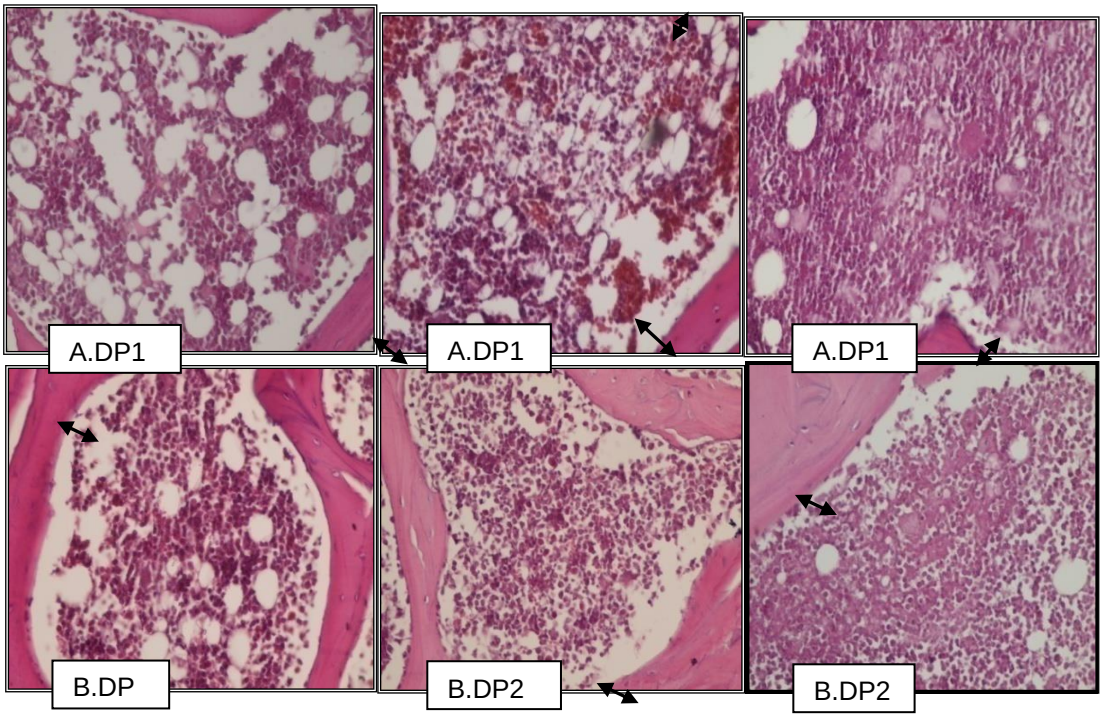
Figure 4. Histological picture of the vertebrae of ovariectomized rats with bone meal and (CaCO3) dose of 400 mg/kg BW/day

Description:

(A.DP1). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 400 mg/kg BW/day on the 10th day during the study (100x magnification).

(B. DP2). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 400 mg/kg BW/day on the 10th day during the study (100x magnification).

(B. DP2). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 400 mg/kg BW/day on the 30th day during the study (100x magnification).



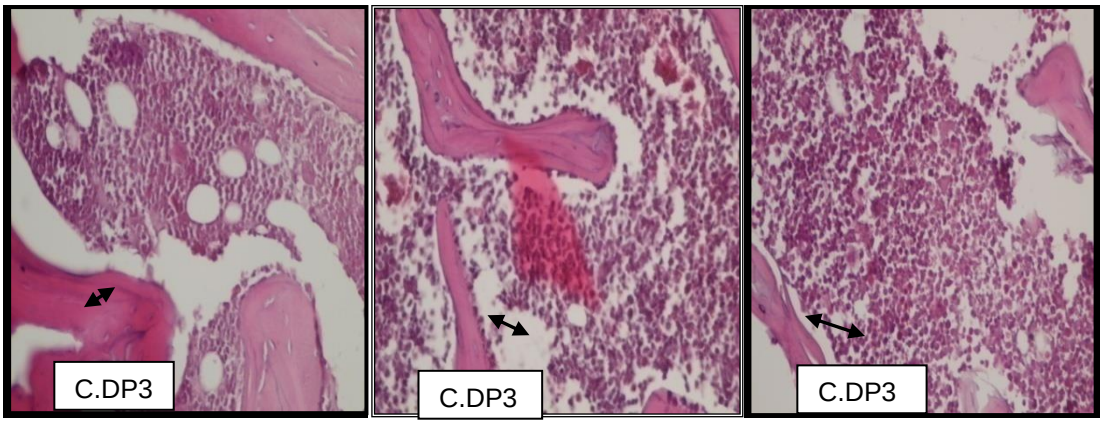


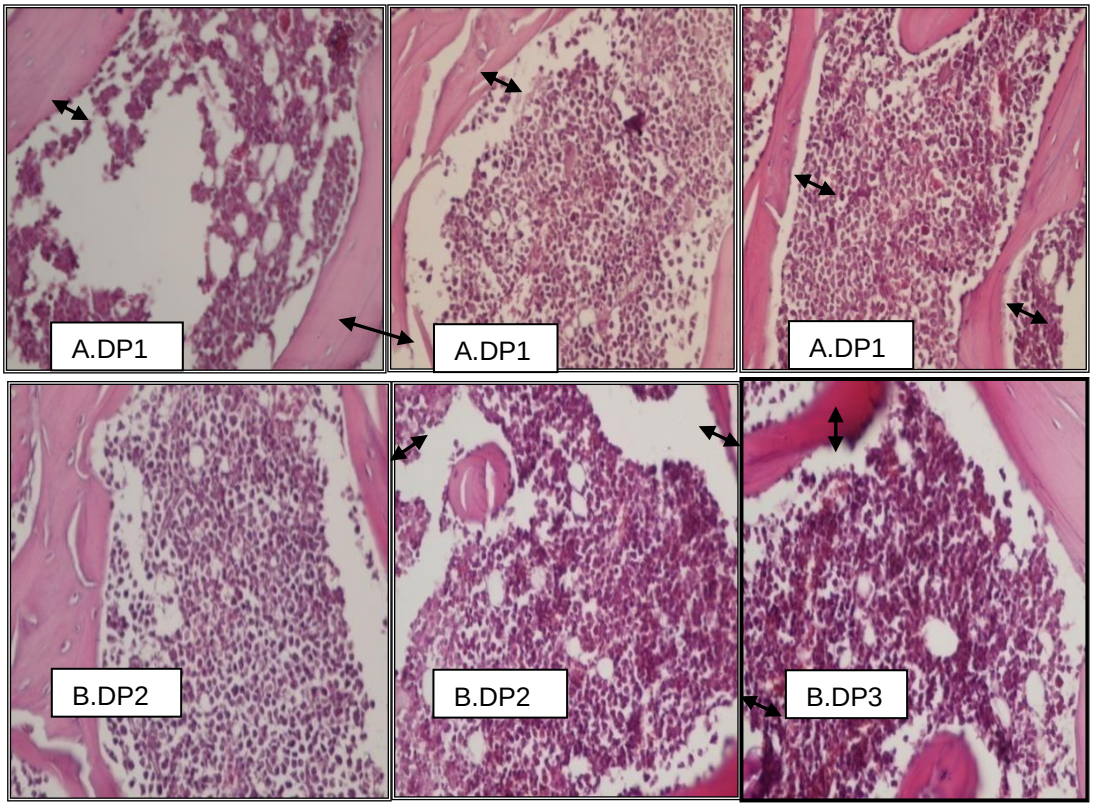
Figure 5. Histological picture of the vertebrae of ovariectomized rats with bone meal and (CaCO3) dose of 800 mg/kg BW/day

Description:

(A.DP1). Histological description of ovariectomized rats treated with fish bone meal at a dose of 800 mg/kg BW/day on the 10th day during the study (100x magnification).

(B.DP2). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 800 mg/kg BW/day on the 20th day during the study (100x magnification).

(C.DP3). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 800 mg/kg BW/day on the 30th day during the study (100x magnification).



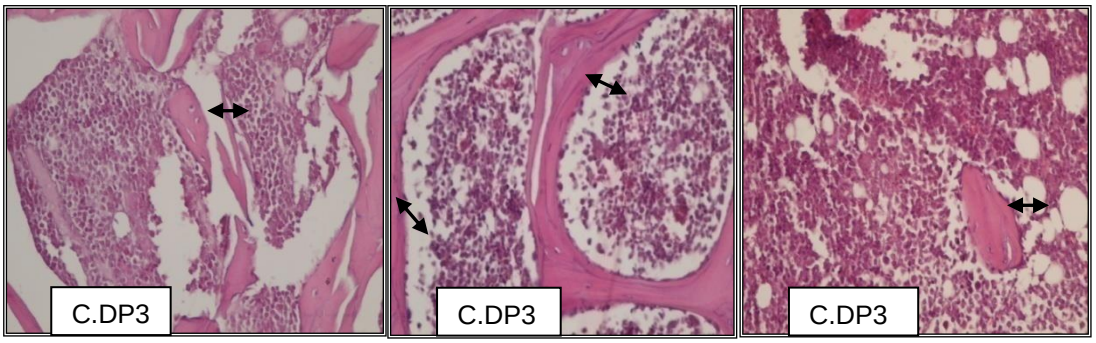


Figure 6. Histological picture of the vertebrae of ovariectomized rats with bone meal and (CaCO3) dose of 1600 mg/kg BW/day

Description:

(A.DP1). Histological description of ovariectomized rats treated with fish bone meal at a dose of 1600 mg/kg BW/day on the 10th day during the study (100x magnification).

(B.DP2). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 1600 mg/kg BW/day on the 20th day during the study (100x magnification).

(C.DP3). Histological picture of ovariectomized rats treated with fish bone meal at a dose of 1600 mg/kg BW/day on the 30th day during the study (100x magnification).

Bone density is determined by the amount of mineral content in the bone matrix, mainly composed of calcium and can determine bone strength. The use of calcium supplements to increase bone density has been widely promoted in society. This is in accordance with (Mader, 1998[15]; Yulianti, 2003)[16], that additional calcium intake aims to prevent a decrease in bone density. Akesson *et al.*, (1998) stated that the addition of calcium helps increase bone density[17]. This is different according to research by Sankaran (2000), which states that dietary calcium doses above 1500 mg per day alone cannot increase bone density[18]. Bone density is part of the results of bone growth in addition to the weight, length and thickness of the bones (Ganong, 2003[19]; National Dairy Council, 2001)[20]. The thickness of the bone mass at any time always changes addition and reduction through the remodeling process (Bostrom, 2000[21]; Manolagas, 2000)[22]. Therefore, steps are needed to maintain bone strength through the consumption of additional natural calcium available in nature. Various studies on the effect of giving calcium on bone mass have been carried out through interventions with fortification into food products and the results can increase bone reabsorption (Akesson et al., 1998[17]; O'Brien, 1998)[23]. Estrogen administration has been known to prevent a decrease in bone mass or estrogen replacement therapy (ERT). Estrogen replacement therapy combined with additional oral calcium can increase bone density (Yulianti, 2003)[16].

The process of bone formation mainly involves osteoblasts as the main cells that produce bone matrix (Bostrom, 2000[24]; Manolagos, 2000)[22]. Osteoblasts also regulate the concentration of calcium ions in the matrix through the release of intracellular calcium (Bostrom, 2000)[21]. The development of osteoblasts is controlled, among others, by growth factors, cytoclines, hormones and mechanical signals (Manologos, 2000)[22]. Increased extracellular calcium through a signaling mechanism via calcium receptors on osteoblasts and can serve as a signal for osteoblast mobilization and proliferation (Huang *et al.*, 2001)[25]. The increase in extracellular calcium, among other things, can come from the release of calcium from hydroxyapatite due to matrix degradation during resorption during the remodeling process (Huang *et al.*, 2001)[25]. Giving additional calcium can increase the concentration of extracellular calcium so that it might also cause osteoblast proliferation as in the calcium signaling mechanism. Osteoblast proliferation causes an increase in matrix synthesis so that bone mass increases and the remodeling process runs fast.

3. Discussion

Doses of deproteinated, non-deproteinated yellowfin bone meal and CaCO3 at dose levels of 0, 400, 800 and 1600 mg/kg BW/day. In rats without ovariectomy it was seen that there was no damage to the vertebrae. This is because the rats without ovariectomy do not lose their ovaries so that the uterine cells are not

disturbed and there is no atrophy in the uterus. Mice with the ovariectomy model have lost their ovaries so that if left untreated, permanent damage is seen during observation. Ovariectomized rats caused an increase in serum calcium levels, which is an indicator of an increase in the process of bone resorption (Djojosoebagio, 1996)[26]. The lowest and highest vertebral body wall thickness was found in the controls with an average value of 44 mm-83 mm. Administration of yellowfin tuna bone meal therapy can reduce TNF-α expression in the femur. The control animal model showed the highest TNF-α expression of the other treatments, this was shown from the brown color that appeared on the preparations (Figure 2). This is when compared with a therapeutic dose of 400 mg/kg BW, TNF-α expression decreased, namely 66-71 mm and further decreased at a therapeutic dose of 800 mg/kg BW, 52-84 mm, and the lowest decrease was in animal models given treatment dose of 1600 mg/kg BW, which has an average thickness of 63-92 mm.

This shows an effect on each treatment group as indicated by the difference in the average value of TNF-α expression, with the highest decrease in TNF-α expression at a dose of 1600 mg/kg BW. The results of staining using the immunohistochemical method (IHK) showed that there was expression of TNF-α in each treatment as seen by the appearance of a brown color. Diaminobenzidine gives a brown color to the expression of TNF-α which reacts with the antibody to a decrease in expression that appears to be brown on the femur, as in the control animal model where the brown color is most striking. Animal models treated with doses of 400, 800 and 1600 mg/kg BW showed a decrease in average expression and a decrease in brown color (Fig. 3.4, 5 and 6).

This brown color is visible in the area between the osteocyte cells in the femur bone. Triskayani (2010), stated that TNF-α expression appears extracellularly because TNF-α is a cytokine that acts on receptors in the extracellular system[27]. TNF-α levels increase after menopause, this is characterized by increased bone resorption and loss of bone mass (Balga *et al.*, 2006)[28]. When the state of bone osteoclasts increases, it causes inhibition of osteoblast production which plays a role in bone formation, so that bone remodeling is disrupted and bone resorption increases. Impaired bone remodeling causes bone loss and this is a sign of osteoporosis. When osteoporosis occurs with an increased state of osteoclasts it is characterized by increased expression of TNF-α. In the ovariectomy animal model, there is a decrease in blood calcium in the body. The body responds to this decrease by increasing PTH production. PTH takes calcium from the bones by demineralizing the bones. When bone demineralization occurs, there will be an accumulation of calcium in the blood, resulting in an increase in the level of calcium in the blood. Osteoclast cells capture bone matrix particles and crystals through phagocytosis which eventually dissolves these objects and releases them into the blood. Then the blood will go to the kidneys, normally the kidneys will release or excrete everything that is excess from the body through urine (Yuniarti *et al.*, 2008)[16].

Then PTH will also affect TNF-α cytokines which are osteoclast precursors to increase activity in the process of bone loss, so that calcium from bones will be used for the production of 25-hydroxycalciferol. 25-hydroxycalciferol will be transported to the intestine (jejunum) to be converted into 1,25-hydroxycalciferol. As stated by Murray *et al.* (2003), 1,25 dihydroxycalciferol acts in the small intestine to stimulate absorption of dietary calcium and together with PTH supports the mobilization of calcium from bones[29]. At the same time, 1,25 dihydroxycalciferol and PTH cause the kidneys to reabsorb more calcium ions, so that the plasma and extracellular calcium will increase to normal levels (normocalcemia). However, when there is no calcium compensation, there will continue to be a release of bone calcium and osteoporosis will continue. Administration of yellowfin tuna bone meal therapy to rats indirectly reduced TNF-α expression. Yellowfin tuna bone meal given to ovariectomized rats can increase calcium levels in the body.

4. Conclusion

The results showed that the ovariectomy group experienced lower levels of bone structure damage and trabecular thickness compared to the controls. The results of therapy with fish bone meal doses (0, 400, 800 and 1600 mg/kg BW/day) which had the highest value in the 1600 mg/kg BW/day treatment in the proteinated treatment (92.24 mm) and the lowest in the non-proteinized treatment. deproteination and calcium carbonate (63 mm); non deproteinated (72 mm). Whereas the ovariectomy group and received bone meal therapy for yellowfin tuna at a dose of 1600 mg/kgBW/day experienced improvements in bone structure and thicker trabecular thickness compared to doses of 800 mg/kg BW/day and 400 mg/kg BW/ day.

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