

Vertebrae Recovery of Ovariectomy Rats Using Fishbone Flour Therapy and Calcium Carbonate (CaCO₃)

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ABSTRACT

Yellowfin fish bone meal contains minerals, especially calcium and phosphorus, which are present in high levels after processing; the benefits are enormous for the human body's needs. This study aimed to determine the recovery of ovariectomized rat vertebrae using yellowfin bone meal and CaCO₃ therapy. This study consisted of three stages: firstly, processing yellowfin fish bone waste into fish bone meal with deproteination, non-deproteinization, and calcium carbonate treatment as a comparison. The second stage involved a physicochemical analysis of yellowfin fish bone meal. In the third stage X-rays were used to confirm the extent of spinal damage in ovariectomized rats treated with deproteinized, non-deproteinized fish bone meal and calcium carbonate. The results revealed a significant development of bone remodelling in osteoporosis, as observed through vertebral observation for four months. The bone is a dynamic tissue that is constantly undergoing remodelling due to mechanical responses and hormonal changes. Bone remodeling occurs in the bone remodelling unit, where there is an emotional balance between osteoclast bone resorption and bone formation by osteoblasts. In comparison, the observation via dual-energy absorptiometry x-ray on day 30 following deproteination therapy at a dose of 1600mg/ kg B.W./ day revealed a very significant change in recovery in vertebral bone radiology.

INTRODUCTION

Fishbone is a waste generated from the fishing industry or home industry activities and can be reprocessed into a fishbone meal with enormous benefits. To get quality fish bone flour, a very long process is required. Firstly, the fish bones are steamed then dried and grounded to produce bone meal. Secondly, the bones are cooked under steam pressure, then removed in a closed vessel to soften them for grinding into flour. Lastly, bone ash is obtained through burning of the bones (Talib *et al.*, 2009).

Bone flour produced from the fishery industry can be fermented into food (Belton & Thilsted, 2013). Fishery industrial waste can be used as additional material in the

processing of food products (**Darmawangsyah & Kadirman, 2018**). One of the fishery products wastes that have the potential to be developed is fishbone waste (**A. Talib & K. Zailani, 2017**). It is mainly found in fish processing, such as processing otak-otak, meatballs, and fish crackers (**Fatimah & Jannah, 2009**). The waste resulting from fish processing usually includes heads, bones, scales, and skin, removed and discarded by the community or the fishing industry, leading to environmental degradation (**Talib et al., 2009**). Fishbone waste processing has not been utilized optimally and has the potential to cause environmental pollution. Even though this waste can provide added value economically and be used in additional food products, as a source of minerals, fiber, and iodine (**Asikin et al., 2019**). Fishbone waste has a high mineral content, especially calcium and phosphorus. It is suitable as a natural mineral source, especially in food products, animal feed, or supplements (**Talib et al., 2014**).

This is in line with research **Santoso et al. (2006)** and **Ido and Kaneta (2020)**, which states that using fish oil and fish meal to feed fish farming is very effective since both contain protein, vitamins, and minerals, which significantly affect fish growth. The processing of yellowfin fish bones into bone meal contains high calcium and phosphorus, especially in the form of critical inorganic elements in the body and the highest amounts. In addition to the mineral content in fish bones, absorption or solubility factors in fishbone meals can affect the absorption rate in the body (**Martindale, 2009**). One of the methods used to reduce the protein and fat content in the fish bones is utilizing the deproteination and non-deproteinated boiling method (**Talib et al., 2014**).

The two methods were chosen to determine the best solubility level in the *in vivo* test using experimental animals. The simulation of the absorption rate of the materials used for the experiment can describe the metabolism in the human body. This study specifically investigated the effects of deprotonated yellowfin fish bone meal, non-deproteinated, and calcium carbonate. A comparison was made using calcium carbonate to determine the effectiveness of fishbone meal in restoring vertebral bones in mice. Calcium carbonate is a well-known inorganic mineral at a low price that is commercially available. The mechanism of calcium carbonate dissolves slowly in the stomach and reacts with hydrochloric acid, carbon dioxide, and water (**Martindale, 2009**).

The doses of fishbone meal and calcium carbonate in multilevel doses are very effective for treating rats with osteoporosis. To ensure the effectiveness of the material used, it is necessary to do an x-ray on the vertebrae of the experimental rats. Mice ovariectomized will slowly develop osteoporosis since estrogen and progesterone's functions are not functioning correctly. This is indicated by the onset of bone deterioration or what is called the menopause phase. Menopause is one of the life stages in a woman when the transition phase from reproductive to non-reproductive occurs. It is defined as the period of cessation of menstruation forever, with an average of 51 years. The diagnosis of menopause is made retrospectively after 12 months of amenorrhea followed by a decrease in the circulating estrogen hormone due to cessation of ovarian

function (**Bulun & Adashi, 2007**). It consists of several stages, namely pre-menopause, menopause, and post-menopause. The decrease in estrogen in this phase causes various complaints and problems in women, which impact the decreasing quality of life and discomfort in daily activities (**Thurston & Joffe, 2011**). With the increasing life expectancy of Indonesian women at the age of more than 70 years, and menopause typically occurring at the age of 50- 51 years, women may spend more than a third of their lives in menopause (**A. Baziad, 2003**). This amount is significantly less when compared with the standard U.S. Dietary Reference intake for daily calcium needs per day. According to the data from the Ministry of Health in 2010, one in three women and one in five men in Indonesia has osteoporosis or bone loss. Osteoporosis is the most common disease in bones. This disease is characterized by decreased bone density and an increased risk of fractures (**Cosman et al, 2014**). The data above shows that osteoporosis is a public health problem that needs serious attention from the government because of its increasing prevalence. According to the **World Health Organization (2003)** and data from **IOF (2010)**, osteoporosis ranks as the second most prevalent disease globally, following cardiovascular disease. Studies indicate that women aged 50 years face a comparable risk of mortality from hip fractures as they do from breast cancer. Osteoporosis decreases bone density and damage to bone micro-architecture that causes bones to become brittle or fracture. The method to assess osteoporosis uses a trabecular bone pattern in the proximal femur (**Taradita et al., 2018**). The world body that deals with health problems, the World Health Organization (WHO), estimates that by 2025 the number of older people worldwide will reach 1.2 billion people, which will continue to grow to 2 billion people in 2050. According to the data from the WHO, it is also estimated that 75% of the world's elderly population in 2025 will be in developing countries (**Suarni, 2017**). The above context is essential for conducting a study titled “The Therapeutic Effect of Yellowfin Fish Bone Meal on Vertebral Bone Recovery in Ovariectomy Rat Models.”

MATERIALS AND METHODS

This research was conducted in Ternate City, North Maluku Province. The research has been approved by the Research Ethics Committee with approval No. 220-KEP-UB.

1. Materials

The raw material used include deproteinated, and the non-deproteinated yellowfin bone meal sourced from a tuna loin fillet company in Ternate City, North Maluku. Additionally, the calcium carbonate material for comparison was obtained from a chemical shop. The chemicals used for making fishbone meals were H₂SO₄, alcohol, NaOH, Na₂S₂O₃, HNO₃, HClO₄, distilled water, tablets, pH 7, and pH 4 buffers KH₂PO₄ (phosphorus standard), 1000ppm Ca salt solution (Ca standard) (**Latimer,**

2016). The tools used to manufacture yellowfin fish bone meal were trays, knives, pans, stoves, ovens, autoclaves, disc mills, 100 mesh sieves, and analytical scales. The physical and chemical analysis involved the use of oven, analytical balance, measuring flask, flask, water bath, Nissei AM-3 homogenizer, AAS (Atomic Absorption Spectrophotometer) brand Shimadzu AA-680, Rheoner brand RE 3350 Yamaden, Poselin cup, and filter paper. Kett Electric C-100-3 whiteness meter, measuring cup, Erlenmeyer, soxhlet, fat-free cotton, pipette, electric stove, furnace, pH meter, funnel, measuring cup, Whatman 42 filter paper, and centrifuge test tube were also used (**Latimer, 2016**). Additionally, the tools used for the second phase of the study were: analytical scales, mouse cages, feeding containers, thermometers, aluminium foil, and vertebral bone radiology using dual energy absorptiometry (DEXA) x-rays.

2. Research stage

This research consisted of three stages: first, processing yellowfin fish bone waste into the fishbone meal with deproteinated, non-deproteinized, and calcium carbonate treatments as a comparison. The deproteination method was used to reduce the protein content in fish bones by boiling using acetic acid. In contrast, the non-deproteination process utilized boiling in water instead.

The second research stage was to conduct a physicochemical analysis of the yellowfin fishbone meal, while the third stage involved using X-rays to confirm the degree of vertebral bone damage in ovariectomy rats treated with deproteinated, non-deproteinated fish bone meal and calcium carbonate. The first stage of the research involved starting with yellowfin fish bone waste with a diameter of 60- 100cm in length, then the bones waste were reduced to an average size of 30- 35cm. The next stage involved washing the dirt and blood, followed by boiling. Boiling was conducted three times, gradually for 4 hours each, totaling 12 hours, at a temperature of 100°C. After the first boiling stage, the bones were rewashed under running water and boiled again at the same temperature for three steps. The second stage of the research was boiling fish bones using 1: 3 deproteination and non-deproteination with a boiling time of 30 minutes. After cooking, the fish bones were washed three times until they were clean and then autoclaved for 2 hours at a temperature of 121°C.

Furthermore, the bones were kept in an oven at 60°C for 8 hours, and the subsequent process was milled with a disc mill and sieved with a size of 100 mesh. The physicochemical characteristics observed included the degree of whiteness (**Guess, 2006**), water absorption (**Fernández & Clothier, 2012**), and density (**Astawan et al., 2016**). Chemical characteristics observed encompassed calcium and phosphorus levels (**Quinn et al., 2013**), as well as moisture, ash, protein, and fat content (**Ramdath et al., 2020; Barakat, 2021**). Additionally, calcium solubility, phosphorus solubility, and pH were assessed. The results of the first and second phase studies were then used for therapy in ovariectomy model mice with therapeutic doses (0.400, 800, and 1600mg/ kg

body weight (B.W.)/ day. Weighing and observation of the test animals were conducted over a period of four months. Weighing was performed every three days, specifically on the 1st, 3rd, 6th, 9th, 12th, 15th, 18th, 21st, 24th, and 27th days of each month. The rats were divided into four groups, each group consisting of 12 rats. The rats were kept alone in non-metabolic cages. The rats were provided with ad libitum access to food and water, allowing them to feed and drink freely, or were continuously provided with food and water every day for a total of 108 days.

3. Statistical analysis

All statistical analyses were performed using SPSS 28.0 software (Konishi & Long, 2021). The differences between groups were compared using Student's t-test, specifically analyzing the distinctions between boiling water and the previous method to determine differences between acetic acid and hydrochloric acid. *P*-values were corrected using the Benjamin & Hochberg method, with a significance threshold set at $P < 0.05$ or adjusted $P < 0.05$, as considered statistically significant (Wang *et al.*, 2021). Additionally, the distribution of boiled water content variables with non-deproteination and deproteination was tested using multivariate regression analysis with a 95% confidence level.

RESULTS

1. Chemical characteristics of yellowfin fish bone meal

In previous research, boiling methods using HCl, NaOH, and CH₃COOH were employed. The results of this research indicated that the highest amounts of calcium and phosphorus were obtained using CH₃COOH. Subsequently, this optimal treatment was selected for *in vivo* testing using experimental mice.

The results of the chemical analysis carried out on yellowfin fish bone meal include moisture, ash, fat, protein, calcium, and phosphorus content, are presented in Table (1).

Table 1. Chemical characteristics of yellowfin (*Thunnus albacares*) bone meal

| Parameter | Boiling media | |
|---------------------|-----------------------------|------------------------------|
| | Non deproteination | Deproteination |
| Water content (%) | 2,98 ± 0.14 ^a | 2,54 ± 0.09 ^b |
| Ash content (%) | 56,65 ± 2. 26a | 58,21 ± 0.08 ^a |
| Protein content (%) | 20,98 ± 2.98 ^a | 17,21 ± 2.58 ^a |
| Fat content (%) | 6,36 ± 0.61 ^a | 6,31 ± 0.56 ^a |
| Ca content (mg/g) | 238,61 ± 56.86 ^a | 165,64 ± 55.58 ^a |
| P content (mg/g) | 152,25 ± 6. 93 ^a | 161,62 ± 23. 20 ^a |

The analysis of variance in this study revealed a significant difference ($P < 0.05$) in the water content parameters between the non-deproteinated and deproteinated boiling

media. However, for the parameters of ash, fat, protein, calcium, and phosphorus content, no significant difference was observed between both non-deproteinated boiling media. Deproteinaton and deproteinaton were statistically not significantly different ($P > 0.05$). The analysis of ash content between non-deproteinated and deproteinated samples, with values of 56.65% and 58.21%, respectively, did not show a statistically significant difference. At the same time, previous research (Talib *et al.*, 2009) utilizing boiling water, acetic acid, and hydrochloric acid as media obtained lower ash content, namely 51.45, 44.95, and 45.21%. The analysis of protein content obtained in this study with non-deproteinated and deproteinated treatments was 20.98 and 17.21%, which were not statistically significant ($P > 0.05$). When compared to previous studies utilizing boiling water, acetic acid, and hydrochloric acid, the results of this study were 8.49, 7.58, and 8.22, respectively. The fat content in this study was smaller even though it was not statistically significant ($P > 0.05$). The highest fat content was found in fish bones by boiling acetic acid, and the lowest was cooking using water. The calcium content analysis in yellowfin fish bone meal with non-deproteinated and deproteinated treatments ranged between 238.62, and 165.64mg/ g bk. Moreover, the results of this study were not statistically significant ($P > 0.05$).

The calcium content analysis in yellowfin fish bone meal with non-deproteinated and deproteinated treatments ranged between 238.62, and 165.64mg/ g. Moreover, the results of this study were not statistically significant ($P > 0.05$). The phosphorus content in this study with boiling water and acetic acid media were 152.25 and 161.62mg/ g, respectively, which were not statistically significant ($P > 0.05$).

2. Results of yellowfin fish bone meal therapy on body weight of mice

Observation results for 108 days from January to April with the treatment of deproteinated, non-deproteinated yellowfin fish bone meal and calcium carbonate at doses of 0.400, 800, and 1600mg/ kg B.W./ day showed a very significant impact over the length of time and maintenance. The results of rats' weighing and weight observations in January and February are presented Figs. (1, 2).

The results of observations and weighing the bodyweight of rats for deproteinated, non-deproteinated, and CaCO₃ treatments showed that on the first day, the bodyweight of rats for deproteinaton treatment was 213g/ kg B.W./ day, while the value of the non-deproteinated treatment was 199g/ B.W./ head, and for CaCO₃, it was 201 gr/ B.W./ head. On the 9th day, the bodyweight decreased with a weight of 155g/ B.W., and in the following month, it increased until the 27th day before necropsy with a weight of 184g / B.W./ day. The weight loss is thought to be due to rats' stress from feeding the yellowfin fish bone meal, which is high in calcium, whereas previously, it was with standard feed, which was high in protein and carbohydrates. It is hypothesized that the increase in the rats' appetite may be attributed to stress from the ovariectomy procedure, which could

have caused bleeding during the operation. The body weights of the rats in March and April are presented in Figs. (3, 4).

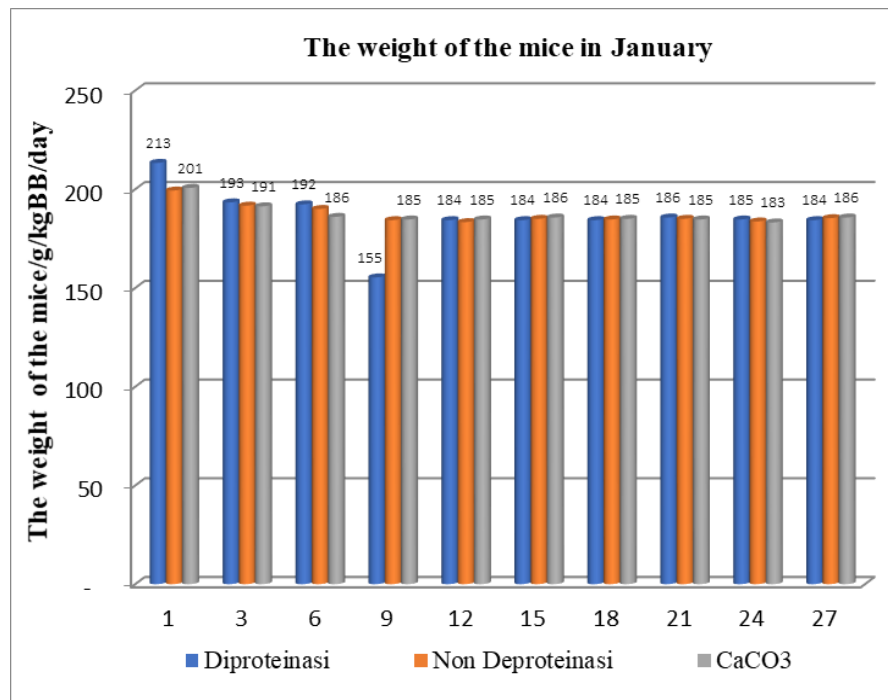


Fig. 1. Diagram of average body weight of rat during January treated with yellowfin fish bone meal and calcium carbonate

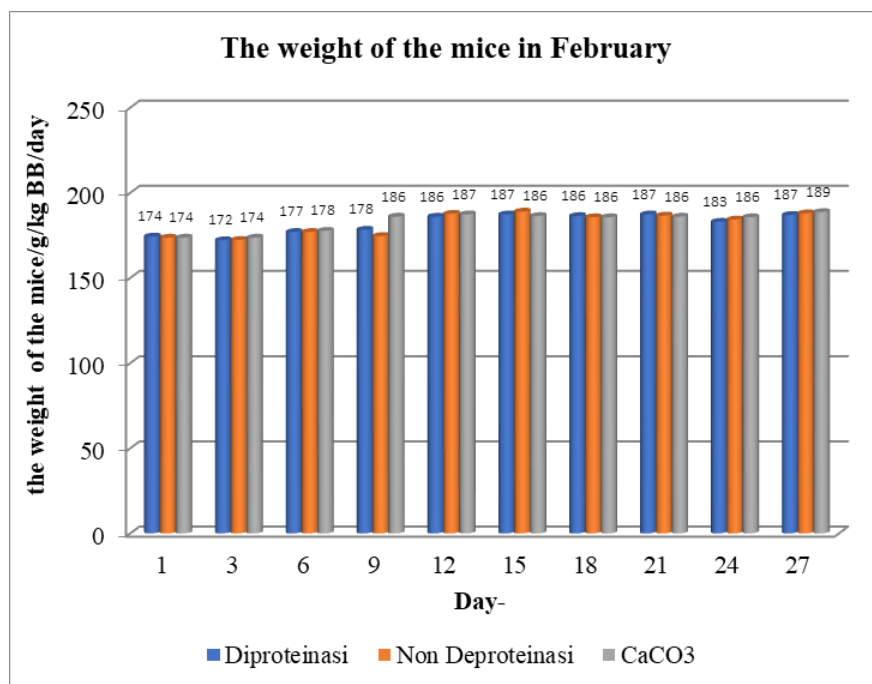


Fig. 2. Diagram of average body weight of rat during February treated with yellowfin fish bone meal and calcium

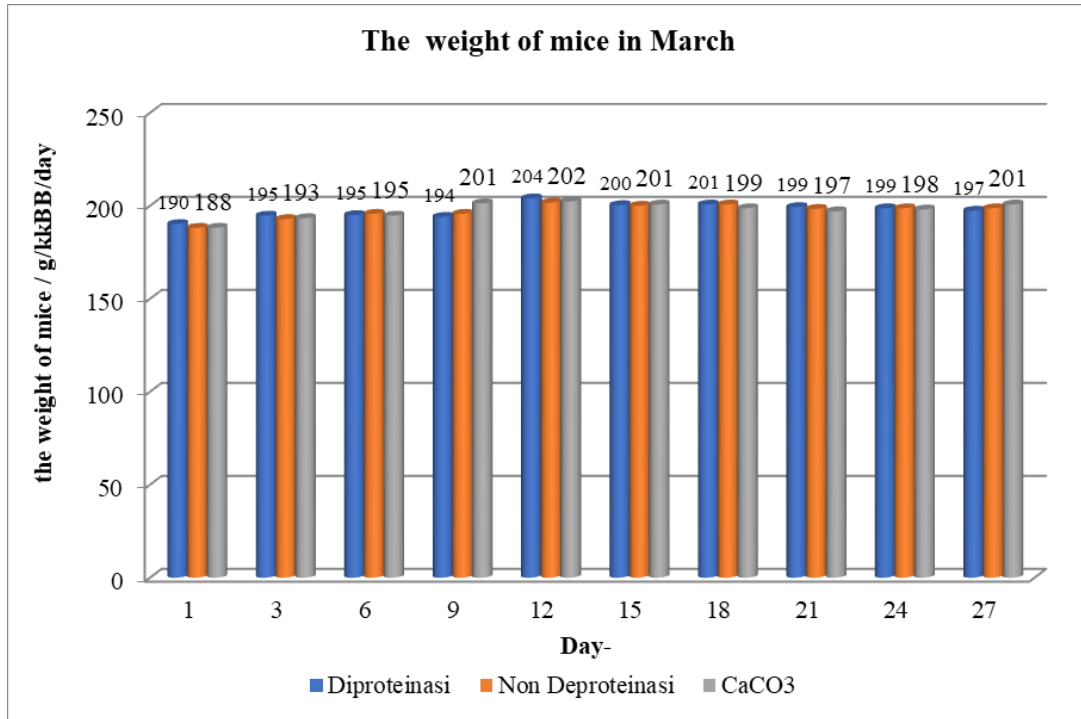


Fig. 3. Diagram of average body weight of rat during March treated with yellowfin fish bone meal and calcium

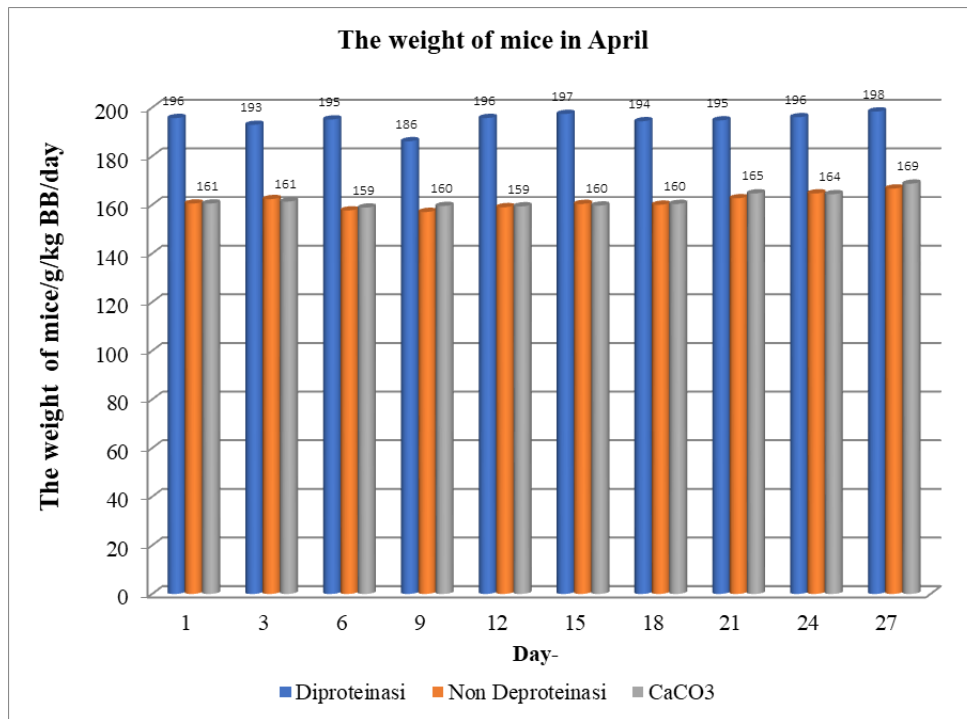


Fig. 4. Diagram of average body weight of rat during April treated with yellowfin fish bone meal and calcium

The same thing happened in the treatment in March and April. The average bodyweight of the rats ranged from 190g/ kg B.W./ day in the deproteinated, non-deproteinated, and CaCO₃ treatments. On day 1, the value of bodyweight was 190g/ kg B.W./ day and it increased on day 12 with a value of 204g/ kg B.W./ day. However, it decreased again on day 27 with a weight of 187g/ kg B.W./ day. In the CaCO₃ treatment, on the 1st day, the weight was 188g/ kg B.W./ day, increasing steadily until the 27th day, reaching 201g/ kg B.W./ day. Similarly, in April, the weight on the 1st day was 196g/ kg B.W./ day, with a slight decrease on the 9th day to 186g/ kg B.W./ day, followed by a continued increase, reaching 198g/ kg B.W./ day by the 27th day. In contrast, for CaCO₃ treatment, the weight on the 1st day was 161g/ kg B.W./ day, decreasing to 159g/ kg B.W./ day by the 12th day, then steadily increasing to 169g/ kg B.W./ day by the 27th day. The same thing happened to the bodyweight of rats with non-deproteinated treatment with the same average value as the CaCO₃ treatment.

3. Vertebrae radiology figure

The results revealed significant bone remodeling developments indicative of osteoporosis upon observing the vertebrae over a four-month period. Bone is a dynamic network that constantly remodels due to mechanical responses and hormonal changes. Bone remodeling occurs in the bone remodelling unit, where there is an emotional balance between osteoclast bone resorption and bone formation by osteoblasts. In humans, bone formation and absorption are in harmony at the age of 30- 40 years. This balance becomes disturbed and heavier towards bone absorption when women reach menopause and men reach 60. In individuals living with osteoporosis, abnormal bone turnover occurs, characterized by an imbalance where bone resorption exceeds bone formation. This increase in bone absorption compared to bone formation, particularly prevalent in postmenopausal women, is partly attributed to estrogen deficiency (Fig. 5).

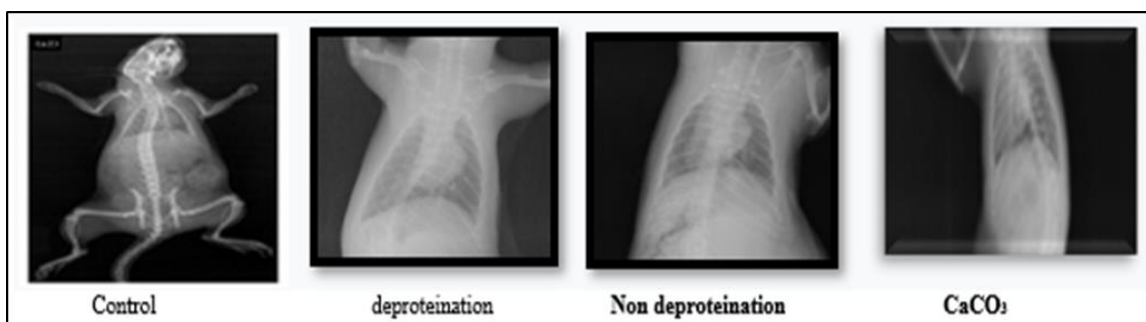


Fig. 5. Ovariectomy control mouse model and treatment of deproteinated, non-deproteinated yellowfin bone meal and CaCO₃

DISCUSSION

The results of chemical analysis for water content parameters of yellowfin fish bone meal with non-deproteinated and deproteinated treatments were 2.98 and 2.54%,

respectively. Compared with previous research by **Thalib *et al.* (2009)**, the water content of yellowfin fish bone meal using boiling water, acetic acid, and hydrochloric acid were 3.516, 3.340, 3.804%. In another study conducted by **Trilaksani *et al.* (2006)**, the water content in skipjack tuna bones was reported as 8.30%. When compared, the water content in this study is significantly lower. The difference in water content is attributed to the deproteinization treatment, which absorbs more water compared to the non-deproteinized boiling medium. Water content in food can be found between cells and inside cells. Free water is present in the tissue, while bound water is usually inside the cell. This difference is also thought to be because the autoclaving time in the previous study was only one hour, but in this study, it was carried out for two hours. The material's durability greatly influences the low water content since the fishbone meal material is more durable in storage. After all, it can inhibit the growth of bacteria and molds. The water content of yellowfin fish bone meal in this study is compared to the maximum standard of 14.5% for wheat flour. As the water content of yellowfin fish bone meal adheres to the required Indonesian National Standard (SNI), it meets the specified standard. Ash content is one of the components of food ingredients. Processing provides a decrease in the ash content after steaming and boiling, but there is an increase in the ash content after cooking the salt (**Salamah *et al.*, 2012**).

Previous research had an autoclaving time of one hour, whereas, in this study, the autoclaving time was carried out for two hours with the aim of losing some of the protein and fat in the material, which in turn could increase the ash content of the final material of yellowfin fish bone meal (**Talib *et al.*, 2014**). Compared to the maximum ash content requirement of 0.6% for wheat flour, the ash content of fishbone meal in this study is not specified within the standard. However, in this study, the focus is on the calcium and phosphorus content, as higher ash content typically correlates with higher levels of calcium and phosphorus in these materials.

The deproteinization treatment was successful in reducing protein content in fishbone meals. Protein content in this study for non-deproteinized and deproteinized treatments was 20.98 and 17.21%, and it was not statistically significant. Previous studies on the protein content of yellowfin fish bone meal using boiling water, acetic acid, and hydrochloric acid ranged from 24.91, 24.62 and 26.11% compared with this study. The protein content in the previous research was more significant than in this study. When compared to the SNI standard for wheat flour protein, which sets the standard at 7. The fishbone meal produced in this study has not entered the specified standard because it has a higher range of numbers, namely 20.98 and 17.21%. The observed protein content is likely attributed to the inherently high fat and protein content of fishbone meal. Despite undergoing boiling treatments with water and acetic acid, the decrease in protein content is not significant, indicating the robustness of the protein structure. The value of protein content in each treatment has decreased, influenced by the type of acid used in boiling

and soaking the raw materials. The longer material is in an acidic environment, the more likely it is that acids break down protein since acids can break down protein (Skrzyszowska & Samiec, 2020).

The fat content analysis in this study was 6.36 and 6.31% compared to previous studies, so this fat content was higher and lower. This was due to the 12-hour cooking process followed by autoclave and boiling using acetic acid and water. Moreover, the content of the yellowfin fish bone meal was reduced. The fat in the bones consists of an acidic structure so that the hydrolysis of fat occurs in the boiling process, which produces glycerol fatty acids. The reduction in fat content significantly affects the durability of the material. If the fat content of the material is high, it will accelerate rancidity due to fat oxidation (Talib *et al.*, 2016). Apart from being influenced by different boiling media, mineral content in yellowfin fish bones is also influenced by ecological factors during fishing, namely season, availability of nutrients, temperature, and salinity (Martínez *et al.*, 1998).

In this study, the calcium content analysis with non-deproteinated and deproteinated treatments was 238, 62, and 165.64mg/ g bk. In previous research by Thalib *et al.* (2014), three treatments were utilized: acetic acid (163.48mg/ g bk), hydrochloric acid (149.30mg/ g bk), and water (159.77mg/ g bk). These results did not show statistical significance when compared. The difference in the value of calcium is thought to be caused by the type of raw material used, which is fresher, and the bone size is much larger than the same material used previously. Fishbones have reasonably high mineral content, especially in the form of calcium phosphate. Calcium phosphate is a mineral that is very important to form bones and teeth and is helpful in the body's metabolism. Therefore, the need for calcium intake and balanced nutrition must be provided every day. Calcium in the body has a very strategic function since it is a macromolecule that is very important in bones and teeth' growth and teeth (Wacker & Holick, 2013). The treatment aims to obtain a high-quality bone meal, low in fat and protein, and high in calcium and phosphorus content.

The results of this phosphorus analysis, compared with previous studies, were that the phosphorus content in this study was higher. The phosphorus levels in yellowfin bone meal with hydrochloric acid boiling medium were lower, with values ranging from 0.66mg/ g bk, while those with water and acetic acid were 0.64mg/ g, and 0.62mg/ g/ bk, respectively. All three values were statistically significant, with no significant difference observed between them ($P < 0.05$). This improvement in results is presumed to be attributed to the refinement of the method used in this study compared to previous research, specifically the extension of autoclaving time from one hour to two hours. The ratio between Ca and P has a strong influence on the absorption process. Optimal calcium absorption in the intestinal cavity requires a calcium to phosphorus ratio of 1:1 to 1:3,

with an intestinal pH of less than 6. A calcium to phosphorus ratio greater than 1:3 will inhibit calcium absorption (**Sediaoetama, 2006**).

1. Fishbone flour therapy on body weight of rats

The results of weighing the rats were conducted every three days for three months. Before ovariectomy, the body weight of the rats was 213g/ kg B.W./ day, while for the non-deproteinated treatment it was 199g/ kg B.W./ day, and for CaCO₃ treatment it was 201g/ kg B.W./ day. However, after ovariectomy, the average weight of the rats decreased. There was a decrease in body weight observed on the 9th day, with a weight of 155g/ kg B.W./ day. Subsequently, there was an increase observed on the 27th day before necropsy, with a weight of 184g/ kg B.W./ day. This decrease in weight was thought to be caused by stress experienced by the mice during the ovariectomy, leading to a reduced appetite and subsequent weight loss. Stress, characterized by the body's reaction to external and internal stressors, can have various consequences, including changes in weight. The results of deproteinated yellowfin, non-deproteinated, and calcium carbonate bone meal therapy greatly influenced the weight gain of rats during the study process and statistically showed significantly different results ($P < 0.05$). These results are consistent with the findings of **Wiryanthini et al. (2017)**, who demonstrated that administering a dose of 280mg per 200 grams of body weight of rats with cocoa bean extract effectively reduced MDA (malondialdehyde) levels and increased other levels in rats experiencing oxidative stress due to psychosocial stress for 14 days. The results regarding Nox (xanthine oxidase) plasma levels are consistent with the findings of **Murphy et al. (2001)**, who demonstrated that administering cocoa powder containing 234mg of flavanols for four weeks inhibited platelet activation and function, thereby potentially preventing atherosclerotic plaque formation. Additionally, high calcium levels in the body were reported to reduce the secretion of parathyroid hormone, increase the hormone calcitonin, and suppress various nutrients such as protein, fat, and several minerals, as noted by **Bhattarai (2020)**. The average body weight of rats ranged from 190g/ kg B.W./ day in the deproteinated, non-deproteinated, and CaCO₃ treatments. On day 1, the body weight was 190g/ kg B.W./ day, which increased to 204g/ kg B.W./day on day 12 and then decreased again to 187g/ kg B.W./ day by day 27. In the CaCO₃ treatment group, the body weight on day 1 was 188g/ kg B.W./day and continued to increase steadily until day 27, reaching 201g/ kg B.W./ day. The bodyweight of the rats fluctuated over time, but some treatments experienced a significant increase because it was suspected that the rats began to adjust rapidly due to the addition of the dose given. Physiologically, the content of ingredients containing protein dramatically affects our bodies' growth process through their roles that other macronutrients cannot replace. Proteins play an essential role in metabolism as enzymes and transport media for molecules such as ions and oxygen. Protein also functions as a regulator of muscle movement, mechanical support of the skin and bones, and body defense or immunization

(Bali & Rafi, 2011). A factor influencing the growth of a rat's body is a diet rich in carbohydrates, protein, vitamins, and minerals. Rats are one of the experimental animals often used for research in laboratories, especially to evaluate the biological value of food and its effects on health. Rats do not have a gall bladder, cannot regurgitate their stomach contents, never stop growing. However, their growth rate typically decreases after reaching approximately 100 days of age (Visakh *et al.*, 2013). The findings from the current research align with those of previous studies. Over the course of 108 days from January to April, it was observed that while some rats experienced an increase in body weight, others exhibited weight loss.

Higher body weight provides a more significant mechanical load, where the mechanical load stimulates bone formation by reducing apoptosis and increasing the proliferation and differentiation of osteoblasts and osteocytes (Cao, 2011; López., 2016). This causes mice with a more significant body weight to have a higher bone density. Observations during the study revealed that the average body weight of rats increased at each dose level of yellowfin fish bone meal therapy. In contrast, rats in the control treatment group (without yellowfin fish bone meal therapy) and the calcium carbonate therapy group showed a decrease in body weight. These results indicate that increasing the dose can affect the body weight of the rats. This is thought to be because yellowfin fish bone meal has a high calcium and phosphorus content to help restore bone damage that has experienced osteoporosis.

The effect of the treatment had an impact on the weight gain of the rats. Another study using crab shell flour showed that the decrease in body weight of rats was higher and statistically significant ($P < 0.05$) at the rate of 20%. This is thought to be related to dietary calcium levels and the ratio of calcium and phosphorus in feed. The higher excretion of calcium in feces will affect the metabolic process in the body, and the rest will be excreted through feces. Bodyweight gain can be used as a criterion for measuring growth, a very complex process that includes both the increase in live weight and the development of all body parts simultaneously and evenly. The value added to bodyweight is obtained by measuring body weight, which is carried out periodically (Savvidis, 2011). The increase in body weight of the rats was influenced by the amount of calcium intake received, both from deproteinized and non-deproteinized yellowfin fish bone meal and calcium carbonate. The data on body weight measurement of rats for 30 days show that the greater the dose of calcium given, the rats' body weight can increase. However, the length of time of the study can affect the reduction in the body weight of the rats because the rats are stressed. After being treated with yellowfin fish bone meal, rats that have osteoporosis can improve bone damage, especially in the vertebrae bones. Contrary to the current study, research conducted by Widodo (2006) demonstrated that a calcium diet led to a reduction in the body weight of male rats

These results are different because the research did not use an ovariectomy mouse model but used male rats. A study utilizing the ovariectomy mouse model showed that the greater the calcium diet is given, the lower the bodyweight of the rats, while the lower the calcium diet was granted, the greater the bodyweight of the rats.

2. Vertebrae radiology

The dual energy absorptiometry (DEXA) results showed that the vertebral bones treated with deproteinated, non-deproteinated yellowfin tuna bone meal and with CaCO₃ from day 0 to day 30 showed different results. The difference was seen from the physical shape of the mice on x-ray on day 0, indicating that bone damage in the vertebrae was striking compared to controls. However, after being treated using yellowfin fish bone meal at doses of 400, 800, and 1600mg/ kg B.W./ day, the changes were visible in the vertebrae. In ovariectomy rats without therapy with yellowfin bone meal, the level of damage was very high, especially in the vertebrae. A different thing is shown in the ovariectomy mouse model that has been treated using yellowfin fish bone meal and CaCO₃. The higher the dose is given, the greater the remodeling process. A different thing is shown in normal mice without ovariectomy. The vertebrae are not damaged when compared to ovariectomy models.

X-ray results in normal rats show that there is no damage to the spine (vertebrae). The difference in results can be attributed to the fact that normal rats do not experience osteoporosis, unlike ovariectomized rats. Ovariectomy performed on normal rats can cause damage to bone tissue, especially in the spine. The ovariectomy process can reduce the levels of the hormone's estrogen and progesterone. This is caused by the ovaries, as the leading producer of the hormones estrogen and progesterone are not functioning, therefore hormone levels in ovariectomy rats will decrease drastically.

Rat bones treated with yellowfin fish bone meal and calcium carbonate experienced a faster remodeling process. The remodeling process usually consists of two stages: forming the matrix and the mineralization process. In bone formation, collagen type I and non-collagen protein are synthesized. The sequence of the bone mass formation process begins with the construction of the osteoid by osteoblasts consisting of collagen and non-collagen proteins that have not been mineralized. The osteoid will then mineralize to form hydroxyapatite crystals which are the main minerals of bone (**Blair, 2017**).

They are giving a dose of a yellowfin fishbone meal. The hormone estrogen will stimulate the release of mediators that affect the activity of osteoclasts, which function as bone absorbing cells. The number and movement of osteoclasts directly cause osteoporosis to absorb bone, influenced by mediators, and estrogen levels. Apart from estrogen levels, nutrition is one factor that influences the bone healing process, and one of them is calcium intake according to the standard human calcium needs per day (**Karpouzos, 2017**). Mineral requirements, especially calcium and phosphorus, play an

essential role in overall bone mass, and studies have shown that calcium deficiency can lead to osteoporosis. The increase in the activity of the parathyroid glands and parathyroid hormone will increase calcium absorption by the intestine and reabsorption of calcium by the kidney tubules and increase the activity of osteoclasia and inhibit osteoclast activity until osteoporosis occurs (**Nakada, 2017**). The cause of osteoporosis is not only due to calcium deficiency but also protein, phosphorus, magnesium, vitamin D, and vitamin K intake and is also an essential factor related to bone health (**Josse, 2013**).

The World Health Organization (WHO) defines osteoporosis as hip bone mineral density (BMD) that is 2.5 SD or more below the mean BMD for healthy young women (**Nicholas & Wilson, 2017**). It is usually measured by dual-energy absorptiometry (DEXA) to determine the density of the spine or hips. This decrease caused the ovariectomy rats to experience osteoporosis which was shown to decrease drastically when without therapy. The post ovariectomy group of rats given a combination of estrogen and progesterone showed a significant increase in muscular area compared to the control group. Administration of a variety of estrogen and progesterone increased estrogen receptor α (ER α) expression in muscular tissue compared to the control group. However, androgen receptors (A.R.) in muscular tissue did not experience a significant increase in rats given the combination of estrogen-progesterone compared to the control group.

Adipocytes, an essential source of estrogen production in menopausal women, inhibit bone absorption by osteoclasts. The increase in adipose tissue, along with the rise in BMI in menopausal women, results in increased estrogen production, which suppresses bone absorption by osteoclasts, increasing bone mass. The protective effect of obesity on osteoporosis is probably due to several mechanisms associated with greater body weight to provide a more significant mechanical load. The automatic load stimulates bone formation by reducing apoptosis and increasing the proliferation and differentiation of osteoblasts and osteocytes. As a result, people with the overweight body will tend to have a higher bone density. Obesity appears as a protective factor against osteoporosis. However, if obesity is measured by the percentage and distribution of body fat, obesity will be a risk factor for osteoporosis (**Migliaccio, 2011**). Obesity is related to insulin resistance, which is characterized by high plasma insulin levels. Elevated plasma insulin levels can cause various disorders, including excess androgens and estrogens in the ovaries. This can lead to increased sex hormones, which increase bone mass due to reduced osteoclast activity and increased osteoclast activity.

Although many studies have shown that obesity, as measured by the BMI (Body Mass Index) indicator, has a protective effect against osteoporosis, BMI estimates obesity is considered inaccurate. This is because body weight is a heterogeneous phenotype consisting of fat, muscle, and bone mass. Hence, it is not known whether fat mass or lean mass is associated with increased bone mass (**Cole, 2013**).

CONCLUSION

The chemical analysis results indicate that the highest calcium content is found in the boiling treatment of yellowfin fish bones using the non-deproteination method, while deproteinated boiling is more effective for phosphorus. Observation of rat body weight over 108 days reveals the highest values in treatment using deproteinated yellowfin fish bone meal therapy on the 12th day of March, reaching 204g/ kg B.W./ day at a dose of 1600mg/ kg B.W./ day. Vertebral bone radiographs of ovariectomized rats treated with deproteinated yellowfin fish bone meal therapy at 1600mg/ kg B.W./ day show significant changes in bone remodeling, marked by the formation of osteoid cells and osteoblasts on the vertebrae.

The implications of the study results suggest potential therapy for individuals with osteoporosis and highlight the environmental benefits of utilizing recycled fish bones into flour to prevent pollution. However, the study's limitations include the use of small rat samples, revealing that higher calcium diets lead to lower rat weights, while lower calcium diets lead to increased rat weights. Further research is needed to optimize calcium intake and assess its effectiveness in individuals with osteoporosis.

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