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Research Article
Effect of Low Protein Diet on Bone Structure of Young Wistar Mice

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Abstract

Background and Objective: Malnutrition and stunting are major unresolved problems in Indonesia. Protein deficiency can cause stunted growth, as well as make physical and cognitive abilities cannot reach their maximum potential. During childhood the need for protein must be fulfilled so that the peak of bone formation during adolescence can be perfect. In malnourished children, a low protein diet will lead to thinning of the bone cortex. Due to the high rate of stunting and malnutrition in children due to protein deficiency, a study was conducted on the effects of feeding low protein diet on rat bones. **Materials and Methods:** Male Wistar rats (n = 10) at 6-8 weeks old (body weight around 250 g), control groups were fed a normal chow diet and low protein diet groups were given low protein chow diet (protein 5%) for 18 weeks, then the rats were sacrificed and the femoral bones were isolated. Body weight, femur weight, femur length were checked and bone density was examined using X-ray. **Results:** The body proportions of the low protein group rats were smaller and thinner than those of the control group. This difference is supported by the significant weight loss starting from the sixth week after low protein feeding. There are significant differences in body weight and femur weight between the control and low protein diet groups. Bone density decreases significantly in low protein diet group. Macroscopically, the femur length of the low protein group was shorter than the control group, however the femur length did not show significant differences statistically between the two groups. **Conclusion:** A low protein diet decreased the body weight of the rats, also causing impaired bone growth characterized by decreasing femur weight. The low protein diet also caused osteoporosis in the bones.

Key words: Low protein diet, femur weight, femur length, bone density, malnutrition

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Malnutrition and stunting are major unresolved problems in Indonesia. Malnutrition or severe wasting is a nutritional status below normal based on the calculation of body weight per age, which is an index of less than -3 SD. Stunting is a condition of chronic malnutrition that makes toddlers too short for their age. According to UNICEF on 2018, Indonesia is ranked 4th in the world for malnutrition and 5th for stunting. Poverty and lack of knowledge are factors that cause malnutrition and stunting and vice versa, these health problems can cause ongoing poverty[1-4]. Indonesia National Basic Health Research (Riskesdas (Riset Kesehatan Dasar)) 2018 shows 13.8% of toddlers with wasting and 3.9% of toddlers with malnutrition (super wasting). This percentage is classified as a high-category problem, namely the wasting rate between 10-14%, according to WHO. Wasting and super wasting are conditions of acute malnutrition characterized by low body weight compared to height. The 1 from 3 toddlers suffers from stunting, which is a problem of chronic malnutrition due to inadequate nutrition during the first 1,000 days of life. In 2017, there were 27.5% of toddler suffering from stunting, which according to WHO this figure includes chronic nutritional health problems, which is above 20%. In accordance with the 2020-2024 RPJMN policy directions, malnutrition and stunting are one of the priorities for health development, with a target of reducing the prevalence of wasting to 7% and stunting to 14% in 2024[1,2,5].

The Food Security Agency noted that the daily protein consumption of the Indonesian population in 2020 was 62.05 grams/capita/day, a decrease of 1.3% from 2019, where the standard figure for protein sufficiency consumption of the Indonesian people is 57 grams/capita/day. Vegetable sources account for 65.7% of the protein consumed. The daily protein consumption is above the standard, especially in urban communities. Rural residents tend not to get enough standard protein. Humans require a minimum of 0.8 g kg^{-1} of protein to maintain body homeostasis. Protein is needed for growth and development to build and maintain bones, muscles, red blood cells and to form enzymes, since in the womb so that the physical condition during adulthood and elderly can be maintained optimally. One-third of bone mass is formed by protein, both collagen and non-collagen[3,4,6,7].

Protein deficiency can cause stunted growth, as well as make physical and cognitive abilities cannot reach their maximum potential, especially in women who will become mothers, malnutrition can be transmitted to the next generations. During childhood the need for protein and calcium must be fulfilled so that the peak of bone formation during adolescence can be perfect. Average bone growth stops at the age of 16-18 years[8-10]. Low protein diets are commonly adopted by people with kidney disease to reduce the workload of the kidneys. In normal people in developed countries, low protein diets are not an option due to the abundance of animal products and lifestyle changes. In developing countries, low protein diets may occur due to poor socioeconomic conditions. This is also influenced by geographical conditions and education levels, where carbohydrate consumption is greater in groups with low education levels[11,12].

Low protein diet will decrease the number and activity of osteoblasts within 14 days. In malnourished children, a low protein diet will lead to thinning of the bone cortex[13,14]. Due to the high rate of stunting and malnutrition in children due to protein deficiency, a study was conducted on the effects of feeding low protein diet on rat bones.

MATERIALS AND METHODS

Study area: This study was conducted in Central Laboratory, Universitas Padjadjaran, Sumedang, Indonesia from October 2021 until May 2022.

Subject: Male Wistar rats at 6-8 weeks old (body weight around 250 g; n = 10) were brought from PT Bio Farma, Bandung, Indonesia. Rats were housed at 24°C, 55% relative humidity and a 12 hrs light dark cycle and given food *ad libitum* and water *ad libitum*. After weaning period, rats were divided into 2 groups, control group and low protein diet group. Control groups were fed normal chow diet and low protein diet groups were given low protein chow diet (protein 5%, carbohydrate 48%, fat 9%, water 10% and calcium 0.8%). All groups were given chow diet for 18 weeks. The body weight was measured every four weeks. By the end of experimental period, the rats were sacrificed and the femoral bones were isolated. Femoral bones were weighed using electronic pocket scale (Camry®) and measured length using digital caliper (TriiCles®). Femoral bones were kept in formalin 10% solution until used.

Ethical consideration: Animal procedures were approved and carried out according to the guidelines of the Animal Ethics Committee of Universitas Padjadjaran Bandung, Indonesia (No. 1398/UN6.KEP/EC/2022).

Bone X-ray: The femoral bones were subjected to X-ray imaging using commercial imaging system (Cube X series, Portable X-ray, JPI Healthcare Co., Ltd., South Korea), 50 kVp,

300 ma, exposure time 0.16 ms, 6.60 mAs and X-ray beam 90 cm. Bone density were analyzed using ImageJ software.

Statistical analysis: Data were analyzed with independent t-test using IBM SPSS statistics version 21 (SPSS IBM, Armonk, New York, USA) and $p<0.05$ considered as significant. Data are expressed as Mean±Standard Deviation (SD).

RESULTS

Mice that received a low protein diet had smaller body than control mice after 8 weeks of treatment as shown in Fig. 1(a-b) depicted the average body weight of rats receiving low protein diet which began to show significant weight loss from the 6th week ($p<0.01$). The body weight of the rats continued to decrease significantly starting from the 14th week ($p<0.01$) and continuing until the 18th week compared to the control rats. On the other hand, the control mice continued to gain weight until the 18th week.

Macroscopically, the femurs of rats that received a low protein diet were smaller and shorter than those of control rats, as shown in Fig. 2(a-c). The results of measurement of the length of the femur using a digital caliper showed non-significant differences in the average length of the femur. The mean femur length of low protein diet rats was 33.85±2.57 mm compared to 36.98±0.42 mm of control rats ($p = 0.071$). The average femur weight of the rats was significantly different, where the average femur weight of the low protein diet rats was 0.742±0.134 g, while the average femur weight of the control rats was 0.986±0.13 g ($p<0.05$).

The X-ray photos anteroposterior of the femurs (Fig. 3a) of the low protein diet rats showed a thinner cortex area and trabecular area are more lucent than the femurs of control rats. It showed an osteoporosis process in rats on low protein diet. From calculations using the ImageJ software, the average bone density area in the low protein diet rats was 30.524±7.66 AU, significantly different from the control rats, which was 49.246±4.005 AU ($p<0.001$) as shown as on graphics on Fig. 3b.

DISCUSSION

In this study, an animal model of malnutrition with low protein diet administration was developed. Results showed that administration of low protein diet during growth period affected bone growth and development. In low protein diet group, bone weight was lower 25% compared to normal chow diet group ($p = 0.020$). To analyse whether bone weight influence bone density, we observed the bone density using X-ray protocol. Bone density in the low protein diet group was lower 38% compared to the control group ($p = 0.001$).

Protein is needed by the body to build and maintain bones, muscles, red blood cells and also to form enzymes. One-third of bone mass is formed by proteins, both collagen and non-collagen[3,8,9]. Bone development is influenced by genetics and environmental factors such as nutrition and exercise[15].

Human bone growth stops at the age of 16-18 years. Rat bone growth reaches peaks at 4-6 months of age. After 6 months of age, bone starts to become porous as extra bone

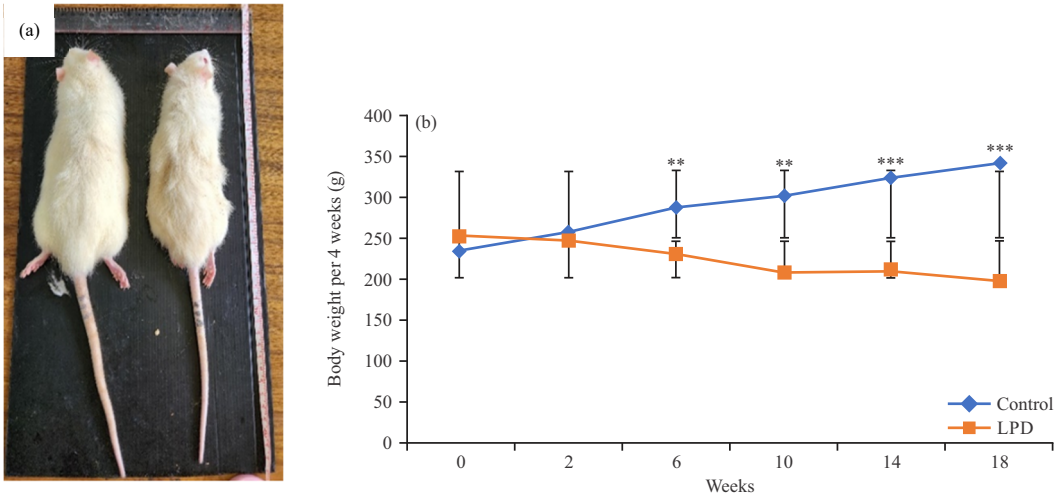

Fig. 1 (a-b): Representative figure (a) of normal rat (left) and low protein diet rat (right), (b) shows body weight per weeks

Significant weight loss in low protein rats starting at 6th week 228.6±28.11 g, n = 5 ($p<0.01$) compared to control rats 287±28.11 g, n = 5, low protein diet group weight continuing to decrease until 18th week 196.4±49.48 g, n = 5, compared to control group 340.4±27.32 g, n = 5, *Means $p<0.05$ significant, **Means $p<0.01$ highly significant and ***Means $p<0.001$ very highly significant

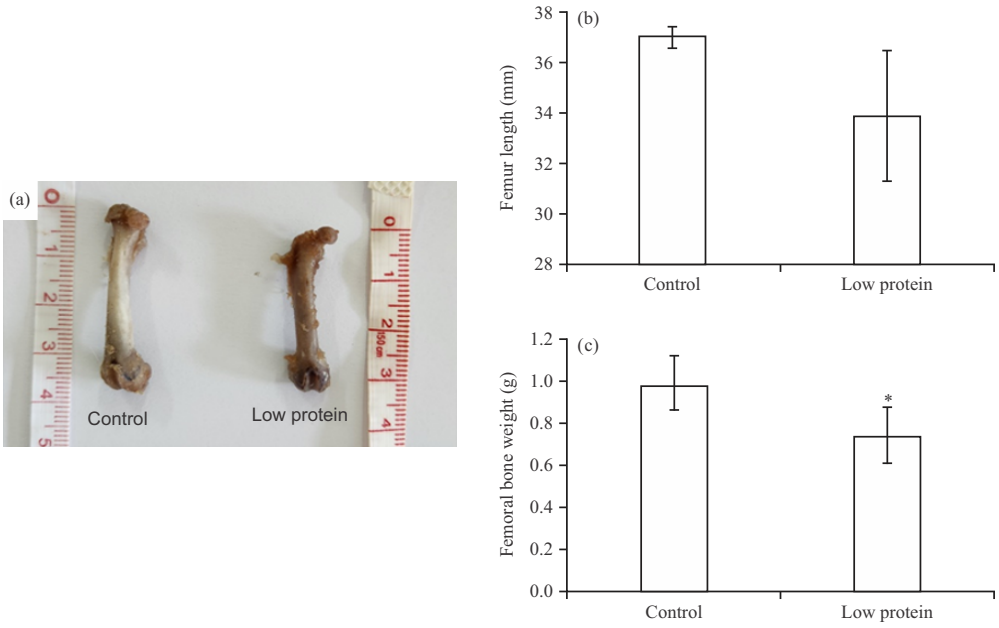

Fig. 2(a-c): Macroscopic differences in bone length (a) between control (left) and low protein diet mice (right), (b) Difference in femur bone length of low protein diet group 33.85±2.57 mm, n = 5, compared to control rats 36.98±0.42 mm, n = 5 ($p>0.05$), (c) Difference in femur bone weight between control rats 0.986±0.13 g, n = 5 and low protein diet rats 0.742±0.134 g, n = 5 ($p<0.05$) and *Means $p<0.05$ significant

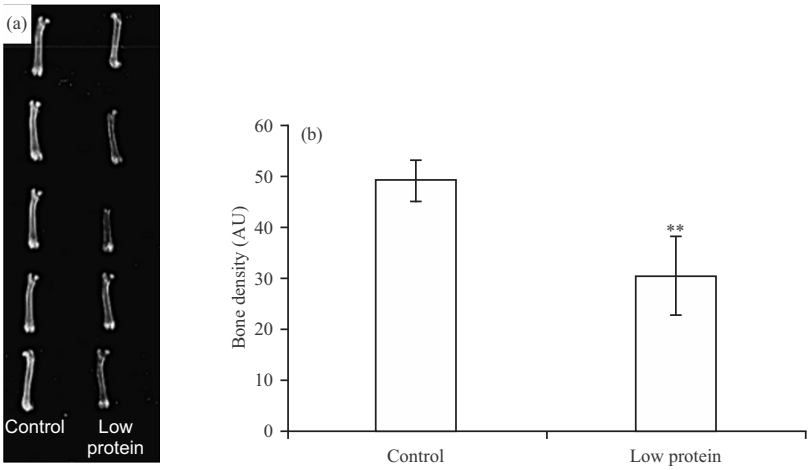

Fig. 3(a-b): X-Ray results of the femur (a) of the control rat (left) compared to the femur of the low protein diet rat (right), (b) Results of bone density calculation using ImageJ software

Bone density in rats on a low protein diet 30.523±7.662 AU, n = 5 decreased significantly compared to control rats 49.246±4.005 AU, n = 5 and **Means $p<0.01$ highly significant

mineralisation, such as blood vessels, begins to occur[16]. In this study, 6-8 weeks old rats were fed a low protein diet for 18 weeks. The age of the rats at the time of bone sampling was 24-26 weeks, equivalent to about 18 years of human age. The initial weight of the rats when they first arrived was between 210-260 g. After 18 weeks of feeding, the group that received the low protein diet experienced a significant weight loss compared to the group that received the normal diet. This also happened in the study of Wu *et al.*[7], where the reduction in body weight in rats given low protein diet was associated with a reduction in deposits of white adipose tissue, brown adipose tissue and liver organs. Protein deficiency conditions

make rats use adipose reserves. Protein deficiency causes decreased serum leptin levels, inhibition of the mTOR signalling pathway, reduced phenylalanine, tryptophan, aspartic acid and reduced hunger which will result in weight loss[7].

Stunting is a state of chronic malnutrition so that the height of toddler is too short compared to their age[2]. Protein deficiency will decrease the expression of BMP2 (Bone Morphogenetic Protein 2) which is a growth factor that acts as a stimulant of mesenchymal cell differentiation into osteoblasts. The BMP2 also plays a role in morphogenesis, proliferation, differentiation, migration and apoptosis of osteoblasts through activating the p38 pathway which will increase the expression of RUNX2 as a transcription factor for bone formation. Reduced BMP2 will reduce bone mass, cause bone to fail to grow wider and result in abnormal bone geometric shape and increase the risk of fracture[15,17,18]. In this study, femur bone weight in the low protein diet rat group were significantly different from the control group. This situation is thought to be because low protein diet will reduce the expression of BMP2 and RUNX2. Low protein diet will also decrease the expression of miR-26a which functions for angiogenesis and osteogenesis during the process of bone regeneration and repair. The miR-26a will regulate osteoblast differentiation through SMAD1 which will activate BMP signalling, allowing osteogenesis to occur. In this case, protein deficiency not only affects the number of osteoblasts but also inhibits the potentiation of osteogenesis[13]. Femur lengths in this study showed differences between the groups, but did not show significant differences by statistic measure. Ribeiro *et al.*[19], used high protein diet to mice and gave swimming intervention, but the length of tibia did not increase. Both low protein diet and high protein diet will make bicarbonate increase, followed by acid-base compensation, because acidic state of the body. Bone also participates in acid-base homeostasis. Bone will release alkaline minerals into body fluids through increased activity of osteoclasts, resulting in bone resorption[20].

In this study, 5% low protein diet was used. Normally rat diet contains about 26% protein. In the X-ray images of the femurs of the low protein rats, the cortex area was thinner, as well as the trabecular area was more lucent than the control, indicating a process of osteoporosis. Mean bone density was also significantly different. Low protein diet for 14 days began to cause a decrease in the number and activity of osteoblasts. It is suspected that there is an increase in osteoclast activity in bone when protein is deficient. More osteoclast activity than osteoblast will lead to reduced bone mass, destruction of bone microstructure and decreased bone strength. Bone marrow mesenchymal stem cells will differentiate into adipocytes, not osteoblasts, under protein deficiency conditions[13,14,21]. This process was similar to the study of Bourrin *et al.*[22] where feeding a 2.5% protein diet will cause reduction in bone mineral density in the lumbar area, femur and proximal tibia. After 24 days of low protein diet, there was 30% decrease in periosteum mineralization area, 67% decrease in bone formation and an increase in osteoclast activity. Research of Mekraldi *et al.*[23] also suggested that feeding low protein diet for 15 weeks caused a significant decrease in bone microarchitecture.

The results of this study show that feeding low protein diet to rats for 18 weeks starting from the age of 6-8 weeks or the equivalent of a toddler, has a negative effect on bone. Protein deficiency conditions need to be prevented in growing children because bone growth will stop around the age of 18.

CONCLUSION

Feeding a low protein diet for 18 weeks decreased the body weight of the rats, also causing impaired bone growth characterized by decreasing femur weight. The low protein diet also caused osteoporosis in the bones. Further research is needed on further interventions if protein deficiency condition has occurred and the correlation with vitamin D.

SIGNIFICANCE STATEMENT

Malnutrition is one of the major problems in developing countries. Malnutrition could lead to various complications such as stunting, cognitive decline and other health problems. Precise cellular and molecular mechanism pathway contribution to malnutrition is still uncertain and involves multifactorial processes nutritional intake and hormonal changes. Thus, it is important to develop an animal model to study the multifactorial process involving malnutrition. In this manuscript, the development of a malnutrition animal model and several measurements of growth retarded such as bone length and weight were presented. This study showed that a low protein diet decreased the body weight of the rats, also causing impaired bone growth characterized by decreasing femur weight. The low protein diet also caused osteoporosis in the bones.

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