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The Bambara Groundnut's Potential for Heart Histological Repair in Protein-Deficient Female Mice

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ABSTRAK

Defisiensi protein diketahui memberikan perubahan histologis yang signifikan pada jantung seperti hipertrofi, penebalan dinding ventrikel kiri, peningkatan deposisi matriks ekstraseluler dan diameter kardiomiosit, serta fibrosis interstisial. Kacang bambara (*Vigna subterranea*) dengan kandungan protein dan asam amino berpotensi untuk menanggulangi defisiensi protein dan memperbaiki perubahan struktur histologis pada jantung. Penelitian ini bertujuan untuk mempelajari pengaruh defisiensi protein 10% dan penambahan kacang bambara terhadap struktur histologis jantung mencit betina galur Swiss-Webster. Lima belas jantung dari 5 kelompok dikumpulkan untuk dipreparasi dengan metode parafin setebal 6 µm, dan diwarnai dengan Hematoxylin-Eosin dan Mallory Acid Fuchsin. Data dalam penelitian ini adalah parameter biometrik, lebar kardiomiosit, dimensi internal ventrikel kiri (LVID), ketebalan dinding posterior ventrikel kiri (LVPW), dan struktur histologis otot jantung. Data kuantitatif dianalisis dengan ANOVA satu arah dan *post hoc* dengan Duncan (p-value=0,05), sedangkan data kualitatif dianalisis menggunakan Kruskal Wallis (p<0,05). Tidak terdapat perbedaan yang signifikan (p>0,05) antara kelompok kontrol dan perlakuan pada parameter biometrik, LVID, LVPW, dan lebar kardiomiosit. Pada parameter struktur histologis otot jantung, kelompok defisiensi protein menunjukkan perubahan berupa lesi atrofi, hipertrofi, nekrosis, dan fibrosis yang lebih banyak dibandingkan dengan kelompok kacang bambara. Dengan demikian, penambahan kacang bambara berpotensi sebagai suplemen protein yang dapat memperbaiki struktur jantung pada kondisi defisiensi protein

Kata kunci: defisiensi protein, jantung, kacang bambara, mencit betina, struktur histologis

ABSTRACT

Protein deficiency is known to cause significant histological changes in the heart, such as hypertrophy, left ventricular wall thickening, increase of extracellular matrix deposition and cardiomyocyte diameter, and interstitial fibrosis. Bambara groundnut (*Vigna subterranea*), with protein and several amino acids, has the potential to overcome protein deficiency and repair the histological changes in the heart. This research aims to study the effect of 10% protein deficiency and adding bambara groundnut on the histological structure of Swiss-Webster female mice's hearts. Fifteen hearts from 5 groups were collected to be prepared with the paraffin method with 6 µm thickness and stained with Hematoxylin-Eosin and Mallory Acid Fuchsin. The data in this research are biometric parameters, cardiomyocyte width, left ventricular internal dimension (LVID), left ventricular posterior wall (LVPW) thickness, and the histological structure of the heart muscle. The quantitative data were analyzed with one-way ANOVA and post hoc multiple comparisons (p-value=0.05), whereas the qualitative data were analyzed using Kruskal Wallis (p<0.05). There were no significant differences (p>0.05) between the control and treatment groups on the biometric parameter, LVID, LVPW, and cardiomyocyte width. In the histological structure of heart muscle, the protein deficiency group shows alteration such as atrophy, hypertrophy, necrosis, and fibrosis lesions that are higher than the group with the addition of bambara groundnut. Therefore, adding bambara groundnut has the potential as a protein supplement to repair the histological structureof the heart with protein deficiency.

Keywords: protein deficiency, heart, bambara groundnut, female mice, histological structure

INTRODUCTION

Protein is an essential macronutrient for the structure and function of the heart. The inadequacy of protein intake may cause protein deficiency that alters the heart muscle's cellular regulation and histological structure (Penitente *et al.,* 2014). This alteration is a part of the compensatory response and serves as an adaptive mechanism to face malnutrition in the body (Mendes *et al*., 2017). Several studies involving rodents as animal models proved the structural change of heart muscle due to protein deficiency. For instance, hypertrophy and the left ventricular wall thickening (6% protein) (Murça *et al*., 2012), a decrease in left ventricular internal diameter, and an increase in extracellular matrix deposition (8.8% protein) (Hennig *et al*., 2019). Additionally, there is an increase in cardiomyocyte diameter and interstitial fibrosis (4% protein) (Ferreira *et al*., 2022).

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is one of the legumes that is primarily produced in Sub-saharan Africa (Adebowale *et al*., 2011). The nutrient-rich legume is well known for its high protein and amino acid content, which can be used as an alternative plant-based protein addition for protein deficiency (Khan *et al*., 2021). Bambara groundnut contains 23.6% protein, with vicilin (7S) and legumin (11S) as the dominant storage protein (Tan *et al*., 2020). 100 g of bambara groundnut protein also contains several essential amino acids, such as leucine (6.7 g), lysin (6.1 g), and phenylalanine (5.28 g), as non-essential amino acids like glutamic acid (15.34 g), aspartic acid (12.34 g), and arginine (8.06 g) (Adebowale *et al*., 2011). Based on the in vitro protein digestibility (IVPD), the raw bambara groundnut has a protein digestibility value ranging from 70%-81%. This value will increase to 87.5% with the cooking process (Tan *et al*., 2020).

Protein deficiency can lead to a significant histological change in the heart muscles. Nevertheless, little is known about the effect of a 10% protein deficiency and bambara groundnut addition on rodent heart muscle's histological structure, especially mouse's heart. Therefore, this research was conducted to study the effect of 10% protein deficiency on the histological structure of the heart muscle in adult female Swiss-Webster mice (*Mus musculus* Linnaeus, 1758) and also learn about the potency of bambara groundnut to repair the histological structure of heart muscle affected by protein deficiency. This research is focusing on the quantitative parameters, such as body weight, heart weight, heart index, cardiomyocyte weight, left ventricular internal dimension (LVID), left ventricular posterior wall (LVPW) thickness, and the histological structure of the heart muscle.

MATERIALS AND METHODS

Animal

The Medical and Health Research Ethics Committee (MHREC) approved the experimental protocol from the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, with the ethics committee approval letter KE/FK/0913/EC/2022. Fifteen female Swiss-Webster mice were obtained from the Integrated Research and Testing Laboratory (LPPT), UGM. They were kept in the Animal House and divided into five groups with three mice per cage (polypropylene cage with size 40 x 50 x 50 cm^3). They were maintained under controlled environments with artificial 12 hours of light/dark cycle, temperature 22°C, and humidity 55%. Before the experiment, 1-month-old Swiss-Webster mice were acclimatized for seven days and fed with *ad libitum* access to a standard diet (AINS-93M) and drinking water (Reverse Osmosis).

Diet Treatments

This research involved five groups with different treatments of diet: 1) control, with a normal protein diet (contains 14% protein); 2) protein deficiency (PD), with the deficient diet (contains 10% protein); 3) PD 100, with the deficient diet and the addition of 100 g bambara groundnut (contains 11.3% protein); 4) PD 200, with the deficient diet and the addition of 200 g bambara groundnut (contains 12.6% protein); and 5) PD 300, with the deficient diet and the addition of 300 g bambara groundnut (contains 13.9% protein). The bambara groundnut (*Vigna subterranea* (L.) Verdc.) type black testae were obtained from the bambara Groundnut Research Center in Gresik, East Java, Indonesia. The calculation of the protein percentage in every group was based on the proximate analysis from Pusat Studi Pangan dan Gizi (PSPG), UGM. The composition of the primary dietary nutrients and minerals in every group is given in Table 1.

Data Collection

After 60 days of treatment, the animals were weighed to collect the body weight data. The animal was anesthetized with 0.5 mL of ketamine (100 mg/kg body weight) and 0.25 mL of xylazine (10 mg/kg body weight) by intraperitoneal administration. The termination is carried out by cervical dislocation undertaken by an expert. For histological analysis, 15 hearts from 5 groups (3 samples each) were removed and weighed to measure the heart index based on the heart and body weight ratio. The longitudinal sections of the whole heart were fixed in 10% Neutral

Buffer Formalin (v/v) before further preparation using the paraffin method (Baudouy *et al*., 2019) with modification. The fixed organs were dehydrated in graded serial ethanol and embedded in paraffin. Blocks were sectioned on a rotary microtome (Microm GmbH Nr 5870) with a 6 μm thickness. The slide was stained with Ehrlich Hematoxylin and Eosin (HE, consists of 1% of Eosin Y in 70% alcohol) for histopathological observation such as hypertrophy, necrosis, atrophy, hemorrhage, and leukocyte infiltration. Mallory Acid Fuchsin (MAF) was also used to determine fibrosis lesions in the heart muscle.

The histopathological observations were performed with an ordinal scoring method with a score ranging from 0-4. "0" score indicated there is no lesion in the field of view, and a "1" score suggests <1% lesion in the field of view, "2" indicates 1%-5% lesion in field of view, "3" indicates 6%-10% lesion in field of view, and "4" shows 11%-15% lesion in field of view. Photomicrographs were taken on a light microscope (Leica ICC50 E) integrated into a digital camera. The measurement of cardiomyocyte width and histopathological observations were performed with 40 x 10 magnification in the right ventricle, interventricular septum, and left ventricle area. Besides that, the left ventricular internal dimension (LVID) and left ventricular posterior wall (LVPW) thickness were measured with 4 x 10 magnification in the left ventricle area. All data were taken with imageJ.

Statistical Analysis

The quantitative data such as body weight, heart weight, heart index, cardiomyocyte width, LVID, and LVPW were analyzed using one-way ANOVA and post hoc multiple comparisons with Duncan ($p<0.05$). The histopathological analysis was performed using the Kruskal Wallis non-parametric test with significant $p<0.05$. All statistical analysis was performed using SPSS version 25.

RESULTS

Biometric Parameter

Biometric parameter measurement aims to determine whether there are changes in body weight, heart weight, and heart index due to protein deficiency treatment and bambara groundnut addition. On biometric parameters, all biometric parameters did not show any significant differences between groups ($p>0.05$) (Table 2.)

Table 1 Composition of female mice's isocaloric diet on control, protein deficiency, and protein deficiency with bambara groundnut addition groups

Diet compositions (g)	Control	PD	PD 100	PD 200	PD 300
Sucrose	100	100	100	100	100
Corn starch	621	661	561	461	361
Casein	140	100	100	100	100
Bambara groundnut	-	-	100	200	300
Corn oil	40	40	40	40	40
Vitamin mix	10	10	10	10	10
Mineral mix	35	35	35	35	35
Choline chloride	3	2.5	2.5	2.5	2.5
Tert-butylhydroquinone (TBHQ)	0	0.01	0.01	0.01	0.01

Table 2 Protein deficiency and the addition of bambara groundnut did not show any change in biometric parameters of female mice

Parameters	Control	PD	PD 100	PD 200	PD 300
Body weight (g)	40.63 ± 4.41	41.03 ± 1.03	45.87 ± 2.05	45.93 ± 5.23	44.40 ± 1.06
Heart weight (g)	0.19 ± 0.04	0.18 ± 0.03	0.21 ± 0.05	0.17 ± 0.04	0.23 ± 0.06
Heart index (10^{-3})	4.9 ± 1.40	4.4 ± 0.70	4.6 ± 1.00	3.9 ± 1.43	5.1 ± 1.51

Data are shown as mean ± SD (n=3, $p>0.05$).

LVID (Left Ventricular Internal Dimension) and LVPW (Left Ventricular Posterior Wall) Thickness Measurement

LVID and LVPW measurements aim to determine whether there are changes in chamber diameter and left ventricle wall thickness due to protein deficiency treatment and bambara groundnut addition when compared to the control group. However, the statistical results showed that the mean LVID and LVPW between groups did not differ significantly (p>0.05) (Figure 1.)

Cardiomyocyte Width

Cardiomyocyte width measurements were carried out to determine the effect of protein deficiency and addition with bambara groundnut on changes in the size of cardiomyocyte width. Based on the results listed in Figure 2, the five groups had an average cardiomyocyte width ranging from 14 to 17 µm. The statistical results showed that the mean width of cardiomyocytes in the three observation areas did not differ significantly between groups (p>0.05).

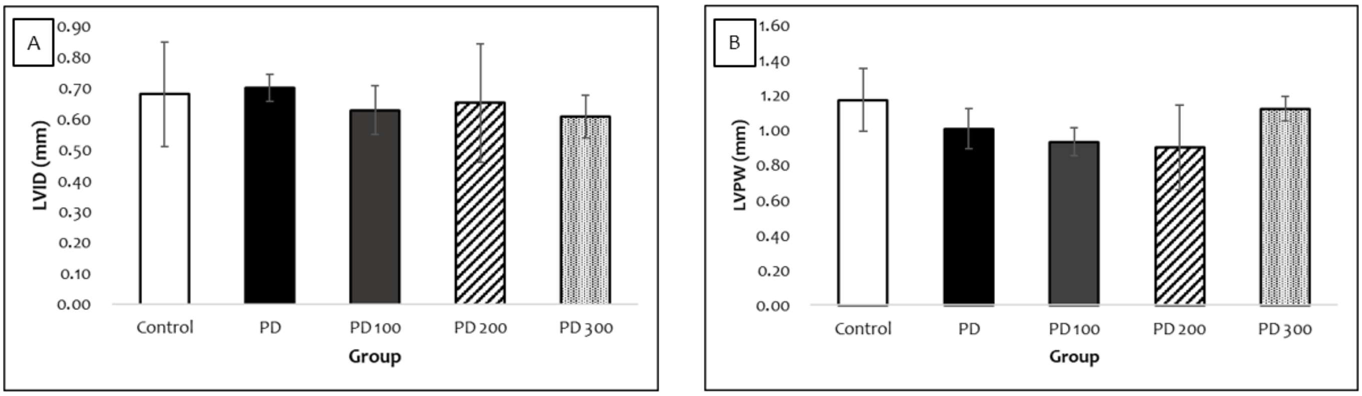

Figure 1 Protein deficiency and the addition with bambara groundnut did not show any change in (A) LVID and (B) LVPW of female mice. Notes: PD = Protein deficiency; PD 100 = Protein deficiency with the addition of 100 g bambara groundnut; PD 200 = Protein deficiency with the addition of 200 g bambara groundnut; PD 300 = Protein deficiency with the addition of 300 g bambara groundnut. Data are shown as mean ± SD (n=3, p>0.05).

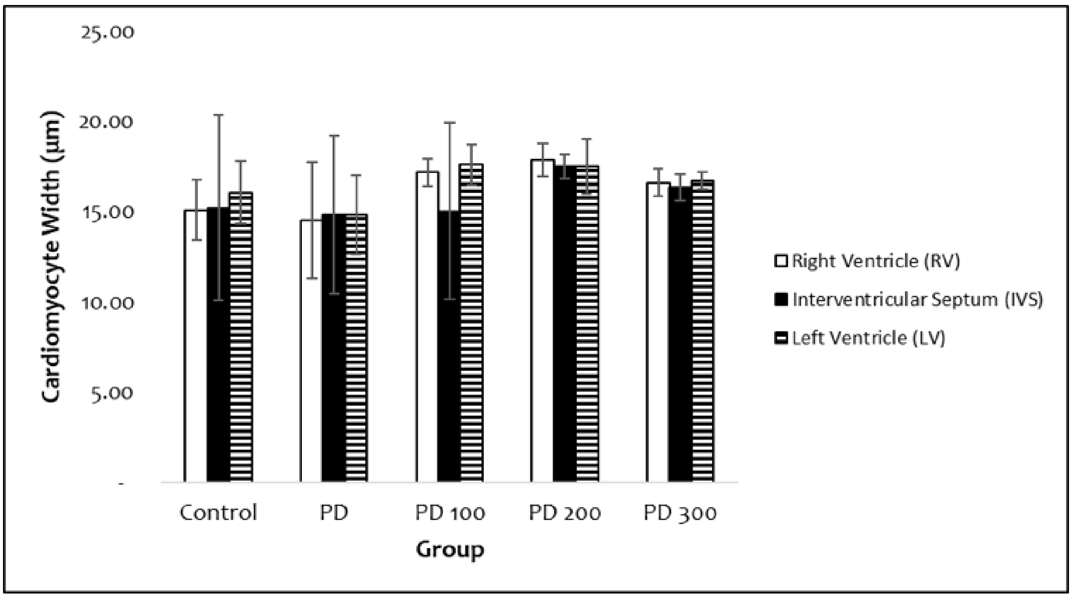

Figure 2 Protein deficiency and the addition with bambara groundnut did not show any change in cardiomyocyte width of female mice. Notes: PD = Protein deficiency; PD 100 = Protein deficiency with the addition of 100 g bambara groundnut; PD 200 = Protein deficiency with the addition of 200 g bambara groundnut; PD 300 = Protein deficiency with the addition of 300 g bambara groundnut.Data are shown as mean ± SD (n=3, p>0.05)

The Histological Structure of Heart Muscle

Observation of the heart muscle's histological structure aims to assess whether there are changes in the structure of the heart muscle due to protein deficiency treatment and the addition of bambara groundnut. The results of comparative descriptive observation of mice heart muscle showed changes in histological structure in the heart muscle of the mice with protein deficiency (Figures 3 and 4.). These changes were evidenced by damage in several areas of atrophy lesions, hypertrophy, necrosis, hemorrhage, leukocyte infiltration, and fibrosis. As

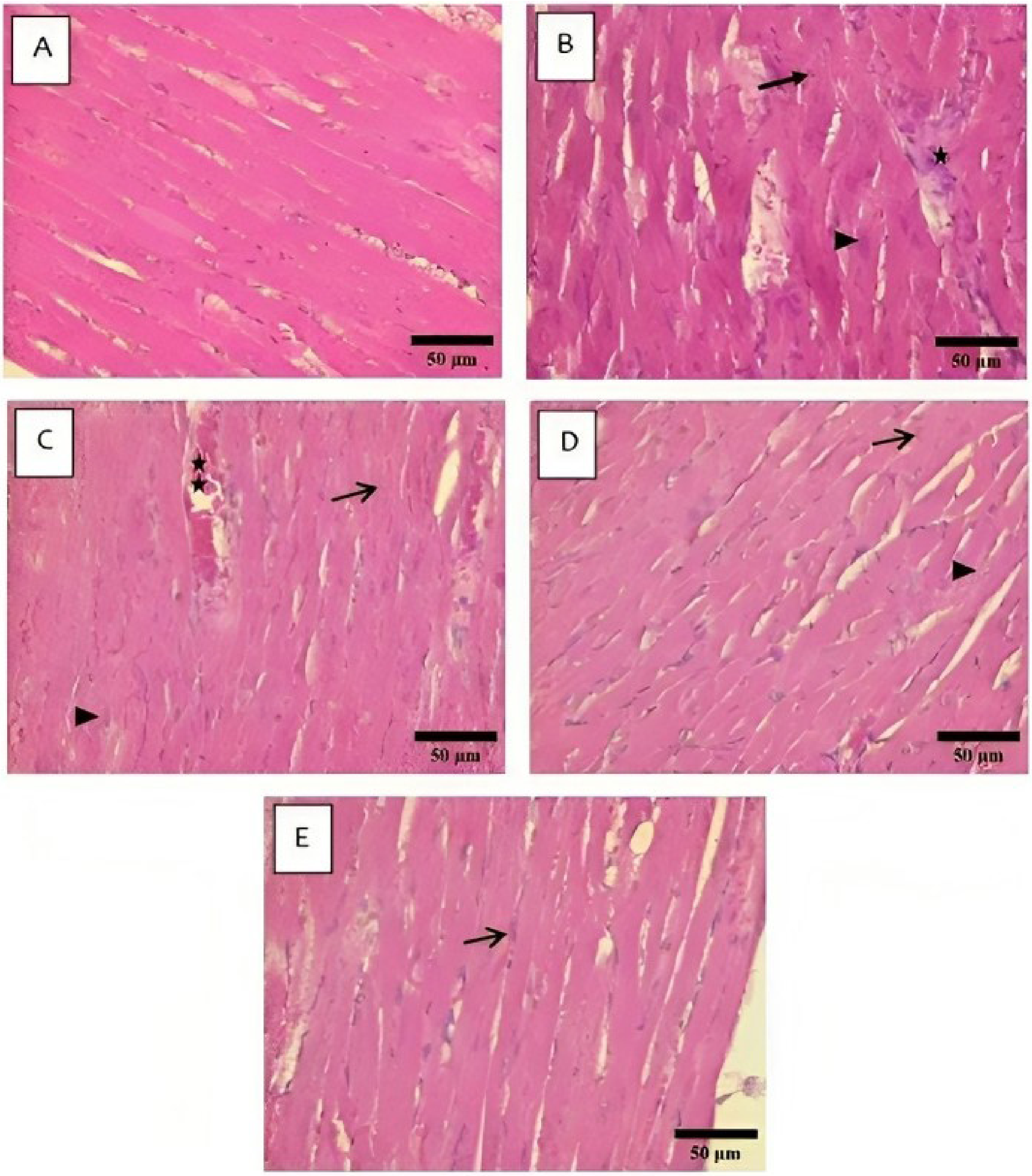

Figure 3 Protein deficiency caused the alteration on the histological structure of mice's heart muscle with HE staining. A. Control, B. Protein deficiency, C. Protein deficiency with the addition of 100 g bambara groundnut, D. Protein deficiency with the addition of 200 g bambara groundnut, E. Protein deficiency with the addition of 300 g bambara groundnut. Notes: atrophy (thick arrow); hypertrophy (arrow head); necrosis (thin arrow); leukocyte infiltration (asterisk); hemorrhage (double asterisk). Scale bar: 50 µm.

for the treatment of protein deficiency with bambara groundnut addition, it was found that mice receiving 300 g of bambara groundnut as part of their treatment showed fewer signs of heart muscle damage and exhibited a structure more closely resembling the control group, compared to mice in the addition group with 100 and 200 g of bambara groundnut. The results showed that the left ventricle had the most severe damage compared to the right ventricular and interventricular septum. In addition to comparative descriptive observations, changes in the histological structure of the heart muscle were also scored

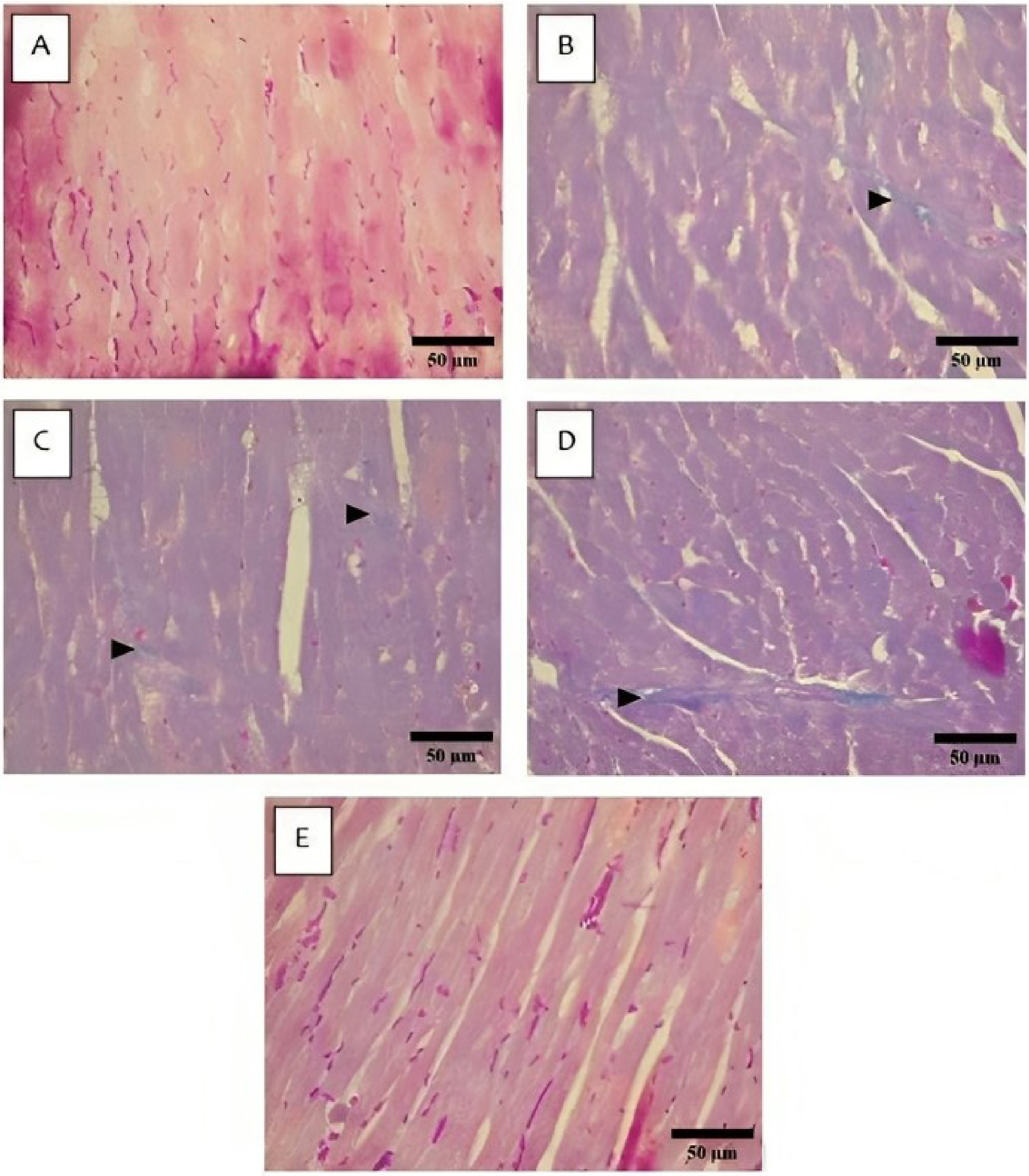

Figure 4 Protein deficiency caused fibrosis lesion on the histological structure of mice's heart muscle with MAF staining (coloring blue with arrow head). A. Control, B. Protein deficiency, C. Protein deficiency with the addition of 100 g bambara groundnut, D. Protein deficiency with the addition of 200 g bambara groundnut, E. Protein deficiency with the addition of 300 g bambara groundnut. Scale bar: 50 µm.

using the ordinal scoring system. This scoring aimed to determine the severity of lesions that indicate damage in the protein deficiency group and addition with bambara groundnut against the control group.

Based on the results listed in Table 3, it was known that all lesions observed in the area of the right ventricle and interventricular septum did not show any significant differences between groups ($p>0.05$). However, the atrophy, hypertrophy, necrosis, and fibrosis lesions The results of Kruskal Wallis analysis showed a significant difference ($p<0.05$) in atrophic lesions between the protein deficiency group and other groups; in hypertrophic lesions between control, protein deficiency, and protein deficiency with the addition of 300 g bambara groundnut group toward protein deficiency with the addition of 100 g bambara groundnut group and the addition of 200 g bambara groundnut group; in necrosis lesions control group toward protein deficiency, protein deficiency with the addition of 100 g bambara groundnut group and the addition of 200 g bambara groundnut group; in fibrosis lesions control and protein deficiency with the addition of 300 g bambara groundnut group toward protein deficiency with the addition of 100 g bambara groundnut group.

DISCUSSION

Protein is one of the nutrients which has an essential role in physiological function in the body. Protein deficiency can affect the metabolism process related to hunger and satiety regulation. A decrease in protein intake will trigger the activity of the ghrelin hormone to stimulate hunger (Abdalla, 2015). The enhancement of ghrelin secretion will cause a change in food intake regulation (Cui *et al*., 2017) in mice with protein deficiency. In this case, mice will increase their daily intake to compensate for protein deficiency and increase hunger (Brennan *et al*., 2012). If the protein deficiency continues, the fat content in the body will increase through the activity of fat-storage enzymes and lipolysis (Abdalla, 2015). This condition will elevate the body and protein deficiency with the addition of the bambara groundnut group.

Table 3 The result of histopathological observation of female mice's heart muscle using ordinal scoring sytem

	Lesion observed					
Group	Atrophy	Hypertrophy	Necrosis	Leukocyte Infiltration	Hemorrhage	Fibrosis
RV						
Control	0	0	0	0	0	0
PD	0	1	2	0	2	0
PD 100	0	0	2	0	2	0
PD 200	0	0	2	0	1	2
PD 300	0	0	1	0	0	0
IVS						
Control	0	0	0	0	1	0
PD	0	0	2	0	0	2
PD 100	0	1	2	0	1	0
PD 200	0	1	2	0	1	2
PD 300	0	0	1	0	0	0
LV						
Control	0	0	0	0	1	0
PD	3	0	2	0	0	1
PD 100	0	1	3	1	0	1
PD 200	0	1	2	1	1	1
PD 300	0	0	1	1	1	0

Notes:
"0" = No lesion
"1" = lesion present less than 1% of area examined
"2" = Lesion present in between 1% to 5% of area examined
"3" = Lesion present in between 6% to 10% of area examined
"4" = Lesion present in between 11% to 15% of area examined
RV = Right Ventricle
IVS = Interventricular Septum
LV = Left Ventricle

The heart can remodel to adapt to changes in the body (Spaich *et al*., 2015). The alteration of the heart muscle histological structure in mice, either adaptive or maladaptive, is an actual mechanism of heart remodeling due to protein deficiency in the body. This research found several lesions, such as atrophy, hypertrophy, necrosis, fibrosis, hemorrhage, and leukocyte infiltration in the ventricle and interventricular septum. The histological structure of mice's heart muscle from the protein deficiency group showed a visible alteration compared to control and protein deficiency with the addition of the bambara groundnut group. Atrophy was the new finding that only occurred in the left ventricle area. Atrophy in this group was caused by a decrease in amino acid contents and protein degradation as a side effect of nutrient deficiency (Baskin *et al*., 2011; Kamalov *et al*., 2013).

Conversely, an increase in cardiomyocyte size (Zachary & McGavin, 2012) through hypertrophy is a compensatory response of the heart muscle to face the elevation of blood pressure caused by protein deficiency (Baskin *et al*., 2011). Protein deficiency triggers blood pressure and thickening of the left ventricular wall (Saheera & Krishnamurthy, 2020). This condition will trigger the heart muscle to enlarge its cardiomyocyte size so the increase in blood pressure could be well compensated. Hypertrophy shown in the protein deficiency group with the bambara groundnut addition both in the ventricle and interventricular septum indicated an adaptive mechanism to maintain the geometrical structure and the performance of the heart muscle (Bech-Hanssen *et al*., 2021).

The limited ability of rodent's cardiomyocytes to regenerate and proliferate (Talman & Ruskoaho, 2016) enables them to trigger the histological structure change that leads to a maladaptive response. This condition causes cardiomyocyte damage that affects cellular death through necrosis. In several areas of the heart muscle observed, necrosis will accompany leukocyte infiltration to the inflamed area. These leukocytes are vital in eliminating the dead cardiomyocytes and stimulating cell repair through fibroblast proliferation (Prabhu & Frangogiannis, 2016). The clear area will be filled with collagen, extracellular matrix, or myofibroblast from the fibroblast transdifferentiation (Frangogiannis, 2015).

A connective tissue deposition will help the heart muscle maintain its integrity, although the elasticity will worsen (Saheera & Krishnamurthy, 2020). Besides atrophy, hypertrophy, necrosis, leukocyte infiltration, and fibrosis, some observed areas of heart muscle also show hemorrhage lesions. This phenomenon involves red blood cells circulating outside the blood vessels, often occurring in an area close to necrosis and leukocyte infiltration.

Based on the study's results, as listed in Table 2, the treatment of protein deficiency and protein deficiency with bambara groundnut addition increased the body weight of female mice insignificantly in the control group. The heart weight and index of female mice in the protein deficiency and protein deficiency groups with bambara groundnut addition showed relatively normal results with no significant difference from the control group. These results align closely with a study on 8.8% protein deficiency (Hennig *et al*., 2019), suggesting that protein deficiency conditions lead to increased food intake without causing significant changes to biometric parameters in female mice.

The results obtained from biometric parameters are also supported by results on LVID and LVPW (Figure 1.) and cardiomyocyte width (Figure 2.). In this study, treatment of protein deficiency and protein deficiency with bambara groundnut addition did not affect the chamber diameter and wall thickness of the left ventricle. This result differs from previous studies that stated that 6% protein deficiency (Murça *et al*., 2012) significantly increased LVPW parameters. The 8.8% (Hennig *et al*., 2019) protein deficiency study showed a significant decrease in LVID size in the protein deficiency group. In cardiomyocyte width parameters, treatment of protein deficiency and protein deficiency with bambara groundnut addition did not cause significant changes in the size of cardiomyocyte width. This result is also different from previous studies that showed an increase in the size of cardiomyocyte width in the protein deficiency group of 4% (Ferreira *et al*., 2022). Based on the results regarding LVID, LVPW, and cardiomyocyte width parameters in this study, it is suggested that the treatment of protein deficiency and 10% protein deficiency with bambara groundnut addition did not result in significant changes to LVID, LVPW, and cardiomyocyte width.

The left ventricle is the area with the most damage rather than the right ventricle and interventricular septum. The mice heart muscle damage, both in the protein deficiency and protein deficiency with the bambara groundnut addition group in this research, is still relatively slight (<25%) based on the ordinal scoring grading literature (Derelanko, 2000). This result aligns with the development of several parameters, such as the heart weight, cardiomyocyte width, LVID, and LVPW, that are not significantly different between groups. Based on the previous research about protein deficiency, the consumption of a low protein diet that ranged from 4%-8% protein (Murça *et al*., 2012; Mendes *et al*., 2017; Hennig *et al*., 2019; Ferreira *et al*., 2022) gave a significant effect on the heart muscle's histological structure. This condition raises an assumption that the 10% protein deficiency diet given to female mice didn't significantly change the heart muscle's histological structure, indeed negatively impacting heart function.

Based on the histopathological observation of female mice's heart muscle with bambara groundnut addition, we noted that the bambara groundnut can repair the histological structure of mice's heart muscle with protein deficiency. This result has been proven with the histological structure of heart muscle in the protein deficiency with the addition of 300 g bambara groundnut group in the ventricle or interventricular septum with a similar structure to the control group. The protein and amino acid contents (Tan *et al*., 2020) contained in bambara groundnut can be used as a supplement to overcome protein deficiency. However, it is essential to note that the utilization of bambara groundnut as protein addition need to be accompanied by the consumption of another protein source, specifically rich in essential amino acid such as methionine, to fulfill the daily need of amino acid that may be unable compensated in sufficient amount with bambara groundnut alone (Ferreira *et al*., 2022).

Based on the results and discussion, it can be concluded that the 10% protein deficiency caused slight alteration in the histological structure of female mice's heart. Besides that, the protein and amino acid contained in bambara groundnut can repair the alteration because of protein deficiency, so that this legume would be potential as an alternative protein supplement.

"The authors have declared that there was no conflict of interest in the research".

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