

Anthropometry, Fatty Liver, Plasma Lipid, and Adipose Tissue on Rat Wistar Induced Low-Protein Diet

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ABSTRACT

Protein energy malnutrition problem still exists in developing country. In fact, Indonesia has faced it as double burden of nutrition problems. This study aims to develop a standardized diet for developing protein energy malnutrition (PEM) on the rat to support nutrition research. Low-protein modified AIN-93M diet (LP) has been given to rats for 12 weeks intervention compared to normal protein diet (ND). Anthropometry, plasma lipid, fatty liver and the weight of adipose tissue have been analysed. The LP group showed a significantly lower body weight, body length and waist circumference ($p < 0.05$), a more fatty liver marker shown in LP compared to ND ($p < 0.05$). This study suggests that giving low protein modified AIN-93M diet promotes protein energy malnutrition in the rat.

Keywords: Protein energy malnutrition (PEM), low protein diet, anthropometry, fatty liver

INTRODUCTION

Energy protein deficiency is a fundamental nutrition problem in developing country including Indonesia. Some studies in the clinical setting have been conducted to identify the problem of energy protein deficiency [1, 2, 3, 4]. Nowadays, the functional food research for combating PEM is demanding. However, there are some limitations of clinical studies for proving the effect of functional food combating PEM. Several ethical and pragmatic constraints associated with using human subjects, while animal experiments are reproducible and robust. Therefore using the animal model as research sample for nutrition intervention is the main concern. For this purpose, the animal model needs a standardized diet to minimize variation during an experiment that resulted from the diet in an animal model [5]. Standardized diet for an animal model, especially rat, is AIN-93M [5] using for the maintenance of growth of rat as an animal model. However, others researcher also use other standard diets such as PARS for maintaining animal model growth.

This project aims to develop low energy protein diet standard that is modified from AIN-93M. Thus this study will examine the effects of low protein diet

on the anthropometry, fatty liver, plasma lipid, and adipose tissue. As an effect of PEM is lowering anthropometry size, developing fatty liver, changing in plasma lipid level and the loss the adipose tissue fat mass in body weight.

MATERIALS AND METHODS

Animals and housing condition

The Ethical committee for Conduction of Animal Studies at Faculty of Medicine, University of Brawijaya (UB) approved the experimental protocol (No. 416/EC/KEPK/07/2014). The animals in these experimental protocols were individually housed in cages.

Animals and diet

Thirty-two male Wistar rats, weighing 177-259 g at ± 3 months. All rats were fed in the Brawijaya University laboratory non-purified diet (PARS) during the acclimatization period. Then, thirty-two rats were divided into two groups ($n = 16$). The first group was continuously given PARS (Wonokoyo, Indonesia) as normal diet (ND) and the second group was given low-protein modified AIN-93M modified (LP). The dietary intervention was carried out ad libitum for twelve weeks.

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Table 1. Percentage of nutrient in PARS (ND) and AIN-93M diet (LP)

Nutrient Percentage	PARS (ND)	Modified AIN-93 M (LP)
Carbohydrate	75.1%	77.66%
Fat	4.78%	11.21%
Protein	15.14%	8.07%
Energy density	2.22%	3.2%

Nutrients composition of both diets are illustrated by Table 1.

Food intake

Food intake was measured every 24 hours by weighing the amount of total food (g) provided to the rats and subtracting the remaining food (g) in the cage after 24 hours.

Anthropometric, plasma lipid, white adipose tissue, and fatty liver analysis

Following the intervention period, Rat was anesthetized and killed by ketamine and xylazine 0.005 mL/g body weight via intramuscular rats. The anthropometric measurement including body weight was weighed weekly throughout the twelve-week intervention period. Others anthropometric measurement are abdominal circumference (AC), thoracic circumference (TC), body length (nose to-anus or anus-to-nose length) were made in anesthetized rat

The white adipose tissue (WAT) comprised visceral fat (epididymal, perirenal, and omental fat) and subcutaneous fat (inguinal fat) were then dissected out and weighed. Plasma samples were obtained by puncturing the right ventricle of heart after euthanasia and were collected in ethylenediaminetetraacetic acid- (EDTA-) coated tubes and centrifuged at 3000× rpm at 22°C for 25 minutes [6]. The whole liver was quickly removed, placed in formalin, and then stored at -20°C until it was analyzed. We examined the liver lipid accumulation using Hematoxylin-Eosin (HE)-stained as modified from previous protocol described previously [7, 8]

RESULTS AND DISCUSSION

Food intake, body weight, AC, TC and body length

PARS (ND) was given to the rats as a control group (normal diet), and Low protein modified AIN-93M diet had been given to rat with ad libitum or as desired. In adaptation phase, every rat had been given PARS diet, but slowly rat in LP was given low-protein modified AIN-93 M diet aiming to adjust the rat with treatment during research. After adaptation phase (2

Table 2. Anthropometry and nutrient intake

Treatment group	ND	LP	p-value
Baseline body weight (g)	204.03 ± 16.65	210.18 ± 19.95	0.35
Final body weight (g)	362.71 ± 41.35	149.50 ± 18.17	0.00
Body weight gain (g)	165.31 ± 40.75	-60.69 ± 15.74	0.00
Body length (cm)	25.44 ± 0.78	21.31 ± 1.11	0.00
TC (cm)	15.9 ± 1.06	11.72 ± 0.81	0.00
AC (cm)	17.50 ± 1.34	13.29 ± 0.98	0.00
Food intake (g)	41.26 ± 5.51	11.34 ± 1.57	0.00
Energy (kcal)	96.5 ± 12.89	38 ± 5.27	0.00
Protein (%)	15.13	8.07	0.00
Fat (%)	4.77	11.2	0.111
Carbohydrate (%)	75.11	77.66	0.00

Note: Amount (n) = 16 samples, ND = PARS Normal Diet, LP = Low Protein modified AIN-93M Diet; TC (thoracic circumference); AC (abdominal circumference)

weeks), PARS normal diet was still given to ND rat, but LP was given low protein modified AIN-93M.

Table 2 showed that rat in PARS normal diet group (ND) encounters a weight gain (165.31 ± 40.75). Meanwhile, a rat in AIN-93 M normal diet standard encounters a weight loss (-60.69 ± 15.74). Similar result is shown in body length, AC, and TC; LP group was significantly lower than ND group, p = 0.00 (α = 0.05).

Rat intake was measured by reducing the given food amount with food leftover that was weighed every day. Then, rat intake was converted into energy value, carbohydrate, protein, and fat for each diet. Intake average, energy total, carbohydrate, protein, and fat can be seen in Table 2.

The food intake in ND group (41.26 ± 5.51) g is higher than intake average in LP (11.34 ± 1.57) g with p-value 0.00 (α = 0.05). Thus, energy average in ND (96.5 ± 12.89) Kcal/day is higher than LP (38 ± 5.27) Kcal/day with p-value 0.00 (α = 0.05). This result revealed a significant difference between energy and food intake in ND and LP. It showed that rat consumed low protein modified AIN-93 diet less than PARS.

Factor that can affect rat intake is energy density and also percentage of fat from the total energy. Rat intake in LP is significantly lower than ND. It is because energy density in LP is higher than ND. A higher energy density caused the rat to feel fullness faster, so it reduces rat consumption [9]. Fat intake in ND contributes 4.77% of total energy. Meanwhile, fat intake LP contributes 11.2% of total energy. There is no significant difference in fat intake with p-value 0.11

Table 3. Plasma lipid and body fat mass

Plasma	ND (mg/dL)	LP (mg/dL)	p-value
Cholesterol	70.69 ± 15.93	65.50 ± 15.98	0.37
Triglyceride	74.88 ± 34.20	47.44 ± 27.09	0.01
LDL	25.08 ± 15.38	24.65 ± 6.61	0.53
HDL	49.04 ± 9.01	43.07 ± 9.13	0.07
White Adipose Tissue	19.42 ± 5.48	2.59 ± 0.87	0.00
Fatty liver	4.23 ± 2.15	35.36 ± 19.59	0.00

($\alpha = 0.05$). It has been reported that high-fat-diet-induced satiety effect longer than normal fat content diet. Thus it decreases food intake.

Carbohydrate intake in ND contributes 75.11% of total energy. Meanwhile, carbohydrate intake in LP contributes 77.66% of total energy. There is a significant difference in carbohydrate intake with p-value 0.00 ($\alpha = 0.05$). It also becomes a reason that consumption in LP is lesser than ND. The higher carbohydrate content in diet can make rat feel full faster, so food intake in LP is lower than ND. A high glucose can stimulate satiety center that is located in the ventromedial nucleus of the hypothalamus and otherwise, a lower glucose, can stimulate neuron in feeding center that is located in the lateral nucleus of the hypothalamus. Stimulation in feeding center increases hunger. Satiety center affects the incidence of satiety after eating. This feeling of satiety due to the increase in blood glucose after we eat causes the increasing glucose that passes through satiety center and activates it [10].

Protein intake in ND contributes 15.13% of total energy. Meanwhile, protein intake in LP contributes 8.07% of total energy. There is a significant difference in protein intake with p-value 0.00 ($\alpha = 0.05$). The recommendation of protein content on rat feed is 14.1%. It is to fulfill rat protein requirement for maintenance their health [5]. LP group contains 8.07% protein, and the other hand ND provides 15% protein from total energy. The difference of protein content affects on total daily food intake of the rat. ND group consumed more food than LP group. This shows that mice did not well accept LP diet.

Malta *et al.* and de Oliveira *et al.* [11, 12] reported that low protein diet (4% total energy) on rat decreased food intake compared to normal protein diet on a rat. The Impact of decreased intake in that research is similar to this research. In this research, the total protein is 8% from total energy, which still contains too low protein in rat food. Both studies showed the decreasing food intake affects weight gain during the administration of low protein diet. Study

from Malta *et al.* and de Oliveira *et al.* revealed, 30 days low protein diet on rat induces hyperleptinemia thus decreases food appetite on rat [11, 12].

Plasma lipid and body fat mass

Rat plasma cholesterol, triglyceride, Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), and white adipose tissue (WAT) were analysed after 12 weeks treatment. Table 3 shows the result of those variables (plasma lipid, body fat mass, and foam cell).

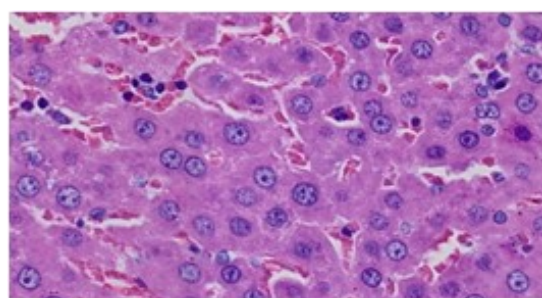
Table 3 shows mean ± standard deviation for each variable. There is no significance different in cholesterol total, LDL, and HDL with a p-value in sequence 0.37, 0.53, and 0.07. For triglyceride there is a significance different between ND (74.86 ± 34.20) and LP (47.44 ± 27.10) with p-value 0.01 ($\alpha = 0.05$). This difference caused by different intake in each group. Whereas, intake in ND diet is higher than low-protein modified AIN-93M.

PARS diet is made from *comfeed PARS* whose contains Distillers Dried Grains with Soluble (DDGS) and feed additive that stimulate growth process in chicken so if it is given to animal model it can stimulate their appetite [13]. In this research, a higher intake possibly was caused by *comfeed* content, and it can cause triglyceride serum in an animal model.

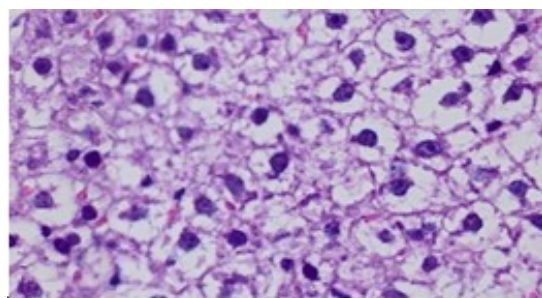
WAT total amount was measured by weighing some part in rat such as perinatal, omental, epididymal, and inguinal. Table 4 shows that WAT in ND (19.42 ± 5.48 g) is higher than LP (2.60 ± 0.87 g) with p-value 0.00. It means that there is a significant difference in WAT between ND and LP ($\alpha = 0.05$).

Meanwhile, in ND group there was an excessive energy, so it was used to increase adipose tissue mass. Moreover, a fat source in LP comes from soy fat with high polyunsaturated fatty acids (PUFA). PUFA in diet can obstruct lipogenesis in the liver. Meanwhile, in ND one of the fat sources is Distiller's Dried Grains with Soluble (DDGS) which have high saturated fatty acid.

In this research, food intake in ND is higher than LP. It was the reason why a total of WAT from four places (perirenal, omental, epididymal, and inguinal) in ND is greater than LP. The lower intake in LP causes fat that has been used as energy source, meanwhile in ND, there is an excessive energy that used to increase e adipose tissue mass. This statement supported by Fantuzzi and Mazzone (2007), stating that the higher rat intake, the amount of excess energy will be higher, so it will be deposited in TAG form in adipose tissue that contributes in WAT enlargement. However, in energy deficiency TAG in adipose tissue will be broken down



(a)



(b)

Figure 1. The effect of the different protein composition diet on lipid droplet deposition of hepatic tissue by HE staining, observed by microscope Olympus photo slide BX51 with 400 \times magnification.

into fatty acid that will be used as energy source by another organ [14]. It can be concluded that adipose cell enlargement results from imbalanced lipogenesis and lipolysis [15].

Fatty liver

ND and LP rat's liver cell can be seen in Figure 1 and there is different in hepatic tissue ND (A) and LP (B). In picture A there are a lot of normal cell. Meanwhile, in picture B liver cell has experienced intracytoplasmic swelling that pressing nucleus position, causing nucleus position in peripheral. As we seen in Table 3, the amount of fatty liver cell in LP is higher than ND with p-value 0.00 ($\alpha = 0.05$).

Lower food intake and protein intake in the rat have an effect on weight loss. It makes rat in kwashiorkor condition lead to fatty liver in rats. Rats in kwashiorkor condition will increase fatty acid serum level and low Very Low-Density Lipoprotein (VLDL) level [16]. The low VLDL level was resulted from low intake amino acid, so it makes Apoprotein B-100 and Apoprotein C synthesis decrease. The low VLDL causes the transport failure of triglyceride from the liver to tissue. This causes an excessive accumulation of fat in the liver, well known as fatty liver [17].

CONCLUSION

In conclusion, It has been shown that low-protein administration modified from AIN-93M standard diet develops protein energy malnutrition that mimics developing human PEM reflected from the changes of anthropometry parameters, fatty liver, plasma lipid and adipose tissue.

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