ORIGINAL ARTICLE

The Effect of a Low Protein Diet on the Expression of IL-6, TNF-α and TGF-β in the Kidney Tissue of Mice Model

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ABSTRACT

Introduction: High prevalence of malnutrition has impact that often causes problems in body organs. Various reports suggest an association between malnutrition and chronic kidney failure. However, the effect of malnutrition on the immunological status of the kidney has not been identified. Objective: to identify the effect malnutrition on the immunological status of the kidney. **Methods:** It was an experimental study with post test only control group design to compare two dietaries intake in two mice groups. Group 1, mice were given with standard intake. Group 2, mice were fed with low protein diet. The variables analyzed were the expression of IL-6, TNF-α and TGF-β in kidney tissue by immunohistochemistry and comparative test using Mann-Whitney test. **Results:** Eleven dead mice were found in the low protein diet group until day 75. At the end of observation, the number of mice in control group remained 10, while, in malnutrition group, there were 9 mice which were still alive but in a state of malnutrition. Based on comparative test between the group receiving and not receiving low protein diet, it was found that the expression of IL-6, TNF-α and TGF-β in kidney tissue showed a significant difference ($p<0.05$). **Conclusion:** Low protein diet in malnutrition affects the immunological status of the kidney as marked by the expression of IL-6, TNF-α, and TGF-β in the kidney tissue of mice model.

Keywords: Low protein diet, Kidney tissue, IL-6, TNF-α, TGF-β expression

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INTRODUCTION

Food plays an important role in maintaining the health and increasing growth. Malnutrition is a nutritional disorder caused by lack of protein or calories, which is often accompanied by deficiencies of other nutrients (1).

The WHO definition for malnutrition is an imbalance between the supply of nutrients and energy and the body's need to support growth, maintenance, and specific functions (2). Shortages of food intake will lead to dangerous malnutrition, especially in children. The risk of disruption of growth and development shows the importance of nutrition, which is essential for children (3). Malnutrition in children may even begin in the womb. Therefore, a mother's diet menu must be maintained by consuming a nutritious balance of food during pregnancy. In the event of malnutrition, the birth weight will be low or abnormal. In case of malnutrition in infancy, the child will have a tendency to experience a variety of chronic diseases in adulthood, such as heart disease, diabetes, and high blood pressure. This is because malnutrition causes enormous damage to the child's body. For instance, heart, kidney, stomach, intestines, lungs, and brain, as well as various organs and other systems would be affected (4-8). Furthermore, damage to such important various organs causes damaged or delayed physical and intellectual development (9). It is clear that the effects of malnutrition, even since fetal life, will last until adulthood and develops into a variety of diseases (10). The high prevalence of malnutrition is often reported in various reports (11-12). The impact of malnutrition reportedly often causes problems in the body (13). Nutritional disorders can even affect as maturity disorder of all organs when it occurs in intrauterine fetal phase (14). Interestingly, low birth weight (LBW) is reported as a risk factor for systemic arterial hypertension and chronic kidney disease (CKD) for several diseases in adulthood (15). Malnutrition also influences the profile of cytokines expression, such as IL-6 and TNF-α (16-20). If malnutrition as the trigger has an acute impact, the hosts quickly become infected with fatal consequence of death (12), and if the trigger is chronic/long lasting, the kidneys will not be able to experience perfect injury healing. In consequence, the

function decreases and leads to chronic renal failure.

It has been widely reported that malnutrition impairs the body's immune status (12, 21). As with other body systems, it is reported that nutritional status is an important modulator of immune response (22-23), determines the risk and prognosis of disorders of the body, and it is directly also affected by infection (24). Malnutrition is reportedly the largest cause of immunodeficiency worldwide (25). The association between biological mechanisms of infection/inflammation and lack of protein has been described in experimental studies (12). Protein deficiency raises inflammatory mediator TNF-α, which is important to activate immune mechanism (26). Abnormal increase in its expression can lead to extensive tissue damage caused by leukocytes that activate chronic inflammation (27).

However, the effect of malnutrition on the immunological status of the kidney has not been identified. This study was intended to identify the effect of malnutrition on the immunological status of the kidney. To disclose such mechanism, this study was conducted using a model of mice fed with low protein diet since the lack of protein is one of the major causes of malnutrition (1). We focused on the expression of TNF-α, IL-6 and TGF-β in the kidney tissue as the expression of these markers in other body organs are found to be influenced by malnutrition (16-20).

MATERIALS AND METHODS

Experimental Animals

A total of 30 pathogen-free male Balb/c mice of 8 weeks old with a body weight (BW) of around 25 g were obtained from the Animal Facility of the Faculty of Medicine, Airlangga University, Surabaya. Firstly, mice were acclimatized during one week for adaptation. The mice were housed in clean metal cages and fed with two kinds of protein diet and tap water ad libitum in an air-conditioned room (23 ± 1°C), under conventional conditions with a 12:12 hr, light : dark cycle. They were kept as outlined in the "Guide for the Care and Use of Laboratory Animals" by The Faculty of Medicine, Airlangga University.

Diets

The experimental mice food used two kinds of protein diet for the low protein diet studies. One was normal protein diet containing 19.9% protein (normal protein diet mice) and a low protein diet containing 8.46% protein (low protein diet mice). The composition of the diet is shown in Table I.

Experimental design

Thirty mice were randomly divided into two groups. The first group of 10 mice was fed with a normal protein diet. The second group, 20 mice were fed with low protein diet. Based on preliminary study, each group received diet treatment for 75 days. Body weight gain was calculated on days 52 and 72 of the treatment. There actually was no specific reason for monitoring the body weights twice only on those days. Since what we expected was the presence of significant weight loss in the experimental group of mice fed with low protein diet, what was underscored in this study were the body weights at the beginning and at the end of the study. On day 75 post treatment, the experimental mice of both groups were killed by decapitation, then the kidneys were removed and fixed in 10% neutral buffered formalin and embedded in the paraffin. This experiment was performed to observe the macrophages expressing IL-6, TNF-α and TGF-β through immunohistochemical staining. The macrophages expressing IL-6, TNF-α and TGF-β were quantified based on the number of cells expressing those cytokines per square area of tissue using calibrating the eyepiece graticule scale.

Table I: Dietary Compositions and Feed Consumption per day of Mice

Ingredient	Normal protein diet	Low protein diet
Protein	19.9% (1.99 g)	8.46% (0.846 g)
Fat	6.0% (0.6 g)	3.82% (0.382 g)
Carbohydrates	69.6% (6.96 g)	81.52% (8.152 g)
Metabolic Energy (ME)	3100 kcal (3.1 kcal)	3500 kcal (3.5 kcal)

Normal protein diet was formulated based on standard mice diet

Immunohistochemical staining

This protocol was carried out to determine the number of macrophages expressing IL-6 in kidney tissue of animal treatment groups. The tissue was sliced 4 to 6 µm then placed on object glass and deparaffinization was performed to attract and/or eliminate paraffin in the tissue. The sliced tissues were inserted respectively into xylol 3 times, each for 2 minutes. Thereafter, they were inserted successively into ethanol with gradually decreased concentrations, starting from 100% ethanol three times each for 1 minute, then 95% ethanol twice, each for 1 minute, then 90%, 80% and 70%, respectively, for 1 minute. After washing with tap water for about 5 minutes, they were put in 3% peroxide for 30 minutes to remove endogenous peroxidase. Then, after washing with tap water, they were rinsed with distilled water, then PBS respectively for 2 minutes. In the next stage, they were put into a solution of 0.25% trypsin in PBS (pH 7.4) for 6 minutes at 37°C, then washed with PBS 3 times, each for 2 minutes. After that, they were put into the primary antibody (anti-IL-6/Rat anti-mouse, BD Pharmingen™) for 30 minutes, then washed three times with PBS, each for 2 minutes. Subsequently, they were incorporated into the secondary antibody of biotinylated labeled Rabbit anti-Rat for 30 minutes. Then, they were washed with PBS 2 times, each for 2 minutes, successively put into HRP labeled streptavidin for 30 minutes, and washed with PBS three times, each for 2 minutes. They were then inserted into the substrate chromogen (DAB solution) for 5 minutes, washed with PBS three times, each 2 minutes, then

rinsed with distilled water. They were then put in Mayer Hematoxylin for 6 minutes, washed with running water, and finally they were mounted and could be observed using a microscope.

The same protocol of immunohistochemical staining for TNF-α and TGF-β was used as IL-6, with primary antibody suitable for each cytokines (anti-TNF-α/Rat anti-mouse and anti-TGF-β/Rat anti-mouse, respectively, BD Pharmingen™).

Data analysis
Body weight gain was recorded as the last body weight on day 75 post treatment. The survival rate was counted by ratio of the number of living mice divided by total mice in each group. SPSS version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data of body weight were obtained in duplicate and mean ± SD was calculated, while data of macrophages expressing IL-6, TNF-α and TGF-β were also obtained in duplicate and their mean ± SE were calculated. A P-value < 0.05 was considered statistically significant. The Mann-Whitney-U test was used to compare the results in different groups.

RESULTS

The survival rate

Within the low protein diet mice group, it was recorded that one mouse died on days 32, 44, 52, 57, 63, 69, 71 and day 75 post treatment, except at day 73 of treatment when 3 mice died. This means 11 mice died in total. In comparison, there was no recorded death of any mice in normal protein diet mice group until day 75 post-treatment. This means the survival rate up to day 75 post treatment was found to be at 45% in the low protein diet mice group, whereas in the normal protein diet mice group, the survival rate 100% (Fig. 1).

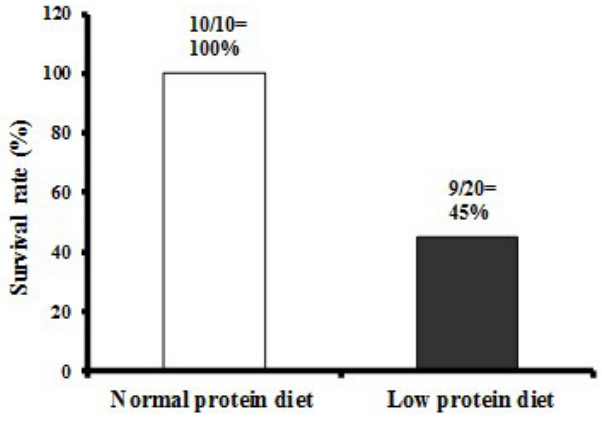

Figure 1: The effect of low protein diet on the survival rates of mice during treatment. The comparison of survival rate percentages between the normal protein diet mice group and the low protein diet mice group until 75 days post treatment. All ten mice from the normal protein diet mice group were all still alive, while only nine out of twenty mice from the low protein diet mice were still alive.

Body weight gain

Calculation of the average of body weight on day 52 of treatment in the normal protein diet mice group was obtained to show 30.22 ± 0.93 grams, while in the low protein diet mice group, it was 14.99 ± 1.09 grams. On day 72 of treatment in the normal protein diet mice group the body weight was found to be 35.34 ± 1.15 grams, while in the low protein diet mice group it was 10.4 ± 1.08 grams. There were significantly differences (P<0.01) on the comparison of body weight gain between the low protein diet mice group and the normal protein diet mice group (Fig. 2).

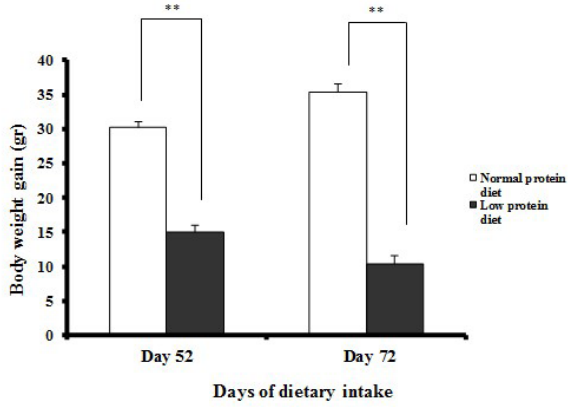

Figure 2: The comparison of body weight gain between the normal protein diet mice group and the low protein diet group at day 52 and day 72 post treatment. There was a significant difference of body weight gain at both mice groups. Each value represents mean and SD of 10 mice. **P<0.01.

Cells expressing IL-6, TNF-α and TGF-β

Quantification of expression of IL-6, TNF-α and TGF-β on day 75 of treatment showed significant differences (P<0.05) between the normal protein diet mice group and the low protein diet mice group. There was a significant increase of the expression of IL-6, TNF-α and TGF-β in the low protein diet mice group compared to the normal protein diet mice group (Table II and Fig. 3).

Table II: Expression of IL-6, TNF-α and TGF-β of Macrophages on Kidney Tissues of Treatment Protein Diet Groups on day 75 of Treatment

Treatment Diet Groups	IL-6	TNF-α	TGF-β
		Mean ± SE	
Normal protein diet mice group	0.07^{a} ± 0.09	0.1^{a} ± 0.17	0.11^{a} ± 0.13
Low protein diet mice group	0.57^{b} ± 0.31	0.4^{b} ± 0.38	1.16^{b} ± 0.57

Superscript is different in same column represented significantly differences (*P*<0.05)

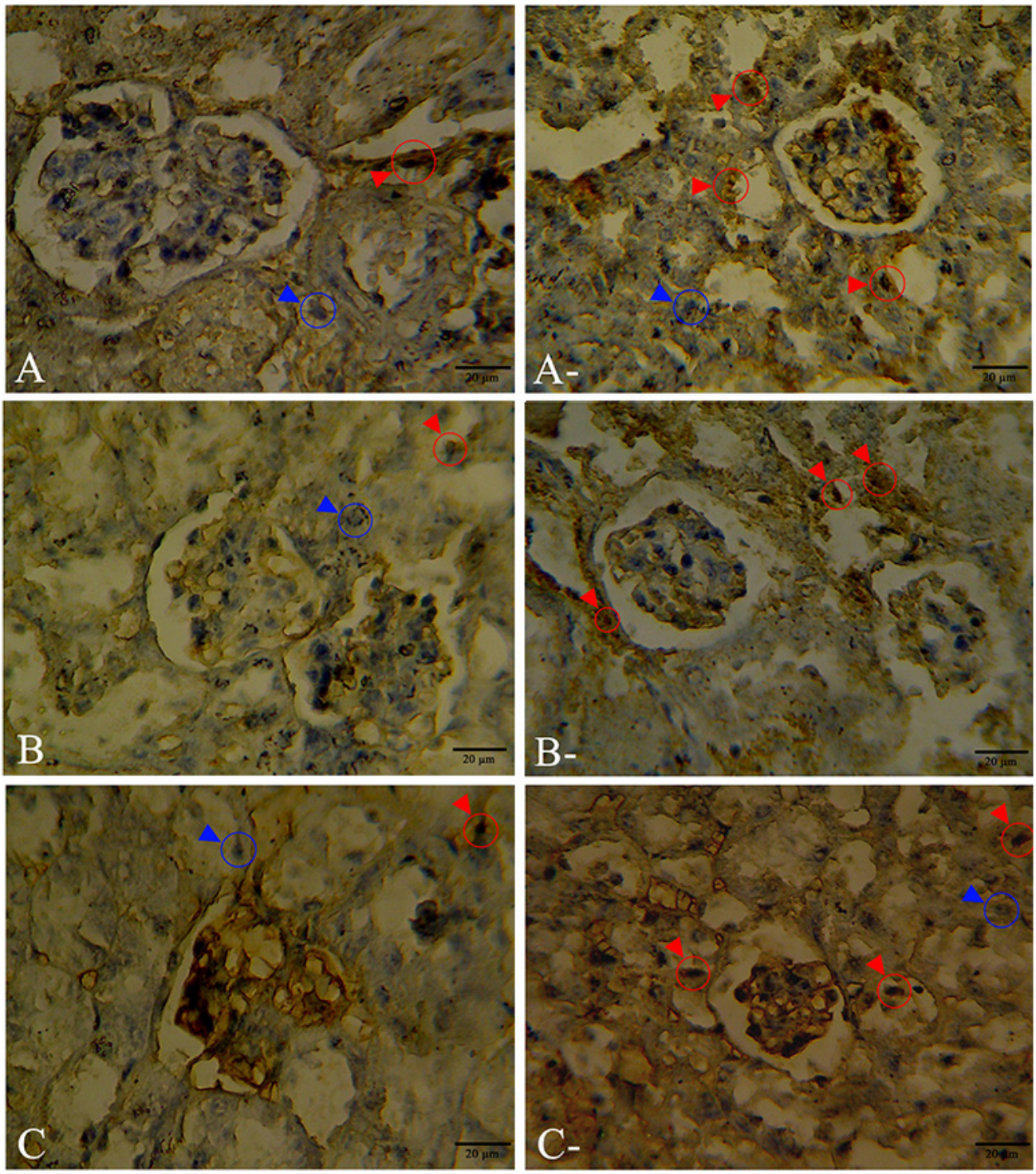

Figure 3: The effect of low protein diet on expression of IL-6, TNF-α and TGF-β in kidney tissue of mice which appeared in mice kidney tissue stained with immunohistochemistry using a monoclonal antibody anti IL-6, TNF-α and TGF-β. Macrophages cell expressing IL-6, TNF-α and TGF-β was changed and were characterized with brown cytoplasm. Macrophages expressing IL-6, TNF-α and TGF-β in kidney tissue of the low protein diet mice group (A-, B-, C-) were more than in kidney tissue of the normal protein diet mice group (A, B, C). blue head arrow, macrophages that do not express IL-6, TNF-α and TGF-β; 400X; red head arrow, macrophages that express IL-6, TNF-α and TGF-β.

DISCUSSION

This study found that the group of mice receiving low-protein diet lost body weight. It seems that weight loss is related to the decrease in muscle mass. Low protein in the body of the mice resulted in disruption of cell metabolism, resulting in impaired balance on enzymatic systems as well as on the expression of cytokines that play a role in the immune system, which ultimately leads to death. The amount of weight loss also strongly suggests that macrophages become activated and enhance the increased expression of TNF-α, IL-6, IL-8, and followed by the TGF-β in susceptible body tissues (28). This study was also found in the kidney of the mice that there was immunohistochemical expression of TNF-α, IL-6. This indicates that the role of TNF-α and IL-6 become more predominant in producing injury of the kidney tissue. This indication confirmed previous studies (29-30). Thus, the amount of weight loss and kidney damage in malnourished group appears to be caused by the disruption of cell metabolism balance or abnormality in the cytokine pathway, causing inability to fight infectious agents.

Our study found many dead mice during the observation period. In fact, in the second month treatment four were found dead, and in the third month there were seven dead mice. This finding confirms further that mice mortality is closely related to malnutrition conditions that may be exacerbated by the length of malnutrition and infection attack in the subjects. It is reported that the vulnerability by the body damage, infection, and death is increasing as a reflection of the decline in immune system (31). In turn, this will aggravate malnutrition itself and eventually lead to death (32). The decrease in the secretion of lysozyme factor also exacerbates the tendency of the inability to survive against infection (33). Bad nutrition creates a high risk of death. The report about the survey on malnutrition shows that it is increasingly clear that children with severe malnutrition are at much higher risk of death (34). Therefore, it is apparent that the causal relationship between malnutrition, infection and immune suppression is exacerbated by the increasing malnutrition.

This study showed that surviving mice also have a loss in muscle mass as evidenced by the decrease in body weight. The process of the weight loss is predicted as a representation of increased metabolic activity. Compared with the control group that survived until the end of the study, it had been strongly suggested that there was increased metabolic activity, which was rapid and continuous until an excessive expansion of cellular injury occurred, though the host was trying to survive through its homeostatic system. This is understandable because of the metabolic disorder will affect the redox system until hyperactive oxidative system is taking place without being able to offset the reduction system (35). This condition induces uncontrolled ROS generation, so that an excessive increase in ROS will damage the body's cells, including the cells of blood vessels and kidneys. Uncontrolled ROS by the redox system will continuously damage the cells, producing a lot of cell debris that would stimulate macrophages to release nitric oxide (36). The macrophages are increasingly becoming very active to release many kinds of cytokine, ie TNF-α, IL-6 (37). As a consequence, TNF-α induces the endothelium to express E-selectin, whose function is to bind neutrophil (38). Furthermore, the neutrophil will release MMP8 to degrade type 1 collagen, with the end result of vascular damage (vasculopathy) (36). Such condition may precede to result in ischemic process which eventually lead to the loss of any organ function, including the kidney.

In this study, increased expression of TNF-α and IL-6 in the kidney tissue of malnourished mice was highly significant compared to that in non-malnourished mice in this study, which also confirmed the previous study (27). Referring to the theory that TNF-α is a pro-inflammatory and pro-apoptotic cytokine, it can be predicted that when there is increased expression of TNF-α and IL-6 in a site, there is an increase of cell death (27). It has also been proved that ROS from any cause triggers cell death, including in cases of malnutrition. In the conditions of prolonged malnutrition ROS will continue to increase and this is also accompanied by increased expression of TNF-α and IL-6, which together will exacerbate and accelerate the destruction of kidney tissue, and this is confirmed by a report that prolonged malnutrition may result in organ damage (39). If so, the kidney in the mice with chronic malnutrition will most likely suffer from damage cell or cell death caused by TNF-α and IL-6, ROS, and a combination of both.

Along the damage process in kidney tissue, either caused by MMP, ROS, TNF-α, the body has a balance system that seeks to regulate against such damage through the process of collagenesis played by macrophages that release TGF-β1 to induce fibroblast to produce collagen (40). If balance between MMP and collagen is disrupted, the kidney tissue may be damaged. In this study we found an increase expression of TGF-β1 in renal tissue of malnourished mice, with a significance level of $P < 0.05$ that can be regarded as having high potential to become fibrosis.

The analysis of the three parameters showed that two parameters were in destruction pathway (IL-6 and TNF-α) and the other one in repair pathway (TGF-β1). All those parameters have increased significantly. Apparently, the three variables were instrumental in the process of kidney tissue damage. This indicates that malnutrition maneuvers from the end of first month showed an increase in the number of dead mice and within two and half months the number of mice that died due to malnutrition was 11 out of 20 malnourished mice. In contrast, those in the other control group did

not experience death until they reached the two and half month stage. It seems that this study, besides showing mortality, also shows the possibility of renal function impairment. Significant weight loss of the experimental mice in this study was strongly associated with decreased or impaired ATP and disturbance in proximal sodium pump and augmented urinary sodium excretion tubes (41). This leads many swelling cells to become dead cells which activate macrophages to express cytokines and phagocytes dead cells.

However, the limitation of this study was that this study did not test the renal function as well as the renal cell death, either caused by TNF-α and IL-6 or by ROS. As we focused on the increased expression of the immunological markers of IL-6, TNF-α and TGF-β in malnourished kidney tissue, other examinations related to renal function, such as the measurement of blood parameters, were not conducted. The monitoring of body weight gain of the experimental animals should have also been performed in more regular basis.

CONCLUSION

Low protein diet in malnutrition causes changed amd affected the immunological status of the kidney as marked by the expression of IL-6, TNF-α, and TGF-β in the kidney tissue of mice model.

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