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Decreased Renal Function Induced by High-Fat Diet in Wistar Rat: The Role of Plasma Angiotensin Converting Enzyme 2 (ACE2)

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Researcheson the effects of High Fat Diet (HFD) on decreased l function withcystatin C (cysC) serum levels biomarker are few and show different findings. Renin Angiotensin System (R plays a key role in controlling renal function and one of the integral components of the RAS is Angiotensin Converting Enzyme 2 (ACE2). Research on the relationship betweenplasma ACE2 levels withserumcysC levels in animals induced by HFD has not been done. We hypothesize that administration of HFD can cause a decline in early stage renal function through ole of ACE2. 30 male wistar rats aged 10-12 weeks (body weight between 170-220 grams) were randomly divided into 5 groups (6 rats/group): baseline,normal diet for 8 we (ND8), ND for 16 weeks (ND16), HFD for 8 weeks (HFD8) and HFD for16 weeks (HFD16). Body weight and naso-anal length were measured to get the index value of obesity and body fat percentage.Obesity index measured are lee index, rohrer indexand TM index.Blood samples obtained by intracardiac for examination of plasma ACE2 levels and serum cysC levels. After 8 and 16 weeks, HFD increases body weight, obesity index and body fat percentage. HFD also increases plasma ACE2 levels and serum cysC levels.Body weight, obesity index and body fat percentage have a positive correlation with plasma ACE2 levels. Plasma ACE2 levels were posi y correlated with serum cysC levels. HFD causes a decrease of early ge renal function as evidenced by the increase in serum cysC levels. Plasma ACE2 levels play a role in the pathogenesis of the decline in early stage renal function induced by HFD.

Keywords: ACE2, Cystatin C, Renal Function, High Fat Diet.

In recent years, HFD has increasingly been seen as a significant risk factor that can lead to disease. However, the mechanism of HFD that has a negative impact on health is still difficult to understand. Current research related to HFD focuses on the impact of HFD on medical conditions, the underlying mechanisms and the development of therapeutic strategies[1].

HFD is a risk factor for kidney disorders[2]. In HFD induced obesity it causes damage to kidney structure, inflammation[3,4]and oxidative stress[5]. Decreased renal function is characterized by increased urinary albumin excretion or albuminuria[6,7], increased plasma creatinine levels[3]and lower creatinine clearance[8]. Howeverresearch on the effects of HFD on renal function with biomarkers of cysClevels was very small and showe different findings. HFD causes an increase in serum cysC levels which implies glomerular and proximal tubular changes[7]. Criniganet *al* (2015) found that although HFD increased serum cysC levels, it was not statistically significant[9].

CysC is a marker for measuring GlomerolusFiltration Rate (GFR)[10,11]and more appropriately used to diagnose kidney damage with a decrease in GFR compared to creatinine clearance[10]. CysC is a low molecular weight protein (13kD) whi s an endogenous cysteine proteinase inhibitors produced by all nucleating cells in the human body at a fairly constant level. CysC is filtered freely by the glomerulus and is not secreted and is almost com tely absorbed back in the proximal tubule[11]. This characteristic makes cysC useful for detecting early stage renal dysfunction. CysC not influenced by age, gender, muscle mass and ethnicity[12].

e of the pathophysiological mechanisms that also plays a central role in the development of ney disease is the activation of RAS[13]. RAS plays a key role in controlling kidney function[14,15]. One of the integral components of the RAS is ACE2[16].

ACE2 is a monocarboxypeptidase that degrades angiotensin (Ang) II to Ang (1-7)[13]and convert angiotensin (Ang) I to Ang (1-9)[17]. alance between increasing levels of Ang II and ACE2/Ang (1-7) as receptor axis contribute to kidney injury[13]. ACE2 is widely expressed in the kidneys especially in proximal tubular epithelial cells[14]and visceral glom us[18]. ACE2 protein and mRNA levels have been shown to change in diabetic kidney disease, hypertensive kidney disease and various injurious renal models[14].

Li *et al* (2015) examined the effect of giving HFD to various RAS components. However, they did not examine the effect on ACE2 which is also one of the components of the RAS[19]. The findings isencouraging research on the effects of HFD to the activation of RAS components through the role of ACE2.Very few data is available about the effects of giving HFD to plasma ACE2 levels. Not even found data related to the relationship between plasma ACE2 levels with serum cysClevels in animals induced by HFD. We hypothesize that administration of HFD can cause a decline in early stage renal function through the role of ACE2.

MATERIALS AND METHODS

Diets

Preparation of animal diet was conducted in Animal Food and Nutrition Division, Faculty of Animal Husbandry, Hasanuddin University, Makassar, Indonesia. The animal diet was arranged th normal diet (ND) compositionconsisting of 3.1% fat, 16.1% protein, 3.9% fi r and 5.1% ash/ mineral. Composition of the high fat diet (HFD) consisted of 21.4% fat, 17.5% protein, 50% carbohydrate, 3.5% fiber and 4.1% ash/mineral[20](table 1). To get rations/feed according to the composition that has been prepared, analysis of the feed samples using the proximate method was carried out. The analysis was done twice to get accurate results (table 2).

Animals

30 malewistar rats aged 10-12 weeks (body weight betwe 170-220 grams) were maintenance in Molecular Biology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia[21,22,23]. After acclimatization for 2 weeks (in the room with sufficient air circulation, room temperature 28±2p , humidity 50±10% and room ligh rranged in a 12-h light and dark cycle), wistar rats were randomly divided into 5 groups (6 rats/group). Group I was the group 0 weeks (baseline), group II (ND8) was the controls group given ND for 8 weeks, group III (ND16) was a control group given ND for 16 weeks, group IV (HFD8) was the treatment group given HFD for 8

weeks and group V (HFD 16) was the treatment group given HFD for 16 weeks. All wistar rats have free access to food and drink (ad libitum). Blood samples were taken through intracardiac at week 0 (after acclimatization) for the baseline group, week 8 for the HFD 8 and ND8 groups and week 16 for the HFD16 and ND16 groups for examination of plasma ACE2 levels and serumcysClevels. All of these procedures were carried out at the Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

Measurement of body weight, body fat percentage and obesity index

The body weight and length of the naso-anal were measured to obtain the obesity index value. The obesity index measured waslee index, rohrer index, and TM index. Rats were declared obese if Lee index value is >0.3, Rohrer index>30, and TM index>50. Body fat percentage is calculated based on TM index[24]. The formula used is:

Rohrer index = {body weight (gram)/naso-anal length (cm)^3}×10^3

Lee index = $\sqrt{\text{body weight (gram)x10}}$

naso-anal lenght(mm)

TM index = body weight (gram)/naso-anal length (cm)$^{2.383}$×10^3

Body fat percentage = 0.581 × TM index–22.03

Collection of blood samples, Examination of Plasma ACE2 Levels and Serum CysC Levels

Rats were restrained to control head and body movements. After intraperitonialanesthesia using ketamine anesthetic agents (100 mg/kg) and xylasine (10 mg/kg), blood was taken as much as 2 ml intracardiacusing a needle 19-21. Blood was inserted into the sample tube. Blood was centrifuged to obtain plasma/serum and stored at -80°C before examination. Plasma ACE2 levels were measured by elisamethod using ACE2 reagents (Rat ACE2/ACE-2 Elisa Kit LS-F33783, LifeSpanBioScience, Inc.). Serum CysC levels were measured by the Elisa method using a cystatin C reagent (Rat CST3/Cystatin C Elisa Kit LS-F21524, LifeSpanBioScience, Inc.). Plasma ACE2 levels and serum CysC were read using Elisa Reader 270 (Biomeriaux, France) with a wavelength of 450 nm for 30 minutes in units of ng/ml. Each the experiment were done in duplicate.

Statistical Analysis

The data obtained was processed using SPSS version 24 for Windows then analyzed with a significance level of <0.05. Before testing the difference hypothesis and correlation, the data normality test was first carried out. The difference test conducted was a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Correlation test performed using Pearson correlation test.

RESULTS

Effects ofHFD on Body Weight, Obesity Index and Body Fat Percentage

As expected, there were differences in body weight, obesity index and body fat

Table 1. The Composition of Animal Diet

Composition	HFD CH (%)	HFD Protein (%)	HFD Fat (%)	HFD Fiber (%)	HFD Ash (%)	ND CH (%)	ND Protein (%)	ND Fat (%)	ND Fiber (%)	ND Ash (%)
Corn	38,11	4,50	2,02	0,74	0,01	2,20		0,09		
Bran	6,32	2,29	1,87	1,06	1,28	48,30	4,72	0,56	0,94	0,20
MBM		1,29	0,33	0,07						
Premix						5,40	7,07	0,50	0,41	1,11
Fish Flour		1,16	0,17	0,08	0,22	6,32	2,29	1,87	1,06	1,28
Soybean meal	5,76	7,54	0,53	0,44	1,18		1,74	0,26	0,12	0,32
Tallow		0,41	16,16		0,02					
Vegetable oil			0,41							
Total	50,19	17,19	21,49	2,39	2,71	62,22	15,82	3,28	2,53	2,91

CH: Carbohydrate; MBM: Meat Bone Meal; ND: Normal Diet; HFD: High Fat Diet

percentage between ND and HFD groups (table 3). The body weight in the HFD8 group was higher than of ND8 group ($p< 0.001$), as well as between groups given HFD16 and ND16 ($p<0.001$), even between ND8 and ND16 groups ($p<0.05$). Body weight in the HFD8 group was higher than HFD16 group.

Obesity index measured was Lee index, Rohrer index and TM index. Lee index in the HFD8 group was higher than ND8 group ($p<0.001$). Likewise between HFD 16 group and ND16group ($p<0.001$). The duration of administration of HFD did not affect the Lee index as evidenced by not finding differences between groups given HFD 8 and HFD16. Interestingly, Lee index after administration of ND or HFD did not show obesity. Rohrer index and TM index were also higher in the HFD group than NDfor both between ND8 and HFD8 and between ND16 and HFD16 (respectively, $p<0.001$ and $p<0.001$). The interesting thing was also the Rohrer index and TM index in all groups showed good value of obesity after given ND or HFD.

Body fat percentage is calculated based on the TM obesity index value . Body fat percentage was also higher in the HFD group than ND for both between ND8 and HFD8 and between ND16 and HFD16 (respectively, $p<0.001$ and $p<0.01$).

This finding means that administration of HFD increased body weight, body fat percentage and obesity index in wistar rats.The longer the HFD administration, the higher the Rohrer index, TM index and body fat percentage.

Effects of HFD on Plasma ACE2 Levels

Plasma ACE2 levels in the HFD group were higher than in the ND group for both between ND8 and HFD8 and between ND16 and HFD16 (respectively, $p<0.001$ and $p<0.001$). Plasma ACE2 levels in the HFD16 group were higher than HFD8 group ($p<0.001$). The longer the HFD is given, the higher the plasma ACE2 levels. Administration of HFD increases plasma ACE2 levels (table 4).

Effects of HFD on Serum CysC Levels

Serum cysC levels in the HFD group were higher than in the ND group between ND8 and HFD8 and between ND16 and HFD16 (respectively,

Table 2. Proximate Analysis Results

No	Sample	Composition					
		Water	Protein	Fat	Fiber	CH	Ash
1	ND1	13,04	20,41	4,67	3,70	67,54	3,68
2	ND2	13,32	20,52	5,34	4,39	66,51	3,24
3	HFD1	10,32	19,85	23,53	3,59	49,02	4,00
4	HFD2	10,35	19,26	24,71	3,81	47,69	4,52

Except for water, all fractions are expressed in dry matter. ND1 and HFD1: results of 1st analysis; ND2 and HFD2: results of the 2nd analysis. CH: Carbohydrate; ND: Normal Diet; HFD: High Fat Diet

Table 3. The Effect of HFD on Body Weight, Obesity Index and Body Fat Percentage

Parameter	Diet Groups				
	Baseline	ND8	ND16	HFD8	HFD16
Body Weight (gr)	185.17 ± 6.75	204 ± 14.77	220.17 ± 5.98^{d***}	339.83 ± 14.77^{a*}	334.33 ± 10.94^{b*}
Lee Index	0.22 ± 0.01	0.24 ± 0.00	0.25 ± 0.00	0.30 ± 0.00^{a*}	0.30 ± 0.00^{b*c*}
Rohrer Index	29.10 ± 2.81	34.03 ± 1.44	33.85 ± 1.00	51.58 ± 2.20^{a*}	54.21 ± 2.82^{b*}
TM Index	49.35 ± 4.62	56.67 ± 0.00	58.66 ± 2.80	59.81 ± 5.35^{a*}	92.86 ± 6.27^{b*}
Body fat percentage (%)	6.44 ± 2.59	10.89 ± 2.30	12.03 ± 1.62	29.95 ± 3.09^{a*}	31.07 ± 5.56^{b**}

Data are presented in the mean ± SD form (n=6). Differences between groups were analyzed using the one-way ANOVA test followed by Bonferroni's test. Significant differences are indicated by superscript: ^{a} between ND8 and HFD8, ^{b} between ND16 and HFD16, ^{c} between HFD8 and HFD16 and ^{d} between ND8 and ND16. $^{*}p<0.001$, $^{**}p<0.01$, $^{***}p<0.05$

p<0.001 and p<0.001). Although statistically it did not show a significant difference, serum cysClevels in the HFD16 group were higher than those ofHFD8 group. Giving HFD increases serum cysC levels (table 4).

Body Weight, Obesity Index, Body Fat Percentage and Plasma ACE2 Levels

Body weight correlated with plasma ACE2 levels , direction of positive correlation with very strong correlation strength (p<0.001, r=0.867).Obesity index of Lee, Rohrer and TM also correlated with plasma ACE2 levels, direction of positive correlation with very strong correlation strength (respectivelyp<0.001, r=0.882; p<0.001, r=0.866; p<0.001, r=0.870). The body fat percentage also correlated with plasmaACE2 levels, the direction of the positive correlation with a very strong correlation strength (p<0.001, r=0.862). The direction of positive correlation means that the higher body weight, obesity index and body fat percentage, the higher the plasma ACE2 levels (Figure 1).

Plasma ACE2 Levels and Serum CysC Levels

Plasma ACE2 levels correlated with serum cysC levels (p<0.001). The direction of correlation was positive with a very strong correlation strength (r=0.918). A positive correlation direction means that the higher plasma ACE2levels, the higher serum cysClevels (Figure 2).

DISCUSSION

Body weight and body fat percentage after the administration of HFD was higher than ND both for 8 weeks and 16 weeks. This finding is consistent with many studies that have been done before. There was an increase in adiposity (body weight, fat mass, percentage of fat and adipocyte size) after administration of HFD both for 8 weeks[20]and for 16 weeks[25,26]. Significant weight gain occurred during 4 weeks of diet and tended to persist until the end of the study[24]. Recent findings indicate that administration of HFD for 8 weeks adds 169% of adipose retroperitoneal tissue and 107% of epididymal tissue[27].Other recent findings prove that tissue adipose visceral weight increases after 6 weeks of HFD administration even to 300% weigher after 24 weeks of HFD administration.Administration of HFD for 6 weeks is considered a short period. However, Crinigan *et al* (2015) reported that administration of HFD short-term (6 weeks) increases visceral adiposity[9].

Increased body weight from several experimental animal studies given HFD varied.This is due to differences in research characteristics such as experimental animal clusters, intestinal microbiota conditions[25], initial body weight, dietary fat composition, method of administration, experimental period and the amount of food intake consumed by experimental animals.Mice C57BL/6 and wistarrat strains are most widely used in research models of HFD administration.

The physiological mechanism by which a HFD can increase body weight is explained in many previous studies.HFD causes hypertriglyceridemia which causes leptin sensitivity.Leptin is a protein secreted by adipocytes, transported across the blood brain barrier and work in the central nervous system to regulate food and energy expenditure. Hypertriglyceridemia induced with HFD inhibits this mechanism. Because of limitations, this study

Table 4. Effects of HFD on Plasma ACE2 Levels and Serum Cystatin C Levels

Biomarker	Diet Groups				
	Baseline	ND8	ND16	HFD8	HFD16
Plasma ACE2 Level (ng/ml)	1.63 ± 0.07	1.92 ± 0.07	2.05 ±0.06	2.20 ±0.06^{a*}	2.37 ±0.07^{b*c*}
Serum Cystatin C Level (ng/ml)	0.99 ± 0.07	1.22 ± 0.08	1.32 ±0.06	1.46 ±0.07^{a**}	1.57 ±0.05^{b*}

Data are presented in the mean ± SD form (n = 6). Differences between groups were analyzed using the one-way ANOVA test followed by Bonferroni's test. Significant differences are shown by superscri[illegible]etween ND8 and HFD8, ^{b}between ND16 and HFD16, ^{c}between HFD8 and HFD16 and ^{d}between ND8 and ND16. *p<0.001, **p<0.01, ***p<0.05

not measure triglyceride levels. In addition, energy from fat has a greater effect in increasing body weight than energy from non-fat. Fat has a very high efficiency in using nutrients compared to protein and carbohydrates. Signs of satiety that are weaker from fat than carbohydrates and proteins also play a role in the desire to consume a HFD[28].

Obesity index after administration of HFD is also higher than ND both for 8 weeks and 16 weeks. The longer the diet is given, the higher the obesity index. The results of this study are consistent with previous research. Giving a high-fat diet for 8 weeks increased the Lee index,Rohrer index, and TM index and body fat percentage. The obesity index increases according to the duration of administration of HFD and the degree of obesity[24]. Giving HFD for 12 weeks increased the lee's obesity index by 10.45%compared to the control group.Increasing adipocyte mass also increases body weight and obesity index[29]. In contrast, previous studies found that administration of HFD for 3 weeks did not increase obesity

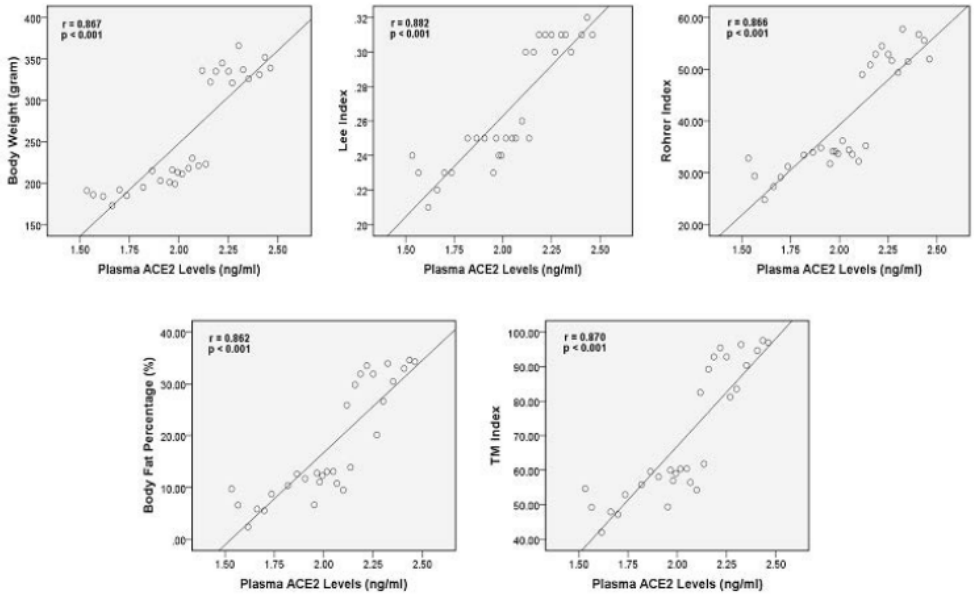
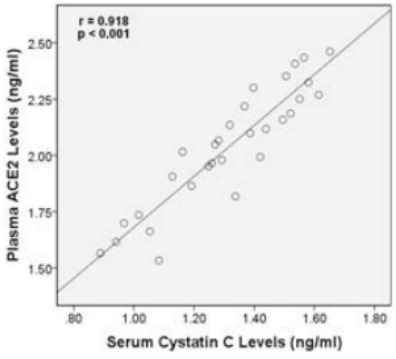


Fig. 1. Correlation between Body Weight, Body Fat Percentage and Obesity Index with Plasma ACE2 Levels



2. Correlation between Plasma ACE2 Levels with Serum Cystatin C Levels

index[30].This difference may be due to the duration of diet in their study which is too short. The macronutrient composition/percentage of fat in the diet given is not mentioned in their publications.

We were the first to research the effect of giving HFD to plasma ACE2 levels and we found plasma ACE2 levels in the HFD16 group were higher than in the HFD8 group. Administration of HFD increases plasma ACE2 levels. This shows the protective role of ACE2[31].

The duration of giving a HFD influences the dynamic ofplasma ACE2 levels both short and long term administration. Provision of a long-term diet is 8 weeks or more[32]. The longer the administration of HFD,

the higherplasma ACE2levels.We found that long-term administration of HFD increasedplasma ACE2 levels. This increase is in response to compensation for renoprotective.

We also found levels serum cysC increased after administration of HFD compared to ND both for 8 and 16 weeks. The results of our study support that the administration of HFD causes a decrease in early stage kidney dysfunction seen in increasing cysC serum levels.This finding is not surprising because damage to renal structure and function caused by HFD has been known in many previous studies. The administration of 15 weeks of HFD in wistar rats caused a decrease in kidney function parameters characterized by urinary albumin excretion and increased plasma creatinine levels[3]. Rats given HFD for 6 weeks showed lower creatinine clearance[8]. Renal injury in rats given HFD for 16 weeks occurs due to lipid accumulation, infiltration of macrophages (inflammation) and oxidative stress in the nose that causes glomerulosclerosis, interstitial fibrosis and albuminuria[5]. Changes in kidney function and structure are also observed in C57BL/6 male mice given by HFD for 12 weeks showed albuminuria and lipids accumulation in the glomeruli and proximal tubules[6]. Changes in structure appear even worse in the renal cortex (glomerulus, tubules, interstitium and blood vessels) due to the administration of longer duration of HFD (18 months)[4]. A recent study found that giving HFD also causes a decrease in the diameter of the capsule of the tubules and the cell volume of bowman's capsule[27].

However,research on the effects of HFD on kidney function with biomarkers cysC levels is very little.In accordance with our findings in this study, recent studies in C56BL/6 male mice given HFD for 22 weeks proved to lead to increased excretion of urine albumin and cysC levels which implies glomerular and proximal functional changes in tubules. Increased urinary cysC excretion was accepted as a biomarker for tubular injury[7]. Other research found different results in which the provision of short term HFD for 6 weeks on sragu-dawley rats did not cause early renal injury. CysC levels in rats given HFD were higher than ND, but not statistically significant. Creatinine and urine protein concentrations also show no difference. The possibility that the duration of HFD administration used in this study is not sufficient to cause a decline in kidney function[9].

Plasma ACE2 levels are positively correlated with levels cysC serum. The direction of positive correlation means that the higher plasma ACE2 levels, the higher the serum cysC levels. We were the first to carry out a study related to the correlation between ACE2 and a decrease in early kidney function disorders using cysC biomarker. Previous studies have reported on the correlation of ACE2 with biomarker of different decrease on kidney function. The mRNA expression of urine ACE2 gene was positively correlated with kidney function parameters, namely the degree of proteinuria and creatinine serum and negatively correlated with eGFR[33]. In a rat model of CKD, ACE2 expression decreased significantly. Inhibition of ACE2 causes a decrease in cortical ACE2 activity, reduces 50% of FITC-inulin clearance and significantly increases urine albumin excretion[34]. The findings in this study indicate that plasma ACE2 levels play a role in decreasing early kidney function, as evidenced by an increase in serum cysC levels.

We also found that body weight, body fat percentage, and obesity index were positively correlated with plasma ACE2 levels with very strong correlation strength. The higher the body weight, the percentage of body fat, and obesity index, the higher plasma ACE2 levels. Obesity is associated with overly active systemic and local RAS including adipose RAS. This proves that there is a relationship between RAS and obesity with special emphasis on the role of adipose tissue RAS in the pathogenesis of metabolic disorders in obesity[35]. Possible findings indicate that HFD-induced obesity activates RAS through the role of ACE2.

CONCLUSIONS

Administration of HFD has been proven to increase body weight, body fat percentage, and obesity index.Administration of HFD increases plasma ACE2 levels. Administrationof HFD causes a decrease in early renal function as evidenced by increase in serum cysClevels. Plasma ACE2 levels play a role in the occurrence of andecrease in early stage renal function induced by administration of HFD. Body weight, body fat

percentage, and obesity index also play a role in increasingPlasma ACE2 levels.

Further research is needed on intervention variationsin the form of conventional or herbal medicine which can inhibit the decline of early renal function due to HFD administration, research on the effects of HFD administration on the cysC gene and other RAS components, and research on the administration of HFD with a longer duration.

Ethical Approval

The research was carried out after obtaining recommendations for ethical appro from The Committee on Ethics of Medical Research, Faculty of Medicine, Hasanuddin University (Recommendation number: 366/H4.8.4.5.31/PP36-KOMETIK/2018) date May 14, 20 Procedures for experimental animals were carried out in accordance with the principles of the Declaration of Helsinki.

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