Journal of Food and Nutrition Research, 2018, Vol. 6, No. 12, 725-730
Available online at http://pubs.sciepub.com/jfnr/6/12/2

DOI:10.12691/jfnr-6-12-2

High Fiber Diet Suppression of TLR4 and NFκβ Gene Expression Correlated with TNF-α and IL-6 Levels in Hypertriglyceridemia Rats

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Received November 10, 2018; Revised December 11, 2018; Accepted December 19, 2018

Abstract Hypertriglyceridemia causes hypoxia and increases the release of free fatty acids in adipose tissue through lipolysis which stimulates an increase in TLR4 and NFκβ levels as well as TNF-α and IL-6 secretion. The high fiber diet had a suppressing effect against TLR4 and NFκβ gene expression and decreased TNF-α and IL-6 levels. This study evaluated the effect of high fiber diet (P1= 1.04 g content of fiber/rat/day, P2= 2.07 g content of fiber /rat/day and P3= 3.11 g content of fiber/rat/day) on TNF-α and IL-6 levels as well as expression of TLR4 and NFκβ in rats induced by high fat and fructose diet for 6 weeks. The findings indicate that the administration of high fiber diet to the hypertriglyceridemia rats could control body weight gain and significantly reduce TNF-α and IL-6 levels (p<0.05) lower than the hypertriglyceridemia groups when compared before treatment. In addition, the treatment of high fiber diet resulted in suppressing the expression of TLR4 and NFκβ gene (p<0.05) when compared with the normal and hypertriglyceridemia groups. The suppression of TLR4 and NFκβ were correlated with TNF-α and IL-6 levels. In conclusion, the administration of the high fiber diet to the hypertriglyceridemia rats was able to decrease TNF-α and IL-6 levels possibly by suppression of TLR4 and NFκB gene expression.

Keywords: *TLR4, NFκβ, TNF-α, IL-6, hypertriglyceridemia, high fiber diet*

Cite This Article: I Gusti Ayu Nyoman Danuyanti, Arta Farmawati, and Sunarti, "High Fiber Diet Suppression of TLR4 and NFκβ Gene Expression Correlated with TNF-α and IL-6 Levels in Hypertriglyceridemia Rats." *Journal of Food and Nutrition Research*, vol. 6, no. 12 (2018): 725-730. doi: 10.12691/jfnr-6-12-2.

1. Introduction

High fat but low fiber dietary consumption and lack of physical activity are factors initiating metabolic syndrome, including hypertriglyceridemia conditions, one of whose main symptoms is the increased triglyceride level in blood [1,2,3]. High fat diet may increase fat in abdominal areas, and hypertrophy of adipocytes cells that is accompanied by increased triglycerides in the adipose tissue. If the high fat diet is accompanied by the intake of fructose as sweetener and consumed excessively over a long period, it leads to insulin resistance through the reduction of insulin receptor sensitivity [4,5,6].

Chronic hypertriglyceridemia as a result of high fat and fructose diet also causes hypoxia in adipose tissues, hence adipocyte cells will secrete pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) to attract immune cells and macrophage infiltration as an inflammatory response [7,8]. Increased TNF-α production will stimulate free fatty acids (FFA) release from adipose tissues which will bind with Toll-Like Receptor 4 (TLR4) receptors in adipose tissues [9,10]. TLR4 signals will activate necrosis factor of NFκβ transcription, a regulator that can cause inflammation. Activation and translocation of NFκβ in the nucleus also causes an increase of pro-inflammatory cytokine production such as TNF-α and IL-6 [11,12].

Hypertriglyceridemia can be optimally managed through therapies of drugs, nutrition, and physical activity. Functional food containing fiber and antioxidants are highly recommended for individuals experiencing hypertriglyceridemia [14]. Fermentation of dietary fiber in the colon produces several products such as methane gas, CO_2, and Short-Chain Fatty Acids (SCFA), especially acetate, propionate and butyrate. Propionate acid plays an important role in obstructing TNF-α production and increasing anti-inflammatory cytokine secretion (IL-4 and IL-10) by reducing immune cell infiltration to adipose tissues so it can suppress inflammation distribution in adipose tissues. Butyrate functions to maintain homeostasis of intestinal epithelial cells and inhibits NFκβ activation pathways in order to reduce secretion production of pro-inflammatory cytokines and adhesion molecules that may cause vascular damage caused by inflammation [15,16]. Acetate is the SCFA

having the largest proportion in the intestines and is involved in lipogenesis processes in adipose tissues with the catalyst of Acetyl CoA synthesis enzymes [15].

This study use of dietary fiber was expected to be able to provide more considerable benefits in managing hypertriglyceridemia. Several previous researches also showed that consumption of dietary fiber could prevent weight gain, change intestinal microbe composition, and improve lipid profile and inflammation response of rats fed with high fat diet [16-21]. Thus, in the current study, we aimed to evaluate the effect of high fiber diet administration to reduce TNF-α and IL-6 levels as well as suppress the expression of TLR4 and NFκβ genes in rats fed with a high fat and fructose diet.

2. Materials and Methods

2.1. Animal and Diets

Twenty-five (25) male Wistar rats (*Rattus norvegicus*) (8 weeks old, 180-200 g) were obtained from BioFarma laboratory in Bandung. This study was approved by the Ethical Committee of Integrated Research and Testing Laboratory, Universitas Gadjah Mada (Approval Number: 00065/04/LPPT//2017).

All rats were individually in cages and maintained under standard conditions (12:12-h light/dark cycle and 22-25°C room temperatures). They were acclimated for 7 days by a control diet prepared in accordance with AIN-93M formulation with slight modification (L-Cystine was substituted by DL-Methionine and Choline bitartrate by Choline chloride) and water *ad libitum* [22]. The diet consisted (g/100 g diet) of corn starch 61.94, casein 14, sucrose 10, corn oil 4, cellulose 5, mineral mix 3.5, vitamin mix 1, DL methionine 0.3, choline chloride 0.25, and tetrabutylhydroquinone 0.008.

Then, the rats were divided into five groups: 1) normal rats (N); 2) hypertriglyceridemia rats (HT); 3) hypertriglyceridemia rats with fiber 1.04 g/rat/day (P1); 4) hypertriglyceridemia rats with fiber 2.07 g/rat/day (P2), and 5) hypertriglyceridemia rats with fiber 3.11 g/rat/day (P3). Groups 2, 3, 4, and 5 were given a high fat and fructose diet for 7 weeks to induce the hypertriglyceridemia condition. High fat and fructose diet was prepared according of the control diet formulation while sucrose was substituted by fructose and modification was done with trans fat to reduce the amount of corn starch [23,24]. The rats were considered as hypertriglyceridemia, if they had triglyceride level in plasma >70.79 mg/dL [25].

The groups of P1, P2, and P3 were fed the high fiber diet, referring to the control diet and substituting 40% corn starch with sweet potato and pumpkin as sources of fiber. The total of fiber content in 100 g of diets for P1, P2 and P3 were 6.88 g, 13.77 g and 20.65 g, respectively. This result was examined by the Center for the Study of Food and Nutrition, Gadjah Mada University.

Each group was given a high fiber diet of 15 g/200 g rat body weight/day, so fiber content given in each treated group was P1=1.04 g/rat/day, P2= 2.07 g/rat/day, and P3= 3.11 g/rat/day for 6 weeks. Before and after administration with high fiber diet, the body weight, TNF-α and IL-6 levels were measured. At the end of the experiment, the rats were euthanized for collection of white adipose tissue samples, which were taken and kept in cryotube that was free from RNAase at temperature of -80°C to be analyzed for TLR4 and NFκβ gene expression.

2.2. Body Weight Measurement and Biochemical Analysis

The body weights were recorded weekly. The Enzyme Linked Immunosorbant Assay (ELISA) method (Fine Test, Wuhan Fine Biotech Co.Ltd) was determined by using the plasma Tumor Necrosis Factor-α (TNF-α) and interleukin-6 (IL-6) levels according to the manufacturer's protocol. The measurement was performed before and after treatment using the high fiber diet.

2.3. RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from frozen white adipose tissue samples by using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. The purity and concentration of the RNA were determined by 260/280 nm absorbance ratio by using Nano Drop Microvolume Spektrofotometer and only samples with a ratio greater than 1.7 were used. cDNA was synthesized by using a reverse transcription kit (Thermoscientific, USA) and stored at -80°C prior to quantitative polymerase chain reaction (qPCR). qPCR analysis was performed by using an SYBR Green PCR kit on a Bio-Rad CFX96 instrument (Bio-Rad). PCR conditions for each primer couple were as follows: 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s during 40 cycles. The results were normalized to β-actin and relative gene quantification was performed by using the $2^{-}\Delta\Delta Ct$ method [26].

The specific primer sequences (purchased from Genetica Science) were listed in Table 2.

2.4. Statistical Analysis

All values were presented as a mean ± standard deviation. Paired t-tests were used to evaluate Tumor Necrosis Factor-*α* (TNF-α) and Interleukin-6 (IL-6) plasma levels before and after administration with high fiber diet. One way ANOVA was used to analyze the differences in body weights, TNF-α and IL-6 plasma levels as well as the expression of TLR4 and NFκβ genes between the groups. Tukey's honest significant difference (HSD) were as post hoc tests. Differences were considered as statistically significant at $p<0.05$. The differences in correlations among variables were analyzed by Spearman rank tests.

Table 1. Primer sequences for qPCR

Primer	Length (bp)	Sense	Antisense
TLR4	110	5'-GCAGAAAATGCCAGGATGATG-3'	5'-AAGTACCTCTATGCAGGGATTCAAG-3'
NFKB	199	5'-GAGAGCCCTTGCATCCTTTA-3'	5'-CTTCCCTTTGGTCTTTCTGT-3'
β-actin	155	5'-ACGGTCAGGTCATCACTATCG-3'	5'-GGCATAGAGGTCTTTACGGATG-3'

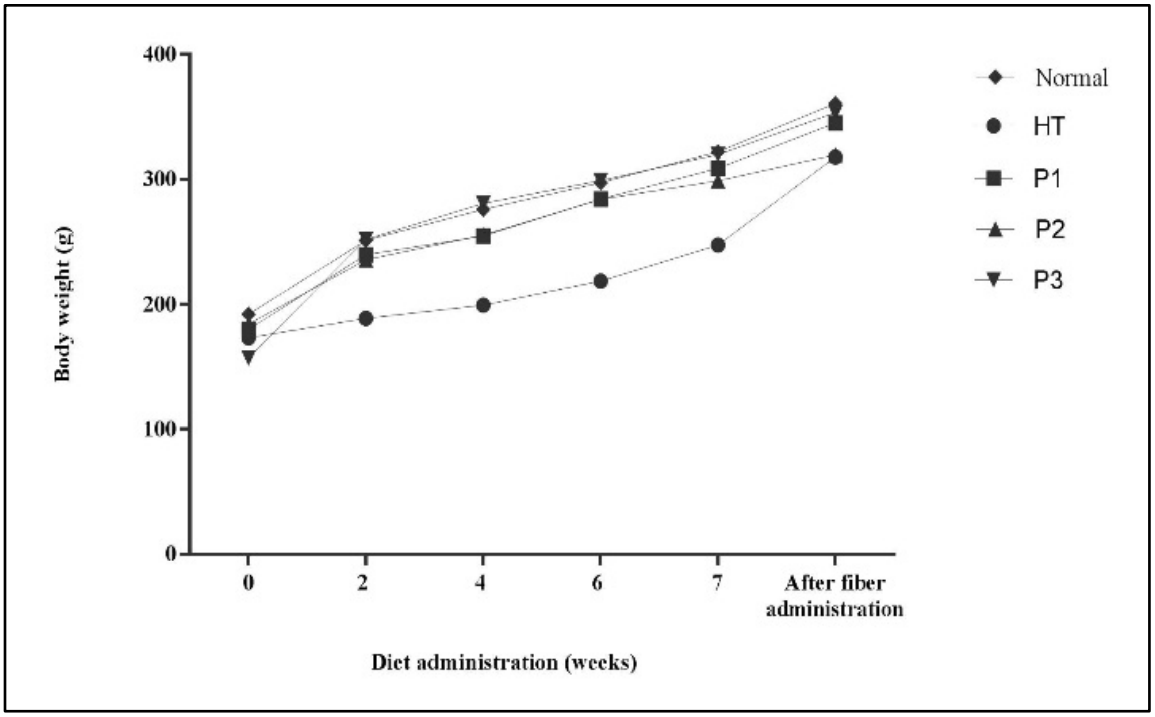
3. Results

3.1. Body Weight Gain Control in Rat Associated with High Fiber Diet

The adminitration of the high fiber diet could control the body weight gain when compared with hypertriglyceridemia groups (P1=11.78%; P2=6.96% and P3=10.51% vs hypertriglyceridemia=28.45%). The P2 group could control body weight gain more than the others even though the body weight of each group was not different. The pattern of rat weight improvement since the beginning of experiment and induction of high fat and fructose diet for 7 weeks and after the high fiber diet was given for 6 weeks are displayed in Figure 1.

3.2. Effect of High Fiber Diet on TNF-α and IL-6 Plasma Levels

The high fat and fructose diet lead to an increase in TNF-α levels as shown in Table 2 and Table 3. Oppositely, TNF-α and IL-6 levels in rat plasma were decreased after high fiber diet administration when compared before treatment and was lower than the HT group (p<0.05).



0: initial body weight
2-7: number of weeks when high fat diet and fructose were given
HT: hypertriglyceridemia; P1: hypertriglyceridemia with fiber 1.04 g/rat/day, P2: hypertriglyceridemia with fiber 2.07 g/rat/day, and P3: hypertriglyceridemia with fiber 3.11 g/rat/day (calculated from 15 g formula high fiber diet/day).
Normal: control diet; hypertriglyceridemia: high fat and fructose diet.

Figure 1. The pattern of rat body weight improvement before and after high fiber diet administration

Table 2. The level of TNF-α after administration with high fiber diet

Groups	TNF-α level (pg/mL)		Δ %	*p* value
	Pretest	*Posttest*		
Normal	74.672 ± 21.85[a]	72.096 ±16.08[a]	3.43	0.739
HT	188.864 ± 25.54	198.604 ± 39.20[b]	5.16	0.290
P1	165.884 ± 20.37	141.630 ± 17.94[bc]	↓14.62	0.020
P2	181.516 ± 15.25	141.724 ± 19.97[bc]	↓21.92	0.009
P3	164.088 ± 29.25	147.472 ± 32.49[bc]	↓10.13	0.012
p value	<0.001[***]	<0.001[***]		

Data are shown as a mean ± SD (n= 5).
HT: hypertriglyceridemia; pretest: before high fiber diet administration; posttest: after high fiber diet administration.
[a] significant difference (p<0.05) compared with HT and P1, P2, P3.
[b] significant difference (p<0.05) compared with normal group.
[c] significant difference (p<0.05) compared with HT group.
significant difference between grous according to ANOVA followed by the Tukey HSD (Honest Significant Difference) test.
↓: % TNF-α plasma level decrease after high fiber diet administrations.
P1: Hypertriglyceridemia with fiber 1.04 g/rat/day, P2: hypertriglyceridemia with fiber 2.07 g/rat/day, and P3: hypertriglyceridemia with fiber 3.11 g/rat/day (calculated from 15 g formula high fiber diet/day).
Normal: control diet; hypertriglyceridemia: high fat and fructose diet.

Table 3. The level of IL-6 after administration with high fiber diet

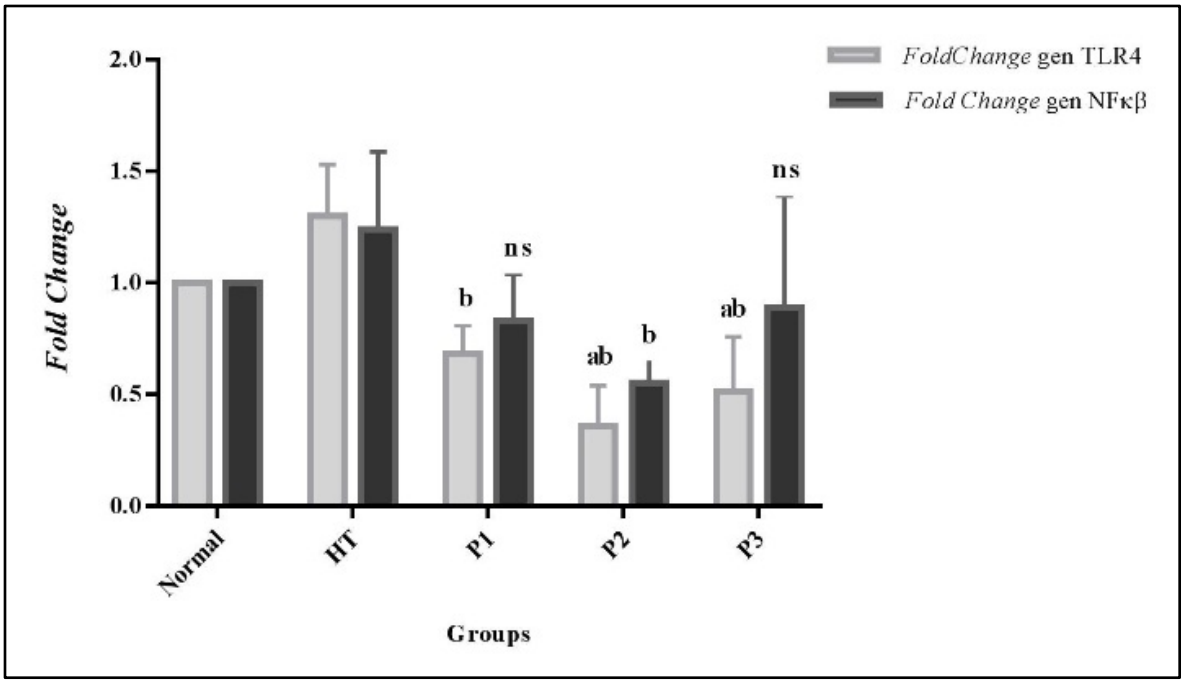
Groups	IL-6 level (pg/mL)		Δ %	*p* value
	Pretest	*Posttest*		
Normal	723.14±180.38[a]	831.80±162.25	15.03	0.159
HT	1349.12±209.01	1603.02±152.61[b]	18.82	0.005
P1	1306.76±110.27	1096.20±110.84[c]	16.11	0.003
P2	1333.08±191.43	925.44±185.39[c]	30.58	0.002
P3	1349.10±130.82	1096.20±85.77[c]	18.75	0.009
p value	<0.001[***]	<0.001[***]		

Data are shown as a mean ± SD (n= 5).
HT: hypertriglyceridemia; pretest: before high fiber diet administration; posttest: after high fiber diet administration.
[a] significant difference (p<0.05) compared with HT and P1, P2, P3.
[b] significant difference (p< 0.05) compared with normal group.
[c] significant difference (p< 0.05) compared with HT group.
significant difference according to ANOVA followed by the Tukey HSD (Honest Significant Difference) test.
↓: % IL-6 plasma level decrease after high fiber diet administrations.
P1: Hypertriglyceridemia with fiber 1.04 g/rat/day, P2: hypertriglyceridemia with fiber 2.07 g/rat/day, and P3: hypertriglyceridemia with fiber 3.11 g/rat/day (calculated from 15 g formula high fiber diet/day).
Normal: control diet; hypertriglyceridemia: high fat and fructose diet.

The decreased levels of TNF-α and IL-6 after the treatment shown between groups had an effect that was not different (p>0.05) with the largest reduction in P2 groups (hypertriglyceridemia rats with fiber 2.07 g/rat/day).

3.3. The Expressions of TLR4 and NFκβ Gene after High Fiber Diet Adiministration

The gene expressions of TLR4 and NFκβ are shown in Figure 2. The decrease of IL-6 level was positively correlated with expressions of TLR4 and NFκβ genes. The gene expression levels of TLR4 in the HT group had a mean expression level 1.3 times higher than the normal group, while the treatment (P1, P2, and P3) groups had lower expression if compared to the normal group (P1= 0.68 fold; P2= 0.36 fold; P3= 0.51 fold) and more significantly decreased (p<0.05) if compared to the HT group without high fiber diet administration (HT= 1.30 fold). The lowest expression was seen in the P2 group (hypertriglyceridemia rats fed with fiber 2.07 g/rat/day).



Data are shown as a mean ± SD (n = 5).
[a] significant difference (p<0.05) compared with normal group.
[b] significant difference (p<0.05) compared with HT group.
[ns] no significant difference (p<0.05) compared with HT group.
Significant difference according to ANOVA followed by the Tukey HSD (Honest Significant Difference) test.
HT: hypertriglyceridemia; P1: hypertriglyceridemia with fiber 1.04 g/rat/day, P2: hypertriglyceridemia with fiber 2.07 g/rat/day, and P3: hypertriglyceridemia with fiber 3.11 g/rat/day (calculated from 15 g formula high fiber diet/day).
Normal: control diet; hypertriglyceridemia: High fat and fructose diet.

Figure 2. Comparison of relative TLR4 and NFκβ gene expression levels after high fiber diet administrations as determined by the quantitative polymerase chain reaction (qPCR) normalized by β-actin.

The suppression of TLR4 gene expression was positively correlated with the expression of NFκβ gene (p=0.02; r=0.452). The expression of NFκβ gene after treatment was lower when compared with normal and HT groups (P1= 0.83 fold; P2= 0.55 fold; P3= 0.89 fold vs HT= 1.24 fold) with the lowest expression in P2 group (Figure 2). The decrease of TLR4 and NFκβ gene expressions among different treatments (P1, P2 and P3) was not different (p>0.05).

4. Discussion

High fat and fructose diet given to rats for 7 weeks caused increases in rat body weight when compared to normal rats (Figure 1). The plasma triglyceride levels had mean 190.91 mg/dL higher than normal rats (data not shown). This result was consistent with previous studies which showed rats given a high-fat diet combined with high sugar caused an increase in body weight, triglyceride and cholesterol levels [11,16]. The administration of high fiber diet could control the rat body weight gain for all treatments (P1, P2, and P3), when compared to the groups of normal and HT rats. This finding may be attributed to the fiber effects from the combination of pumpkin and sweet potato in the high fiber diet intake. Soluble fiber plays an important role in making a viscous solution, so it tends to inhibit gastric emptying and nutrition absorption including glucose [27]. In addition, SCFAs as a result of fiber fermentation were able to reduce visceral adiposity, body weight gain and fat accumulation in adipose tissues by activating G protein receptors (GPR43) [28]. Adam et al. [30] showed the body weight gain was decreased after rats were fed fiber diet with pectin content of 3% when compared with normal group without fiber diet administration for 8 and 28 days.

This finding was in line with the result of our research showing that there was more significant increase in TNF-α and IL-6 levels in rat plasma (p<0.05) after it was induced by high fat and fructose diet, if compared to normal group without high fat and fructose diet induction (Tables 2,3). Increased TNF-α secretion was positively correlated with IL-6 levels (p = 0.000; r = 0.774). Masi et al. [8] also reported that the combination of high sugar diet (sucrose) and fat fed to rats for 8 weeks caused increased pro-inflammatory cytokines. Hypoxia in adipose tissue was caused by hypertriglyceridemia that would increase pro-inflammatory cytokine secretion (such as TNF-α and IL-6) as a form of inflammatory response [7,10].

The increased pro-inflammatory cytokine secretion (such as TNF-α) induces FFA releases from adipose tissues through lipolysis processes [7,10]. Increased fatty acids directly circulated would bind with TLR4 receptor to induce the expression of TLR4 gene, then activating and translocating NFκβ transcription factor to the nucleus. Therefore, proinflammatory cytokine synthesis in adipose tissues might increase [7,8,10,30]. In high-fat diet fed mice, pro-inflammatory cytokines in the liver have been found to increase with lipid levels resulting in NF-κB activation and downstream cytokine production causing increased inflammation [31].

The tendency of reduction of TNF-α and IL-6 levels was found more effective when the rats were given the diet with fiber content of 2.07 g/rat/day (P2). On the other hand, there was more suppression of the expression of TLR4 gene in each treatment (p<0.05), if compared to normal group and HT groups (Figure 2). The expression of NFκβ was decreased in groups P1, P2 and P3 after treatment, with the lowest expression in group P2 when compared with the normal and HT groups. The reduction of TLR4 gene expression was also positively correlated with NFκβ gene expression (p=0.02, r=0.452) after high fiber diet administration.

Previous research showed that a combination of soy milk and fiber diets could reduce the expression of TLR4 gene in rat intestines with high cholesterol diet [19]. This finding relates to the role of SCFA as a product of fiber fermentation resulting in the cecum and colon to be brought from intestinal lumen into the circulation in order to be absorbed by a particular organ/tissue. It has the function to inhibit molecules substrate or signaling [29].

In adipose tissues, SCFAs (acetate, propionate, and butyrate) could decrease lipolysis processes through activation of FFAR2 mainly by acetate and propionate, then it causes dephosphorylayion and deactivation of lipase sensitive hormones in adipose tissues, so lipolysis could be inhibited. Inhibition of lipolysis causes FFA concentration in circulation to decrease, so it could inhibit the activation of TLR4 gene resulting in deactivation of NFκβ and reduction of pro-inflammatory cytokines production. Propionate acid also has an important role in inhibiting TNF-α and IL-6 secretion and increasing pro-inflammatory cytokines secretion (IL-4 and IL-10) by reducing immune cell infiltration to adipose tissues, so it could suppress inflammation distribution in adipose tissues [14].

5. Conclusion

In this study, we found that the administration of the high fiber diet was able to suppress TLR4 and NFκβ gene expression which positive correlated with decreases in TNF-α and IL-6 levels in hypertriglyceridemia rats. There were no significantly different effects between different treatments (P1, P2 and P3), but the most effective was when the rats were given diet with fiber content of 2.07 g/rat/day.

Acknowledgements

This research was supported by Politeknik Kesehatan Mataram and Badan Pengembangan dan Pemberdayaan Sumberdaya Manusia, Ministry of Health Republic of Indonesia.

Statement of Competing Interests

The authors have no competing interests.

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