The Effect of Soya and Genistein Supplementation on Serum Lipid Profile of Male Dawley Sprague Rat

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ABSTRACT

Background: Non-pharmacological treatment is needed to reduce serum lipid levels and prevent atherosclerosis. Foods that contain Isoflavones can improve lipid profiles in the serum.

Aim: To determine the effect of soya and genistein supplementation in various doses on total cholesterol, Low Density Lipoprotein Cholesterol (LDLC), High Density Lipoprotein Cholesterol (HDL), Triglycerides in male Sprague-Dawley rats.

Methods: This study of 35 male Sprague-Dawley rats aged 6-8 weeks were divided into 7 groups, control group; low, medium, and high doses of soy milk; and low, moderate, and high doses of genistein. The treatment was given orally for 60 days. The control used was mice with standard feed. On day 61, serum and hepatic tissue samples were taken and checked for lipid profiles using the Friedewald formula. Statistical analysis was carried out by one-way ANOVA and Tukey's posthoc test and Dunnet test, then regression analysis.

Results: There was a significant increase in total cholesterol levels ($p < 0.05$) and LDLC levels in low and moderate doses of soy milk group compared to all groups of genistein. There was no significant differences in HDL levels in soya and genistein groups. There was significant increase in Triglyceride levels in all groups of soy milk and low and moderate doses of Genistein compared to high doses of genistein.

Conclusion: In this study, Soya and genistein supplementation had increased effect on the Total Cholesterol, LDLC and Triglycerides but no effect on HDL.

Keywords: soy, genistein, cholesterol, lipoprotein, triglycerides

INTRODUCTION

The prevalence of coronary heart disease (CHD) in Indonesia in 2013 based on physician's diagnosis was around 883,447 people.⁽¹⁾ The most common cause of CHD is atherosclerosis, which is a chronic inflammatory response to cholesterol deposition on the walls of arterial blood vessels.⁽²⁾

Currently, atherosclerosis therapy includes anti-inflammatory drugs such as cyclooxygenase (COX) inhibitors and immunosuppressant drugs, but these drugs only work to inhibit the progression of existing atherosclerotic plaques, not prevent their formation.⁽³⁾ Soybeans contain genistein isoflavone phytoestrogens which can reduce cholesterol levels in the blood by increasing

HDLc, lowering LDLc and triglycerides in the blood.⁽⁴⁾

In 2015, soybean consumption in Indonesia reached 2.54 million tons of dry beans, consisting of direct consumption of 2.3 million tons of residents, 39,000 tons of seeds, 446,000 tons of non-food industry, and 49,000 tons of soy milk, indicating easy access for residents. Indonesia against soybeans.⁽⁵⁾

METHODS

The design study is Post Test Control Group Design with regression analysis. This study used experimental animals, Sprague Dawley mice. Inclusion criteria: male mice, age 6-8 weeks, body weight 160-200 grams, have not experienced any treatment or have not received any chemical intake, and are in good health with active. Exclusion criteria: mice do not want to eat, rats that have decreased physical condition or die.

A total of 35 experimental animals were divided into 7 groups, the control group; low doses of soy milk (0.8 ml), medium doses of soy milk (1.6 ml), and high doses of soy milk (2x1.6 ml); and low doses of genistein (0.8 ml), moderate doses of genistein (1.6 ml), and high doses of genistein (2x1.6 ml). The treatment was given orally for 60 days. The control used was mice with standard feed.

On the 61st day, rats were sacrificed, blood samples were taken, then checked for lipid profiles using Friedewald's formula and made liver histological preparations by staining with Oil Red O. The making of slides and reading of slides was carried out at the Histopathology Laboratory of the Faculty of Medicine, Universitas Brawijaya. The study was carried out in February-April 2019.

All technical data processing results were analyzed in a computerized manner using the Statistical Product and Service Solution

software, IBM SPSS Statistics 20 with a significance level or a probability value of 0.05 ($p = 0.05$) and a confidence level of 95% ($\alpha = 0.05$). The steps of the comparative hypothesis test are as follows: data normality test using the Kolmogorov-Smirnov test, variant homogeneity test using the Barlett homogeneity test, One-way ANOVA test, and post hoc test using the Tukey-HSD test.

RESULTS

The study used 35 male Sprague Dawley rats, divided into 7 groups, each consisting of 5 rats. Four subjects were excluded from the study because they died from infection. Characteristic between groups is shown at **Table 1**.

At the end of the study there were 31 subjects for data analysis which were divided into each group as follows: Group K1: 5 subjects, K2: 4 subjects, K3: 5 subjects, K4: 5 subjects, K5: 3 subjects, K6 4 subjects, K7: 4 the subject. Subjects who had extreme body weight scores at the start and end of the study were excluded from the data analysis. Determination of extreme values using Box Plot analysis.

Effect of Soybean Milk and Genistein on Lipid Profiles

Effect of Soybean Milk and Genistein on Lipid Profiles and Lipid Infiltration is shown at **Table 2**. There is a significant effect of giving soy milk and genistein on total cholesterol with different doses. Based on the results of Tukey's 5% test, comparison of the Control group (K) with all treatment groups with soy milk and genistein, the p -value was less than 0.05 ($p < 0.05$) for 0.8 ml and 1.6 ml soy milk. From this test it was proven that the mean total cholesterol of soy milk giving 0.8 ml and 1.6 ml was higher than the control group.

Table 1. Characteristic between Group

Characteristic	K1	K2	K3	K4	K5	K6	K7
N	3	4	4	3	3	4	4
Initial Weight (gr)	166.9±1.4	201.3±10.9	219±5.3	202.5±10	186.7±7.3	189.8±7.1	206.4±5.4
Final Weight (gr)	325.4±8.7	334.2±9.4	344.1±15.9	333.8±1.4	263.6±18.7	329.3±15.4	344.6±13.4
Initial Size (cm)	16±0.2	17.9±0.2	17.7±0.13	17.9±0.2	17.8±0.2	17.42±0.5	18.07±0.21
Final Size (cm)	20.4±0.08	20.3±0.2	20.6±0.2	20.03±0.1	19.7±0.5	20.2±0.1	20.5±0.1
Initial BMI	0.65±0.02	0.62±0.02	0.69±0.02	0.63±0.03	0.59±0.01	0.62±0.02	0.63±0.01
Final BMI	0.78±0.05	0.8±0.02	0.81±0.04	0.83±0.01	0.67±0.02	0.81±0.03	0.81±0.02

K1 = control group, K2 = low doses of soy milk (0.8 ml) group, K3 = medium doses of soy milk (1.6 ml), K4 = high doses of soy milk (2x1.6 ml), K5 = low doses of genistein (0.8 ml), K6 = moderate doses of genistein (1.6 ml), and K7 = high doses of genistein (2x1.6 ml). Data are presented in the form of Mean + SEM (Standard Error of Mean). One Way ANOVA test showed no significant differences in final body weight, body length and BMI between the control and treatment groups.

Starting from the control group, it appears that the mean total cholesterol is higher in all treatment groups with soy milk and genistein, except that the 2x 1.6 ml of genistein has lower total cholesterol than the control group. The highest average total cholesterol was found in the provision of 1.6 ml soy milk and the lowest average total cholesterol was found in the administration of genistein 2x 1.6 ml, namely 31.16 ± 20.58 mg/dL.

The effect of soy milk and genistein doses on total cholesterol levels forms a non-linear pattern. Increasing the dose of soy milk and genistein does not always increase or decrease total cholesterol levels. It is shown in **Figure 1**.

Figure 1. Regression analysis effect of soya milk and genistein on total cholesterol levels

It can be seen that there was an increase in total cholesterol levels at the addition of 1.6 ml doses of soy milk and genistein. Then, by increasing the dose of genistein 2x1.6 ml, there was a decrease in total cholesterol levels.

Table 2. Comparison of Lipid Profiles and Lipid Infiltration

Treatment	Total Kolesterol		HDL		LDL		Triglycerides	
	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p
Control	35.14 ± 13.79 ^{ab}		42.04 ± 10.93		19.33 ± 5.87 ^{ab}		0.58 ± 0.08 ^a	
Soya 0.8 ml	114.56 ± 42.46 ^{cd}		33.38 ± 2.73		26.32 ± 4.29 ^b		0.66 ± 0.04 ^{ab}	
Soya 1.6 ml	170.87 ± 35.99 ^d		44.14 ± 4.88		49.17 ± 7.04 ^c		0.72 ± 0.11 ^{ab}	
Soya 2x1.6 ml	100.8 ± 6.25 ^{bc}	0.000	53.1 ± 13.5	0.000	49.41 ± 7.9 ^c	0.006	0.93 ± 0.07 ^b	0.000
Genistein 0.8 ml	41.34 ± 22.46 ^{ab}		82.74 ± 34.07		19.78 ± 24.08 ^{ab}		1.02 ± 0.36 ^b	
Genistein 1.6 ml	75.83 ± 24.92 ^{abc}		57.85 ± 10.4		46.87 ± 3.17 ^{bc}		1.04 ± 0.22 ^b	
Genistein 2x1.6 ml	31.16 ± 20.58 ^a		113.94 ± 2.24		7.51 ± 1.61 ^a		0.47 ± 0.08 ^a	

The effect of genistein dose on HDLC lipid levels forms a non-linear pattern.

Soy milk affects total cholesterol by 52.05%, while the remaining 47.95% is explained by errors and other factors not examined. Genistein affects total cholesterol by 51.01%, while the remaining 48.99% is explained by errors and other factors not examined.

There is also a significant effect of giving soy milk and genistein on HDLC levels at different doses. Based on the results of the Dunnet T3 5% test, comparison of the Control (K) group with all treatment groups with soy milk and genistein, the p-value was less than 0.05 ($p < 0.05$) in all groups. From this test it was proven that the mean HDLC levels of soy milk and genistein were not significantly different from the control group. Starting from the control group, it was seen that the mean HDLC lipid content was higher in the treatment group with 1.6 ml and 2x 1.6 ml soy milk, but this increase was not statistically significant. When compared with the genistein group, an increase in all genistein doses was shown, but it was not statistically significant.

The effect of soy milk dose on HDLC lipid content formed a linear pattern. Increasing the dose of soy milk increases HDLC lipid levels. It can be seen that there is an increase in HDLC lipid levels along with the addition of the soy milk dose. Soy milk has an effect on HDLC lipid content by 55.88%, while the remaining 44.12% is explained by errors and other unexamined factors. It is shown in

Figure 2.

Figure 2. Regression analysis effect of soy milk and genistein on HDLC.

while the remaining 29.66% is explained by errors and other factors not examined.

There is a significant effect of giving soy milk and genistein on LDLC lipid levels at different doses. Based on the results of the 5% Dunn test, it was shown that the Control group had an average LDLC lipid content of 19.33 ± 5.87 with the letter "ab" notation. When compared with the soy milk group with various doses, it was shown that there was an increase in LDLC lipid levels at all doses of soy milk and a significant increase occurred in the 1.6 ml and 2x 1.6 ml soy milk. This can be seen from the mean $\pm$ SD for the soy milk group of 1.6 ml and 2x 1.6 ml higher and has a different letter than the control group.

In the comparison between the Control group and the group given genistein, It was shown that the 0.8 ml and 1.6 ml genistein group had a higher mean than the control group. This proved that there was an increase in LDLC lipid levels in the two doses of genistein but it was not statistically significant. This can be seen from the mean $\pm$ SD of the group giving genistein 0.8 ml and 1.6 ml higher but it has the same letter as the control group.

Starting from the control group, it was seen that the mean LDLC lipid content was higher in the treatment group with all doses of soy milk, and statistically significant increases occurred at doses of 1.6 ml and 2x 1.6 ml. When compared with the genistein group, it was shown that there was an increase in the administration of genistein 0.8 and 1.6 ml but it was not statistically significant.

The effect of soy milk and genistein doses on LDLC levels formed a non-linear pattern. Increasing the dose of soy milk and genistein does not always increase or decrease LDLC levels. It is shown in **Figure 3**.

Figure 3. Regression analysis effect of soya milk and genistein on LDLC

It can be seen that there is an increase in LDLC levels at the addition of 1.6 ml doses of soy milk and genistein. Then, by increasing the dose to 2x1.6 ml, there was a decrease in LDLC levels.

Soy milk has an effect on LDLC lipid content by 80.34%, while the remaining 29.66% is explained by errors and other factors not examined. Genistein affects LDLC lipid levels by 72.88%, while the remaining 27.12% is explained by errors and other factors not examined.

There is no significant effect of giving soy milk and genistein on Tryglicerida levels with different doses. Based on the results of the Tukey 5% test, the comparison of the Control group (K) with all treatment groups giving soy milk and genistein, obtained a p-value more than 0.05 ($p > 0.05$) when giving Genistein 0.8 ml and 1.6 ml. The difference in mean value is negative, indicating that the mean of the control group is lower than the group giving Genistein 0.8 and 1.6 ml. From this test, it was proven that the average Tryglyceride levels were 0.8 and 1.6 ml higher than the control group.

Starting from the control group, it appears that the mean Tryglicerida levels were higher in the treatment group with all doses of

soy milk and 0.8 ml and 1.6 ml of genistein. The average decrease in Tryglicerida levels was found in the group given 2x 1.6 ml of genistein with an average Tryglicerida level of 0.49 ± 0.06 mg / dL, but the decrease was not significant ($p > 0.05$).

The effect of soy milk and genistein doses on Tryglycerida levels formed a linear pattern. Increasing the dosage of soy milk and genistein increases TG levels. It can be seen that there is an increase in triglyceride levels along with the addition of soy milk and genistein doses. It is shown in **Figure 4**.

Figure 4. Regression analysis effect of soya milk and genistein on Triglycerides

Soy milk has an effect on Triglyceride levels by 73.68%, while the remaining 26.32% is explained by errors and other factors not examined. Genistein affects Triglyceride levels by 73.68%, while the remaining 26.32% is explained by errors and other factors not examined.

DISCUSSIONS

In this study, 4 subjects were excluded due to death. The proportion of sample deaths reached 11.3%, exceeding the initial estimate of 10%. In group 5, there were 2 rats that died due to infection so that the number of samples was only 3, less than the minimum sample requirement in the calculation of the Federation formula. Data from subjects who

had extreme body weight scores at the start and end of the study were not used in the data analysis.

In this study, the total number of samples used for data analysis was 25 samples. So there are several groups that do not meet the minimum sample requirements, namely groups K1, K4, K5 ($n = 3$). One way ANOVA test results showed the effect of giving soy milk and genistein on body weight ($p = 0.022$).

Post hoc test with Tukey HSD, showed no effect of soy milk on body weight and BMI between the control and treatment groups at all doses. Genistein also did not significantly affect differences in body weight and BMI between control and treatment groups at all doses. Study by Wang *et al* showed that supplementation of soy milk and genistein in large doses in male rats tended to lose weight. Whereas at low doses it tends to increase body weight.⁽⁶⁾

However, there is a gap between invitro and invivo studies due to the overlapping of transcription factors, metabolism and the stability of active substances. The use of soy milk and genistein also gave different results, because in soy milk there is another isoflavone, daidzein, which influences lipogenesis through the PPAR- α independent pathway in hepatocytes rather than through estrogen receptors in adipocyte tissue. Microarray gene analysis showed that adipogenesis in low-dose genistein was influenced by the phospholipase A2 group 7 gene and phospholipid transfer protein. Meanwhile, the anti-adipogenic activity of genistein and down-regulation of the adipogenic gene require expression of $Er\beta$.⁽⁷⁾

In this study all groups of mice were obese. This result is not in accordance with the initial study design, because the control used is

a negative control, so it is expected that obesity does not occur in the control group. This study did not calculate the amount of leftover feed, and the physical activity of rats. Obesity that occurred in all groups in this study could occur due to excess calorie intake compared to daily calorie requirements compared to physical activity of rats. Daily calorie requirements for male Sprague Dawley rats 110 kcal ME/BW 0.75kg/day.^(8,9)

While the food intake given in this study in all groups was 70-80 g/cage/day which was equivalent to 120-137 kcal/rat/day.

The Effect of Soybean Milk and Genistein on Lipid Profile

From the results of this study, it was found that giving soy milk to male Sprague dawley rats, the results were more increased total cholesterol, LDLC and triglycerides; and HDLC decreased more than genistein.

Several studies have shown that supplementing the diet with soy protein will lower blood cholesterol and reduce chronic disease in populations in the West and in Japan.⁽¹⁰⁾

Isoflavones increase HDLC and reduce total cholesterol, LDLC and TG, presumably due to the role of soy isoflavones, namely genistein, daidzein and glycitein, which have biological activity as phytoestrogens, antioxidants and antimutagen.⁽¹¹⁾

In the liver, isoflavones are presumed to bind to ER, thereby increasing the density and number of LDLC receptors via an up-regulation mechanism. So that the uptake of LDLC in plasma by the liver increases and decreases the total cholesterol fraction in the blood.⁽¹²⁾

Isoflavones also aid in the transport of LDLC to steroidogenic tissues such as the adrenal glands, testes, and ovaries. After

binding to the estrogen receptor on lipoproteins, cholesterol is absorbed by steroidogenic cells and then stored and transferred to the steroid synthesis site. Increased HDLC by isoflavones, presumably by means of isoflavones that bind to ER in the liver will increase the production of nascent HDL. Nascent HDLC will approach macrophages to take cholesterol stored in macrophages. After taking up cholesterol in macrophages, nascent HDLC turns into mature, spherical HDLC remnant. Furthermore, some of the cholesterol esters carried by HDLC will take two pathways¹³: The first route is to the liver and is captured by the class B type 1 (SR-B1) receptor scavenger. The second pathway is that cholesterol esters in HDLC will be exchanged with triglycerides and VLDLC and IDLC with the help of cholesterol ester transfer protein (CETP).⁽¹³⁾

Apart from being estrogenic, isoflavones also act as antioxidants. Soy isoflavones can reduce the Thiobarbituric Acid Reactive Substance (TBARS), a radical that causes mutagenesis, carcinogenesis and cell death.⁽¹⁴⁾

Isoflavones interfere with the hydrogen peroxide reaction by donating hydrogen atoms to free radicals, so that free radicals do not form. This is very useful when there are excessive levels of LDLC in the plasma, most of the LDLC will be oxidized and then eaten by macrophages to become foam cells. Isoflavone antioxidants prevent LDLC oxidation, so that a lot of LDLC is captured by liver cells, so that total cholesterol falls. The lower the foam cell, the lower the risk of atherosclerosis, so that isoflavones are also called cardioprotective. Soybean intake can reduce the direct absorption of cholesterol and TG in the

intestine, so that cholesterol ester and TG levels decrease. This also results in a decrease in total cholesterol levels in the blood.⁽¹⁴⁾

The reduction in total cholesterol levels studied varied, depending on the factors and treatment of the study, including the form and type of isoflavones, isoflavone intake levels, study time, gender of the subject, lipid levels and menopause status.⁽¹⁵⁾

Another form of isoflavones are tablets. In one isoflavone tablet contains daidzein, genistein, biochanin, formonectin or contains daidzein and genistein or contains only genistein. From the results of study on isoflavone tablet form shows that Isoflavone tablets, up to 150 mg per day, have a significant effect in lowering serum total cholesterol, LDL-cholesterol and triglycerides. There are also significant benefits over serum HDL-cholesterol.^(16,17)

The results were consistent when the tablet was introduced as isolated genistein only, but the mixture of genistein and daidzein, or a mixture of genistein, daidzein, formonectin and biochanin did not give a significant effect. It is thought that each composition of the isoflavone combined influence each other so that it gives insignificant results.⁽¹⁶⁻¹⁹⁾

A meta-analysis of 17 studies by Yeung, showed that the level of isoflavone intake (<50g/hr; 51-100g/day; and 101-150g/day) had no significant effect on reducing total cholesterol. It is suspected that isoflavones, especially genistein, which bind to estrogen receptors, have a low affinity, besides that isoflavones must compete with endogenous estrogens against estrogen receptors.^(20,21)

Treatment time also affects total cholesterol levels in isoflavone intake. The treatment time of 11-20 weeks gave significant

results compared to 2-10 weeks, this is thought to be an adaptation time until it reaches a certain affinity for the estrogen receptor. While the treatment time of 21-30 weeks gave insignificant results. This is presumably because the period 21-30 is the end of the duration of the isoflavones in the blood which will then be excreted through the kidneys.⁽²²⁻²⁶⁾

The effect of gender turned out to have an effect on total cholesterol levels in isoflavone intake. A man has a decrease in total cholesterol that is more significant than a woman. This is presumably because isoflavones in women have to compete with endogenous estrogens whose levels are higher than endogenous estrogens in men. This was the exception to the variable for women with peri- or postmenopausal status (hypoestrogen).^(25,27)

Apart from these factors, it is also necessary to consider several factors that have not been discovered or researched. These factors, namely body weight and lipid distribution, intake of other proteins, and physical activity may have an effect on reducing total cholesterol in the blood.

Limitations

There are several limitations: 1. The number of samples is too small. In this study, the sample size was reduced because the deaths that occurred exceeded the estimated number and there were extreme values that were not used in data analysis. 2. Negative control in study becomes obesity due to factors that are beyond the knowledge of researchers and are not considered when making study designs and proposals. 3. The content of other isoflavones in soy milk was not checked. Examination is necessary because the isoflavone compounds other than genistein in

soy milk include phytoestrogens with different affinities.

CONCLUSION

There was an increase in total cholesterol, Low Density Lipoprotein Cholesterol (LDCL) and Triglycerides in Sprague Dawley male rats in the soy milk and genistein supplementation group. There was no effect on High Density Lipoprotein Cholesterol (HDCL) and liver lipid infiltration in Sprague Dawley male rats in the soy milk and genistein supplementation group.

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