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Original Article

The effects of genistein-rich edamame on LDL, Hs-CRP level, Neutrophil-Lymphocyte Ratio and Monocyte-Lymphocyte Ratio: Experimental study in atherosclerosis induced male rats

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Abstract

Background : Atherosclerosis is related to hypercholesterolaemia and inflammation. Edamame is one of the soybeans with high levels of genistein which protects the formation of atherosclerosis. This research aimed to evaluate the effects of genistein-rich edamame on LDL, hs-CRP, Neutrophil-Lymphocyte Ratio (NLR) and Monocyte-Lymphocyte Ratio (MLR) on atherosclerosis-induced mice.
Methods : This research is a true experimental study with posttest only-controlled group design. Thirty male rats were divided randomly into five groups: normal control group without any intervention, negative control group with atherosclerosis induction, positive control group with atherosclerosis induction administered with atorvastatin, first intervention group with edamame extract supplementation, and second intervention group with genistein-rich edamame extract supplementation. After 28 days, serum LDL, hs-CRP, LMR and NLR were measured.
Results : There were significant differences (p<0.001) of hs-CRP values between the second intervention group compared to the negative control, positive control and first intervention group. Edamame extract lowered LDL cholesterol levels in the second intervention group compared to positive control, but the difference were not statistically significant. There were significant differences (p<0.05) of MLR values between the second intervention group and the negative control and also the first intervention group, and the second intervention group was significantly lower compared to the negative control and also the first intervention group. There was no significant difference on NLR values between all groups.
Conclusion : The administration of genistein rich edamame may reduces the inflammation in the blood vessels better than edamame extract represented by lower hs-CRP and MLR values compared to the edamame extract.

Keywords : Genistein rich-edamame, LDL, hsCRP, neutrophil-lymphocyte ratio, monocyte-lymphocyte ratio

INTRODUCTION

Atherosclerosis is an inflammatory disease of arteries which characterized with imbalance and abnormal accumulation of lipids, inflammatory cells, matrix deposits and proliferation of smooth muscle cells in the walls of medium and large caliber arteries.[1] This accumulation is most often detected during the second decade of life and develops further along with age.[1] The development of vascular lesions results in a reduction or cessation of blood flow through blood vessels to the tissues.[1] Important manifestation of atherosclerosis is *atherosclerotic vascular disease* (AVD), a serious contributor to mortality and morbidity in the world than any other disease.[2]

According to world statistics, there are 9.4 million deaths each year caused by AVD, and it is estimated that this number will increase to 23.3 million by 2030.[3,4] In Indonesia AVD is the most cardiovascular diseases which have mortality rates and high morbidity.[5] According to the Health Research and Development Agency, Ministry of Health of the Republic of Indonesia, the death rate due to AVD is 12.9% of all deaths.[6] The prevalence of diseases caused by AVD based on doctor's diagnosis in 2013 Basic Health Research (Riskesdas) is 0.5%, while based on doctor's diagnosis or symptoms is 1.5%. The results of *Riskesdas* showed that AVD is the seventh highest Non-Communicable Diseases (PTM) in Indonesia.[7]

Hypercholesterolemia is closely related to atherosclerosis because the pathogenesis of atherosclerosis starts from the formation of *fatty streaks* in blood vessels. One type of lipoprotein particle, low-density lipoprotein (LDL), carries cholesterol to various places in the body, including the arterial wall.[8] LDL itself is the main source of cholesterol which can accumulate to form *foam cells* which are the initial stages of the development of atherosclerosis.[8]

Inflammation plays an important role in every stage of atherosclerosis from the beginning of the development of plaque to the rupture of the plaque that can cause thrombosis.[9,10] Activated inflammatory cells are found in coronary plaque in patients with acute coronary syndrome[11] and signs of inflammation are detected in coronary artery blood.[12] Accumulation of monocytes and phagocyte monocyte derivatives in arterial walls, contributing to the formation process, chronic inflammation, exacerbation and complications of atherosclerosis.[13] *Lymphocyte Monocyte Ratio* (LMR) is calculated from the amount of lymphocytes and monocytes, two types of white blood cells.[14] Lymphocytes play an important role in lipid deposition in the formation of atherosclerotic plaque.[15] A high monocyte count is associated with an increase in the thickness of the media intima.[16] Monocytes play an important role in the pathophysiology of atherosclerosis, thus LMR can also be a useful marker for knowing the process of atherosclerosis.[17]

One *marker* that can be used to see inflammation is *C-reactive protein* (CRP). CRP examination can detect acute inflammation and severe tissue damage. Examination of high sensitivity C-reactive protein (hs-CRP) can be used to detect at an early stage inflammatory processes of coronary heart disease (CHD), including stable CHD, because this measurement method has a very high sensitivity so that it can measure the levels of CRP in a very small quantity.[18] *The American Heart Association and Centers for Disease Control* (AHA / CDC) recommend the use of hs-CRP as a marker to assess the risk of cardiovascular events and are an independent predictor for cardiovascular disease that plays an active role in the development of atherosclerotic plaque. Hs-CRP level >3 mg / dL is considered as a high risk for CHD.[19]

The neutrophil lymphocyte ratio (NLR) has also been shown to be associated with cardiovascular events.[20] The superiority of NLR's predictive value on cardiovascular risk is caused by two main factors namely, first, that NLR is not affected by conditions such as in vitroactivity and treatment of blood specimens that can affect the absolute value of leukocyte subtypes.[14,21,22] The second and most important factor is that the NLR illustrates the detrimental effects of neutrophils that play a role in nonspecific inflammation that will secrete inflammatory mediators such as elastase, myeloperoxidase and oxygen free radicals that play a role in atherosclerosis and can cause plaque rupture.[14,21,22] Therefore, NLR is more predictive than the measurement of each leukocyte subtype itself.[14,21,22]

Prevention efforts through early detection of risk factors and control efforts are very important. One form of control efforts is through diet, such as consumption of fat and foods that have an antihypercholesterolemia effect, as example is soy consumption.[23] Edamame is a soybean variant that has a higher isoflavone content compared to other food.[23] In Indonesia, edamame has been widely known and consumed by the public as vegetable soybeans or Japanese soybeans since 1995.[24] Indonesian people are very often consume edamame together with other food ingredients.[24]

Genistein is an isoflavone that is widely found in Edamame.[25] Genistein has the potential to prevent atherosclerosis by suppressing inflammation of blood vessels. The protective effect of soy isoflavones on atherosclerosis works through a variety of mechanisms, such as preventing oxidation of LDL, increasing blood vessel reactivity, inhibiting proinflammatory cytokines or cell adhesion proteins and reducing platelet aggregation.[26]

Studies on the effect of genistein on inflammatory markers are still very limited. Therefore, in this study the researchers wanted to know the effect of giving genistein edamame (Glycine max (L) Merrill) to LDL levels, hs-CRP

levels, lymphocyte neutrophil ratio and lymphocyte monocyte ratio in (Rattus novergicus) male rats induced atherosclerosis.

METHODS

This study was a *true experimental* study with *post test only with control group design*. This research was conducted in three places, Semarang STIFAR Laboratory as a place of making experimental materials, LPPT UGM as a treatment place for experimental animals and Prof. Soeparwi Hospital for analysis of LDL levels, hs-CRP levels, neutrophil lymphocyte ratio and lymphocyte monocyte ratio in rats.

This study used 30 Wistar strain male rats (Rattus novergicus) age 15-week-old weighing 180–220 grams, divided into 2 treatment groups and 3 control groups with 6 rats in each group. The groups were normal control group without any intervention, negative control group with atherosclerosis induction, positive control group with atherosclerosis induction administered with atorvastatin, first intervention group with edamame extract supplementation, and second intervention group with genistein-rich edamame extract supplementation. Rats were kept in *stainless steel* cages with a 12-hour lighting cycle. Rat food was given *ad libitum*. The treatment in this study was the genistein-richedamame extract (Glycine max (L) Merrill) in rats induced atherosclerosis and administration of atorvastatin as positive control. Genistein levels were obtained as much as 78% percent of the total extract composition. The outcomerecorded in the study was the level of LDL, hs-CRP levels, neutrophil lymphocyte ratio, and monocytes lymphocytes ratio.

Data obtained from the research was LDL levels, hs-CRP levels, lymphocyte neutrophil ratio and lymphocyte monocyte ratio. Analysis of the research data were processed usingcomputer program.

RESULTS

Analysis of Research Results and Statistical Tests

LDL cholesterol

Statistical test results and mean LDL cholesterol levels were shown in Table 1, while comparisons between groups are presented in the bar graphin Figure 2. *T-test* was conducted between the normal control group and the negative control group, between negative control group and positive control groups, as well as between the negative control group and the two treatment groups; and shown in Table 1. Genistein-rich edamame extract administration reduced LDL levels lower than edamame extract in treatment group 1 and positive control group,

TABLE 1
LDL levels

Groups	LDL levels (mg/dL) Mean ± SD	*p**
Normal control	7.33 ± 1.633	
Negative control	10.00 ± 6.00	
Positive control	7.50 ± 1.975	0.290
Treatment 1	8.60 ± 2.302	
Treatment 2	8.33 ± 2.805	

**p* = tested with One Way Anova, significant if < 0.05

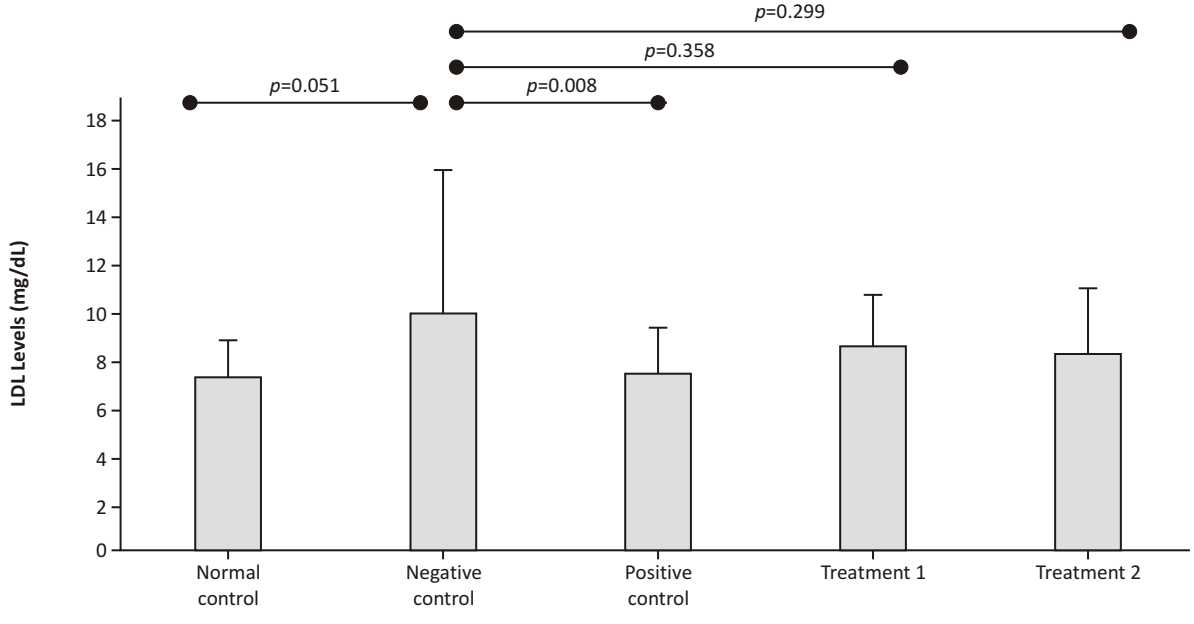

Figure 1. LDL levels among groups

but the difference was not statistically significant.

In one-wayANOVA test, significance was obtained p = 0.290, which means that there was no difference in all groups in LDL levels of all study groups.

hs-CRP

Comparisons between groups are presented in the bar graphin Figure 2. From the data on the results of descriptive statistics, it can be seen that the levels of hs-CRP were found highest in negative control group and were found lowest in the positive control and treatment 2 groups.

From the Post-Hoc test results, there were significant differences between the normal control group and the negative control group, treatment 1 and treatment 2; between the negative control group and the normal control, positive control, treatment 1, and treatment 2 group; between the treatment group 1 with the negative control, positive control, treatment 1, and treatment 2 group; between treatment group 2 with normal control, negative control, and treatment 1 group; and between positive control group with normal control, negative control and treatment 1 group.

***Neutrophil-Lymphocyte Ratio* (NLR)**

Data from the NLR examination results were shown in Table 3 below, while comparisons between groups were presented in the bar graphin Figure 3. T-testwascarried out between the normal control group and the negative control group, as well as between the negative control group with positive control and the two treatment groups.

From the NLR data, it can be seen that administration of genistein-rich edamame extract can reduce NLR levels more than edamame extract alone, viewed from the data in the positive control group as a baseline. However, this difference was not statistically significant.

TABLE 2
Analysis of differences of hs-CRP levels

Groups	hs-CRP (mg/dL) level Mean ± SD	*p**
Normal control	0.033 ± 0.003	
Negative control	0.044 ± 0.003	
Positive control	0.020 ± 0.001	<0.001*
Treatment 1	0.030 ± 0.002	
Treatment 2	0.020 ± 0.001	

*p< 0.05

TABEL 3
Analysis of NLR differences

Groups	NLR Mean ± SD	*p**
Normal control	0.3318 ± 0.0835	
Negative control	0.3970 ± 0.1518	
Positive control	0.2430 ± 0.1096	0.177
Treatment 1	0.3708 ± 0.1001	
Treatment 2	0.3161 ± 0.0828	

*p = tested with One Way Anova, significant if < 0.05

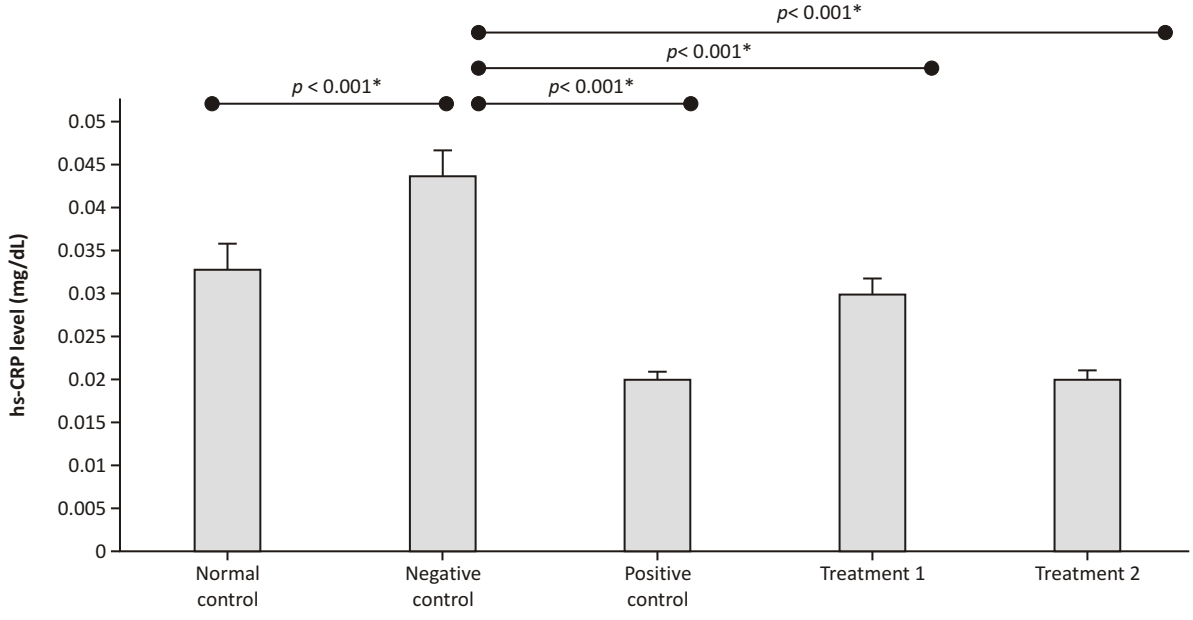

Figure 2. hs-CRP levels among groups

TABEL 4
Means and SD of MLR among group

Groups	MLR Mean ± SD	*p**
Normal control	0.0222 ± 0.0108	
Negative control	0.0323 ± 0.0071	
Positive control	0.0122 ± 0.0072	0.002
Treatment 1	0.0250 ± 0.0088	
Treatment 2	0.0133 ± 0.0084	

*p = tested with One Way Anova, significant if < 0.05

Monocyte-Lymphocyte Ratio (MLR)

The MLR examination data are shown in Table 4 below, while the comparison between groups was presented in the bar graphic Figure 4. Figure 4 showed the mean and standard deviation of MLR values obtained from the diff count measurements of the five study groups. Testing of T-test wasperformed between normal control group and negative control group, and between the negative control with positive control and two treatment groups; and was shown in the line above Figure 4.

In one-way ANOVA test, the significance of p = 0.002 was obtained. Thus, Bonferonni Post-Hoc test was done to determine differences between groups. From the

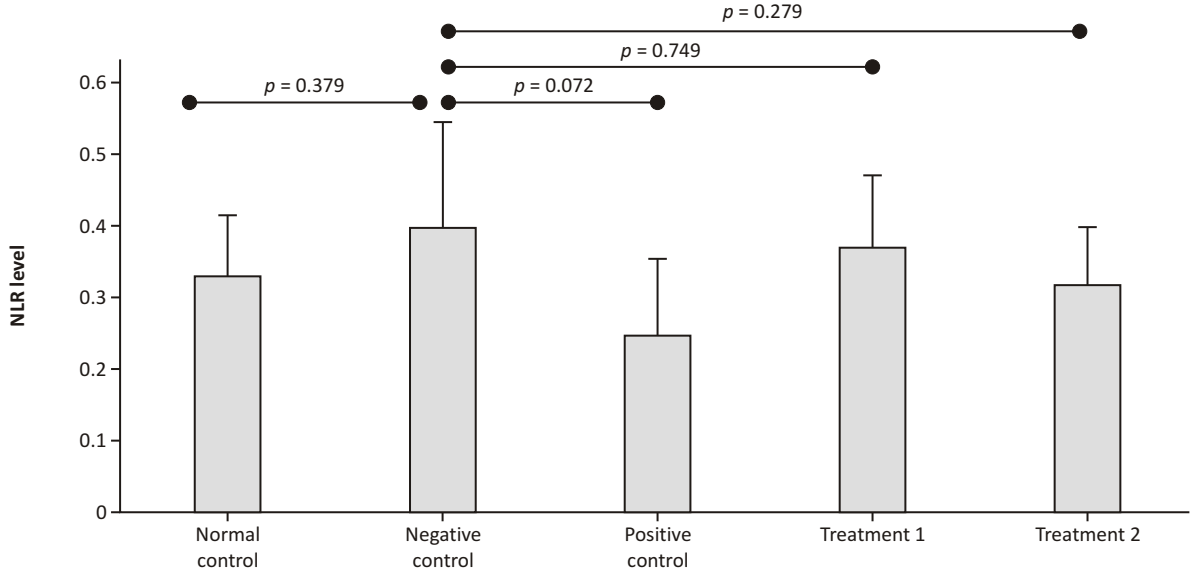

Figure 3. Bar Graphof NLR levels

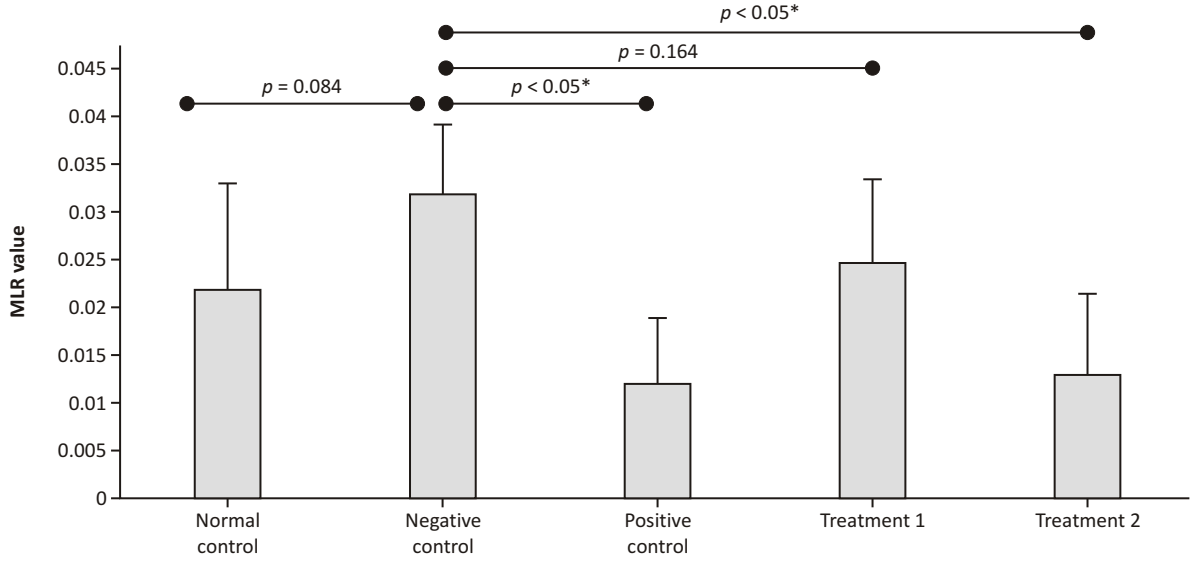

Figure 4. Bar Graph of MLR values

Post-Hoc test results, a significant difference was found between the negative control group with positive control and treatment 2 group. There were no significant differences between positive control and treatment 2 group.

DISCUSSION

Effects of genistein-rich edamame extract on serum LDL levels

Atherosclerotic lesions are associated with serum LDL status because fat is a major component of fatty streak. The process of fatty streakformation begins with LDL uptake by macrophages. This process is getting worse when LDL is oxidized, oxidized LDL uptakeis easier compared with normal LDL uptake.[27] Low-density lipoprotein (LDL) carries cholesterol to various places in the body, including arterial walls .[8] LDL cholesterol itself is a major source of foam cells formation, which is an initial stage of the development of atherosclerosis.[8] Foam cells that occur are actually fat cells that are eaten by macrophages, which are monocyte forms after they have been developed and withdrawn by MCP-1. Thus, LDL pathogenesis in atherosclerotic plaques formation is closely related to monocytes and MCP-1.[9,27] Kapiotis *et al* found that genistein can act as an LDL antioxidant and can inhibit the LDL oxidation process. Inhibition of this oxidation process results in endothelial cell protection against further damage that occurs due to atherogenic LDL.[28]

In this study, the group that received induction of atherosclerosis without any therapy had the highest LDL levels, while the group that received therapy with genistein-rich edamame extract, edamame extract alone, or those that received atorvastatin showed lower LDL levels. However, there were no significant differences between each groups. This is similar to the findings from Taku *et al* who found that intake of isoflavone extract did not significantly reduce LDL cholesterol levels obtained from a meta-analysis of 12 studies, as well as study by Gonzalez *et al*. who found that administration of isoflavone did not reduce HDL and LDL levels in diabetic patients.[29,30] Shim *et al* also found that decreased lipid levels after administration of soy isoflavones did not have a consistent value, because some studies showed a decrease in lipid levels, and several other studies did not.[31]

This is thought due to the beneficial effect of soy isoflavones which may require synergistic interactions with various other components. In addition, the extraction process might reduce the effect of isoflavones, in this case genistein, to exert influence on the lipid profile; Clarkson who stated that alcohol-washed soy protein extract did not have the same effect as soy protein itself,in which soy protein did not experience alcohol washing.[29,31] Taku *et al*. Found that soy protein which is undergone isoflavones depletion also reduced LDL levels similar to isoflavone-rich soy protein.[32] Conversely, intake of isoflavone extract alone without soybean has no effect on the lipid profile. This suggests that certain activating factors may be needed for soy protein to allow soy isoflavones, such as genistein, to exert influence on lipid profiles; or synergistic interactions between soy isoflavones and other soy components are needed.[29–32] Sacks *et al*. also stated the same thing; where the effect of reducing lipid levels may be caused by non-protein components of soy, and not soy protein itself.[33] In addition, conditions where the decrease was not significant could caused by the insufficient induction which did not produce significant higher LDL levels, thus the decrease by administration of genistein extract was not significant either. Errors that occur may caused by ingredients requirement of other than egg yolks to increase LDL levels.

Effects of genistein-rich edamame extract on serum hs-CRP levels

CRP and hs-CRP are related to atherosclerotic pathogenesis because CRP enhances the regulation of Plasminogen Activator Inhibitor-1. The existence of CRP facilitates the formation of foam cells from LDL.[27] TheAmerican Heart Association and Centers for Disease Control (AHA / CDC) recommend the use of hs-CRP as a marker to assess the risk of cardiovascular events and are an independent predictor for cardiovascular disease that plays an active role in the development of atherosclerotic plaque. Hs-CRP level> 3 mg / dL is considered as a high risk for CHD.[19]

In this study, there was a significant difference in the value of hs-CRP in treatment group 2 to the value of hs-CRP in rats compared with the control group, both normal and negative control groups. These results are similar to studies conducted by Farsi *et al*., who found that administration of genistein reduced CRP levels in diabetics.[32] An increase in hs-CRP affects the expression of vascular endothelial plasminogen activator inhibitor-1 (PAI1) and other adhesion molecules after LDL uptake by macrophages.[33] Thus, an increase in hs-CRP predicts cardiovascular events. A study examined the effect of statins on C-reactive protein and LDL shows that statin administration decreases CRP and LDL, and also decreases the incidence of major cardiovascular events.[34] In this study, it was also found that administration of genistein-rich edamame extract in treatment group 2 decreased hs-CRP equivalent to atorvastatin in positive control group, so it can be concluded that the effect of reducing the risk of atherosclerosis represented by hs-CRP levels in the administration of genetein-rich edamame extract was equivalent with atorvastatin.

Effects of genistein-rich edamame extract on NLR

Neutrophils have an important role in the progression

and instability of atherosclerotic plaque that can trigger ACS. Neutrophils will attached to endothelial to prevent ischemic reperfusion from capillaries and also release autochoids that induce vasoconstriction and platelet aggregation.[27] In contrast, lymphocytes have a protective role against atherosclerosis. Increasing of lymphocytes in some rodents is associated with an increase in anti-inflammatory cytokines, IL-10, and suppressing pro-inflammatory cytokines such as IL-6 and TNF-α.[35] Pro-inflammatory cytokine production occurs along with increasing sympathetic nervous system activity, and stimulation of the parasympathetic nervous system influences lymphocyte formation; thus, a decrease in lymphocytes might represent a decrease in the activity of the parasympathetic nervous system and an increase in sympathetic nervous system activity.[36] Hence, as atherosclerosis increases, neutrophil values continue to rise and lymphocyte values continue to fall; then, the ratio between neutrophils and lymphocytes (shown through NLR) will also increase. Lee *et al* found that genistein can inhibit atherogenesis by improving endothelial dysfunction and stabilizing atherosclerotic lesions by inhibiting MMP-3 expression.[37]

In this study, there were no significant differences in NLR values between each groups in this study. This result showed that the increase in NLR values was not quite different (not high enough / not low enough) when compared to other groups. In addition, it can also be concluded that the administration of genistein-rich edamame extract was considered insignificant in reducing NLR levels in treatment group 2 compared to the control group, both normal and negative controls. Although the difference was not significant, the highest NLR mean values were found in the negative control group. The results of previous studies by Mayer *et al* found that an increase in neutrophils is directly proportional to an increased risk of cardiovascular events in patients with coronary heart disease and peripheral artery disease.[38] The lowest NLR mean in this group was found in the second comparison group, which was in atorvastatin group.

The mean value of NLR in treatment group 2 was one level above the positive control group. The mean value of NLR in treatment group 2 was quite low, although as we can see in the results of the difference tests, there were no significant differences to show that the administration of genistein-rich edamame extract in treatment group 2, could significantly reduce the NLR value in rat induced atherosclerosis. This shows that the increase in NLR values is not quite different when compared to other groups. In addition, it can also be concluded that the administration of genistein-rich edamame extract was considered insignificant in reducing NLR levels in treatment group 2 compared to the control group, both normal and negative controls.

Effect of genistein-rich edamame extract on MLR

A high monocyte count is closely related to an increase in the thickness of the intima and media tunica.[16] This is caused by the occurrence of endothelial dysfunction in early atherosclerotic plaque lesions. Endothelial dysfunction causes increased endothelial permeability of blood vessels, thus allowing inflammatory cells (monocytes and T lymphocytes) and lipids to enter the intima tunica. Monocytes that enter the endothelium will be bound to the endothelium, then develop into macrophages and accumulate lipids from lipoproteins.[39] Macrophages derived from monocyte derivatives are one of the main cells that play a role in atheroma. In the early stages of atheroma plaque formation, interactions between endothelial and macrophages exacerbate endothelial dysfunction, which results in loss of hemostatic control.[40] In addition, the lipids that enter the intima tunica will be oxidized to oxLDL. In response to oxLDL, endothelial cells produce monotyte chemotactic / chemoattractant protein type 1 (MCP-1) which is able to stimulate monocytes that are bound to endothelium to enter the subendothelial space.[9,27] Thus, more monocytes enter the endothelium and sub-endothelium, causing blood vessels to thicken and atherosclerotic plaque to develop. An increase in the number of monocytes can be a sign of the development of new atherosclerotic plaques.[41]

Lymphocytes, as discussed in the previous section, also have a role in the pathophysiology of atherosclerosis, especially in the condition of lymphopenia. Because both lymphocytes and monocytes play an important role in the pathophysiology of atherosclerosis, MLR can also be a useful marker for knowing the process of atherosclerosis.[17]

From One Way ANOVA - Post Hoc Bonferonni test, there was a significant difference between the negative control group with positive control and Treatment 2 group. This result showed that genistein-richedamame extract can provide a significant decrease in MLR compared to edamame extract in Treatment 1. There was no difference of MLR values found between positive control and Treatment 2 groups. This again proved that genistein-rich edamame extract was as superior as atorvastatin in reducing MLR values compared to other control and comparison groups.

The findings in this study were in line with the findings of Gottstein et al who found that genistein significantly inhibited gamma interferon (IFN-γ) and LPS-induced NO production in macrophages. In addition, genistein also suppresses platelet aggregation, TNF-α secretion and monocyte chemoattractant protein type -1 (MCP-1).[40] Similar results were also found by Babu, Si, Fu, Zhen and Liu who found that genistein significantly suppressed the adhesion of monocytes to endothelial cells by 87% and significantly reduced MCP-1 levels.[42] Because *monocyte chemoattractant protein type–1* is able to stimulate monocytes attached to the endothelium

to enter deeper into the sub-endothelium and cause more severe damage, suppression of this molecule can prevent further damage and thickening of the blood vessels walls mediated by monocytes.[9,27] In addition, MLR can also be an independent risk factor and predictor of the severity of coronary artery disease; thus, a decrease in MLR produced by genistein supplementation is associated with a reduced risk and severity of coronary artery disease compared to the control group and edamame extract group alone.[43]

This study had several limitations, first the concentration ofgenistein-rich edamame extract used in this study was only a single dose, so it could not distinguish the effect of graded doses of the substance. This research was only carried out for four weeks, so there was still a bias because the process of atherosclerosis in rats has a different speed with humans, also because of this short period of time we could not determine the end result (end-point surveillance) whether the decrease in hs-CRP in the administration of genistein actually reduced the rate of atherosclerosis or not.

CONCLUSION

Administration ofgenistein-rich edamame extract *(Glycine max (L) Merrill)* does not reduce LDL levels, reduced hs-CRP levels, does not reduce the ratio of neutrophil lymphocytes, and also decreases the ratio of lymphocyte monocytes in male rats *(Rattus novergicus)* induced atherosclerosis.

Thus, administration of genistein-rich edamame extract may reduce inflammation in blood vessels better than edamame extract alone, which is indicated by the lower levels of hs-CRP and MLR counts.

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