Original Article

Effect of Genistein-rich Edamame Extract on Eosinophil-Lymphocyte Ratio Experimental Study on Atherosclerosis Induced Male Rats

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Abstract

Background : Atherosclerosis is associated with hypercholesterolemia and inflammation. Edamame, a high-genistein soybean variant, is believed to have protective effects against atherosclerosis. This study aimed to determine the influence of edamame extract rich in genistein on the eosinophil-lymphocyte ratio (ELR) levels in rats induced with atherosclerosis.

Methods : This research utilized a true experimental design with a post-test only control group. Thirty male rats were divided into five groups: negative control, positive control, treatment 1 with edamame extract supplementation, treatment 2 with edamame extract rich in genistein supplementation, and treatment 3 receiving atorvastatin. After 28 days, the ELR levels were examined from rat blood. Data were analyzed using One-Way ANOVA - Bonferroni, Kruskal-Wallis Mann-Whitney, and Pearson correlation tests.

Results : There were no significant differences in ELR levels (p>0.05) among all treatment groups compared to the negative and positive control groups. T-tests between the negative control and positive control groups, as well as between the positive control and the three treatment groups successively, showed non-significant results (p: 0.376; 0.856; 0.169; 0.066).

Conclusion : The administration of edamame extract rich in genistein did not significantly reduce inflammation levels in blood vessels compared to edamame extract alone, as indicated by non-significant results in the ELR difference analysis.

Keywords : Genistein Rich Edamame, Eosinophil-Lymphocyte Ratio

INTRODUCTION

Atherosclerosis is an inflammatory disease of the arteries characterized by 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. Atherosclerotic lesions are very susceptible to occurring in the curves of blood vessels and their branches.[1] The manifestation of atherosclerosis is atherosclerotic vascular disease (AVD), which is a serious contributor to mortality and morbidity in the world compared to other diseases.[2] In Indonesia, AVD ranks first among the cardiovascular diseases that have high mortality and morbidity rates.[3] According to the Health Research and Development Agency, Ministry of Health of the Republic of Indonesia, the death rate due to manifestations of AVD is 12.9% of all deaths.[4]

Inflammation plays an important role in every stage of atherosclerosis, from the beginning of plaque development to plaque rupture which can cause thrombosis.[5,6] Previous research showed that eosinophils and lymphocytes have a role in inflammation, atherosclerosis and endothelial dysfunction. The eosinophil to lymphocyte ratio (ELR) is a novel inflammatory biomarker that has been demonstrated to show an association between ELR and poor outcomes in patients with cancer. ELR was also found to be a predictor of the severity of isolated coronary artery ectasia.[2,7]

Prevention efforts through early detection of risk factors and control efforts are very important. One of them is through limiting the consumption of fats and types of food that have anti-hypercholesterolemic effects, one of which is soybeans. Edamame is a variant of soybeans that has a higher isoflavone content than other food crops.[8] In Indonesia, edamame is widely known and consumed together with other food ingredients.[9] Genistein is an isoflavone that is abundant in Edamame, has the potential to prevent atherosclerosis by suppressing blood vessel inflammation. Studies on the effect of genistein on inflammatory markers are still very limited. Therefore, in this study, researchers wanted to determine the effect of administering genistein edamame (Glycine max (L) Merrill) on the eosinophil lymphocyte ratio levels in male rats *(Rattus novergicus)* which were induced by atherosclerosis.

METHODS

Research Design
This research is a true experimental research with post test only with control group design. Edamame extract (Glycine max (L) Merrill) rich in genistein administered to mice with induced atherosclerosis and administration of atorvastatin as a positive control, while the outcome was the level of eosinophil lymphocyte ratio.

Research Sample
Male *rattus novergicus* rats aged 15 weeks, with body weight around 180–200 grams were selected as the inclusion criteria. Mice were kept in stainless steel cages with a 12-hour light cycle. Rat food was given ad libitum. The minimum sample size is determined based on Federer's experimental sample size formula, namely (t-1)(r-1) ≥ 15, where t is the number of treatment groups and r is the sample size for each treatment group.[10] In this study, there were four treatment groups and one control group. From this formula, it was determined that the sample size for each group was 6 mice. Thus, the total number of samples in this study was 30 mice.

Time and location of Research
Research and data collection were carried out for 2 months. This research was carried out in three places, namely the STIFAR Semarang Laboratory as a place for making experimental materials, LPPT FK UGM as a place for treatment of experimental animals and RSH Prof Soeparwi for analysis of eosinophil lymphocyte ratio levels in mice.

Research Variable
The independent variable of this research is edamame extract (Glycine max (L) Merrill) rich in genistein and the dependent variable is eosinophil lymphocyte ratio levels.
Research Implementation

Thirty male Sprague Dawley rats that met the inclusion and exclusion criteria were adapted for 7 days. Sample group 1 as a negative control (K-) was not given any treatment apart from being given standard food and drink ad libitum. Group 2 as a positive control (K+) was injected with adrenaline 0.006 mg/200 gr BW once on the first day and egg yolk was given on the following day at a dose of 5 gr/200 gr BW once a day every day for 28 days. Sample group one (P1) received treatment similar with that in the positive control group, but with added edamame extract rich in genistein 38mg/200 gr BW. Second group (P2) received treatment similar with that in the positive control group, but with added edamame extract rich in genistein 5mg/200 gr BW. Meanwhile, the third group (P3) was given treatment similar with that in the positive control group, with an addition of atorvastatin 1.5mg/ 200gr BW. After 28 days, eosinophil lymphocyte ratio (ELR) levels were measured.

The standard edamame extract process begins with drying for 3 days, weighing, maceration with MeOH, filtering with a rotary evaporator, then evaporation using a water bath. Extraction was carried out using n-hexane, collecting the extract, and measuring the genistein levels in it.[10,11] ELR calculations were carried out from the results of peripheral blood smears, calculated in the acute phase (< 4 hours). Blood smears were made using Giemsa staining method. The smear

was aired until dry, then dripped with methanol for 5 minutes, stained using drops of Giemsa that had been diluted with phosphate buffer (1:4) for 30 minutes. The preparations are then rinsed with distilled water and dried. Leukocyte count were observed in blood smears using a binocular microscope at 100x objective magnification with the addition of immersion oil. The differential count was carried out in an area where the erythrocytes were evenly distributed. ELR was calculated from the ratio between the number of eosinophils and lymphocytes from the peripheral blood smear.[12]

Data Analysis

Data obtained from research observations are in the form of eosinophil lymphocyte ratio levels. The data was tested for normality using Shapiro Wilk test, then continued with statistical testing. The data normally distributed were tested with ANOVA, while data not normally distributed were tested with Kruskal-Wallis test. The *p value* of significant differences was <0.05. If the test results show that H0 fails to be rejected (no differences between groups are found), a post hoc test is not carried out, if a difference is found, a post hoc test is carried out. The post hoc test carried out depends on the results of the Test of Homogeneity of Variances. If the same variance is obtained (significance value >0.05), a post-hoc test is carried out using the Bonferonni method. If different variations are obtained (significance value <0.05), a post-hoc test is carried out using Games-Howell. Data processing was carried out with SPSS 25 for Windows software.

RESULTS

Measurement of Genistein Rich Edamame Extract

After going through extraction and collection process, genistein content was calculated in genistein-rich edamame extract by comparing the standard genistein reagent. Genistein levels were found to be 78% of the total extract composition.

Eosinophil-Lymphocyte Ratio (ELR)

Shapiro-Wilk and Levene Test showed the data was normally distributed and homogeneous, so difference test was continued with one-way ANOVA. The ELR results data are displayed in Table 1, while the comparison between groups is presented in the bar graph in Figure 1.

From ELR data, it can be seen that there are inconsistencies in ELR results, where the ELR value was highest in the negative control group and the lowest in treatment group 3. Giving genistein rich edamame extract did not reduce ELR levels more than edamame extract alone, in fact it was significantly higher compared to edamame extract alone. However, this difference was not statistically significant. Because the results of the one-way ANOVA test were not significant, a post-hoc test was not carried out.

T-test was carried out between the negative control group and the positive control group, as well as between the positive control group and the three treatment groups as shown in Table 2. There were no significant results between the negative control group

TABLE 1
ELR Analysis

Group	ELR Mean ± SD	p*
K-	0.1115 ± 0.1015	0.435
K+	0.0087 ± 0.1593	
P1	0.0170 ± 0.1597	
P2	0.3135 ± 0.0511	
P3	0.0042 ± 0.0065	

Description: *Significant (*p* < 0.05)

TABLE 2
LSD Post HOC test

Group	K-	K+	P1	P2	P3
K-	−	0.376	−	−	−
K+	−	−	0.856	0.169	0.066

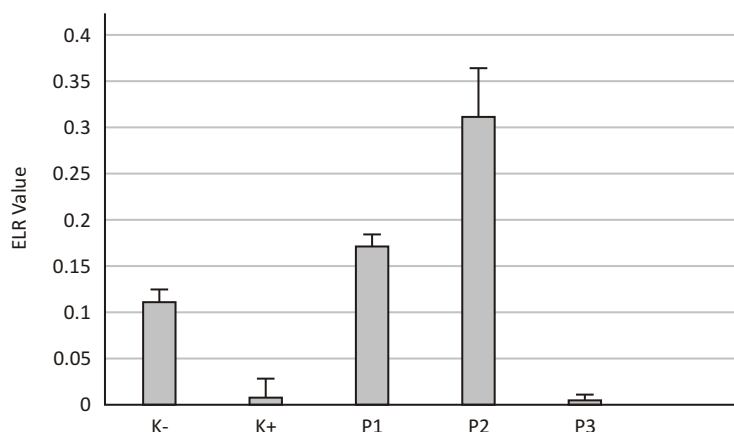Description: *Significant (*p* < 0.05)

Figure 1. ELR Value Bar Graph

and the positive control group.

DISCUSSION

Genistein is claimed to exert many beneficial effects on health, such as protection against osteoporosis, reduction in the risk of cardiovascular disease, alleviation of postmenopausal symptoms and anticancer properties. Apart from that, genistein exerts evident anti-inflammatory properties by affecting granulocytes, monocytes, and lymphocytes that can serve as a novel source of potential phytotherapeutic agents for anti-inflammatory therapies.[14]

Genistein in edamame has an effect of inhibiting the formation of atherosclerotic lesion by becoming an active ingredient that inhibits proliferation of TGF-β1 cell signal. It has anti atherogenic properties in blood vessels, antiplatelet aggregation, help oncrease excretion of bile acids or neutral sterol and interferes with the formation of micelles. Genistein also has an active form from tyrosine kinase inhibitor. It plays an important role in LDL cholesterol catabolism.[15]

The eosinophil-to-lymphocyte ratio (ELR) is a new inflammatory bioindex that takes into account both eosinophil and lymphocyte levels. Eosinophils and lymphocytes have been implicated in inflammation, atherosclerosis, and endothelial dysfunction in previous investigations. An increased ELR was closely related to inflammation and atherosclerosis.[16,17]

Eosinophil-Lymphocyte Ratio (ELR) data in this study has been analyzed using statistical tests. ELR is the ratio between the number of eosinophils and lymphocytes in blood, which reflect the inflammatory response in the body. The results of statistical tests, including the Shapiro-Wilk test and Levene Test, showed that the ELR data had a normal and homogeneous distribution, so one-way ANOVA was chosen as the difference test. However, the ANOVA test did not show significant differences between groups, with p value of 0.435. High variability in ELR results was seen in negative control group which showed the highest value, while treatment group 3 (P3) showed the lowest value. Although there were inconsistencies in these results, the differences were not statistically significant.

In previous studies there was an association between ELR and cardiovascular health, especially in patients with isolated coronary artery ectasia (CAE) who showed higher ELR.[7] Eosinophils, as the main component of ELR, have role in inflammatory responses and atherosclerosis. Previous studies suggest that increased eosinophil counts may be associated with the risk of future cardiovascular events. Another study found elevated serum levels of immunoglobulin E, basophils, and eosinophils in patients with coronary artery disease compared with healthy control subjects. Other studies also show that eosinophil cationic protein (ECP), which is a sensitive marker of eosinophil activation, is associated with coronary atherosclerosis.[4] Eosinophils can exhibit pro-atherosclerotic properties through proteins stored in prominent cytoplasmic granules. In previous studies, it was found that eosinophils are involved in inflammatory processes and endothelial dysfunction, strengthening the argument that ELR may reflect cardiovascular health conditions.[1,4] Although not statistically significant, the high variability of ELR in the study groups is interesting and indicates variability in response of inflammation that may influence cardiovascular prognosis.

This study has several limitations involving the concentration of genistein-rich edamame extract used in this study were not able to differentiate the effect of multiple doses on the test parameters. This research was only carried out for four weeks, so there is still bias because the process of atherosclerosis in mice occurs at a different rate than in humans, yet it can't determine the final results (end-point surveillance).

CONCLUSION

Administration of genistein rich edamame extract did not significantly reduce inflammation severity in blood vessels compared to edamame extract alone (p = 0.435) as indicated by non-significant results in the ELR difference analysis.

Ethical Approval
This research has been approval by the Health Research Ethics Committee, Faculty of Medicine, Diponegoro University with Ethical Clearance.

Conflicts of Interest
The authors declare no conflict of interest.

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Author of Contributions
RDP, EB, AS were involved in planning and supervised the work, RDP performed the measurements, processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. RDP performed the xyz calculations and statistical analysis. RDP, EB, AS aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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