The Efficacy of Genistein-Rich Edamame as a Prevention of Atherosclerotic Lesion in Abdominal Aorta: Study in Rats Model of Atherosclerosis

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ARTICLE INFO

Keywords:
Genistein-rich edamame
Abdominal aortic wall thickness
Foam cells count

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All authors have reviewed and approved the final version of the manuscript. https://doi.org/10.32539/bsm.v5i10.414

ABSTRACT

Background: Atherosclerosis is the main cause of ischemic heart disease which leads to death for people aged more than 40 years old. Genistein is an important isoflavone compound which may protect the blood vessels from endothelial injury. This research is to observe the efficacy of genistein rich edamame as a prevention for atherosclerotic abdominal aortic lesions that seen from abdominal aortic thickness and foam cells count.

Method: Thirty rats were divided randomly into five groups and treated for 28 days. The negative control group was given food and drink ad libitum. The positive control group was induced for atherosclerosis using adrenaline 0.006 mg / 200 gr BW injected on the first day and then the next day was given egg yolk 5 gr / 200 gr BW on next day for 28 days. All of the treatment groups were induced for atherosclerosis and treated with genistein-rich edamame extract 5 mg / 200 gr BW, edamame extract 38 mg / 200 gr BW and atorvastatin 1.5 mg / 200 gr BW. Data were analyzed using One Way ANOVA - post hoc Bonferroni test, Kruskal Wallis - Mann Whitney test, and Pearson correlation test.

Results: There were significant differences (p<0,001) in abdominal aortic thickness and foam cells count between positive control group and treatment genistein-rich edamame extract, edamame extract and atorvastatin. There was a significant correlation between the abdominal aortic wall thickness and foam cells count (correlation coefficient value 0,753; p<0,001).

Conclusion: The administration of genistein-rich edamame extract can prevent the thickening of abdominal aorta and foam cell formation. Genistein-rich edamame can prevent foam cells formation better than atorvastatin.

1. Introduction

Atherosclerosis is a chronic process of thickening and loss of arterial wall elasticity caused by yellowish pile of plaque containing cholesterol and other lipid substances in moderate and large arteries intimal and medial layer.¹ Globally, the mortality rate caused by Atherosclerosis Vascular Disease (AVD) between 1994-2004 was about 30% for 30-55 years age group population and 50% for >55 years age group.² In 1975, based on Survei Kesehatan Rumah Tangga Nasional (SKRTN), diseases manifested from atherosclerosis was the 11th cause of death in Indonesia, and in 1992 until now increased as the major cause of mortality in more than 40 years old people.³

Prior studies suggested that soy protein significantly reduced cholesterol level in blood and prevented atherosclerosis, either in animal models or humans. Edamame (Glycine max (L) Merrill) is one of the soy variants which contains high flavonoids compared with other similar foodstuffs. In Indonesia, edamame has been widely known and consumed by people as vegetable soy or Japanese’s soy since 1995. Indonesian people used to consume edamame with other foods.⁴

Genistein in an important active ingredient in Edamame. Genistein is included in isoflavones categories which is know to have an antilipidemic, anticancer, and antioxidant property. Genistein, which is derived from isoflavones compound, was highly contained in an important compound in edamame.
proinflammatory genes expression and inhibiting free radicals production.\textsuperscript{5}

One of the methods to evaluate atherosclerosis in animal model is to measure the thickness of lesion at the arterial blood vessel wall. Moreover, foam cells formed by macrophages modulation in abdominal aorta as seen in immunohistochemistry examination as CD68 expression is one of the signs to measure atherosclerosis. Prior studies suggested that abdominal aorta was good enough to show any atherosclerotic lesions in anatomic pathology examinations.\textsuperscript{6}

2. Methods

This was a true experimental study with post-test only. The treatment was supplemented with genistein-rich edamame extract, edamame extract, and atorvastatin, with abdominal aortic wall thickness and foam cells count in male rate induced with atherosclerosis as the outcomes.

Wistar strain male rats (\textit{Rattus novergicus}) aged 15 weeks and weighted 180-220 grams were used in this study. Wistar strain male rats were chosen as samples because they would not be influenced by estrogen hormone, in which may affect atherosclerosis formation. Wistar strain rats may also showed active compound reactions from substances, thus may give more specific results. \textit{Rattus novergicus} rats also had a similar blood vessel characteristic with humans.

Minimal sample count was done using experimental sample count formula from Federer. There were five groups in this study, consisted of six rats in every group. The groups were allocated as treatment groups, one group was allocated as the negative control group, and one group as the positive control group.

This study was performed in three places, including animal laboratory at Gadjah Mada medical school as treatment place for animal model, Collage of Pharmacy Laboratory Semarang as treatment material manufacture place and Anatomical Pathology Laboratory at Surakarta University to analyze the abdominal aortic wall thickness and foam cell counts.

Data about abdominal aortic wall thickness and foam cells count in each group underwent editing, coding, and entering processes into SPPS software version 25.0. The data was tested for their normality using Sapiro Wilks test. Inferential statistic test for abdominal aortic wall thickness using Oneway ANOVA followed by post hoc Bonferroni test and foam cells count using non-parametric Kruskal-Wallis test and continued with Mann-Whitney test. Correlation test for abdominal aortic wall thickness and foam cells count was performed using Pearson correlation.

3. Results

In this research, all samples did not die or weight loss which caused dropped out. Genistein levels were calculated on edamame rich genistein extracts measured by comparing genistein standard reagent. Genistein levels were found in 78\% of the total composition of edamame extract.

Abdominal aortic thickness

The microscopic result from abdominal aortic wall thickness were measured by two anatomic pathology specialist. Calculation of abdominal aortic wall thickness is the result of the average measurement of abdominal aortic wall thickness in the four zones of atherosclerotic lesions in the cross section of the abdominal aorta. Measurements were measured from the tunica intima to the thickness of the athersclerotic lesions that formed which led to the tunica media. The microscopic result of abdominal aortic wall thickness can be seen in Figure 1.
A. Negative control  
B. Positive control  
C. Atorvastatin  
D. Genistein rich-edamame extract  
E. Edamame extract

Figure 1. Abdominal aortic wall thickness. The picture is seen from 4 zones with the end result of abdominal aortic thickness measured from the average abdominal aortic wall thickness at four zones of atherosclerotic lesions in the cross section of the abdominal aorta with a magnification of 40 times.

Data was normally distributed ($p>0.05$) when Shapiro-Wilk test was performed, thus One Way ANOVA test was performed to found any differences in abdominal aortic wall thickness between treatment groups, with results in table 1.

Table 1. Comparative analysis of abdominal aortic wall thickness

| Group                                | Abdominal aortic wall thickness (µ) (Mean ± SD) | $p$   |
|--------------------------------------|-----------------------------------------------|-------|
| Negative control (K-)                | 100,67±13,18                                  |       |
| Positive control (K+)                | 189,96±10,20                                  |       |
| Genistein-rich edamame extract (P1)  | 138,04±6,13                                   | <0,001*|
| Edamame extract (P2)                 | 148,10±13,38                                  |       |
| Atorvastatin (P3)                    | 124,05±4,99                                   |       |

* Tested with One Way ANOVA ($p<0.05$ was considered significant)

From the Oneway ANOVA test, the $p$ value < 0.05, concluded that there was a significant difference in abdominal aortic wall thickness in every group. Post hoc Bonferroni test was performed as the following to evaluate the differences between groups.
Foam cells count

Foam cells count seen from CD68 expression were measured by two anatomic pathology specialist. Foam cells count was taken from the same zone where atherosclerotic lesions was thought to be the most prominent as in abdominal aorta thickness measurement before. The microscopic result of foam cells count can be seen in figure 3.

Figure 2. $p$ value for post hoc Bonferroni Analysis for abdominal aortic wall thickness $*p<0.001$

Figure 3. Foam cells count resulted by CD68 expression. The picture shown with the arrow is a picture of foam cells expressed by CD68 through brown imaging with a magnification of 400 times.
Data was abnormally distributed (p<0.05) when Shapiro-Wilk test was performed, thus, non-parametric Kruskal-Wallis was applied to evaluate any differences in foam cells count as seen in CD68 expression between treatment groups, with results as below:

Table 2. Comparative analysis of foam cells count

| Group                                      | Foam cell count (Mean ± SD) | p       |
|--------------------------------------------|-----------------------------|---------|
| Negative control (K-)                      | 0±0                         |         |
| Positive control (K+)                      | 4.83±1.47                   | <0.001* |
| Genistein-rich edamame extract (P1)        | 0±0                         |         |
| Edamame extract (P2)                       | 0.33±0.52                   |         |
| Atorvastatin (P3)                          | 0.17±0.41                   |         |

* Tested with non-parametric Kruskal-Wallis (p<0.05 was considered significant)

From the non-parametric Kruskal-Wallis test, there was a significant differences between foam cells count in all groups with p value < 0.05. Then, Mann-Whitney test was used to evaluate the differences between each variables.

![Figure 4. p value for Mann-Whitney test Analysis of foam cells count. *p=0.002; **p=0.003](image)

**Correlation between abdominal aortic wall thickness and foam cells count**

The results from normality test that the data was normally distributed (p>0.05), thus correlation analysis was followed by Pearson test. From Pearson test, p value < 0.05, thus there was a significant correlation between abdominal aortic wall thickness and foam cells count. Based on de Vaus's classification of the strengths between the two variables shown from the correlation coefficient, the relationship between the strength of the abdominal aortic wall and the number of foam cells that have a correlation coefficient of 0.753 is a very strong relationship.
4. Discussion

The prevention of atherosclerotic lesions was demonstrated by inhibition of abdominal aortic wall thickening and prevention of foam cell formation that compared with positive controls. Even though all treatments can be antiatherosclerosis agent, edamame-rich genistein extract was able to inhibit foam cells better than atorvastatin or edamame extract.

Genistein in edamame has an effect of inhibiting the formation of atherosclerotic lesions in aorta abdominal by becoming an active ingredient that inhibits proliferation of TGF-β1 cell signals so it has antiatherogenic properties in blood vessels. Genistein also has anti-platelet aggregation properties, helps increase excretion of bile acids or neutral sterols and interferes with the formation of micelles.\(^7,8,9,10\) Genistein has an active form from tyrosine kinase inhibitor, which plays an important role in LDL cholesterol catabolism. Moreover, genistein also influence SREBPs that manage genes in HepG2 cells which increase plasma cholesterol and LDL breakdown. Through the process, foam cells formation is inhibited, thus may be an indicator for anti-atherosclerosis.\(^9,10,11,12\)

In the study of Kamal and friends (2016) which resulted that edamame extract did not affect serum triglyceride levels in experimental animals.\(^13\) This is not in accordance with this study. This difference possible because the dose of edamame used in this study was 38 mg / 200 grBB while in the Kamal study using a dose of 33.5 mg / 200 grBB. Triglycerides are conditions that cause high LDL and low HDL. It was known from the theory that previously oxidized LDL exposure would make macrophages aggregated to foam cells in the intima layer that seen as fatty streaks. Edamame contains flavoid compounds that can be antioxidant so that it can prevent oxidized LDL. Besides being able to inhibit oxidized LDL, edamame flavonoids can increase blood HDL levels.\(^9,14,15\) Furthermore, flavonoid antioxidant also has a role as anti-inflammatory which inhibits neutrophil cells migrations to tissue and changed to macrophages.\(^15\)

When compared with edamame extract, genistein-rich edamame extract is better at preventing atherosclerotic lesions, which means that genistein in edamame has been shown to be an active ingredient that can prevent atherosclerotic lesions.\(^16\) Although the results of prevention of atherosclerotic lesions are not as good as genistein-rich edamame extract, edamame extract is also able to prevent the occurrence of atherosclerotic lesions which are demonstrated by inhibiting thickening of the abdominal aortic wall and preventing formation of foam cells. Edamame has high flavonoids. Edamame flavonoids can act as an anti-inflammatory which can inhibit the migration of neutrophil cells to the tissue so that the thickening of the abdominal aortic wall can be inhibited and foam cell formation can be prevented.\(^15,16,17,18,19\)

Although the average thickness of abdominal aorta in edamame extract is not as good as atorvastatin, edamame extract can be considered as an alternative phytopharmaca as antiatherosclerosis. This is in accordance with Stach’s research and friends that Edamame may prevent atherosclerosis by inhibiting LDL oxidation. Flavonoid in edamame has an antioxidant property which may inhibit LDL oxidation in atherosclerosis. This inhibition process was showed by VCAM-1 excretion at the vessel’s endothelium caused by oxidized LDL. Antioxidant also may inhibit LDL toxicity effect in smooth muscle cells and macrophages. Moreover, antioxidant is also important to reduce oxidative degradation by nitric oxide. From Zeipina’s research and friends, Bei’s research and friends, edamame which is rich in vitamin C also has a role in strengthening arterial walls through collagen synthesis and preventing leukocyte adhesion which results in arterial damage.\(^20,21,22\)

In the results of the study of abdominal aortic wall thickness and number of foam cells, administration of genistein-rich edamame extract can prevent the formation of foam cells better than atorvastatin administration, although administration of atorvastatin is better at preventing thickening of the abdominal aortic wall. Although physiologically the formation of foam cell and abdominal aortic thickening takes place simultaneously, this can be due to the formation of foam cells more influenced by monocyte activity that will be phagocytes by macrophages while abdominal aortic thickening is more affected by
myocyte proliferation in blood vessels.\textsuperscript{18,23,24}

In the correlation analysis between abdominal aortic wall thickness and foam cells count in every group, we found that there was a correlation between both variables. The correlation of the strength relationship is a very strong. This result can be explained because foam cells is an initial proof of atherosclerosis process. Thus, more foam cells may cause abdominal aorta thickening.\textsuperscript{18}

However, this study has a weakness that only focuses on genistein activity so that other factors that play a role in preventing atherosclerotic lesions cannot be explained in detail. Furthermore, atherosclerosis process in rats was faster than in human, thus atherosclerosis process bias in rats cannot be avoided. The other weakness of the study that cannot be avoided is the diversity of the points of abdominal aorta that were used as observational samples of abdominal aortic thickness and the number of foam cells that might be a research bias. This is because the sample curving points are taken from several parts of the abdominal aorta that are worthy of anatomical pathology.

5. Conclusion

Genistein-rich edamame supplementation may prevent atherosclerotic lesion showed in abdominal aortic wall thickness and foam cells count in male rats induced with atherosclerosis. Genistein-rich edamame supplementation may prevent atherosclerotic lesion formation better than edamame extract. Edamame-rich genistein can prevent the formation of foam cells better than atorvastatin, but edamame-rich genistein is not better in preventing abdominal aortic thickening than atorvastatin.

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