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Expression of VCAM in Male Wistar Rats (*Rattus norvegicus*) with Hypercholesterolemia Supplemented with Pumpkin Seeds (*Cucurbita moschata* Duch) Extract

Jujuk Proboningsih[1(CA)], Bambang Wirjatmadi[2], Kuntoro Kuntoro[3], Merryana Adriani[4]

[1(CA)]Faculty of Public Health, Airlangga University / Health Polytechnic of Surabaya, Indonesia; jujuk_sriyono@yahoo.com (Corresponding Author)

[2]Faculty of Public Health, Airlangga University, Indonesia; wirjatmadi@fkm.unair.ac.id

[3]Faculty of Public Health, Airlangga University, Indonesia; kuntoro2@yahoo.com

[4]Faculty of Public Health, Airlangga University, Indonesia

ABSTRACT

Treatment of cardiovascular diseases tends to be expensive and requires long-term therapy. Investigation about the effect of pumpkin seeds on prevention of endothelial dysfunction of blood vessel has never been reported yet, accordingly, bodies of research are required to know its effectiveness. This study aimed to analyze the expression of Vascular Cell Adhesion Molecule (VCAM) in male Wistar rats (*Rattus norvegicus*) with hypercholesterolemia supplemented with pumpkin seeds extract (*Cucurbita moschata* Duch). This was an experimental laboratory study with randomized post-test control group design. The independent variable was pumpkin seeds extract, and the dependent one was expression of VCAM. Data analysis employed chi-square test to test the difference between control and treatment groups. Study results showed that there was significant difference (p=0.000) between VCAM expression in group-K2 (positive control) as compared to group-K1 (negative control) and group-K5 (treatment group-dose 3) α=0.001, whereas no significant difference was found between that of as compared to group-K3 (treatment group-dose 1) α=0.125 and group-K4 (treatment group-dose 2) α=0.05. The study showed reduction of VCAM expression in treatment groups consisted of male Wistar rats with hypercholesterolemia supplemented with pumpkin seeds (*Cucurbita moschata* Duch) extract.

Keywords: Pumpkin seeds, VCAM expression, hypercholesterolemia

INTRODUCTION

Background

Each year cardiovascular disease (CVD) causes over 4 million deaths in Europe and over 1.9 million deaths in the European Union (EU), and almost half CVD causes 47% of all deaths in Europe and 40% in the EU[(1)]. National data from Basic Health Research (*Riset Kesehatan Dasar*) 2013 has reported that prevalence of hypertension in Indonesia should receive greater attention. This is due to the prevalence remains high that over 25.8% people aged ≥18 years have cardiovascular diseases. High-risk food consumption behaviour, such as unhealthy high-fat foods, has been reported over 40.7% people aged ≥10 years. Consequently, this kind of behaviour lead to hypercholesterolemia over 35.9% people aged >15 years which presented abnormal total cholesterol according to their blood test[(2)]. Treatment of cardiovascular diseases tends to be expensive and requires long-term therapy. Adverse drug reactions such as dizziness, cough, heartburn, headache, arm pain, flu-like symptoms, blurred vision, memory loss, irritability and fatigue are the reason the cardiovascular patients stop their treatment. Reducing morbidity and mortality of cardiovascular disease as well as controlling the risk factors are the main priority for both primary and secondary prevention[(3)].

Endothelial cells are single layer cells (monolayer) that line the lumen of blood vessel, and are essential in regulating the vascular tonus. Endothelial cells may release both constricting and dilating substances when stimulated by blood-bome substances or by shear stress associated with the flow of blood. Important endothelial vasodilators include endothelium-derived relaxing factor (EDRF, known as nitric oxide, compound containing nitroso), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF). The major endothelial vasoconstrictors are endothelin-1, thromboxane A_2 (TXA_2) and prostaglandin H_2[(4)].

Result of amino acid analysis test from pumpkin seed (Cucurbita moschata Duch) through observation of labortorium conducted at Central Ilmu Hayatin Laboratory Universitas Brawijaya by HPLC (High Performance Liquid Chromatography) has an L-arginine content of almost 5% (4.91%) in every 100 grams of its powder Arginine is the biologic precursor of nitric oxide (NO), an endogenous messenger molecule involved in a variety of endothelium-dependent physiological effects in the cardiovascular system. As the precursor to nitric oxide, many of arginine's clinical effects are thought to be mediated by its effects on endothelial-derived relaxing factor. An immense quantity of research has explored the biological roles and properties of nitric oxide, which appears to be of critical importance in maintenance of normal blood pressure, myocardial function, inflammatory response, apoptosis, and protection against oxidative damage [(5)].

The condition of the body resulting in hypercholesterolaemia, will activate ROS (reactif oxygen space, occurs lipid peroxidation / MDA) which will activate LDL oxidation. ROS will also cause NO inhibition where NO has a role also to protect LDL from oxidative. A decrease in the amount of NO will cause the oxidation of LDL that is difficult to control, leading to endothelial dysfunction characterized by increased VCAM (Vascular Cell Adhesion Molecule) expression,Amino acid composition including Arginine is rich in pumpkin seeds. Arginine content was found as 16.4% of total protein in pumpkin seeds extract[(6)].

Purpose

This study aimed to describe mechanism of endothelial dysfunction in laboratory animals supplemented with pumpkin seeds extract (*Cucurbita moschata* Duch).

METHODS

Method used in the present study was experimental laboratory study with randomized post-test control group design, and the experimental animals were male Wistar rats (Rattus norvegicus) as the subject of the study. Study population was male Wistar rats (Rattus norvegicus), ±2 months of age, weighing ± 200 grams, with sample sizes of about 5 rats per group. Sampling technique used in the study was random allocation. The care of the experimental animals was done in cage available at Biochemical Laboratory, Department of Basic Medicine, Faculty of Medicine, Airlangga University, Surabaya, on June-July 2017. A total of 25 male Wistar rats aged 2-3 months were adapted first for 2 weeks before involved in the study. Thereafter, those male Wistar rats were divided into 5 groups, which consisted of group-K1 as negative control group, group-K2 as positive control group, group-K3 as treatment group treated with pumpkin seeds extract at dose 0.27 grams/day, group-K4 as treatment group treated with pumpkin seeds extract at dose 0.36 grams/day, and group-K5 as treatment group treated with pumpkin seeds extract at dose 0.54 grams/day. Pumpkin seeds used in the study were pumpkin variety (Cucurbita moschata Duch) grown in Kedungadem subdistrict, Bojonegoro regency, East Java, obtained from seeds collector at Kapas subdistrict Bojonegoro. The pumpkin seeds were dried at room temperature, then crushed into powder. Pumpkin seed powder was then soaked in plenty of 95% ethanol for 3x24 hours at room temperature 25°C, and filtered using filter paper. Subsequently, the filtrate was evaporated using rotary vacuum evaporator at 400°C and in the end of the process brown viscous liquid was obtained as pure extract. High-cholesterol diet was needed to increase blood cholesterol levels in the experimental animals. Accordingly, that kind of diet was made of some composition as follows: 5% egg yolk, 10% pork oil, 1% palm oil, and regular diet ad 100%. In order to obtain hypercholesterolemic rats, they were fed with high-cholesterol diet for 38 days. Thereafter, they were sacrificed, and samples were taken and examined. Administration of pumpkin seeds extract (Cucurbita moschata Duch) to the experimental animals and feeding them with the high-cholesterol diet were done in the same time, which means it started on the first day until the last day of the treatment. The extract was administered using a sonde at each dose once daily to the treatment groups. Aorta and heart were then stored in the specimen bottle filled with 10% neutral buffered formalin for histopathological examination. The next steps were sample preparation and staining for immunohistochemical examination to observe the expression of VCAM.

Examination of VCAM expression was performed at Immunohistochemical Laboratory, Faculty of Medicine, Airlangga University, Surabaya. Positive immunoreaction for VCAM expression was detected on aortic endothelial cells by the presence of brown cytoplasmic staining. Quantification was performed under light microscope at 1000x magnification, 10 fields of view, and the mean was calculated.

RESULTS

Blood cholesterol levels of all experimental animals were tested before being sacrificed to confirm the presence of hypercholesterolemia. Blood test was carried out on day 21 and day 38. Means blood cholesterol levels of rats on day 38 are presented in the table below (Table 1).

Table 1. Blood cholesterol levels (mg/dl) of in both control and treatment groups male Wistar rats (Rattus norvegicus) supplemented with pumpkin seeds (Cucurbita moschata Duch) extract.

Groups (n=5)	Mean (mg/dl)
K 1	50.8
K 2	68
K 3	57.4
K 4	61.4
K 5	56.6

Notes:
K1: Negative control group, experimental animals were fed with standard diet only
K2: Positive control group, experimental animals were fed with high-cholesterol diet
K3: Treatment group – dose 1, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.27 grams/day/rat
K4: Treatment group – dose 2, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.36 grams/day/rat
K5: Treatment group – dose 3, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.54 grams/day/rat

Based on Table 1, only group-K1 which had normal mean blood cholesterol level, whereas the four other groups have abnormal levels as more than 54.0 mg/dl. According to Kusumawati, normal blood cholesterol level of Wistar rat (*Rattus norvegicus*) is 10.0-54.0 mg/dl[(7)].

The study results showed that mean VCAM expression among negative control group was not detected, whereas positive control group showed very high expression of VCAM, as well as in treatment group – dose 1 (0.27 grams/day). However, treatment group – dose 2 (0.36 grams/day) and treatment group – dose 3 (0.54 grams/day) showed decreased expression of VCAM. It is presented in Table 2 below.

Table 2. Mean VCAM (Vascular Cell Adhesion Molecule) expression of in both control and treatment groups male Wistar rats (*Rattus norvegicus*) supplemented with pumpkin seeds (*Cucurbita moschata* Duch) extract.

Groups (n=5)	Mean	Standard Deviation
K 1	0.00	0.0000
K 2	1.25	0.42362
K 3	1.6	0.43526
K 4	0.798	0.44796
K 5	0.382	0.16468

Notes:
K1: Negative control group, experimental animals were fed with standard diet only
K2: Positive control group, experimental animals were fed with high-cholesterol diet
K3: Treatment group – dose 1, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.27 grams/day/rat
K4: Treatment group – dose 2, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.36 grams/day/rat
K5: Treatment group – dose 3, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.54 grams/day/rat

The results of chi-square test showed that there was significant difference between VCAM expression in positive control group as compared to treatment groups and negative control group. The detailed results are presented in Table 3.

Table 3 showed that there was significant difference (α=0.000) between VCAM expression in group-K1 (negative control group) as compared to group-K2 (positive control group), group-K3 (treatment group – dose 1), and group-K4 (treatment group – dose 2; α=0.002), whereas no significant difference was found between that of as compared to group-K5 (treatment group – dose 3; α=0.096).

There was significant difference (α=0.000) between VCAM expression in group-K2 (positive control group) as compared to group-K1 (negative control group), group-K5 (treatment group – dose 3; α=0.001), whereas no significant difference was found between that of as compared to group-K3 (treatment group – dose 1; α=0.125) and group-K4 (treatment group – dose 2; α=0.05).

There was significant difference (α=0.000) between VCAM expression in group-K3 (treatment group – dose 1) as compared to group-K5 (treatment group – dose 3), and group-K4 (treatment group – dose 2; α=0.002),

whereas no significant difference was found between that of as compared to group-K2 (positive control group; α=0.125).

There was significant difference (α=0.002) between VCAM expression in group-K4 (treatment group – dose 2) as compared to group-K1 (negative control group), and group-K3 (treatment group – dose 1), whereas no significant difference was found between that of as compared to group-K2 (positive control group; α=0.052) and group-K5 (treatment group – dose 3; α=0.071).

There was significant difference (α=0.000) between VCAM expression in group-K5 (treatment group – dose 3) as compared to group-K3 (treatment group – dose 1), and group-K2 (positive control group; α= 0.001), whereas no significant difference was found between that of as compared to group-K1 (negative control group; α=0.096) and group-K4 (treatment group – dose 2; α=0.071).

The analysis indicated that pumpkin seeds extract had influence in reducing VCAM expression of the hypercholesterolemic experimental animals.

Table 3. Results of cross tabulation with significance level α=0.05 in comparing the difference between groups male Wistar rats (*Rattus norvegicus*) supplemented with pumpkin seeds (*Cucurbita moschata* Duch) extract.

Groups	K1	K2	K3	K4	K5
K1	-	0.000	0.000	0.002	0.096
K2	0.000	-	0.125	0.052	0.001
K3	0.000	0.125	-	0.002	0.000
K4	0.002	0.052	0.002	-	0.001
K5	0.096	0.001	0.000	0.001	-

Notes:
K1: Negative control group, experimental animals were fed with standard diet only
K2: Positive control group, experimental animals were fed with high-cholesterol diet
K3: Treatment group – dose 1, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.27 grams/day/rat
K4: Treatment group – dose 2, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.36 grams/day/rat
K5: Treatment group – dose 3, experimental animals were fed with high-cholesterol diet and supplemented with pumpkin seeds extract at dose 0.54 grams/day/rat

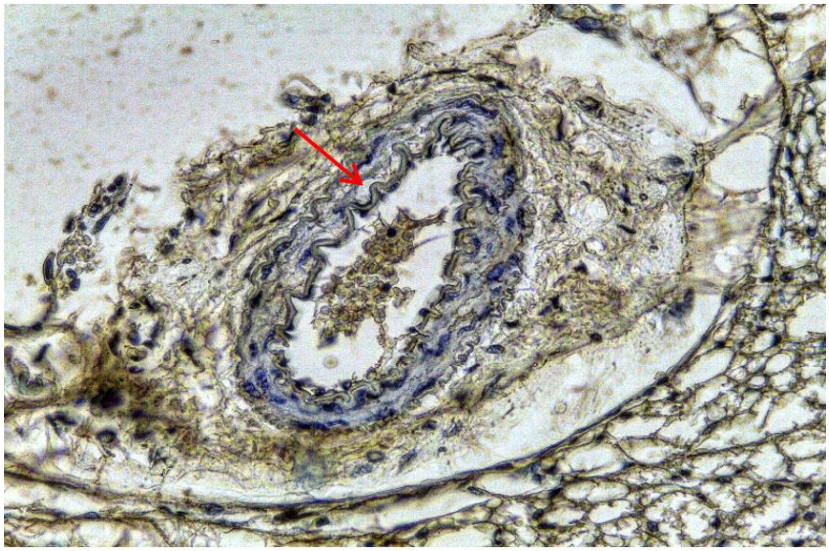

Figure 1. Representative photomicrograph of normal aortic endothelial cells of male Wistar rat (negative control group); the cells demonstrate no expression of VCAM (red arrow indicates negative reaction for VCAM).

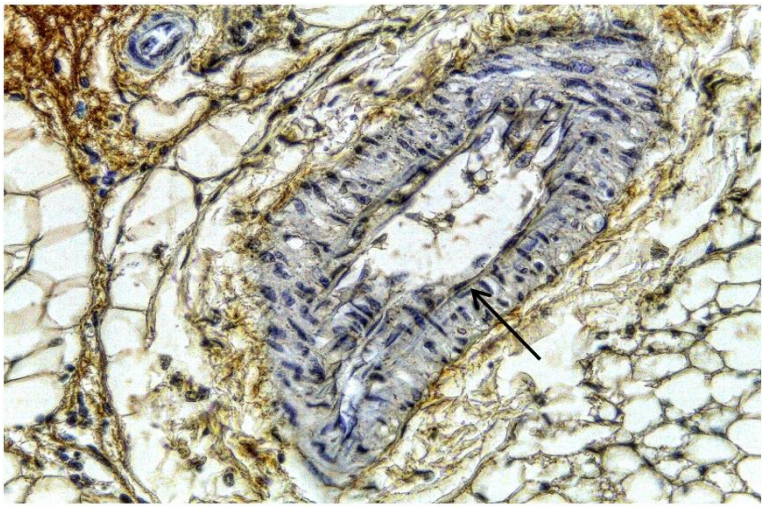

Figure 2. Representative photomicrograph of aortic endothelial cells of male Wistar rat in positive control group; the cells demonstrate expression of VCAM (black arrow indicates positive reaction for VCAM).

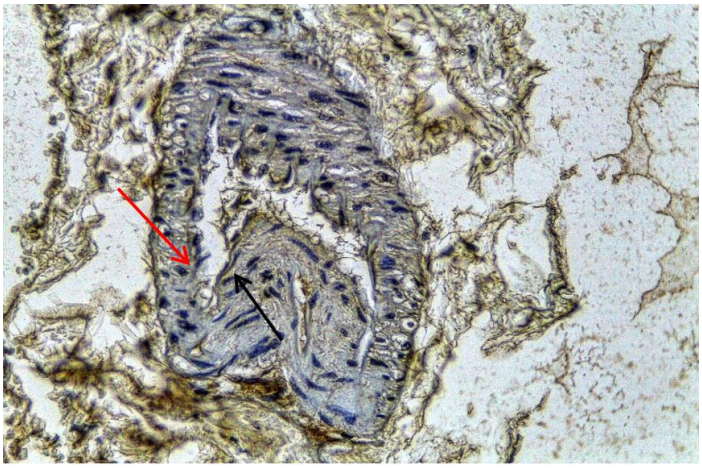

Figure 3. Representative photomicrograph of aortic endothelial cells of male Wistar rat in treatment group (dose 0.27 grams/day); the cells demonstrate expression of VCAM (black arrow indicates positive reaction for VCAM). The expression level of VCAM is increased.

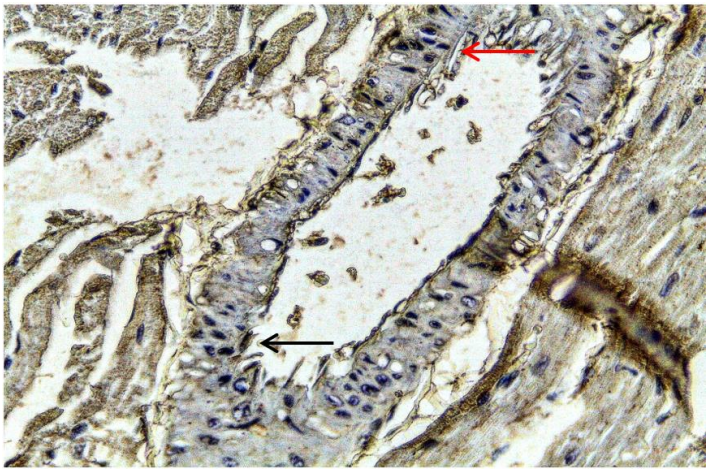

Figure 4. Representative photomicrograph of aortic endothelial cells of male Wistar rat in treatment group (dose 0.36 grams/day); the cells demonstrate expression of VCAM (black arrow indicates positive reaction for VCAM). The expression level of VCAM is decreased.

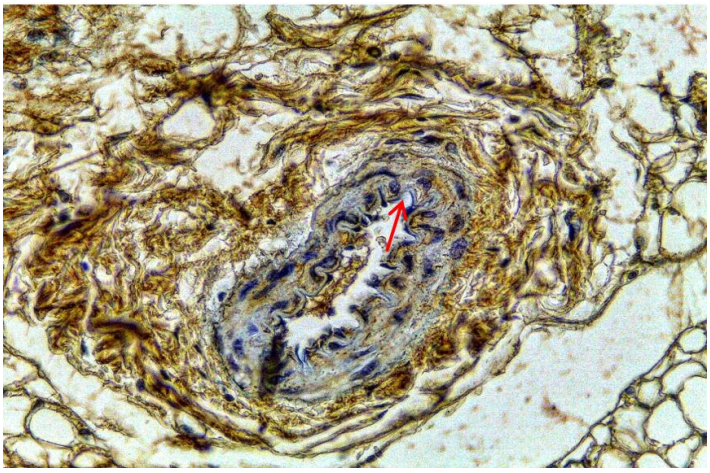

Figure 5. Representative photomicrograph of aortic endothelial cells of male Wistar rat in treatment group (dose 0.54 grams/day); the cells demonstrate no expression of VCAM (red arrow indicates negative reaction for VCAM).

DISCUSSION

Blood cholesterol levels of experimental animals are highly influenced by type of diet, so are blood glucose levels, etc. When blood cholesterol level exceeds normal limit, it tends to raise lipoprotein. Elevated blood cholesterol level induced by high-cholesterol diet is a warning of endothelial dysfunction and atherosclerosis formation due to lipid oxidation that result in endothelial cell (blood vessel wall) adhesion process[8]. The study results showed that there was decreased mean VCAM (Vascular Cell Adhesion Molecule) expression among the experimental animals fed with high-cholesterol diet and supplemented with pumpkin seeds extract. The experimental animals fed with high-cholesterol diet and supplemented with pumpkin seeds extract demonstrated that the higher the dose of extract, the lower the expression levels of VCAM. Moreover, the group of experimental animals fed with high-cholesterol diet without supplementation of pumpkin seeds extract and treatment group – dose 1 demonstrated the highest expression level of VCAM, while the group of experimental animals fed with standard diet without high-cholesterol ingredients demonstrated the lowest expression level of VCAM.

The results of statistical analysis showed significant difference between VCAM expression in the group of experimental animals fed with high-cholesterol diet (positive control group) as compared to the group fed with standard diet (negative control group) and those supplemented with pumpkin seeds extract at dose 3, whereas no significant difference was found between that of as compared to the treatment groups – dose 1 and dose 2.

According to the results obtained, there is correlation between blood cholesterol level and VCAM expression. However, in the other hand it was found that although the blood cholesterol levels of experimental animals treated with pumpkin seeds extract at dose 3 were considered as high, but there was no increased expression level of VCAM, even no significant difference was found as compared to negative control group which did not fed with high-cholesterol diet.

VCAM has been studied by some previous researchers, as reviewed by Ley Klaus and Yuqing Huo that VCAM is not expressed under baseline conditions but is rapidly induced by proatherosclerotic conditions in rabbits, mice, and humans, including in early lesions[9].

Review of VCAM by Bacchi *et.al* revealed that VCAM-1 was expressed by endothelial cells due to transplant rejection and recognized by antibodies to be suppressed, hence the rejection progress could be prevented[10].

The previous study has described that atherosclerosis-induced hypercholesterolemia state is recognized by our body as foreign substance that may cause abnormal state, consequently, it is targeted by antibody and lead to VCAM (Vascular Cell Adhesion Molecule) expression.

This study revealed that subjects with hypercholesterolemia do not always present increased expression of VCAM, due to inhibition of atherogenesis by nitric oxide before VCAM is produced. Atherogenesis process involves oxidation of LDL and recruitment of macrophage and other inflammatory cells into subendothelial space. This process could be inhibited by nitric oxide (NO) produced by eNOS from L-arginine contained in pumpkin seeds.

CONCLUSION

It can be concluded that there was decreased expression of VCAM in rats with hypercholesterolemia supplemented with pumpkin seeds extract. The image of endothelial cells from experimental animals who

received pumpkin seed supplementation looked good and had excellent function, as evidenced by the lack of VCAM. Dijelaskan singkat bagaimana gambaran sel endotel yang mendapatkan suplementasi. Pumpkin seeds supplementation may be of considerable value in the treatment of hypercholesterolemia by controlling other risk factors.

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