

Increasing Atherosclerosis in Streptozotocin-Induced Diabetes into Four Groups of Mice

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Abstract

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AIM: To study the protective effect of medicines on the formation of atherosclerosis in mice, it is needed to conduct the study in mice which is not genetically diabetic mice induced by streptozotocin (STZ) to produce hyperglycemia and atherosclerosis, compared with mice treated by yolk or its combination.

MATERIAL AND METHODS: Fifty-six mice, Double Deutch Webster strain, male, receive 10 weeks, 20 - 30 gr bodyweight were divided into 4 groups (n = 14) i.e. control (do not received any agents), STZ (45 mg/kg/BW was injected intraperitoneally for 5 days), yolk (0.2 cc orally daily for 6 weeks), and combination of STZ and yolk (STZ: 45 mg/kg/BW intraperitoneally add 0.2 cc yolk orally). All animals were executed in the 42nd day. Then, the aorta of the mice's heart tissue was histopathology examined. Blood glucose and cholesterol levels were determined every week.

RESULTS: Hyperglycemia occurred in mice induced by STZ injection with the highest BGL (521.8 ± 48.2 mg/dl; 188.4%) in the 4th-week observation; after that BGL decrease. We found that, except the control, all treatment groups with STZ, egg yolk, and combination underwent atherosclerosis.

CONCLUSION: The present study was able to demonstrate the occurrence of atherosclerosis in mice treated by STZ accompanied with increasing blood glucose and cholesterol level.

Introduction

Diabetes mellitus is a chronic metabolic disorder, identified by the increase in blood glucose level (BGL, hyperglycemia) [1]. Chronic hyperglycemia is the initial cause of microvascular complication, such as retinopathy, neuropathy and nephropathy, as well as macrovascular complications, especially cardiomyopathy [2]. The atherosclerosis risk factors, explain just of a minor part of the excess incidence of vascular disease among diabetic patients, diabetogenic factors themselves must contribute to the development the arterial disease. In particular, hyperglycemia, the primary clinical manifestation of diabetes, is thought to contribute, to diabetic complication by altering vascular cellular metabolism, vascular matrix molecules, and circulating lipoproteins. For instance, hyperglycemia increases diacylglycerol levels and activates protein

kinase C activity in the aorta of streptozotocin (STZ) induced diabetic rats and dogs [2]. Thickening of the basement membranes in renal glomeruli and peripheral capillaries has been observed in STZ - induced diabetic rats and diabetic patients [3].

Hyperlipidemia is a feature of drug-induced diabetes in rats and rabbits, as well as poorly controlled diabetes in humans. Alterations in lipoprotein-cell interactions are also seen in vitro upon glycation of circulating lipoproteins. The role of each of these mechanisms in the pathogenesis of macro - and microangiopathy needs to be clarified [2] [3]. The data from the World Health Organization in 2004 showed that 30% of mortality rate was caused by heart and blood vessel diseases, and 60% of that percentage was caused by coronary heart disease [4]. In Indonesia, the mortality rate caused by coronary heart disease has increased significantly: from 16% (in 1986) to 26.4% (in 2001) and then up to 59.5% (in

2007) [4] [5] [6]. This has highlighted the importance and urgency of studying the mechanism of diabetic atherosclerosis and exploring therapeutic options [5] [7] [8].

Due to its unique advantages over other animal models, the mouse is the most used model for studying the mechanism of diabetes-accelerated atherosclerosis and exploring effective therapeutic approaches. Their advantages are a small size, short generation time, and ease of induction of diabetes and atherosclerosis by diet, drug treatment (streptozotocin or alloxan) or a genetic approach and cost-effectiveness. In the past decade, several diabetic atherosclerosis mouse models have been established [7].

Because in Indonesia it is difficult to obtain transgenic mice, the present study focused on to make healthy mice become diabetes by injecting STZ. Kunjathoor et al., (1996) had shown accelerated atherosclerosis in response to hyperglycemia in STZ - induced diabetic mice [3]. Kostogrys et al. (2012) found that the blood cholesterol level (BCL) was significantly increased in egg yolk diet mice and promoted atherosclerosis [9].

Prior studies have found a positive relationship hypercholesterolemia in STZ - induced diabetic rats as a result from increased intestinal absorption and synthesis of cholesterol. This was followed by the formation of atherosclerosis, which resembles that occurs after high cholesterol diet (egg yolk diet) [10] [11]. Canadian experts had been suspicious of the involvement of egg yolk in atherosclerosis formation and then warned people not to consume egg yolk [12]. On the contrary, Voutilainen et al., found that egg consumption was not associated with increased risk of myocardial infarct, where the relative risk (RR = 0.87 (95% CI 0.71 - 1.07, p = 0.73) in the highest (> 46 g/day) vs the lowest (< 15 g/day) cholesterol diet [13].

Various studies have been conducted to prevent and treat diabetic atherosclerosis by using antioxidants [14] [15] [6] and angiotensin receptor blockers [18] [19] [20].

Therefore, prior to conducting a study on the benefits of natural materials, such as caffeic acid phenethyl ester (CAPE) and other antioxidants in preventing and treating the formation of atherosclerosis, it is necessary to conduct a study on the effect of STZ injection in mice on the incidence of hyperglycemia, hypercholesterolemia, and atherosclerosis formation compared to that occurs in mice ingested with egg yolk or its combination.

Material and Methods

Fifty-six male mice (*Mus musculus*) of certified Double Dutch Webster lineage, 8 to 10 weeks old, and 20 - 30 gram of body weight were used. Before conducting the study, they were adapted to a place with light controlling of 12 hour - daylight (6:00 A.M. – 6:00 P.M.) and 12 dark hours (6:00 P.M. – 6:00 A.M.) with standard diet (eat and drink ad libitum). Their food came from Chroen Phosphate. The mice were placed in the natural temperature and humidity for 6 weeks. They were weighed once in a week to avoid stress. The study began after approved by the Committee of Research Ethics of the Faculty of Mathematics and Science University of Sumatera Utara Medan.

After an adaptation period, the mice were randomly divided into four groups with each group consisted of 14 mice. Group I did not get any treatment as the control, group II obtained STZ injection (45 mg/kg weight, intraperitoneal for five days), group III obtained egg yolk (0.2 cc per - oral for six weeks), and group IV obtained the combination of STZ injection (45 mg/kg weight, intra-peritoneal for five days) and egg yolk (0.2 cc per-oral for six weeks). STZ was obtained from Nicalai - Japan (Batch no. 32238 – 91/2).

Laboratory Analysis

The execution was performed each week by cervical dislocation in each group which consisted of two mice until the end of the study (six weeks). The mice's tails were cut off each week to get their blood for examining glucose level by using digital glucometer (Accu-check® Advantage, Roche Diagnostic, Germany) and for cholesterol level by using digital cholesterol test (Easy touch®GCU, Taiwan). The incidence and the severity level of atherosclerosis were proved by examining its result, using lighting microscope with the magnification of 40-400 times. The parameter was the thickness of aorta wall, the present of foam cells and sclerosis in mice's aorta tissues. The severity of atherosclerosis was differentiated into four groups according to histopathology findings (Figure 1):

1. Normal, when the following pathological matters were not found;
2. Mild, when monocyte adherent, foam cells, lipid core, proliferation of smooth muscle cells were found;
3. Moderate, when the above matters were found plus fibrous plaque, sclerosis, and fibrous cap;
4. Severe, when all the matters above were found, followed by rupture and thrombus.

All data were presented in the Mean ± SEM.

The difference of the average of Blood Glucose Level (BGL) and Blood Cholesterol Level (BCL) was analysed with ANOVA, followed by a post-hoc Bonferroni multiple comparisons, correlation of BGL and CBL with Pearson's regression, and the difference of the incidence of atherosclerosis among the groups with χ^2 test. While for data not normally distributed were analysed by Kruskal Wallis. A difference or correlation was stated significant when $p < 0.05$.

Results

In this present study, the change in the BGL (Table 1), BCL (Table 2), and the incidence of atherosclerosis (Table 3) were documented.

Table 1: The profile of blood glucose level (BGL mg/dl; mean \pm SEM) of all treatment groups, related to time (week)

Groups	Week (wk)							P (Sig)
	0 N=14	1 N=12	2 N=10	3 N=8	4 N=6	5 N=4	6 N=2	
Control	157.1 \pm 10.2	148.1 \pm 6.9	151.6 \pm 9.6	144.3 \pm 7.5	149.2 \pm 9.3	146.0 \pm 11.25	192.0 \pm 15.0	0.515
STZ	180.7 \pm 7.2	192.3 \pm 13.5	258.2 \pm 41.3	302.8 \pm 47.2	521.8 \pm 48.2	310.0 \pm 86.4	**	0.000
Egg Yolk	174.9 \pm 7.5	156.0 \pm 4.2	164.2 \pm 8.7	145.9 \pm 4.9	146.0 \pm 14.3	145.0 \pm 14.0	145.0 \pm 5.0	0.000
Combination	167.3 \pm 6.5	181.5 \pm 18.5	277.2 \pm 39.2	294.3 \pm 73.9	392.3 \pm 119.9	367.0 \pm 108.3	190.5 \pm 1.5	0.014
P	0.123*	0.096	0.006	0.001	0.000	0.115	0.291	

*Kruskal Wallis; ** death mice.

Table 1 showed no significant change of BGL in control group until the 5th week, except in the last week (in the 6th week BGL is 192.0 \pm 15.0 mg/dl), BGL slightly increased (22.3%). In the STZ group, BGL increased significantly (188.4%) in the 4th week; it then decreased after the 4th week, and before the 6th week (the 39th day) the mice died before the execution. In the groups where the mice obtained egg yolk, BGL significantly decreases to 17.1% until the end of the study. As demonstrated in STZ group, in the combination group, BGL increased to 134.7% until the 4th week, and it then decreased again by the end of the study, this BGL alteration is statistically significant.

Table 1 also showed the difference of BGL among the groups, related to time, where significant difference started from the 2nd week up to the end of the study. The most significant difference of BGL was found in the 4th week. However, the difference between BGL in STZ group and the combination group was not statistically significant in all observed weeks.

Table 2 showed that the average of BCL in all treatment groups tended to increase, including in the control groups. In the control group, BCL increased from 150.2 \pm 15.5 mg/dl in the 1st week to 216.0 \pm 37.0 mg/dl in the 6th week, where the highest CBL in

the 4th week (227.0 \pm 22.7 mg/dl; 33.9%).

Table 2: The profile of blood cholesterol level (mg/dl; mean \pm SEM) of all treatment groups, related to time (week)

Groups	Week (wk)							P
	0 N=14	1 N=12	2 N=10	3 N=8	4 N=6	5 N=4	6 N=2	
Control	152.5 \pm 13.3	150.2 \pm 15.5	156.5 \pm 11.6	178.4 \pm 15.6	227.0 \pm 22.7	178.0 \pm 5.7	216.0 \pm 37.0	0.039
STZ	120.8 \pm 6.0	122.1 \pm 6.8	148.3 \pm 7.2	175.5 \pm 17.5	215.0 \pm 13.8	119.8 \pm 8.3	**	0.000
Egg Yolk	139.8 \pm 7.4	141.2 \pm 8.5	151.0 \pm 11.6	181.1 \pm 9.4	165.3 \pm 13.7	145.0 \pm 21.0	135.5 \pm 122.5	0.289
Combination	168.8 \pm 12.7	176.3 \pm 13.5	152.6 \pm 8.6	169.8 \pm 13.8	181.8 \pm 4.7	163.3 \pm 23.1	166.5 \pm 7.5	0.895
P	0.244*	0.188	0.220	0.421	0.461	0.461	0.433	

*, Kruskal Wallis; **, death mice.

In the STZ group, the highest increase of BCL (76.2%) occurred in the 4th week; after that, it was decreased. The same profile to the egg yolk group which the highest increase of BCL (17.9%) was occurred in the 4th week, while in the combination group, the highest increase of the CBL (6.8%) occurred in the 4th week, and after that, it decreased lower than the initial level. However, the changes of BCL in the yolk and combination groups were not significant. Furthermore, based on ANOVA statistical analyses there was no significant difference in CBL between groups for each weeks observation, with an exception for the data of BCL at week-0 was analysed by Kruskal Wallis.

Table 3: The incidence of atherosclerosis

Severity	Control	STZ	Egg yolk	Combination
Normal	13	0	4	0
Mild	1	3	4	1
Moderate	0	11	6	12
Severe	0	0	0	1

$\chi^2 = 45.05$; $df = 9$; $P < 0.0001$.

Table 3 showed that the incidence of atherosclerosis was statistically different ($p < 0.0001$). Atherosclerosis was hardly found in the control group. In the STZ group, all mice underwent atherosclerosis, and the most serious incident was found in the combination group. In the egg yolk group, some mice did not undergo atherosclerosis.

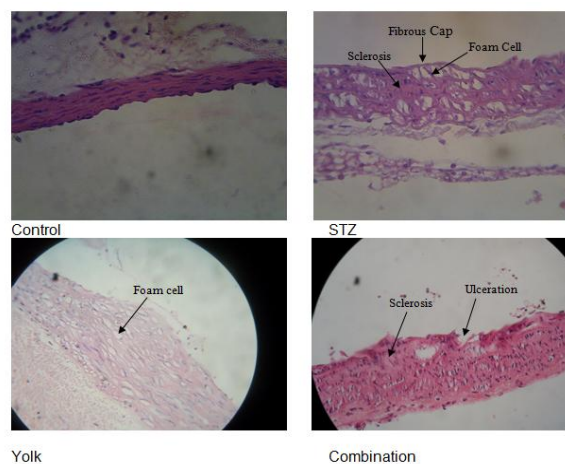


Figure 1: Microscopic Classification of the Atherosclerosis Severity between groups; i.e. Control, STZ, Egg yolk and Combination by magnification 400 times

Furthermore, in the present study, there was a significant positive correlation ($p < 0.05$) between BGL and CBL in STZ group ($r = 0.8469$), while in egg yolk group there was a significant negative correlation ($r = -0.8476$). On the contrary, there was no significant correlation between BGL and BCL in the combination group ($r = 0.1170$).

Discussion

The present study was successful in demonstrating that hyperglycemia occurred in mice induced by STZ injection (Table 1) with the highest BGL (521.8 ± 48.2 mg/dl; 188.4%) appeared in the 4th-week observation; after that BGL decrease. Unfortunately, before the 6th week (i.e. the 39th day), the mice died before the execution. This might be probably caused by the inability to consume food and drink by them. The condition of dehydration accompanied by high BGL in mice may lead to the occurrence of ketoacidosis followed by death [21].

In the present study, we used STZ (45 mg/kg BW intraperitoneally for 5 consecutive days) to induce diabetes in mice. Streptozotocin enters the pancreatic cell via a glucose transporter - GLUT2 and causes alkylation acid and then destruct DNA [22]. Twenty years ago, Kunjathoor et al. (1996) have demonstrated that injection of STZ (40 mg/kg BW intraperitoneally for 5 consecutive days) in mice may increase BGL (250-420 mg/dl), as the result of the decrease in plasma insulin level [3]. Arora et al. (2009) demonstrated that single injection of 180 mg/kg BW STZ will induce diabetes type 1 mice, and 100 mg/kg bw failed to produce diabetes except for sustained hyperglycemia. However, in multiple low doses STZ 40 mg/kg, BW for 5 days constitutively will induce diabetes type 2 [23]. While Mansor et al. (2013) demonstrated that administration STZ intraperitoneally in various doses 15, 20, 25 mg/kg BW gave no effect on BGL and did not change insulin concentration in the rat. In contrast dose of 30 mg/kg, BW STZ induced hypoinsulinemia, hyperketonemia and weight loss. It appears there is a positive dose-dependent relationship between the dose of STZ and BGL [24].

In the control group, there was no significant change of BGL, except in the last week of the study (the 6th week) there was a slight increase of the BGL (22.3%). The similar profile of the BGL happened in the egg yolk group, but BGL tends to decrease lower than the initial BGL (17.1%) at the end of the study. Jung et al. have given fatty diet to mice and found that BGL, BCL and insulin increased [8]. Theoretically, an increase of insulin level should be followed by the decrease in BGL. Although our study did not examine insulin level, the report of Jung et al. could explain why in the present study the decrease of BGL was

found in the egg yolk group as the result of the increase of insulin secretion. [8]. As happened in the STZ group and combination group there was a significant increase of BGL (134.7%) in the 4th week, after that BGL decrease although still higher than basal level (13.8%) at the end of the study.

There was a contradictory of the highest percentage increase of BGL at the 4th week of STZ group (188.4%) and combination group (134.7%), the effect of the increasing BGL by STZ was hampered by egg yolk which decreased BGL. Mansor et al. (2013) demonstrated that high-fat diet or in combination with STZ did not increase BGL. High cholesterol diets produce hyperinsulinemia mice [24]. A combination of various doses of STZ (15, 20, 25 and 30 mg/kg/BW) with high - fat diet, demonstrated that combination of 25 mg/kg STZ was able to rise in BGL [25].

The present study demonstrated that the BCL tended to increase, related to time, in all treatment groups, including the control group. In the control group, BCL increased up to 33.9%, while in the STZ and egg yolk group the increase in of BCL was twice as much as that in the control group. The BCL increased up to 76.2% in the STZ group was occurred in the 4th week; after that, BCL decreased. In the egg yolk group, the highest BCL of 70.9% occurred in the 6th week. Unfortunately, the alteration of BCL was not statically significant. On the contrary in the combination group there was a slight increase of BCL (2.8%), and at the last week, BCL decreases lower than initial level. This data demonstrated that egg yolk diet has minimal effect on BGL although co-administration with STZ. The mechanism of negative interaction should be investigated.

Spence et al. found a significant difference of the area of atherosclerosis plaque ($p < 0.0001$) in patients who consumed a different amount of egg yolk. People who consume fewer than two eggs a week (125 ± 129 mm²) will have a smaller area than those who consumed three eggs or more a week (132 ± 142 mm²) [12]. They recommended that the patients with the risk for cardiovascular disease do not consume egg yolk continuously. Consuming cholesterol-rich diet should be consumed less than 200 mg per day while egg yolk of a big egg contains about 275 mg cholesterol which is more than daily need. From the study on the animal experiment, it was found that cholesterol-rich diet will weigh down the accumulation of macrophage in adipose and atherosclerosis tissues in mice and increase systemic inflammation [12].

Although egg yolk was rich in cholesterol, consuming it in some subjects did not have any effect on BCL. Nevertheless, oxidised cholesterol could increase atherosclerosis although BCL was normal. Oxidized LDL plays an important role in initiation and progress of atherosclerosis. Meanwhile, a Finland researcher reported that consuming egg yolk regularly would not affect the area of carotid plaque or acute

myocardium infarct in Finland males [13].

Even though the previous investigators gave controversial information, the finding in the present study showed that, except the control, all treatment groups with STZ, egg yolk, and combination underwent atherosclerosis. The incidence of atherosclerosis was hardly found in the control group although one mouse was executed in week 0. This incidence might be probably caused by a genetic factor. In the STZ group, all mice underwent atherosclerosis in which the most serious incidence was found in the combination group of STZ and egg yolk. In the egg yolk group, there were some mice which did not undergo atherosclerosis; they were executed in week 0 and the 1st week. The combination of STZ and egg yolk cholesterol-rich diet gave synergic effect in forming atherosclerosis. Chono et al. had researched the benefit of an anti-atherosclerotic medicine which was tested on atherogenic mice, not on usual mice. In fact, Chono study took longer time (14 weeks) to form atherosclerosis after cholesterol-rich diet²⁵ compared with the findings in the present study (4 weeks) [25].

This present study showed significant positive between BGL and BCL in the STZ group at R-value = 0.8469 which indicated the increasing of BGL will followed by the increase in blood cholesterol level. However, in the egg yolk group, there was a negative correlation at R-value = -0.8476 in which BGL decreased will follow by the increase in BCL. On the contrary, there was no significant correlation between BGL and BCL in the combination group ($r = 0.1170$).

Based on the data obtained in the present study, the next study of the benefit of antioxidant CAPE and angiotensin receptor blocker telmisartan on the incidence of atherosclerosis in diabetic mice induce by STZ could be conducted for 4 week observation. In the 4th week treatment, there was a significant change in histopathology (atherosclerosis) and laboratories (hyperglycemia, and hypercholesterolemia) after giving of STZ to non-transgenic mice.

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