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Atherosclerosis Vaccine Using Bacteria *Salmonella typhimurium* on Rat Models

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Authors' contributions

This work was carried out in collaboration between all authors. Author SW is the supervisor of the study, designed the study, completed and edited the manuscript in English. Authors RBNY and MPZ designed the study and wrote the first draft manuscript in Bahasa Indonesia. Authors TSL and FT managed the literature searches. Author AMI analyzed the research data. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To prove the effect of *S. typhimurium* vaccine on inhibiting foam cell formation and arterial wall thickness, and also to decrease body weight and abdominal visceral fat.
Study Design: This experimental research was conducted using rat models.
Place and Duration of Study: Faculty of Medicine, Brawijaya University, Indonesia, between February – May 2011.
Methodology: The vaccine was 10^8 CFU of heat-killed *S. typhimurium*/100µl vaccine per rat. The adjuvant was CFA-IFA 100µl per rat. Twenty Wistar rats were divided into five

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groups: a negative control group (have normal diet), and four treatment groups which were given with atherogenic diet. The four treatment groups were positive control group (atherogenic diet only), vaccine + adjuvant group (added with the vaccine + adjuvants), vaccine group (added with vaccine only), and adjuvant group (added with adjuvant only). The vaccines were injected intraperitoneally, five times in two-week intervals.
Results: There was no significantly difference in the average diet intake every day among the groups (*P*=0.17). The administration of 'vaccine + adjuvants', 'vaccine only' and 'adjuvants only' could decrease foam cell formation and arterial wall thickness compared to the positive control group (*P*= .00). The 'vaccine alone' treatment returned the foam cell numbers to be a normal value just like negative control (*P*=.15), but 'vaccine + adjuvants 'and 'adjuvant alone' did not (*P*=.01). There was a strong and significantly correlation between the foam cell formation with arterial wall thickness (R=0.842, *P*=.00). In addition, administration of 'vaccine only' decreased the rats' body weight and abdominal visceral fat accumulation significantly compared to the positive control (*P*=.04 and *P*=.00 respectively).
Conclusion: The heat-killed *Salmonella typhimurium* vaccine without CFA-IFA adjuvant decreases foam cells expression and aortic wall thickness, body weight, and abdominal visceral fat accumulation in rat-induced atherogenic diet. In suggestion, heat-killed *S. typhimurium* is a potential antigen to be developed as an atherosclerosis vaccine in the future.

Keywords: Vaccine; S. typhimurium; foam cell; arterial wall; body weight; abdominal fat.

1. INTRODUCTION

Cardiovascular disease such as coronary artery disease (CAD) is one of the biggest health problem in the world. Based on WHO (2004), CAD was responsible for 29.34% of all deaths in the world [1] The National Health Survey in Indonesia (2007) showed cardiovascular disease had the highest rank other than infectious diseases [2] CAD is caused by the obstruction of coronary blood vessels in the heart called atherosclerosis. Atherosclerosis is a chronic inflammation in the blood vessels that causes thickening and narrowing of the blood vessel walls [3].

Atherosclerosis is characterized by the accumulation of lipids in the subendothelial tissue of blood vessels [4]. The lipid accumulation is due to its high levels of LDL (Low Density Lipoprotein) in the blood. LDL is oxidized by inflammatory cytokines, free radicals, and certain enzymes to become oxLDLs (oxidized LDL) [3]. The oxLDL expresses PC (phosphorylcholine) on its outer membrane. The PC with oxLDL is recognized and internalized by macrophage [5,6], which in turn forms foam cells. The foam cells secrete proinflammatory cytokine and growth factor, and the dead cells caused by apoptosis contribute to the narrowing of blood vessels in atherosclerosis. Subsequently, the atherosclerotic plaque are formed and may continue to evolve into a more progressive lesion that will block the vessels lumen resulting in symptoms and complication [7] High levels of oxLDL also can cause obesity, because oxLDL is able to induce adipocytes proliferation and makes the exacerbation of the adipocytes accumulation [8].

Therapy for atherosclerosis that includes drug only inhibits the progression of existing atherosclerotic plaques, but doesn't prevent the plaque formation [9]. Atherosclerosis is called as "a silent killer" and new manifestations arise when there has been a complication.

Therefore, an effort such as healthy lifestyle, antioxidants, and essential vitamins are important in preventing of atherosclerotic events [10]. However, the preventive treatment are carried out often too late.

Until now, research on the development of vaccine to prevent atherosclerosis is still rare. Caligiuri, attempted to develop a new vaccine that can induce protective immunity for atherosclerosis by using a specific antibody against phosphorylcholine (PC) in oxLDL [6]. Shenckein et al. showed a cross reactivity between antibody response to PC expressed by bacteria with antibody response to PC expressed on oxLDL membrane due to molecular mimicry of the two antigens [11]. Other study reported that *Salmonella typhimurium* (*S. typhimurium*) bacteria express PC [12].

This research aimed to find out the role of *S. typhimurium* vaccine in inhibiting atherosclerosis progression by measuring foam cell expression, aortic wall thickness, abdominal visceral fat and body weight. This research hopes to identify a new intervention to prevent cardiovascular disease.

2. MATERIALS AND METHODS

2.1 Experimental Design

This research was a true experimental with randomized posttest only control group design and carried out on rat models. Twenty male Wistar rats (6-8 weeks) were divided into five groups: a group that had normal diet and no vaccination (negative control group) and the four other groups treated with an atherogenic diet (treatment groups). The treatment groups were a positive control group (atherogenic diet only), added with 100µl *S. typhimurium* vaccine + 100µl of CFA-IFA (vaccine + adjuvant) group, added with 100µl *S. typhimurium* vaccine without adjuvants (vaccine only) group, and added with 100µl of CFA-IFA (adjuvant only) group. For both diets were weighted 40 grams a day for each rat and were given for 50 days.

2.2 Chemical and Equipment

2.2.1 Chemical

Atherogenic diet was comprised of 75% wheat flour, 8.0% pig-fat oil, 10% sheep-fat oil, 1% coconut oil, 0.125% cholic acid, 5% duck egg (yolk only), and 0.825% water. Normal diet was comprised of 64% pellets, 35% wheat flour, and 1% water. Brain Heart Infusion liquid medium, Bismuth Sulfite Agar, Gram stain, Microbact system. Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA). Propylene glycosolution, 0.5% oil red O solution, 10% formalin, Meyer's hematoxyllin solution, glycerine jelly and aquadest. Chloroform, ethanol, xylol, paraffin, and hematoxyllin-eosine stain.

2.2.2 Equipment

Analytical balance, stirrer, measuring cup, feed grinders, trays, vortex, incubator, eppendorf. Falcon tube, spectrophotometer, plate. Plastic containers, insulin syringes, and vacutainer. Surgical scissors, pin, steroform and cotton buds, object glass, cover glass, bright field microscope. Microtome, tweezers, automatic tissue processing.

2.3 Procedures

2.3.1 Bacterial identification and culture

The *S. typhimurium* was a human isolate and obtained from Microbiology Laboratory, Faculty of Medicine, University of Brawijaya. It was cultured in Bismuth Sulfite Agar medium and then, inoculated into Brain Heart Infusion (BHI) liquid medium for 24 hours. Gram stain and biochemical test kit (Microbact System) was used to identify *S. typhimurium.*

2.3.2 Vaccine production

The *S. typhimurium* was separated from BHI medium using centrifugation at 5000 rpm for 15 minutes. The vaccine dosage for each injection was the 10^8 CFU bacterial cells in 100µl PBS (Phosphate Buffer Saline) and boiled it in 100ºC waterbath for 45 minutes.

2.3.3 Vaccine injection

Rat was given primary injection subcutaneously and four booster injections in two week intervals intraperitoneally.

2.3.4. Foam cells and aortic wall thickness measurement

The frozen section method was used for foam cell preparation. At the day 50^{th}, rat was executed and the aorta was taken out. After the sliced aortas were stained with oil red O, the foam cells were count for ten visual fields under microscopes with 400x magnification. To measure the aortic wall thickness, the sliced aorta stained by hematoxyllin-eosine and measured by a micrometer under microscope for eight visual fields with 400x magnification.

2.3.5 Body weight and visceral abdominal fat measurement

On the day 50^{th}, after the body weight was measured, the rat was executed and the abdominal visceral fat was separated and weighted.

2.4 Data Analysis

The gained data were analyzed by ANOVA and correlation test at 95% of confidence level (α=.05).

3. RESULTS AND DISCUSSION

3.1 Mean of Diet Intake Everyday

According to know whether the rats of treatment groups consumed the atherogenic diet in the same amount, the diet intake was measured every day. The diet intake every day was calculated as 40 grams of the feed regimen minus residual feed. Mean of the diet intake everyday, then, was analyzed statistically by Kruskal-Wallis test. The result of the statistical test obtained *P*=.17. It means the all treatment groups consumed the atherogenic diet in the same amount (see Fig. 1.), that so the effect of the treatments can be compared.

Fig. 2 and Fig. 3 showed that the number of foam cells on the aortic vessel walls in rats treated by *S. typhimurium* + CFA-IFA (vaccine + adjuvant), *S. typhimurium* (vaccine only) and CFA-IFA (adjuvant only) decreased significantly compared to the positive control group (atherogenic only without vaccine). However, treatment with the 'vaccine + adjuvant' and 'vaccine only' group returned to foam cells number which was the same as the negative control group (normal diet). In contrast, the treatment with only adjuvants did not.

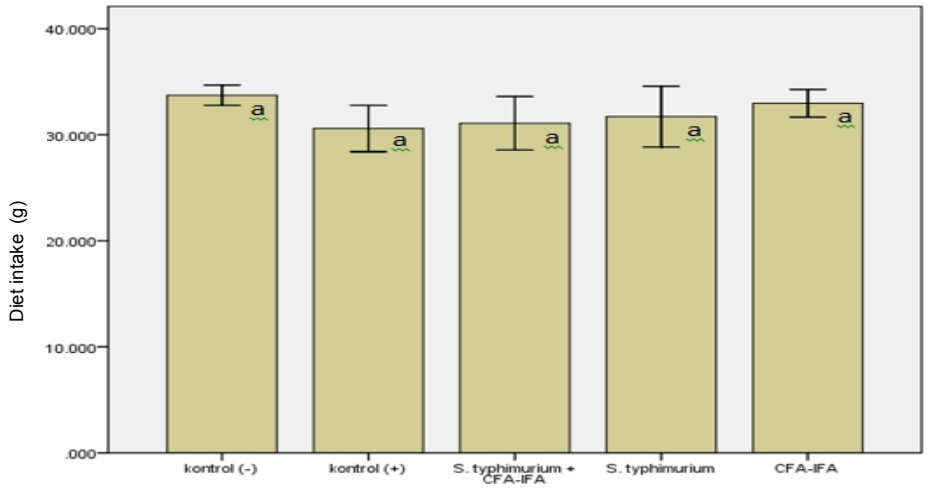

Fig. 1. Mean of diet intake every day
There were no significant differences among all the groups (P=.17)

2.2 Foam cells expression and Aortic wall thickness

The foam cells expression was shown and counted after oil red O staining as a red color cells (see Fig. 2). The aortic wall thickness was evaluated after hematoxyllin-eosine staining (see Fig. 4).

Figs. 4 and 5 showed the aortic wall thickness after vaccination. The result showed that *S. typhimurium* + CFA-IFA (vaccine + adjuvant)), *S. typhimurium* (vaccine only), and CFA-IFA (adjuvant only) decreased aortic wall thickness significantly compared to positive control group (atherosclerosis diet without vaccine). Furthermore, the 'vaccine + adjuvant' and 'vaccine only' treatment could repair the aortic wall thickness to a normal condition just like the negative control group (normal diet), but treated with only adjuvant did not. Also, it was not significantly different between vaccine + adjuvant and vaccine only.

Using Pearson correlation test, it was obtained R = 0,842 and *P*=.01; so there is a strong correlation and it mean the decreasing of foam cells number caused the reduction of aortic wall thickness.

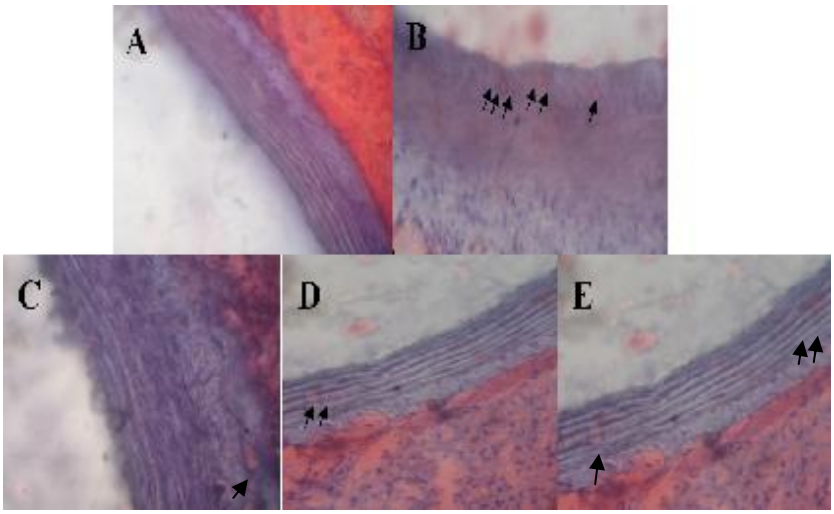

Fig. 2. Foam cell expression in rat's aortic wall (oil red staining; black arrow showed the foam cell)

In 'negative control' showed there is no foam cells (A), but in the 'positive control' can be seen more numbers of foam cell (B). Treatment by 'vaccine + adjuvant' (C) or 'vaccine only' (D) reduced the number of foam cells. Treatment by 'adjuvant only' (E) also decreased foam cells formation but the numbers are still much more than treatment with 'vaccine only'

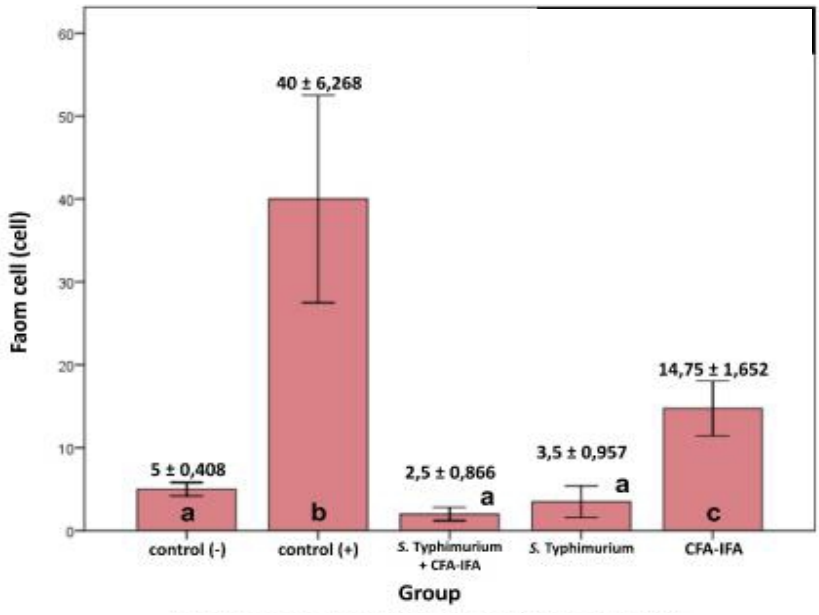

Fig. 3. Foam cells number in rat's aortic walls

The 'negative control', 'vaccine + adjuvants', and 'vaccine only' were not significantly different (P= .15), whereas 'adjuvants only 'and the positive control group each was significantly different with the other groups (P= .00)

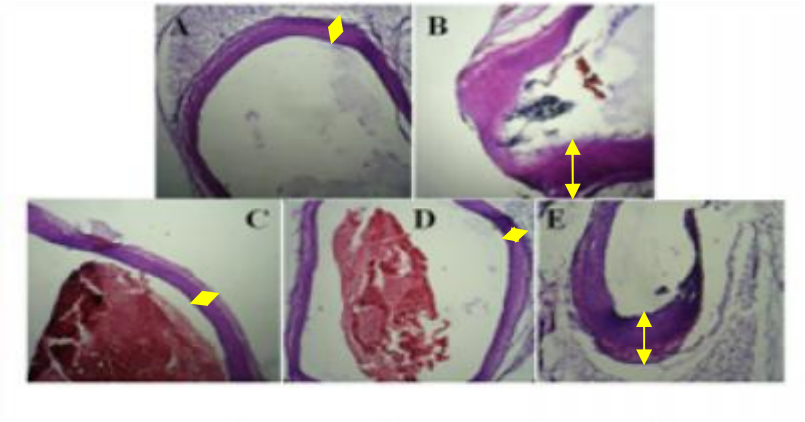

Fig. 4. The rat's aortic wall thickness

(hematoxyllin-eosine staining; yellow arrow showed the aortic wall thickness)
In positive control showed a very thick of the aortic wall (B). Treatment by vaccine + adjuvants' (C) or 'vaccine only' (D) returned the aortic wall as thick as normal (A), but 'adjuvants only' did not (E)

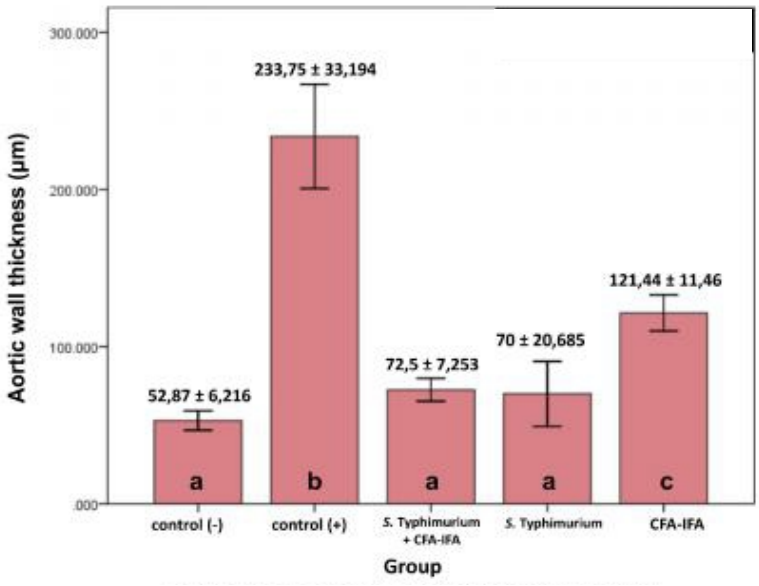

Fig. 5. Mean of rat's aortic wall thickness

The 'negative control', 'vaccine + adjuvants', and 'vaccine only' were not significantly different (P=.09), whereas the 'positive control' group was significantly different with the other groups (P=.00), also 'adjuvants only' was significantly different with the other groups (P=.01),

Clearly, the findings indicated that the *S. typhimurium* heat-killed vaccine decreased the foam cells formation and also the thickness of blood vessels returned to a normal condition. *S. typhimurium* is a Gram negative bacteria that express PC on the bacterial cell wall [12], so vaccination with *S. typhimurium* can induce the anti-PC antibody. The *S. typhimurium* vaccine may induce a specific anti-PC antibody through T cell independent and T cell dependent pathways. In the T cell independent pathway, PC is expressed on the surface of

S. typhimurium can be recognized by B cells. The PC interacts with B cell receptor (BCR), and it will stimulate the proliferation and activation of B cell to produce IgM specific anti-PC antibody. However, the IgM has no long term atheroprotective effect [13]. The other pathway is T cell dependent. In this process, the bacteria vaccine is phagocyted by the antigen presenting cell (APC) such as macrophages and dendritic cells. The PC, then, is presented by APC via CD1 receptor to Natural Killer T cells (NKT cells). This interaction stimulates NKT cells in secreting interleukin-4 which can induce the proliferation and activation of B cells to produce IgG specific anti-PC antibody. As Caligiuri reported that the anti-PC antibodies can cross-react with oxLDL (because oxLDL express PC); so that, the complex between the anti-PC antibodies with oxLDL prevents the oxLDL uptake by macrophages [6]. Also, Kearney reported that the immune complexes will inhibit the adhesion of oxLDL on vascular endothelium, thereby reducing the amount of oxLDL in the intima [14]. Since the vaccination method (four times periodically) will induce IgG production mainly than IgM [15], and the IgG antibody has the long term atheroprotective effect (unlike IgM antibody) [16], therefore, the heat-killed *S. typhimurium* could be considered as candidate vaccine against atherosclerosis.

Usually, in vaccine components, besides the main antigen, it is also important to add an adjuvant to maintain the antigenicity, and to enhance the immune response, also to prevent the tolerance mechanism against the antigen [16]. In the current study, it was shown that the treatment with CFA-IFA (adjuvant only) can also decrease the foam cell formation and aortic wall thickness, although it was not to be a normal condition. This phenomenon may be caused of the CFA-IFA which are composed of lipid that induce lipid peroxidation process which results a malondialdehyde (MDA) as the end product. The MDA can react with oxLDL becomes a MDA-LDL form; and, when this MDA-LDL is uptaken by macrophage, it becomes foam cell. Furthermore, Binder et al. reported that the MDA produced by CFA-IFA can induce the releasing of specific anti-MDA antibody, and the antibody may reacts with the MDA-LDL [5]. Kametzu et al. also mentioned in their research that MDA-LDL has an atheroprotective activity due to the producing MDA-LDL antibody which is able to inhibit MDA-LDL uptaken by macrophage [17]. The consistent result also was reported by Hansen et al. that CFA-IFA which is a lipid-based adjuvant also has protective effect against atherosclerosis, and it was proven that the adjuvant is able to reduce the amount of foam cells and vascular wall thickness [18].

3.2 Body weight and Abdominal visceral Fat

Body weight and abdominal visceral fat can show the obesity progression. Furthermore, the obesity correlates with atherosclerosis. The rat's body weight measured on groups were reported as the weight gain (the difference between the weight before and after treatment) (see Fig. 6.). The abdominal visceral fat weight was reported as Fig. 7.

Similar with the result of the increasing of the body weight, the abdominal visceral fat weight showed that there were significantly different between the *S. typhimurium* (vaccine only) group with the other groups, but interestingly, *S. typhimurium* + CFA-IFA (vaccine + adjuvant) and CFA-IFA (adjuvant only) did not. The findings showed that the addition of adjuvant doesn't always improve the effect of vaccine, but it is depend on the kind of adjuvant and the pathomechanism of disease which will be prevented. In this case, the addition of adjuvant composed of lipid may cause lipid peroxidation and produce a substance called 4-Hidroxynonenal (HNE). Furthermore, HNE will interfere lipid anabolism

and induces the fatty acid synthesis and suppresses β-oxidation process, so that triglycerides will be accumulated in adipose tissue [19,20].

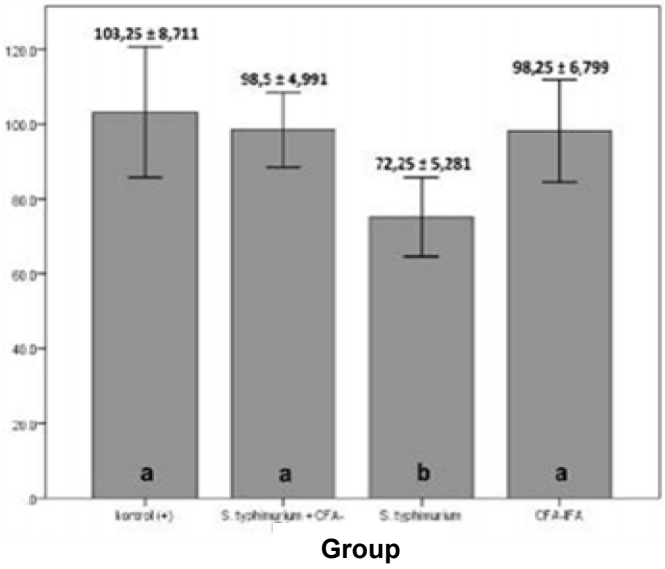

Fig. 6. Mean of increasing body weight (g)

The 'positive control' group, 'vaccine + adjuvants', and 'adjuvants only' were not significantly different (P= .11), 'but vaccine only' group was significantly different with the others (P=.01)

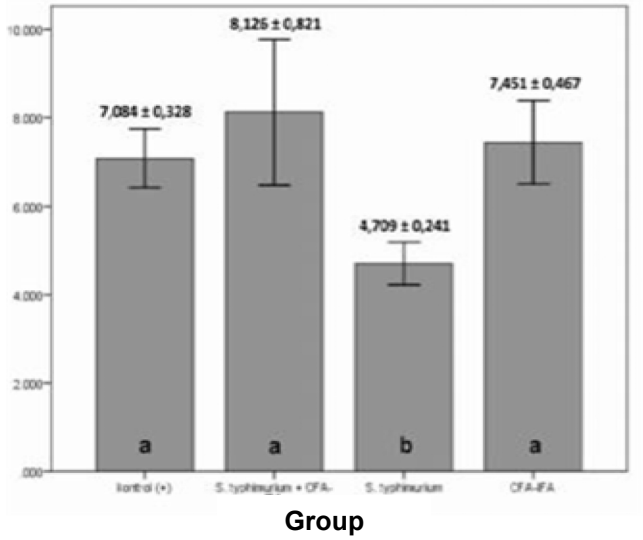

Fig. 7. Mean of the visceral fat weight (g)

The 'positive control' group, 'vaccine+adjuvants', and 'adjuvants only' were not significantly different (P= .11), but 'vaccine only' group was significantly different with the others (P= .01)

The correlation test showed that there was no correlation between the increasing of abdominal visceral fat with the body weight (R=0.072, *P*=.35). The result is consistent with

the definition of obesity. Obesity is a condition of visceral fat accumulation rather than an increase in body weight. Therefore, the visceral abdominal fat weight is a standard parameter that is often used in determining the progression of obesity. The body weight is usually less reliable in determining the severity of obesity although obesity usually is measured using body mass index (BMI), because the increasing of total body weight is also influenced by many other factors, such as increasing of bone mass, organ, or fluid in the body. The statement is supported by the result of the current study, that there is no correlation between the increasing of abdominal visceral fat with the body weight gain.

3.3 *S. typhimurium* Vaccine against Obesity and Atherosclerosis

Obesity and atherosclerosis are interconnected. Albright and Stern [21] has shown that there is a relationship between obesity and atherosclerosis. The relation between the two conditions is mediated by various substances, such as oxLDL and triglycerides (TG). The oxLDL can lead stack of adipose tissues. The oxLDL is able to induce obesity directly and indirectly. Directly, oxLDL is able to induce the adipocytes proliferation and aggravating the stack of the adipocytes [8]. Indirectly, the oxLDL is uptaken by SR or CD36 cells will induce the release of monocyte chemoattractant protein-1 (MCP-1) cytokine, which plays a role in macrophage infiltration process into the adipose tissues [22]. The macrophage infiltration into adipose tissues increases the production of some inflammatory cytokines such as TNF-α and IL-6. This proinflammation condition on adipose tissues will decrease the mRNA expression of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). These enzymes play a role in the resolving of TG to be a free fatty acid (FFA) and glycerol in adipose tissues. Consequently, the process in adipose tissues will be inhibited and the lipid stack will be increased progressively. As a result, TG accumulates in adipose tissues that can be measured as visceral fat and body weight [23,24,25]. The oxLDL also induce TG production through LPL expression and FFA accumulation on adipose tissues. Moreover, the FFA is capable to induce the accumulation of ceramide that contributes the occurrence of adipose hyperplasia [22].

The current study showed that treatment with *S. typhimurium* vaccine (without adjuvant) has an advantage to reduce the foam cells expression, blood vessel thickness, body weight and abdominal visceral fat. However, based on the relationship between obesity and atherosclerosis mechanism above, the current result still can not explain how the really mechanism of the *S. typhimurium* vaccine can prevent atherosclerosis. For this reason, according to use *S. typhimurium* for preventing atherosclerosis, it is suggested to do the further study about the cytokine roles, the level of antibody against oxLDL and TG production, also the proper adjuvants that can be used.

However, related to the results and discussion above, the *S. typhimurium* bacteria vaccine is recommended as a new approach to protect the atherosclerosis process.

4. CONCLUSION

The heat-killed *Salmonella typhimurium* vaccine without CFA-IFA adjuvant decreases foam cells expression and aortic wall thickness in rat-induced atherogenic diet. Also, the vaccine is able to reduce the rat's body weight and abdominal visceral fat. Hence, the heat-killed *Salmonella typhimurium* vaccine has the potential ability to become a new approach for preventing atherosclerosis in the future.

ETHICAL APPROVAL

Ethical clearance to use animal (rats) in this research was provided by the Ethical Committee at Faculty of Medicine University of Brawijaya - Indonesia.

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COMPETING OF INTEREST

The authors declare that they have no competing of interest in the conduct of this study and the production of this manuscript.

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