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Plasma endothelin-1 role on initial atherosclerotic lesion formation in young obese wistar rats: An experimental study

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Abstract
Background: Obesity has a strong relationship with the acceleration of initial atherosclerosis lesion formation that marked by increasing of some biomarkers such as endothelin-1 (ET-1). Obesity also becomes a risk factor for the emergence of various metabolic diseases like hypertension and type 2-diabetes. This study aims to reveal the relation of endothelin-1 serum levels with the early lesion of atherosclerosis.
Results: There were an increasing value in serum ET-1 level and aorta foam cells count in obese group compared to normal group. Furthermore, there is a strong positive correlation between serum ET-1 level and the amount of aorta foam cells (r = 0.628) in young obese rats.
Conclusions: ET-1 showed an important role to triggering the emergence of early lesions in of atherosclerosis in young age obese rat.

Keywords: Obesity, endothelin-1, atherosclerosis, aorta

Introduction
The increasing prevalence of overweight and obesity in children and adolescents has become a national and international health issue because it risk factor for the emergence of various metabolic diseases, such as hypertension and type 2-diabetes [1-3]. Obesity in childhood and adolescence is closely related to early atherosclerotic lesions, in the form of fatty streak and carotid intima-media thickness (IMT), which carries a risk for cardiovascular disease in adulthood [4]. Various proteins are assumed to play a role in the development of metabolic diseases in obesity such as Endothelin-1 (ET-1). Endothelin-1 is a very strong endogenous vasoconstrictor that mainly produced by vascular endothelium, it stimulates the contraction of vascular smooth muscle and cardiac muscle through its interaction with endothelin receptors on the surface of muscle cells [5]. In various organs, ET-1 acts as a paracrine or autocrine toward two receptors: Endothelin A Receptor (ETA), acts in smooth muscle contraction and Endothelin B receptor (ETB), stimulates Nitric Oxide (NO) production in endothelial cells [6, 7]. ET-1 involvement in atherosclerosis lesion development of adults had been reported in several studies both in animals and in humans [8, 9]. However, studies in young age subjects were still rare. Therefore, this study aims to investigate the role of endothelin-1 biomarker in the development of early atherosclerotic lesions in young obese rats.

Materials and Methods
Study design and subject selection
This study is an experimental study with a posttest only control group design. We used ten healthy, male, white Wistar rat (*Rattus Norvegicus*) at the age of 21-30 days (± 2-4 years in human age) as animal model and followed up until the age of 150-180 days (equal with 17-20 years in human age) [10, 11]. We were divided subject into two groups that consist of 5 rats each: control group (wild type/non-obese rat) and case group (obese induction rat). All animal were kept in a standard cage with 20-22 ^{0}C room temperature and 12 hours light and dark cycle.

Obesity induction procedures

The obese group rats were given a high carbohydrates and high fat diet (High-fat CP551 feed + Instant Milk) at the age of 30 to 90 days, while the non-obese group rats were given a standard diet. After 90 days of age, we measured the obesity induction result based on Rohrer index [12] in the group that was given a high-carbohydrate and high-fat diet. If the Rohrer Index value is ≥30, then the obesity condition will be maintained until the age of 150-180 days. Meanwhile, the non-obese group rats were still given a standard diet until the age of 180 days. At the age of 180 days both groups of rats were then terminated.

Endothelin-1 measurement

Plasma ET-1 level was carried out using enzyme-linked immune absorbent assay (ELISA) using blood samples that taken from rat's heart following the standard protocol (MyBiosource, USA, Cat.No MBS162631). To collect the plasma specimen, we used Ethylene diamine tetra-acetic acid (EDTA) as an anticoagulant on the blood tube. After that, we centrifuge samples for 20 minutes at 2000-3000 rpm and collect the supernatant without sediment. Furthermore, we add sample and ELISA reagent into each well. Incubate for 1 hour at 37 °C. Wash the plate 5 times. Add substrate solution A and B. Incubate for 10 minutes at 37 °C. Add stop solution and color develops. Read the optical density value within 10 minutes.

Histopathology examination

The early atherosclerotic lesions were determined by foam cell count in the thoracic aorta using histopathological examination with Haematoxylin-Eosin staining. We collect the tissue by cutting 1 cm thoracic aorta and put it into 10% formalin buffer liquid. After that, we did deparaffinization of dried preparations in xylol 2 times (5 minutes each). Dip in 96% alcohol 2 times (5 minutes each). Then washed with sterile water until the alcohol is clear. Put it in the hematoxylin solution for 5-10 minutes. Then wash with sterile water for 10 minutes. Dip into 0.4% HCl solution 3 times for decolorization. Then wash again with sterile water for 10 minutes. Put it in the Lithium Carbonate solution for 30 seconds and then wash it with water for 10 minutes. Put it in the Eosin solution for 1-2 minutes, then wash it with running water for 10 minutes. Put it in the Ethanol solution for 15 seconds 3 times and for 1 minute 2 times. Soak in the Xylol solution for 5 minutes 3 times. Put the tissue cover (deckglass) and then read the result under a light microscope.

Statistical analysis

The data obtained were analyzed using SPSS 23 using independent T- test and Pearson correlation tests.

Results

Table 1: Subject characteristic

Variables	Non- Obese	Obese	P*
W (gram ± SD)	213 ± 40.31	441 ± 25.56	<0.001
L (cm ± SD)	20.1 ± 0.74	22.6 ± 0.54	<0.001
RI (gr/cm-2)	26.83 ± 1.8	42.07 ± 2.19	<0.001

*Independent t test (sig. p<0.05)
W=Body Weight, L=Body Length, RI = Rohrer Index.

Table 1 showed that after administration of high-fat and high-carbohydrate diet for ± 21 weeks (150 days), the obese group showed more than double-fold weight gain and increasing by more than two centimeters body length compared to non-obese group (p<0.001). This result showed that the subject met the obesity criteria based on the Rohrer index.

Table 2: Plasma ET-1 levels and foam cell counts

	Non-Obese (SD)	Obese (SD)	P*
ET-1 (pg/ml)	16.58 ± 5.56	19.83 ± 10.25	0.550
Foam cell count	1.6 ± 2.3	29.2 ± 17.655	0.008

*Independent t test (sig. p<0.05)

Table 2 showed that there is an increase of ET-1 plasma levels as much as 20% (± 4 pg/ml) in obese group even it was not statistically significant (p=0.550). However, in contrast to the ET-1 levels compared to non-obese group. However, the foam cell count in thoracic aorta of obese group showed a statistically significant (p<0.008) increased of foam cell count approximately 18-fold compared to foam cells that found in non-obese group (Fig.1 and 2).

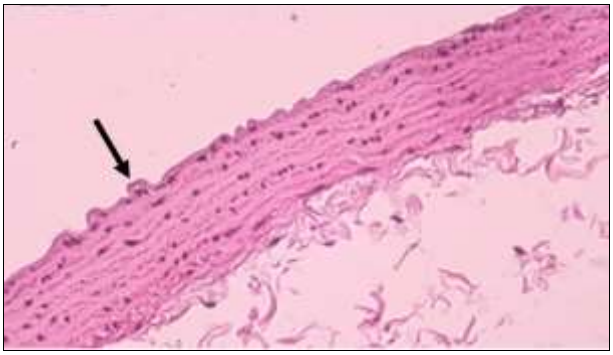

Fig 1: Histology of normal aortic vessel of rat (control group). Black arrow: foam cell.

Fig 2: Histology of aortic vessel post induce obese rat (case group). Black arrow: foam cells.

Table 3: Relationship of ET-1 plasma level and foam cell count

	Value	ET-1
Foam cell count	r	0.628
	p	0.050

r: Pearson correlation value, p: Pearson correlation significance.

Table 3 showed a strong positive relationship between plasma ET-1 level and foam cell count in aortic blood vessel. This result showed that increasing of ET-1 level has a linear correlation with increasing of the foam cell formation (r-0.628, p 0.050).

Discussion

In this study, it was found that all subject with obesity had

more foam cell formation in the aortic wall compared to normal weight group. This result was explained that the atherosclerotic lesions could had been accumulated since an early age and demonstrating the potential of children or adolescence to initiate atherogenesis formation. This result also revealed in several previous studies concerning obesity in children and adolescents. Many study reported that abnormal adipokine cells, inflammation, insulin resistance, and dyslipidemia are considered to become underlying mechanism to illustrating an initial formation of atherosclerotic lesions [13-16]. This study also found that there was a minimal increase in plasma ET-1 levels by 0.2 times in the obesity group compared to the non-obese group. However, it was not a significant difference ($p > 0.05$). Sanchez A. *et al.* (2014) had a quite similar result that showing elevated ET-1 levels in penile arteries of obese mice, while Traupe T *et al*. (2002) discovered that the vasoconstrictor expressed from endothelial cells such as ET-1 became more active in normotensive obese mice. Furthermore, Kubota *et al.* (2017) concluded that obesity is a condition causing the imbalance between vasorelaxant and vasoconstrictor which induces insulin resistance [17-19]. The slight rise of ET-1 level in this study might be influenced by the presence of mild endothelial dysfunction [20]. This dysfunction remained mild because subject that we used in this study were still on a young age (<1-month rats). Goettsch *et al*. (2001) reported that aged would be consequently followed by an increase in vascular ET-1 levels. On the contrary, this correlation would reduce the amount of vasodilators [21, 22]. The number of foam cells in aorta showed a strong association with ET-1 levels in this study (r=0.628). Higher levels of ET-1 induce more formation of foam cells in the thoracic aorta of obese rat. Therefore, this finding suggests that ET-1 plays an essential role in the early onset of atherosclerotic lesions not only in older rat but also in young age [9, 23, 24]. The role of ET-1 as an atherogenic agent is achieved through several mechanisms, such as suppressing the activity of NO, activation of endothelial cell and lectin-like oxLDL receptor-1 (LOX-1), increasing Reactive Oxygen Species (ROS), and stimulating inflammatory process [25-28].

Conclusion

Endothelin-1 has an important role in atherosclerosis by triggering the emergence of early lesions in obese rat at a young age. It is proved by the increase of ET-1 level has a linear correlation with the increase of foam cell formation.

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