Original article

Diagnostic Test of Urine Proteome during Atherosclerotic Process Among White Rat (*Rattus Novergicus* Strain Wistar) Induced by Atherogenic Diet

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Abstract

Introduction: Around twenty five million people in United State have at least one complications of atherosclerosis such as cardiovascular disease. Therefore screening in atherosclerotic process is important. Atherosclerosis is a multistage process that begins with damage to the endothelium due to LDL exposure. Angiography Coronary is the primary diagnostic tool for atherosclerosis. Imaging, as one way of non-invasive diagnosis, have limitation in sensitivity, specificity, and cost. Proteome urine analysis is alternative for atherosclerosis diagnostic. ***Objective:*** The aim of this study was analyse diagnostic performance of urine proteome among white rat (*Rattus Novergicus* strain wistar) induced by atherogenic diet. ***Material and methods:*** This study was quasi experimental in laboratory with randomized posttest only for control group design. The rat samples were divided to two groups i.e. group with atherogenic diet and non-atherogenic diet. Each group consisted of 10 rats. The treatment was done for 15 weeks. Urine sample collection was performed in week 16 of treatment. Urine proteome, collagen type I, was analyzed by ELISA to obtain biomarkers of atherosclerosis. Then determined sensitivity and specificity of the proteome urine analysis was compared with histopathology findings of the aortic arch and its branches. ***Result:*** In week 16, the researchers collected sample from 16 rats i.e. 11 rats with atherogenic diet and 5 ratswith non-atherogenic diet. Urine proteome, collagen type I, had area under the receiver operator with the characteristic curve of 98.2% (95% IK 92.6%-100%), p 0,003. The bestcut offis 0.9025 ng/ml with sensitivity of 100% and specificity of 90.9%. ***Conclusion:*** Urine proteome analyse, collagen type I, had high accuracy for atherosclerosis diagnostic.

Keywords: Urine proteome analysis; Atherosclerosis; Collagen type I

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Introduction

Around twenty five million people in USA have at least one of complications from atherosclerosis as cardiovascular disease[1]. According to data from the Ministry of Health of Indonesia, the prevalence of coronary heart disease based on interviews about the symptoms is about 1.5% from the total population in Indonesia. Those diagnosed by doctor is only 0.5%[2]. However, the proportion of deaths caused by heart disease was 4.6% from the total cause of deaths from all age[2].Thus, the diagnosis of atherosclerosis before the occurrence of complications is very important.

Atherosclerosis begins with endothelial damage due to exposure to LDL. LDL then goes into the intima layer of endothelial and undergoes oxidation. Oxidized LDL will activate receptors on leukocytes, especially vascular cell adhesion molecule-1 (VCAM-1) and release proinflammatory cytokines. Both of these processes resulted in the recruitment of leukocytes, especially monocytes and lymphocytes.

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Monocytes will transform into macrophages that will take LDL within tunica intima. LDL-rich Macrophage is called foam cells. Foam cells and lymphocytes will secrete fibrogenic mediators like growth factor. These mediators will stimulate replication of the extracellular matrix in the vascular muscle tissue. Foam cells also produce proteolytic enzymes that will degrade collagen in fibrous tissue. Atheroma plaque becomes thin and easily ruptured. Rupture of plaques will activate trombocytes that will form thrombus[3, 4, 5].

Coronary angiography is the primary diagnostic tool for atherosclerosis. Imaging, as one way of non-invasive diagnosis, has drawbacks in terms of sensitivity, specificity, and cost[1, 6]. Biomarker test on the blood, such as VCAM-1, selectin and integrin are considered as one of the potential diagnostic tool[7].

Proteome are proteins and peptides that exist in all parts of the body. Proteome is specific to a particular cell or tissue. Unlike the genome, proteome is dynamic following environmental changes. Proteome can be detected through body fluids (such as urine and blood) and tissues. About 100,000 proteome are in the urine proteome, 5000 of them are detected with high frequency. This protein does not only contain information from the kidneys and urinary tract, but also biomarker of systemic diseases. Analysis of proteome in urine can be used to find biomarkers of atherosclerosis[8, 9]. Research conducted by Zimmerli *et al*. on foam cells in vivo identified a specific protein in patients with cardiovascular disease in the form of α-1 collagen (type I, III) [10]. The sensitivity obtained is 98% with specificity of 83%[10]. The early stages of atherosclerosis in humans are difficult to identify. The study from Von Zur Muhlen *et al.* in mice Apo E - / - found 16 specific polypeptide. However, only nine can be identified[11]. Therefore, this study aims to determine the diagnostic performance of the urine proteome in the process of atherosclerosis on rats (*Rattus Novergicus*strain wistar) induced by atherogenic diet.

Materials and Methods

Design of this study was quasi experimental in laboratory with randomized posttest only for control group design. This study conducted at the Research Laboratory of Medicine Faculty in Universitas Islam Indonesia for 16 weeks (1 week of acclimatation and 15 weeks of experiment). The study has been approved by the Ethics Committee of Medicine Faculty, Universitas Islam Indonesia.

Subjects of this study are white rat (*Rattusnovergicus*strain Wistar) male, healthy, weighing 150-200 g, aged 2-3 months came from the Laboratory of Pharmacology, Faculty of Mathematics and Natural Science, Universitas Islam Indonesia Yogyakarta[12].A total of 22 rats divided randomly into two groups by simple random sampling: 12 in experimental groups and 10 in control groups. Experimental groups fed with atherogenic diet (feed supplemented with 10% lard and provision of Propylthiouracil (PTU) mixed with drinking water of rats to induction of atherosclerosis) and others were fed with non-atherogenic diet. Prior to dosing, the animals were acclimated to laboratory for one week. During the adaptation process, the animals were fed a standard food and *ad libitum* drink. The animals were housed in groups of 5-6 per cages.

Urine sample collection was performed after 15 weeks of diet (week 16) for quantitative measurement collagen type I as peptide marker of atherosclerosis. For sampling, the rats were placed into individual cages. Sterile plastics were placed on the bottom of the cage. Urine was observed every 30 minutes over a period of four hours. Samples were centrifuged for 20 minutes at speed of 1000xg. The particles were removed and can be used immediately or stored in aliquot with -20 °C or -80°C.

Reagent component and samples were placed at room temperature (18-25^{o}C) for 30 minutes before use. Reconstitute the Standard with 1.0mL of Standard Diluent, kept for 10 minutes at room temperature, shake gently(not to foam). Standard concentration in a stock solution was 40ng/mL. The concentration of the standard in the stock solution is 40ng/mL. Firstly dilute the stock solution to 20ng/mL and it was used as the highest standard. Then 7 tubes containing 0.5 mL of Standard Diluent were prepared based on the standard and dilution was repeated. Therefore, dilutions were resulted with the concentration of 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng / mL, 1.25ng/ mL, 0.625ng/mL, 0.312ng/mL, and the last tube was 0ng/mL. Wash buffer solution with times dilution was prepared. Stock solution of reagent A and B were also prepared. Dilute both reagent with ratio 1:100 using *Assay Diluents*. Dilution was done in 10 minutes before use.

This study prepared 7 wells for the standard and 1 well for blank. Add 100μL each of dilutions of

standard, blank and samples into the appropriate wells. Cover with the Plate sealer and incubated for 2 hours at 37°C. The liquid in well was removed. Then added 100 ml of Detection Reagent A to each well and incubated it for 1 hour at 37°C after covering it with the Plate sealer.

Investigator then aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or auto washer, and let it sit for 1-2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. After the last wash, remaining Wash Buffer was removed by aspirating or decanting. Invert the plate and blot it against absorbent paper.

About 100μL of Detection Reagent B working solution was added and incubated it for 30 minutes at 37°C after covering with the Plate sealer. Used same step like detection Reagent A. This step is performed 5 times.

Afterward, 90μL of substrate solution was added, covered with a Plate sealer, and incubated for 15 -25 minutes at 37°C. It was kept away from light. The liquid will turn blue by the addition of a substrate solution. Then 50μL of stopping solution was added and the liquid turned yellow. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. Any drop of water and fingerprint on the bottom of the plate were removed and should confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately using ELISA for quantitative measurement of type I collagen. Results about will be analyzed by computer software.

After these procedures, the animals were killed using overdosed ether then retrieve the aortic arch and coronary arteries for histopathology test on forming atherosclerotic plaque using Oil Red-O (OR-O) and Hematoxylen Eosin (HE). Then the tissues were sent to the Anatomical Pathology Laboratory to be analyzed by the Specialist Doctor on Anatomical Pathology.

On this study, we measured type I collagen as peptide marker of atherosclerosis, which was found on analytical observation of the urine proteome compared with histopathologic observation. Sensitivity, specificity, diagnostic accuracy, positive predictive value (NDP), negative predictive value (NDN), likelihood ratio positive (LR +), likelihood ratio negative (LR -) of urine proteome analysis were calculated. The ROC curve and AUC values were analyzed by SPSS 21.

Results:

The study is conducted on February 8 - 30 May 2015 (16 weeks), with one-week of adaptation and 15 weeks of experimental. A total of 22 rats divided randomly into two groups. After 15 weeks of treatment, there were 6 rats died during treatment (5 rats of control group and one rat of experimental group). Age of animals was in the range of 2 months (Table 1).

Table 1. Subject Baseline Characteristics

Characteristic of Subject	Total	Control (n=10) (%)	Treatment (n=12) (%)
Sex			
1. Male	22 (100%)	10 (100%)	12 (100%)
2. Female	0 (0)%	0 (0%)	0 (0%)
Age			
1. <2 months	0 (0%)	0 (0%)	0 (0%)
2. 2 months	22 (100%)	10 (100%)	12 (100%)
3. > 2 months	0 (0%)	0 (0%)	0 (0%)
Weight			
4. <150 gram	0 (0%)	0 (0%)	0 (0%)
5. 150-200 gram	22 (100%)	10 (100%)	12 (100%)
6. >200 gram	0 (0%)	0 (0%)	0 (0%)

The measurement results of type I collagen in urine by ELISA showed that the average is higher in the treatment group. The mean of experimental group is 1.181ng/mL and those of control group is 0.237ng/ml. Results of histopathological measurement on aortic arch and branches with Hematoxylen Eosin (HE) staining shows five samples of the treatment group showed histopathologic changes based on analyze from a doctor of anatomic pathology. Changes that occur is in the form of thickening some parts of tunica intima of aortic arch and presence of

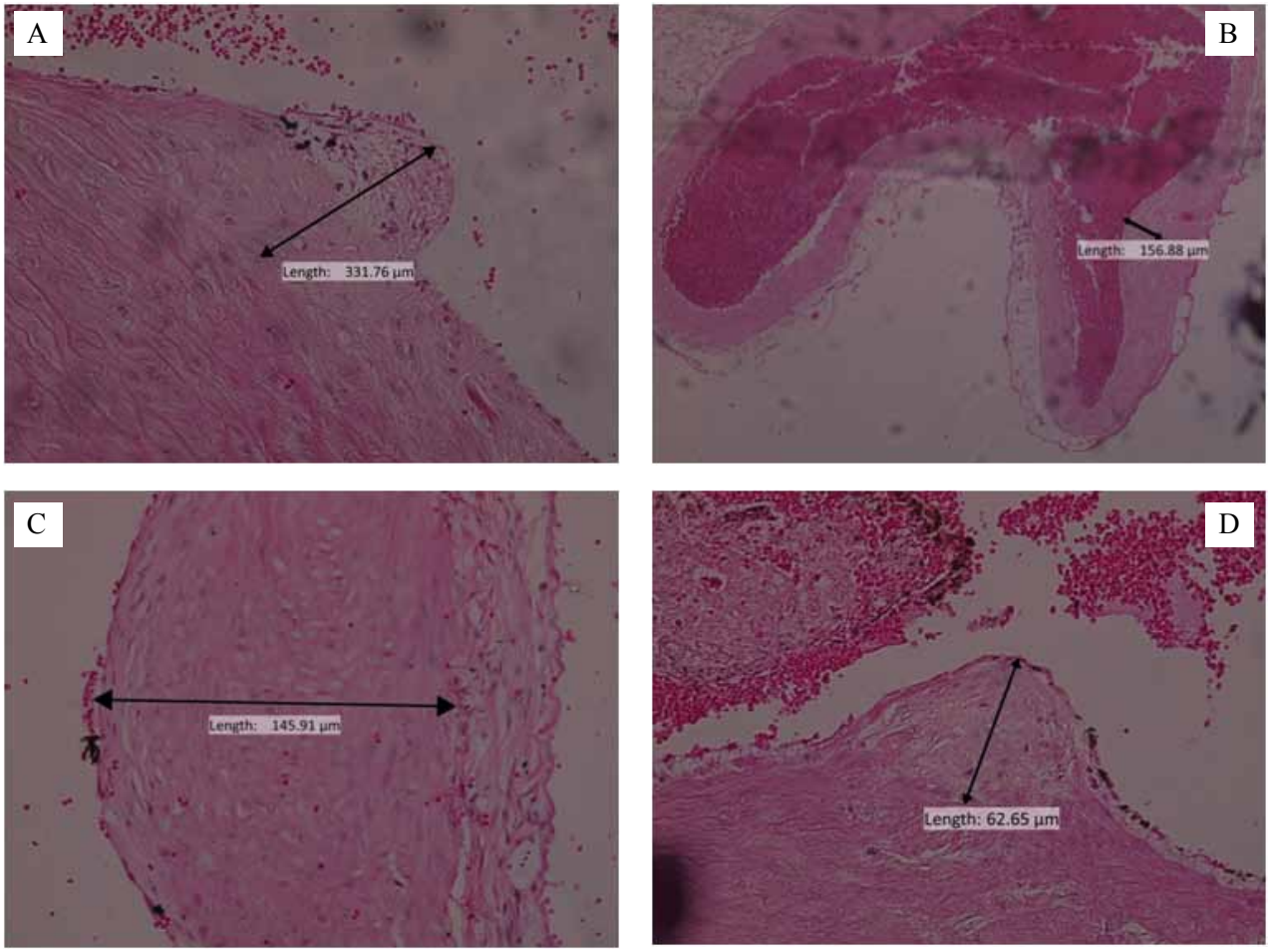

Figure 1. Presence of thickening of tunica intima and presence of *foam cell* observed by microscope with HE staining (40x40). A=5, B=7, C=8, D=11.

foam cells in subjects 5, 7, 8, 9, and 11 (Figure 1).

The results of data analysis regarding the sensitivity (Sn), specificity (Sp), diagnostic accuracy, positive predictive value (NDP), negative predictive value (NDN), likelihood ratio positive (LR +), likelihood ratio negative (LR -) on measurement of collagen type I in urine in various grades by ELISA at the end of the treatment compared with histopathology findings of the aortic arch and its branches is presented. (Tabel 2) Based on study conducted by Papasotriou, type I collagen levels in nomal rat's urine are<0.57 ng/ml. [13] When using that cut off point, type I collagen in urine by ELISA had a sensitivity of 100% but has a specificity of 63.6%. Positive predictive value of 55.5% and a negative predictive value of 100%. About 100% of patients with atherosclerosis would be detected but fairly had high false positive results 25%, so that the collagen type I on this value can be both as a screening tool but not ideal as a diagnostic tool.

Table2. Performance of Measurement Type I Collagen by ELISA Compared with Histopathologic Observation

Level of Collagen Type I (ng/ml)	Sn (%)	Sp (%)	Diagnosis Accuracy	NDP (%)	NDN (%)	LR +	LR -
0	100	9,01	0,37	33,3	100	1,10	0
0,25	100	27,2	0,5	38,4	100	1,37	0
0,50	100	45,4	0,62	45,4	100	1,83	0
0,75	100	63,6	0,68	55,5	100	2,70	0
1,00	80	90,9	0,87	80	90,9	10,9	0,22
1,25	80	100	0,93	100	91	~	0,2
1,5	80	100	0,93	100	91	~	0,2
1,75	60	100	0,87	60	100	~	0,6
2,00	40	100	14,3	100	100	~	0,6

Data were analyzed by SPSS 21 shows the ROC curve has good diagnostic value for the curve away from the line of 50% and nearly 100% (Figure 2). AUC values were obtained for 98.2% (95% CI 92.6% -100%), p 0.003. The statistical value of the relatively strong AUC. A p value <0.05 were obtained showed AUC values obtained significantly different with 50% AUC values.

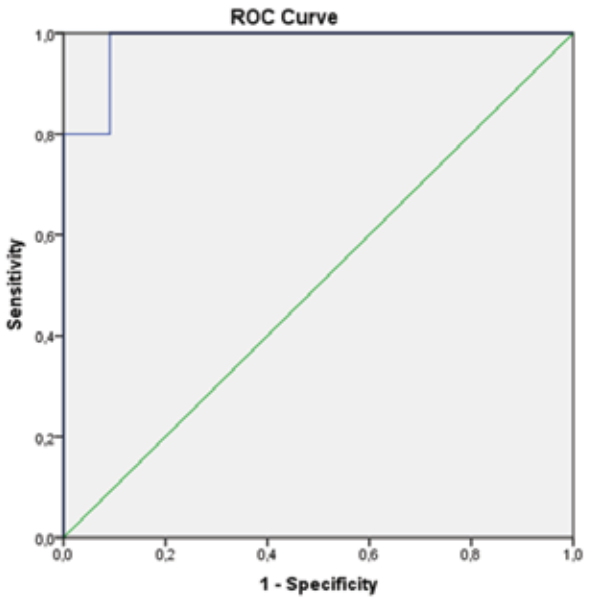

Figure 2. ROC Curve of Type I Collagen in Urine

Selection of the best cutoff for distinguishing atherosclerosis and not of atherosclerosis in this study was done through plotting sensitivity and specificity. Based on the existing curve (Figure 3), the best cut-off value was obtained at 0.9025 ng/ml with a sensitivity of 100% and a specificity of 90.9%.

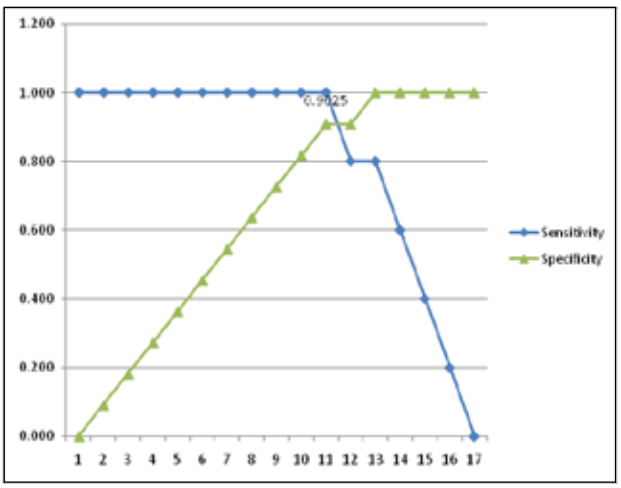

Figure 3. Intersection Curve between Sensitivity and Specificity of Diagnostic Test on Collagen Type I in Urine

Discussion

Atherosclerotic plaque is a complex of vascular cells and extracellular components. In addition to smooth muscle cells and endothelial cells, inflammatory cells are plasma proteins, extracellular matrices are formed, cellular debris and oxidation of fat or protein. It can be seen from body tissues and fluids. Approximately 345 proteins associated with cardiovascular function have been found. A sign of inflammation or cardiovascular disease is related to coagulation proteins, signaling growth, differentiation, and vascular remodeling. [14]

Collagen is the composer of 60% of the total protein plaques that contribute to the growth of plaque.[15] Collagen type I and type III is a protein of the extracellular matrix constituent largest artery. Collagen plays an important role for the stability of atherosclerotic plaque. Degradation of collagen is caused by enzymes metalloproteinase (MMP), one mechanism of collagen-rich plaque rupture.[16] This mechanism is likely to cause collagen fragments detected in patients with atherosclerosis.[17]

In this study, the mean concentration of collagen in the treatment group was higher by (1.3 ng/ml) than the mean of the control group (0.23 ng/ml). Measurements were made at week 16 after treatment for the provision of atherogenic diet. Research conducted by Von Zur Muhlen *et al.* in mice ApoE - / - induced by high-fat diet gained 16 specific polypeptides for atherosclerosis; including collagen type I. Similar results were obtained when confirmed by the discovery in human urine with atherosclerosis. Type I collagen is widely expressed in the urine.[11]

Research on humans with atherosclerotic disease also showed 238 specific proteins. Biomarkers are found including fragments of α1-antitrypsin, a fragment of type I collagen and type III, granin-like neuro endocrine peptide precursor, membrane-associated progesterone receptor component 1, sodium/ potassium-transporting ATPase gamma chain and fibrinogen- α chain.

Five specific polypeptidesare fragment of type I and III collagen. Number of specific markers returned to normal levels after treatment with long-term Ibesartan. Its sensitivity is 98% (95% confidence interval, 88.7-99.6) and specificity is 88% (95% confidence interval, 51.6-97.4.[18]

Fach, *et al.* stated that there are 59 proteins elevated in the urine after in vitro foam cell model. Foam cells are stimulated by giving LDL than HDL. There

are 9 major proteins i.e. fatty acid-binding proteins, chitinase-like enzymes, cyclophilins, cathepsins, proteoglycans, urokinase-type plasminogen activator receptor, and macrophage scavenger receptor. Collagen also increased but in a minimal amount. In this study, after being confirmed by histopathology as the gold standard, only 22.2% of the samples were positive on examination of the urine proteome, which was also positive on histopathologic examination. Meanwhile, false negatives are not obtained in this study.[19]

Collagen is not specific to atherosclerosis. Type I and III collagen can also be detected in patients with diabetes, chronic renal failure, and prostate cancer. Other studies show type I collagen is found in cancer patients with bone metastases, the bone metabolic disease, liver abnormalities, and some other chronic inflammation.[17, 20]

Research conducted by Von Zur Muhlen *et al.*, on mouse ApoE - / - induced by high-fat diet shows that collagen expression can occur because of the general effect of high-fat diet.(11) Other organ disorders are also affected (kidneys, heart, muscles, lymph nodes, spleen, and liver). The amount of collagen are excreted also do not indicate the amount of plaque. The process of plaque collagen degradation is strongly influenced by MMP enzymes. Elimination of collagen also affects the quantity of collagen. If there are abnormalities of the liver, it will increase the amount of collagen. Poor kidney filtration rate causes increased amount of collagen.[17]

According to Lopez, *et al.,* there are three limitations of collagen as a biomarker of cardiac abnormalities. First, collagen is the largest protein in the body and its metabolism is very dynamic. Secondly, there is no clear relationship between collagen as a biomarker with the pathology of heart abnormalities. Third, there has been no proper methodology for measuring collagen.[17]

This research still has still a lot of limitations. The first is for the treatment. Giving atherogenic diet with formula and term as research was not enough to induce atherosclerosis. Therefore, further research needs diet formula that is faster to induce atherosclerosis. Second, the lack of examination of internal organs outside the heart muscle to ensure atherosclerosis, which occurs only in the heart. Further research is expected to examine overall organ.

Conclusion

Analysis of urine protein in the form of collagen type I has good diagnostic capability on atherosclerosis process with a sensitivity of 100% and a specificity of 90.9%.

Conflict of Interest

Writers declare that there is no conflict of interest on this research.

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