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THE ROLE OF ENDOTHELIAL MICROPARTICLE IN CORONARY HEART DISEASE AS THE COMPLICATIONS OF DIABETES MELLITUS

Eka Fithra Elfi[1], Yose Ramda Ilhami[2], Eryati Darwin*[3]

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[1,2]Department of Cardiology, Faculty of Medicine, Universitas Andalas

[3]Department of Histology, Faculty of Medicine, Universitas Andalas. Jl. Perintis Kemerdekaan 94 Padang 25172, telp/fax. 075-131746/075-132838

e-mail:
[1]*ekafithra@yahoo.com*
[2]*yose_milanistio@yahoo.co.id*
*[3]*eryatidarwin@med.unand.ac.id*

**Corresponding author*

Abstract. *Coronary heart disease (CHD) is caused by obstruction of coronary blood flow due to endothelial dysfunction triggered by various genetic and non-genetic risk factors such as hyperlipidemia, hypertension, hyperglycemia and obesity. Endothelial cell activation due to hyperglycaemia in diabetes mellitus induces production of pro-inflammatory factors that damage the cell membrane triggering the formation of membrane particles called microparticles. Endothelial microparticles contain proteins including endothelial nitric oxide synthase (eNOS) which plays a role in the production of nitric oxide (NO). To determine the role of microparticles in the occurrence of coronary heart disease in diabetes mellitus due to endothelial dysfunction, a study was conducted by comparing the levels of eNOS and NO in DM patients who had CHD with DM patients who had no CHD. Blood samples from 20 DM patients who had CHD and 20 DM patients who had no CHD of the outpatients in Cardiology Department and Internal Medicine department of regional public hospital were included in this study. All patients were fulfilled inclusion and exclusion criteria and diagnosed by the appropriate specialist. The eNOS and NO levels were measured using the ELISA method. The results of this study show that eNOS levels in the group of DM patients who had CHD (21,292±12,415 ng/ml) were significantly lower ($p < 0.05$) than those in the group of DM patients who had no CHD (29,721±11,952 ng/ml). Nitric oxide levels in DM patients who had CHD (0,053±0,021 nmol/µl) were not statistically different to the levels in DM patients who had no CHD (0,047±0,032 nmol/µl). From the results of this study we concluded that endothelial microparticle protein eNOS plays a role in the occurrence of CHD due to the complications of diabetes mellitus.*

Keywords: *coronary heart disease, diabetes mellitus, endothelial dysfunction, endothelial nitric oxide synthase, nitric oxide*

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INTRODUCTION

Coronary heart disease (CHD) is the major cause of death in the world with mortality rate reaching 12.8% in the past two decades, especially in developed countries (Lopez et al., 2006). According to World Health organization (WHO) this disease is not only about people in developed countries, but its prevalence is seen to increase in developing coun-

tries and third world countries (WHO, 2008). Deaths from CHD in Indonesia has reached 5.1% and more than 30,000 patients are treated each year (Hasnawati et al., 2009).

Coronary heart disease is generally an obstruction in the coronary arteries due to atheroma plaques. The build-up of plaque narrows the coronary arteries and decreases blood flow to the heart which leads to signs and symptoms of coronary artery disease. The inflammatory process plays an important role starting from endothelial dysfunction that initiates atheroma plaque formation to the onset of plaque instability that results in acute myocardial infarction. The degree of inflammation is a critical component in assessing plaque stability and progression of atherosclerosis (Hansson, 2005; Packard & Libby, 2008; Sayols-Baixeras et al., 2014).

Cardiovascular risk factors are considered to play an important role in endothelial dysfunction, especially hypertension, hyperlipidemia and hyperglycemia. Hyperglycemia (High blood sugar) affects people who have diabetes mellitus (DM) (Calles-Escandon & Cipolla, 2001; Funk et al., 2014). Hyperglycemia and diabetes mellitus cause injury to the vascular system by directly damaging endothelial function and structure. Although the molecular mechanism is not fully understood, several hypotheses have been tested extensively including increased glucose flow in the polyol pathway, increased production of advanced intracellular glycation end products (AGEs) and expression of AGEs receptors, activation of protein kinase C (PKC) and over activation of the hexosamine pathway. All of these mechanisms occur as a result of oxidative stress. In studies with experimental animals Hyperglycemia was associated with the formation of an initial fat streak. Hyperglycemia increases pro-inflammatory cytokines such as IL-6 which generates c-reactive protein (CRP) which causes chronic inflammation and reduces bioavailability of nitric oxide (NO) (Esper et al., 2006; Deanfield et al., 2007; Van de Oever et al., 2010).

Vascular endothelial cells play an important role in vascular homeostasis because they regulate coagulation factors, antiplatelet and fibrinolytic processes of vascular cells, in other words they regulate the intravascular blood clotting process. Anti-thrombosis, anti-inflammatory and anti-proliferation of the endothelium are regulated by NO, S-nitrosyl (S-NO), the signal between pericytes cell and endothelium and the shear stress signal to the glycocalyx on the endothelial surface (Packard & Libby, 2008; Libby, 2012). Pro-inflammatory factors attracts the pericytes toward the endothelial cells. The interaction between endothelium and pericytes cells induces this cells to produce proteases which damage the basement membrane, generating apoptosis or necrosis of endothelial cells or a detached of endothelial cells from the basement membrane (Vidigal et al., 2012; Schiro et al., 2014).

The constitutive calcium-calmodulin-dependent enzyme nitric oxide synthase (NOS) will synthesize NO from the amino acid L-arginine in endothelial cells. Nitric oxide has a various of biological properties including maintaining vascular homeostasis and protecting vessels from injury. In different clinical disorders generating the inflammatory response, the main source of plasma NO is associated with activated eNOS and iNOS. Reducing the release of nitric oxide to the arterial wall either because of excessive oxidative synthesis or degradation causing atherosclerosis associated with various risk factors. The decrease of NO production in pathological conditions causes serious problems in endothelial equilibrium (Förstermann & Münzel, 2006; Tripathi et al., 2007; Matheus et al., 2013).

Microparticles (MP) were first discov-

ered as a fragment of platelets (thrombocytes), termed as 'platelet dust' and were released by activated and apoptotic cells. Other than platelets, microparticles can be actively induced from endothelium, vascular smooth muscle cells, leukocytes, erythrocyte lymphocytes and hepatocytes. Microparticles play an important role in physiological and pathological processes such as inflammatory processes, coagulation and endothelial dysfunction (Agouni et al., 2008; Lovren & Verma, 2013).

The microparticle membrane consists mainly of lipids and proteins and the composition and size of a microparticle depends on the origin of the cell and the process that generates its formation (Agouni et al., 2008). Endothelial microparticles are formed by vesiculation of endothelial plasma membranes and created by disruption of the stability of membrane structures containing actin, vinculin and talin. The mechanism of endothelial dysfunction by microparticle is due to a lack of production of NO and causes vascular inflammation that generates a pro-thrombotic state of atherosclerosis. Endothelial microparticles contain endothelial proteins such as eNOS, cadherin, adhesion molecules and vascular endothelial growth factor receptor (VEGF-R2) (Tramontano et al., 2010; Dignat-George & Boulanger, 2011; Owens & Mackman, 2011)

The purpose of this study was to determine the role of endothelial microparticle proteins eNOS and NO in the occurrence of coronary heart disease due to complication of diabetes mellitus as a risk factor for CHD. The benefit of this study is to inform that high blood sugar levels or hyperglycaemia in diabetes mellitus will trigger endothelial damage that can cause CHD. This is related to the formation of free radicals. High blood sugar in diabetes mellitus is harmful to the arteries, and are at a much higher risk of coronary heart disease. The condition tends to run in families, but it can be controlled by prevent blood sugar from rising such as maintain healthy lifestyle.

MATERIALS AND METHODS

This cross-sectional study was conducted on 40 subjects divided in two groups, the first group is composed of 20 DM patients who had CHD and the second group include 20 DM patients who had no CHD. They were outpatients in Cardiology Department and Internal Medicine Department of regional public hospital. Diabetes mellitus and CHD patients of this study were diagnosed by appropriate specialist, based on clinical, electrocardiogram and laboratory finding. For all subjects who fulfill the inclusion and exclusion criteria, characteristics data such as age, sex, socio-economic and other CHD risk factors were recorded. The collected peripheral blood samples from cubital veins of patients were subjected to measure eNOS and NO levels using monoclonal antibody for ELISA methods following the manufacturer's protocol, carried out in the Biomedical laboratory of the Medical Faculty of Universitas Andalas. All subjects gave written informed consent and the procedures were approved by the Research Ethics Committee of Faculty of Medicine Universitas Andalas.

Data are expressed as means ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) with the Bonferroni post hoc test were used to evaluate the significance of differences in the mean values between different samples. The Student t test is used parametric data to compare the means of quantitative variables between two groups. Patient characteristics were analyzed using nonparametric the Mann–Whitney U test. Statistical significance was assumed at $P < 0.05$.

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RESULTS AND DISCUSSION

Among the 40 DM patients studied, 20 patients had CHD and the remaining 20 had no CHD. The characteristic of patients included in the study obtained from anamnesis and laboratory finding (Table 1). There were the risk factors that cannot be changed such as age, gender and family history, and the risk factors that can be changed such as blood sugar levels, hypertension and body weight. The ages of DM patients had CHD and DM patients had no CHD were in the range of 35-55 and 32-55 years old and there were not significantly different, as well as in gender differences ($p>0.05$). Smoking, overweight and obesity were not different ($p>0.05$) between DM patients had CHD and DM patients had no CHD. There were also a similar numbers of patients with other risk factors such as hypertension and obesity. In both groups there were patients who also had other risk factors that can be changed such as hypertension and hyperlipidemia. Coronary heart disease family history of the DM patients who had CHD seems tend to be higher than in the DM patients who had no CHD, although there were not significantly different ($p>0.05$). The development of atherosclerotic CHD is gradually increases and spread across all ethnicities and regions worldwide. This is related to several risk factors such as smoking habits, abnormal blood lipid levels, overweight and obesity, diabetes mellitus and high blood pressure (Ali et al., 2010). From these risk factors, diabetes is a risk factor that has a typical relationship with CHD, in which diabetic patients have a two to four-fold higher risk of developing heart disease than people without diabetes. Besides that CHD accounts for 65-75% of deaths in people with diabetes (Aronson & Edelman 2014). Diabetes mellitus and CHD are closely associated and generally coexist in a complex medical status (Ali et al., 2010).

Table 1. Characteristics of subjects based on the coronary heart disease (CHD) risk factors in diabetes mellitus (DM) patients who had CHD and DM patients who had no CHD

No	Risk Factors		DM had CHD (n=20)	DM had no CHD (n=20)	p
1	Age		34-55 th	32-55 th	
2	Gender	Male	14	11	>0.05
		Female	8	9	
3	Smoking	Smoking	11	13	
		No smoking	9	7	
4	CHD family history	Father	5	2	
		Mother	3	1	
		Father and Mother	1	1	
		No CHD family history	11	15	
5	DM	DM	17	18	
		DM+other risk factors	3	2	
6	IMT	20 - <25	11	12	
		25 - < 30	6	7	
		> 30	3	1	

Hyperglycemia induces activation of the vascular endothelium that cause endothelial dysfunction, characterized by reduced nitric-oxide (NO)-dependent phenomena such as vasodilation and angiogenesis. The long-term effects of diabetes mellitus include cellular injury, inflammation and various organ failure complications, where one of the complications of diabetes is heart artery disease associated with endothelial dysfunction (Avogaro et al., 2011).

Energy deficiency, oxidative stress and the final product of the glycation process that occurs during the process of endothelial dysfunction can cause cell damage and apoptosis so that microparticles are formed. Circulating microparticles were increase in patients with metabolic syndrome. In vivo research by injecting microparticles from these patients into experimental animals caused a disturbance of vascular relaxation that was dependent on the endothelium of the aorta and decreased eNOS expression due to an increase in reactive oxygen species (ROS) (Martinez et al., 2005).

The eNOS level in the DM patients had CHD was 21.292 ± 12.415 ng / mL, lower than the DM patients who had no CHD (29.721 ± 11.952 ng / mL) with significant differences statistically ($p < 0.05$) (Table 2). Endothelial dysfunction is the initial occurrence of atherogenesis and involves microcirculation, wherein the inflammatory response associated with the formation of atherosclerotic plaques that causes CHD, can produce NO-induced enzymes (iNOS).

Endothelial nitric oxide synthase and NO as a product, play an important role in the control of vascular homeostasis. Most of the microparticles in the circulation originate from platelets and endothelial cells, both of which express eNOS, so it can be assumed that endothelium is an eNOS storage (Mahmoud et al., 2017). In this study eNOS levels in the diabetic patients who had CHD were lower than in the DM who had no CHD. Decreasing of eNOS protein expression and NO release causes disruption of blood vessel walls, circulating cells that express this enzyme, including platelets, endothelial progenitor cells and microparticles.

Based on the central role of endothelial cells and eNOS in controlling vascular homeostasis, it is thought that eNOS activity of microparticles is through a role in maintaining this equilibrium, and is associated with the production and bioavailability of NO. Endothelial NOS is a homeostatic regulator that is important in a variety of cardiovascular functions by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells thereby causing vasodilation of blood vessels. In patients with cardiovascular disease, disorders of vascular vasodilation are associated with decreased eNOS expression and activity in microparticles (Horn et al., 2013).

Study conducted by Helbing et al. (2014) showed that high glucose exposure on glomerular endothelial cells increased the expression of eNOS protein, but reduced release of NO. The reduction in NO bioavailability seems to be related to excess superoxide production and L-arginine deficiency. All of these changes can contribute to a decrease in plasma NO concentration in patients with essential hypertension. Decreasing of NO synthesis can also caused by abnormal processing of intracellular calcium and consequently a decrease in NOS activity (Helbing et al., 2014). But whether endothelial NO production dysfunction is the cause, or the results of atherosclerotic lesion formation are still debated. Most of the evidence supports the hypothesis that the release of NO constitutive endothelium protects against atherogenesis by preventing the proliferation of smooth muscle cells

and leukocyte adhesion. Insulin that has been linked to type II diabetes enhances the PI3K/AKT pathway. The pathway is highly regulated by multiple mechanisms, often involving cross-talk with other signaling pathways. Shi et al. (2013) studied that the specific inhibitor of PI3K not only significantly downregulated the expression of phosphorylated Akt, but also downregulated the phosphorylation of eNOS. This suggested that the PI3K-Akt signal pathway might participate in modulating the activity of eNOS. The limitation of this study is that there was no measurement of P13K-Akt activity.

Table 2. Average of eNOS levels (ng / ml) in the DM patients who had CHD and the DM patients who had no CHD

No	Groups	n	Mean±SD	p
1	DM had CHD	20	21.292±12.415	< 0.05
2	DM had no CHD	20	29.721±11.952	

The NO levels in the DM patients who had CHD were 0.053 ± 0.021 nmol / µl and in the DM patients who had no CHD group were 0.047 ± 0.032 nmol / µl (Table 3). With the normality test, NO levels were not normally distributed, and after data transformation and continued with non-parametric tests using Mann-Whitney Test, there were no differences in NO levels in DM patients who had CHD with DM patients who had no CHD ($p>0.05$). Some studies report an increase in NO levels in diabetic patients, while other studies report otherwise. Vapaatalo & Mervaala (2001) showed that there was a decrease in NO bioavailability in diabetics patients caused by an increase in oxidative stress. In addition, studies conducted on obese and DM animal model, have a decrease in bioavailability of NO (Assman et al., 2016). Hyperglycemia and other metabolic changes can reduce NO production. Hyperglycemia in diabetes stimulates the production of AGEs and protein kinase C (PKC) which can cause oxidative stress, producing ROS production, forming peroxynitrite anions as oxidants which are toxic and cause tissue injury. Injury to endothelial tissue triggers inflammation and formation of MP. It is likely that if frequently repeated this hyperglycemia-induced endothelial damage will have significant adverse clinical consequences.

Nitric oxide can have a beneficial or harm effect depending on its concentration. NO induces relaxation of blood vessels that reduce blood pressure, prevents platelet aggregation and adhesion, smooth muscle cell proliferation and decreases the expression of pro-inflammatory genes that associated with atherogenesis. Nitric oxide interacts with O_2^- which leads to NO inactivation and peroxynitrite production, which modifies post-transcription protein and reduces its function. This stimulates the production of inflammatory mediators and lipid peroxidation causing an increase in vascular permeability resulting in endothelial dysfunction (Förstermann, 2010). Blood vessels are protected against thrombosis and prevented from releasing platelet-derivative growth factors that stimulate smooth muscle proliferation and matrix molecular production. Potential inhibitors of platelet aggregation and cells adhesion to the blood vessel walls are NO that released into the vascular lumen. Endothelial NOS is an important enzyme in adaptive remodeling as a response to chronic vascular disorders.

Study about relationship between hyperglycemia and NO in diabetes mellitus have shown that individuals with metabol-

ic syndrome have increased numbers of microparticles compared to healthy people and microparticles are associated with endothelial dysfunction, due to decreased eNOS expression and increased release of ROS (Adela et al., 2005).

Table 3. Average of NO levels (nmol/µl) in the diabetes mellitus (DM) patients who had coronary heart disease (CHD) and the DM patients who had no CHD

No	Group	n	Mean±SD	p
1	DM patients had CHD	20	0.053±0.021	> 0.05
2	DM patients had no CHD	20	0.047±0.032	

From this study we concluded that eNOS and NO which are endothelial microparticles proteins play a role in the occurrence of coronary heart disease. It could be associated with the biological properties of eNOS and NO that maintain vascular homeostasis to protect endothelium from hyperglycaemia due to complication of diabetes mellitus as a risk factor for coronary heart disease.

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