Different Contribution of Estrogen Receptors, ET-1/ETBR and Superoxide Dismutase and in eNOS Availibility based on Sexual Dimorphism in Early Stage of Kidney Diabetic Rats

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Abstract: Background Diabetes mellitus is metabolic diseases which is influenced by multifactorial conditions. Many factors may contribute to DM progression, such as vasoconstrictor and vasodilator balance, as underlying factor of endothelial dysfunction. Sex difference in male and female phenotype has not been elucidated in DM progression, especially in eNOS availibility which associate with endothelin/endothelin receptors and estrogen signaling. This research aims to elucidate the expression of ERα and Erβ, ppET1 via ETBR as vasodilator properties, then SOD1 and SOD2 as antioxidant in male and female DM rats. Diabetes Mellitus was induced in male and female rats with Streptozotocin 60mg/Kg.BB single injection intraperitonially. Control group was injected with NaCl 0.9%. Rats were terminated at the 1st month. Proteinuria score and histological structure were determined in all groups. Reverse Transcription-PCR was performed to know mRNA expression of eNOS, ETBR, ERα and Erβ, SOD1 also SOD2 from kidney. All groups showed that there were no significant differences in proteinuria score and histological structure related to persistent of eNOS mRNA expression which confirmed by RT-PCR analysis. There were no significant differences between eNOS mRNA expression in DM to control groups in each sex. Furthermore, ERα and ERβ mRNA expression in female were significantly higher than male diabetic rats. Nevertheless, ETBR mRNA expression was significantly higher in male compared to female diabetic rats. Then, female diabetic groups had higher SOD1 and SOD2 mRNA expression compared to male. ERs and SOD upregulation in early diabetic female rats and ETBR upregulation in early diabetic male rats might be associated with persistent eNOS expression in early diabetic condition.

1 INTRODUCTION

The early stage of Diabetes Mellitus (DM) is characterized by hyperfiltration, caused by endothelial dysfunction in glomerular afferent arterioles (Anderson et al., 1993). Endothelial dysfunction triggers the diabetic nephropaty which as a Chronic Kidney Disease (Cheng et al., 2014). There is a study that classified the stages of diabetic nephropaty into two groups, early diabetic nephropaty based on the beginning of GFR (Glomerular Filtration Rate) enhancement, and the

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advanced stage of diabetes nephropaty starting from the decrease of GFR (Eleftheriadis et a., 2013). This classification easily determine the patient based on the differences of vascular function in chronic kidney disease, which nowadays still have a limited data. Therefore there is a need for the approval of vascular function in intial chronic kidney disease (Forbes et al., 2003).

DM causes the endothelial dysfunction which is affected by the decrease of Endothelial Nitric Oxide (eNOS). eNOS helps relaxation of vascular smooth muscle cells by activating Nitric Oxide directly (NO). Stadler et al. (Stadler et al., 2003) revealed the enhancement of eNOS in the early stages. There are several potential vasoactive to increase NO production, namely as estrogen hormone through the estrogen receptors and endothelin-1 through the ETBR. Increased oxidative stress is recognized as the major metabolic abnormality involved in the development of diabetic nephropaty. ROS production is increased in endothelial and renal cells in hyperglycemic conditions.

Increased oxidative stress also is recognized as the major metabolic abnormality involved in the development of diabetic nephropaty. ROS production is increased in endothelial and renal cells in hyperglycemic conditions. ROS can be dishminis by antioxidant enzyme enzymes as though superoxide dismutase, SOD1 and SOD2. But, The overproduction of ROS diminishes expression of the antioxidant. SOD1 is located in the cytoplasm, SOD2 in the mitochondria (Alejandra et al., 2016).

There is a study that suggests the possibility of gender differences in the pathophysiology of the disease. As in line as the studies, found that nondiabetic premenopausal women have a lower risk of having CKD and a slower rate of progression to end-stage kidney disease compared to non-diabetic men with the same age. There is a study that also mentioned that sexual dimorphism affected the NO system. Female rat has a higher NO level than males, this is because eNOS can be converted by estrogen into NO large amounts (Pesce et al., 2005).

There is no study about the expression of eNOS, ET-1 ETBR, SOD1, SOD2, and Estrogen Receptors based on sex and in the early stages of diabetes mellitus. Therefore this study was conducted to assess the differences in vasoprotector aspects namely as estrogen receptor and ET-1 via ETBR which can affect the changes of eNOS in female and male diabetic rats in the early stages of diabetes. And also SOD1 and SOD2, important antioxidant defense in endothelial cells.

2 MATERIALS AND METHODS

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3 MATERIALS AND METHODS

3.1 Samples

This research was conducted in Animal House Anatomy Laboratory Universitas Gadjah Mada, November 2019 – Januari 2020. This study was approved by the Ethics Commission of the Faculty of Public Health and Nursing at Universitas Gadjah Mada with the number KE/FK/1445/EC/2019. Sprague-Dawley rats (Rattus norvegicus) were used in this study. The treatment group was divided into 4 groups with 6 rats in each group. This calculation uses the Frederer formula. The division of these groups are Control Female (CF), Diabetes Mellitus Female (DMF), Control Male (CM) and DMM (Diabetes Mellitus Male).

3.2 Animal Care

In this study, Sprague Dawley rats (3 months, 200-300 grams) were obtained from the Animal Research Unit. Rats are kept in plastic cages measuring 30x40x20 cm, at 23-25 ° C, 40-70% humidity, and dark:light cycle every 12:12 hours. The rats were acclimated for 1 week by adlibitum, given the standard feed AIN-76A and boiled drinking water. Type 1 DM models were prepared by injection of Streptozotocin (60 mg / kgBB dissolved in 0.1 M citric acid pH 4.5) with a single dose intraperitoneally. Whereas the control group was injected with NaCl. STZ injection was done after acclimation for 7 days (8th day). Success was seen by measuring glucose levels over 300 mg/dL. Tissue collection was carried out after 1 month of animal care. Right kidney for transcriptomic testing, fixed

using RNAlater (Ambion) and immediately put in the -20oC freezer while the left kidney was having a fixation using formalin neutral buffer for histological preparation.

3.3 RNA Extraction, cDNA Synthesis, and Reverse Transcriptase Polymerase Chain Reaction

Kidney tissue was extracted using Genezol RNA solution (GENEzol™, Cat. No. GZR100) based on the protocol from the manufacturer. The RNA concentration was quantified using a nanodrop. The RNA was synthesized into cDNA using cDNA kit (Toyobo, Cat. No. TRT-101) with PCR. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to amplify the following specific cDNAs.

RT-PCR was performed by mixing cDNA and Taq Master Mix (GoTaq®Green Master Mix, Cat. No. M7122). The PCR products were analyzed on 2% agarose gel along with a 100-bp DNA ladder (Bioron, Germany, Cat. No. 306009). The expression of the genes was quantified with a densitometry analysis using the ImageJ software. B-actin expression was used to normalize the expression

Table 1. Primers for PCR Analysis

Gen		Sequence (5' -> 3')
ppET-1	Forward	CTGGCTCTATGTAAGTCATGG
	Reverse	GCTCCTGCTCCTCCTTGATG
eNOS	Forward	AAATCCACCCGAGCCACAAT
	Reverse	GGGCTGCCTTTTTCCAGTTG
ET_BR	Forward	TCTCAGCCTTTTGTCCGAGC
	Reverse	CGCCGTTTTCAGTCTCGCA
SOD1	Forward	GCGGTGAACCAGTTGTGGTG
	Reverse	AGCCACATTGCCCAGGTCTC
SOD2	Forward	ATGTTGTGTCGGGCGGCGTGCAGC
	Reverse	GCGCCTCGTGGTACTTCTCCTCGGTG
ERα	Forward	CACACACGCTCTGCCTTGAT
	Reverse	GAGCCACCCTGCTGGTTCAA
ERβ	Forward	GCCAATCATGTGCACCAGTTCCTT
	Reverse	AAAGCCAAGAGAAACGGTGGGCAT
β-actin	Forward	GCAGATGTGGATCAGCAAGC
	Reverse	GGTGTAAAACGCAGCTCAGTAA

3.4 Periodic Acid Schiff Staining

Deparaffinization was done by dipping the glass in xylol and then dipping it in alcohol level 96% to 30% after organ embedded in paraffin. The preparations were then washed using distilled water (Aquades). Then dipped in 1% alcian blue for 5 minutes. Then dipped it in 1% periodic acid for 5 minutes. Dipped it in Schiff reagent for 3-5 minutes then washed it using flow water for 10 minutes. Then the level of alcohol dehydration was done from 30% to 96%. Mounted using an entelan.

3.5 Data Analysis

All data from RT-PCR were tested for Saphiro Wilk's normality and continued with the One-way Anova or Kruskall Wallis test, comparing CF and DMF, CM and DMM, DMF and DMM.

4 RESULTS

4.1 Proteunia Score, Blood Gucose Levels and Kidney Histology

Measurement of protein levels was also carried out to determine the function of kidney filtration [8]. The results of blood glucose and protein levels are as follows:

Blood glucose levels in the DM group both males and females had levels >300 g / dl and the value was significant for the control group ($p<0.05$), which means this group had hyperglycemic conditions. While the protein content in the urine in the DM group was not significantly different compared to the

control group in each gender. it could be said that the kidney was considered to be able to carry out its functions properly. This can be proven with the following histological preparations:

Table 2. Protein levels in urine (mg / 100mL) and blood sugar (g / dL) mice after being injected with STZ 1 month before

Level	CF	DMF	CM	DMM
Blood glucose (g/dL)	106,33 ± 11,5	531,33 ± 99,25#	231,5 ± 29,13	497 ± 103, 15#
Urine protein (mg/100mL)	16,67 ± 15,05	25 ± 12,24	15 ± 16,43	25 ± 12,24

Values in the same line show significant differences ($p \leq 0.05$) versus controls of the same gender. Mean ± Standar deviation.

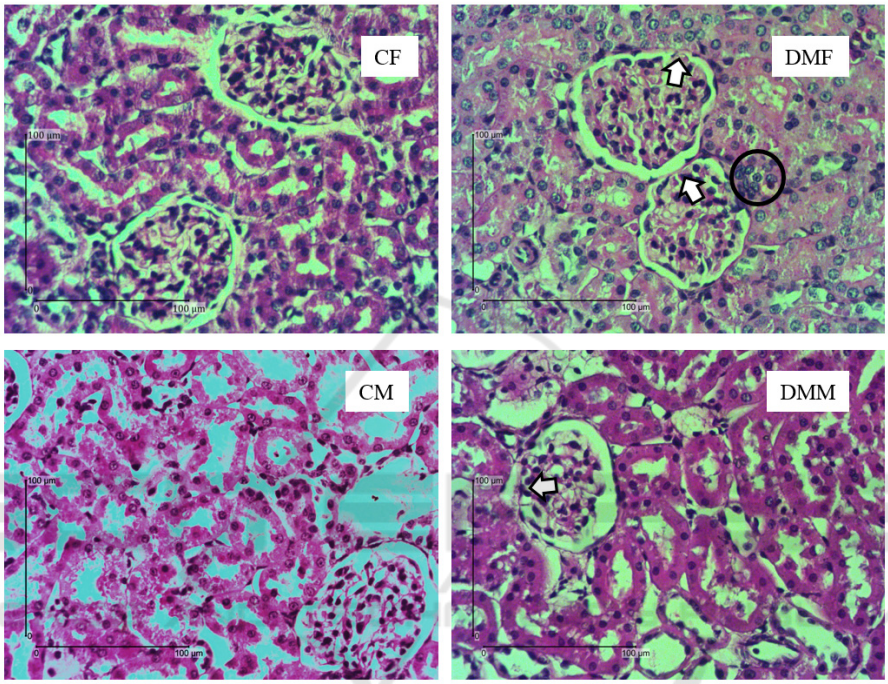

Figure 1. Histological structure of rat kidneys in the early stages of diabetes by PAS staining. The mark of ⇨ shows synechiae, ○ shows infiltration of imflamatory cells.

4.2 eNOS mRNA Expression

The picture above implies that there have not been any noticeable changes in the glomerulus and renal tubules in the diabetes group when compared to the control group in both males and females. There was no visible loss of brush border in proximal tubule and intraluminal cast which is a marker of tubular injury. However, in DMB there were accumulation of inflammatory celss in the insterstitial area. It did not occur in DMJ. In addition, although the glomerulus of the DM group had synechiae, there was no visible thickening of the basement membrane (Setyaningsih et al., 2017; Haryono et al., 2018).

The results obtained from eNOS gene amplification showed that there was no significant difference in the STZ injection treatment compared with controls in each gender ($p > 0.05$) as well as between the male DM rat group and the female DM group ($p > 0.05$).

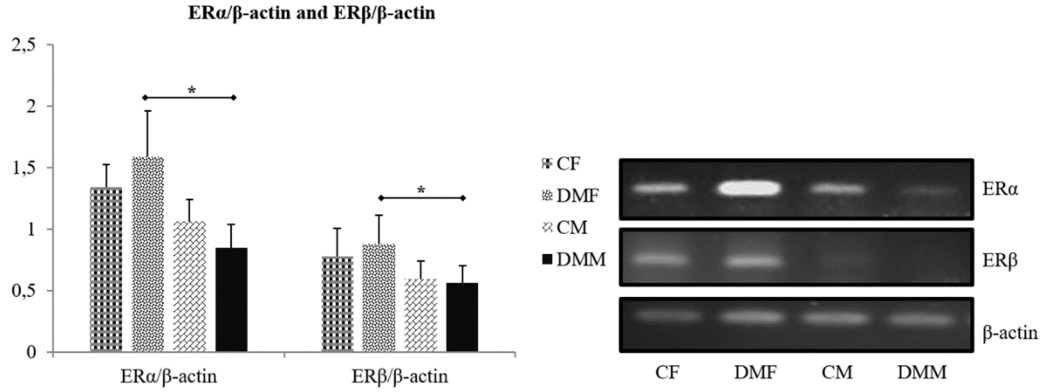

Figure 2. eNOS mRNA Expression in Rat Kidney in Early Stag Diabetes Models

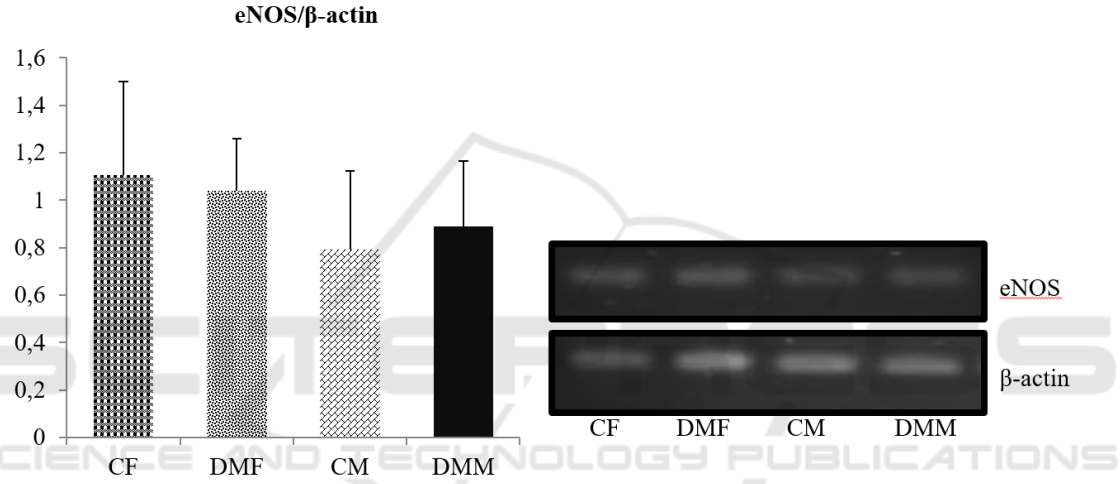

Figure 3. Estrogen receptor mRNA expression in rat kidney in early stage diabetes models. * shows a significant difference ($p \leq 0.05$).

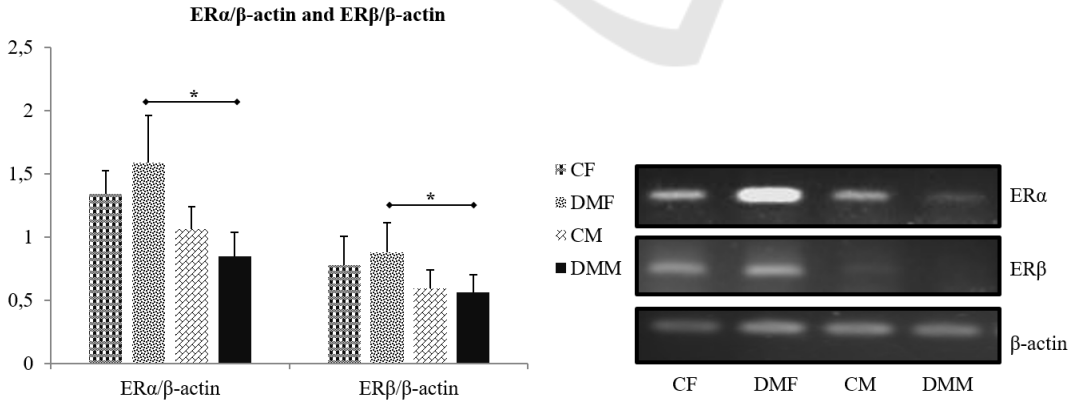

Figure 4. Estrogen receptor mRNA expression in rat kidney in early stage diabetes models. * shows a significant difference ($p \leq 0.05$).

4.3 Estrogen Receptors mRNA Expression

The result showed that there was no significant differences between the control group and the DM group in the ERα and ERβ genes in both male and female gender ($p > 0.05$). In female, estrogen receptor gene amplification in the DM group showed an upward trend, whereas in male species tended to decrease.

4.4 ppET-1/ ETBR mRNA Expression

The ppET-1 mRNA expression showed that there was no significant differences between the control groups of female and female DM, male and male DM control and between female DM and male DM ($p > 0.05$). Whereas in the ETBR gene the results obtained showed significance in the male DM group compared with the female DM group. Male DM group had higher gene expression than female DM groups ($p \leq 0.05$). These results can be chosen from the following histogram:

4.5 SOD1 and SOD2 mRNA Expression

From the picture above, there was no significant difference in SOD1 mRNA expression in the female groups, whereas the DMM was lower than their control. The figure also showed that DMF mRNA expression was higher than DMM. Meanwhile, the expression of SOD2 mRNA in the DM group was higher than the control group in each sex. While the male and female DM groups did not have significant differences, but had a tendency for DMF to be higher than DMJ.

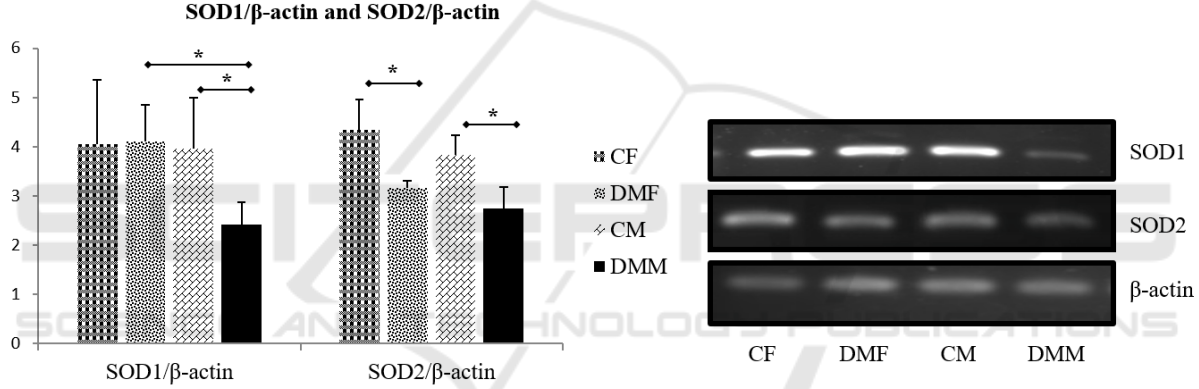

Figure 5. Expression of SOD1 and SOD2 mRNA in rat kidney in early stage diabetes models. * shows a significant difference ($p \leq 0.05$)

5 DISCUSSIONS

In the early stages of diabetes, levels of Nitric Oxide (NO) increase while in the advanced stages of diabetic nephropaty, the NO levels decrease (Nakagawa et al., 2008). The study was confirmed by the research of Stadler et al. (2003) which stated that an increase in eNOS works as a vasodilator of blood vessels.

The results obtained from eNOS gene amplification showed that there was no significant difference in the STZ injection treatment compared with controls in each gender as well as between the male DM rat group and the female DM group. The results obtained are not in accordance with the theory, it is possible because the kidneys used in this study was the entire kidney, which consist of the cortex and medulla while according to the study of Han et al., (Han et al., 2005) stated that there are a differences in the concentration of eNOS expression in the renal cortex and medulla. The expression of eNOS in the renal medulla is higher than the cortex, especially in the arcuate and interlobular arteries. This can be taken into consideration because there may be the NO donors between the cortex and medulla (Nakagawa et al, 2008). Kidney Vascular Tree protein isolation also proves that there is a significant increase in eNOS in the early stage of diabetic rats rather than isolation of all parts of the kidney by immunoblotting methods (De Vriese et al., 2001).

In diabetic kidney, there are hemodynamic changes characterized by hyperfiltration (Hadi et al., 2007). The enhancement of GFR is associated with glomerular hyperfusion caused by the reduction of intrarenal vascular resistance and hypertension from glomerular capillaries resulting in the decreased blood flow of preglomerular resistant blood vessels

compared to postglomerular. Although renal (kidney) vasodilation experiments in diabetes are still under debate, some researchers suggest that an enhancement in NO associated with hyperfiltration in the initial diabetes is supported by the administration of L-arginine which is an endogenous inihibitor eNOS and the administration of NOS blockers that prevent excessive hypefiltration and decrease normal GFR in the early stages of animals testing the of diabetes (Diederich et al., 1994).

Another consideration of the causes of eNOS results that are not significant between treatments is the evaluation of other factors that may be responsible for the changing response (both increasing and decreasing) in the early stages of diabetes. One example of an experiment that caused no change in eNOS in the early stages of diabetes in the study was the inability of daltobran, which is an antagonist of the Tromboxan A2 receptor, in blood vessel relaxation in the 8th week. This happens because vasoconstrictor agents are still stronger than daltobran (Wells et al., 2005).

In this study, gender also affects the differences in eNOS concentrations. Estrogen through estrogen receptors modulating eNOS to relax blood vessels is also one of the factors that can be evaluated, whereas in the male sex, estrogen is not found in abundant quantities. The result showed that there was no significant differences between the control group and the DM group in the ERα and ERβ genes in both male and female gender. In female, estrogen receptor gene amplification in the DM group showed an upward trend, whereas in male species tended to decrease. However, there were significant differences between female DM groups and male DM groups. The male DM group was lower than the female DM group on the amplification results of the two estrogen receptor genes. This is possible due to the mention that sexual dimorphism affects the NO system. Female mice have a higher NO level than males (Stadler et al., 2003)..

Wells et al., (Diederich et al., 1994) confirmed this study by stating that the condition of hyperglycemia in animal models was followed by a tendency for increased expression of ERα protein in the female DM group and a decreased tendency in the DM group with male. Similarly, the expression ERβ. The study also mentions that there has been a decrease in estrogen levels in blood in male and female DM rats that are not directly caused due to hyperglycaemia, but are directly caused by the absence of insulin which can reduce the ability to convert androgens to estrogen or decrease aromatase activity (Barkhem et al., 1998). The relations between DM and estrogen can be proven by the presence of menstrual disorder that often occurs in diabetics who cause abnormal ovarian hormone synthesis. This implies that the regulation of estrogen receptors is not directly caused by hyperglycemia but is caused by the hormone estrogen itself. In addition, the increase in estrogen receptors in female DM groups may be due to estrogen through estrogen receptors which are known to regulate glucose uptake and increase insulin sensitivity (Weels et al., 2005).

Endothelin-1 is also one factor that must be considered because Endothelin-1 is a powerful vasoconstrictor with mitogenic, prooxidative and proinflammatory abilities of vascular function that is significant to vascular function. Excessive production and functional improvement of ET-1 are reported to be agents of development of diabetic nephropaty (Pernow et al., 2012). Whereas ET-1 regulation through ETBR will cause relaxation of blood vessels. The ppET-1 mRNA expression showed that there was no significant differences between the control groups of female and female DM, male and male DM control and between female DM and male DM. Whereas in the ETBR gene the results obtained showed significance in the male DM group compared with the female DM group. Male DM group had higher gene expression than female DM groups.

There was no differences in ppET-1 mRNA expression in the early stages of diabetes mellitus in each treatment group. This is different from the theory that gender affects the expression of ET-1. Pesce et al. mentions female rats have higher NO levels than males. This is proven by the administration of testosterone in transsexuals causing increased levels of ET-1 (Polderman et al., 1993). Expression of ppET-1 gene that was not significantly different between DM groups in each gender was possible because estrogen did not affect ppET-1 levels at mRNA levels. This was evidenced by expression of ECE (Endhotelin Converting Enzyme) which did not differ between treatments (Nuedling et al., 2003).

ETBR expression in the male DM group was higher than females. It might be said that the stable state of eNOS in the DM group compared to the control in the male group was due to high ETBR expression. So that it can activate eNOS and produce NO. ETBR is expressed in endothelial cell muscle, ETBR is responsive to all ET isoforms that cause vasodilation by releasing NO and PGI2 (Soe et al., 1994). This evidence allows that ETBR is used as a vasodilator for blood vessel protection in male rats. In addition, estrogen has the ability to derive ETBR regulation through structures known from the ERE

(estrogen response element) found in the 5 'upstream region of the ETBR gene (Cheng et al., 1993).

Diabetes can increased oxidative stresses that occurs when reactive oxygen species (ROS) leviated. Then, antioxidant was formed as defence system. 4, 25. Enzyme superoxide dismutase can formed hydrogen peroxide as a stable product of ROS from free radicals in response to inflammatory conditions 32. Based on the results, mRNA expression SOD1 and SOD2. in DMF groups was higher than DMM. It might be associated with estrogen can act as potensial antioxidant which could contribute to eNOS persistent 22, even SOD1 in DMF is noteworthy than CF. Less of SOD is needed in women compared to men who will not benefit from the antioxidant properties of estrogen. Hamed et al. found that SOD can restores NO production and ability of glucose-stressed endothelial progenitor cells.

6 CONCLUSIONS

Estrogen receptors and superoxide dismutase upregulated in early diabetic female rats. They can provide signal for good function of endothelial cell. Moreover, ETBR upregulated in early diabetic male rats might be associated with persistent eNOS expression in early diabetic condition. ETBR knockout in diabetics rats can be used to demonstrate ppET-1/ ETBR signaling for vascular relaxation via eNOS in males.

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