ORIGINAL ARTICLE

Centella asiatica Improves Kim-1, eNOS and GPX-1 Kidney mRNA Expression in Diabetes Mellitus Rat Model

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ABSTRACT

Introduction: Diabetic nephropathy is one of the most common complications in diabetes Mellitus. Hyperglycemia and chronic inflammation cause abnormal oxidative stress deposition that leads to the decrease of glutathione peroxidase-1 (GPx1) and endothelial Nitric Oxide synthetase (eNOS). It was reported that *Centella asiatica* has an anti-hyperglycemic and anti-inflammatory effect. However little is known about *Centella asiatica* effect in the kidney of DM. The objective of this study was to know the effect of *Centella asiatica* extract on Kim-1 (marker of kidney damage), GPx1 and eNOS mRNA expression in the kidney of DM rat model. **Methods:** Wistar DM rat model was divided into 6 groups namely non-DM group, DM group , DM with captopril and another DM group treated with *Centella asiatica* with three different dosages (250, 500 and 1000 mg/kg BW). The treatment was given for 8 weeks. The Kim-1, GPx1 and eNOS expression was measured using semi-quantitative PCR. **Results:** The DM group showed higher Kim-1 kidney mRNA expression but lower GPx1 and eNOS kidney mRNA compare to those on the non-DM group. Administration of *Centella asiatica* improves the expression of Kim-1, GPx1 and eNOS kidney mRNA expression in DM rat model. **Conclusion:** *Centella asiatica* has the potential to prevent kidney damage in DM rat model by improving Kim-1, GPx1 and eNOS kidney mRNA expression.

Keywords: Diabetes nephropathy, Kim 1, GPx1, eNOS, *Centella asiatica*

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INTRODUCTION

Diabetic nephropathy is a multifactorial disease, where many factors take their roles in deteriorating kidney condition. It is a set of characteristics of structural and functional kidney problems in the diabetic patient. The abnormalities including kidney hypertrophy, glomerular basement membrane thickness increase, nodular and diffuse glomerulosclerosis, atrophy of tubular, and fibrosis interstitial (1,2). Diabetics nephropathy development imvolves several factors such as hyperglicemia and inflammation which further leads to another biological changes that finally deteriorate the kidney. Hyperglycemia and chronic inflammation cause abnormal oxidative stress deposition that leads to the decrease of glutathione peroxidase-1 (GPx1) and endothelial Nitric Oxide synthetase (eNOS). The GPx is known to have a protective function from oxidative damage. In nephropathy, there are abnormal cells oxidative stress conditions. Oxidative stress is a condition which results from imbalance redox state in which pro-oxidant is overwhelming antioxidant capacity. Hyperglycemia state that is one of the main causes of diabetic nephropathy, is found to provokes the increase of ROS. It was found that animal with nephropathy has lower GPx activities than it was at normal control animal (3, 4). There are six known GPx isoforms existed, GPx1 until GPx6. The GPx1 expression is the most abundant in almost all tissues (5). Meanwhile, eNOS is the major enzymes related to nitric oxide production in kidney vasculature. In diabetic kidneys, eNOS expression has been proven to be upregulated in early stages (1-6 weeks), followed by downregulation as the disease become progressive. However, even though eNOS expression is upregulated in the beginning, NO activity and eNOS are decreased in diabetic nephropathy (6).

Kidney Injury Molecule-1 (KIM-1) is a protein that increased in kidney damage especially in tubular injury including those secondary to diabetic nephropathy (7). KIM-1 is expressed by ischemic or toxic injured proximal tubule epithelial cells and functions as a regulator of cell adhesion and endocytosis in the regeneration process of the kidney (8,9).

Centella asiatica has been shown its potential as antioxidant, anti-inflammatory, anti fibrotic and other

medicinal activities. In vivo and in vitro studies has been shown *C. asiatica* ability to improve diabetic wound healing, cardio-renal protection, and other pathological conditions(10). However little is known about its renal protection effect mechanism especially its effect on Kim-1, eNOS, and GPx1 expression. This study aimed to examine the effect of *C. asiatica* effect on Kim-1, eNOS, and GPx1kidney mRNA expression of diabetes Mellitus rat model.

MATERIALS AND METHODS

Rats were divided into non-DM groups, DM groups, DM treated with captopril, DM treated with DOSE 1 (250 mg / kg BW / day), DOSE 2 (500 mg / kg BW / day), and DOSE 3 (1000 mg / kg BW / day). The DM was induced by 120 mg/kg BW Nicotinamide (NA) injection and of 60 mg/kg BW Streptozotocin (STZ) injection (6). Rats were divided into non-DM groups, DM groups, DM treated with captopril (DMC), DM treated with DOSE 1 (250 mg / kg BW / day) (DMD1), DOSE 2 (500 mg / kg BW / day) (DMD2), and DOSE 3 (1000 mg / kg BW / day) (DMD3). Treatments were given for 8 weeks.

The rats was terminated by overdose of anesthetic agent. The kidneys were collected and preserved in RNA preservation solution before RNA extraction. The RNA from kidney tissue was isolated with FavorPrepTM Tissue Total RNA Purification Mini Kit (Favorgen, FATRK001-1) 2.2. The cDNA was produced with High capacity cDNA Reverse Transcription Kit (Applied Biosystem, LT-22041)

The PCR was processed with Kim-1, GPx1, and eNOS specific primers for each procedure. The Kim-1 primer consisted of forward (5′-TGGCACTGTGACATCCTCAGA -3′) and reverse (5′-GCAACGGACATGCCAACATA-3′) primers. PCR was then performed with 40 cycles of 95°C for 60 seconds, 57°C for 60 seconds, and 72°C for 60 seconds with final extension phase at 40°C for 10 minutes, and then stored at -200°C.

The Gpx-1 primer was consisted of forward primer (5′-TCC CTT GCA ACC AGT TCG -3′) and reverse primer (5′- CTT GAG GCT GTT CAG GAT CTC -3′). The PCR is performed with 35 cycle of 95°C for 60 seconds, 57°C for 60 seconds, and 72°C for 60 seconds with final extension phase at the end then stored at -20°C.

The eNOS mRNA expression test was done using a forward primer (5′- CCG GCG CTA AGA ATG -3′) and reverse primer (5′- AGT GCC ACG GAT GGA AAT T-3′). PCR was done by using 94°C starter for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. The PCR was ended with a final extension at 72°C for 10 minutes and 4°C for 10 minutes. The solution was then stored at -20°C.

The GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as the housekeeping gene. PCR were done with combination of forward primer (5′- CATCCTGCACCACCAACT G -3′) and reverse primer (5′- GGATGCAGGGATGATGTT-3′). The reactions was done in 35 cycles of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute. All the PCR products were processed through the electrophoresis in 1.5% agarose gel. The agarose gel is then read using UV light and the picture of the elctrophoresis result was documented. The density of the PCR product band in the picture were measured using densitometry ImageJ software. The data presented density ratio of the density of the PCR band of GPx or eNOS or Kim-1 and divided with the density of the PCR band of GAPDH.

The data is presented as mean ± Standard Deviation (SD). The mean of each group were compared using ANOVA continued by post hoc analysis.

RESULTS

The expression of Kim-1 mRNA can be seen in the electrophoresis result in Fig 1A. The expression measured and presented as Non-DM (0.9177+ 0.0255), DM (1.1890 + 0.1455), DMC (1.1065 + 0.0795), DMD1 (0.9863 + 0.0323), DMD2 (0.9457 + 0.0806), DMD3 (0.7972 + 0.0384). The data presented in Fig 2A. Kim-1 expression is significantly lower in a group treated with the highest dose of *C. asiatica* (DMD3) compare to those on DM group.

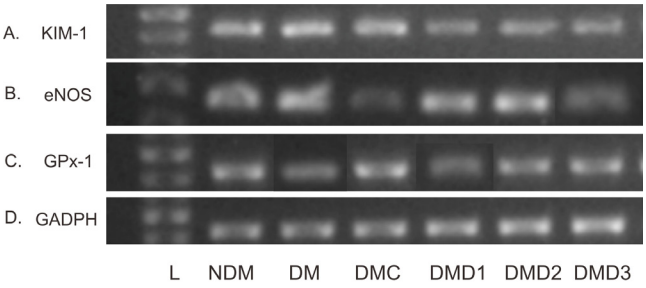

Figure 1: Representative image of electrophoresis result from PCR product of (A)KIM-1, (B) e-NOS, (C) GPx-1, and (D) GAPDH kidney mRNA expression of Wistar rat diabetic model. NDM= normal group; DM= DM group without therapy; DMC= DM group with captopril; DMD1= DM group with extract dose 1, DMD2= DM group with extract dose 2; DMD3= DM group with extract dose 3

The expression of eNOS gene after 8 weeks of treatment is shown in Fig 1B. The data are presented in Fig 2B. The highest mean was in the DM group and the lowest mean was in DMD3 group. Similar with Kim-1 expression, the DMD3 group showed significantly lower expression of eNOS compare those on DM group.

Kidney GPx1 mRNA expression level was also measured after 8 weeks of treatment. The result of kidney GPx1 mRNA expression level is presented in mean SD on

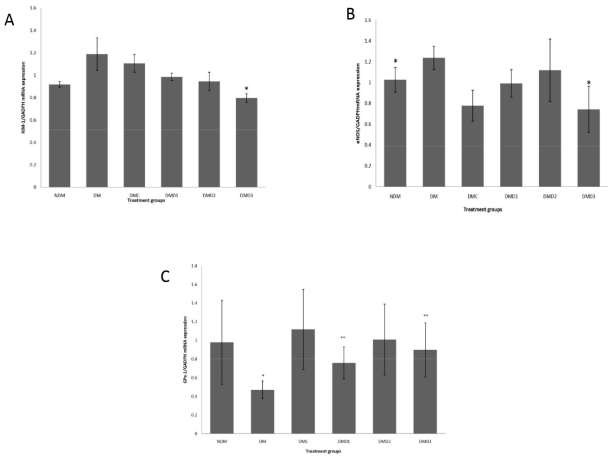

Figure 2: (A)Mean of Kim-1/GAPDH ratio in all groups. *p<0.05 compare to DM group. (B)Mean of eNOS/GAPDH ratio in all groups. *p<0.05 compare to DM group. **(C)Mean of GPx-1/GAPDH ratio in all groups.** *p<0.05 compare to NDM and DMC group. **p<0.05 compare to DM group. Data presented as mean+SD. NDM= normal group; DM= DM group without therapy; DMC= DM group with captopril; DMD1= DM group with extract dose 1; DMD2= DM group with extract dose 2; DMD3= DM group with extract dose 3

the Fig 2C. The lowest kidney GPx1 mRNA expression level happened to be in the DM group, and the highest is on the DMD2 treatment group. The PCR product representative picture can be seen in the electrophoresis result in Fig 1C.

DISCUSSION

The increased expression of Kim-1 occurred especially in injured renal proximal tubule epithelial cells and associated with the regeneration process of the cell. The extracellular domain of Kim-1 consists of a structure that homolog to some adhesion protein showing the possible role of Kim-1 in cell adhesion (4). However, our result shows that KIM-1 is also expressed in Non-DM group. The previous study by Ahmed and Hamed suggest that there is an association of Kim-1 and sex (12).

The study results showed that *C. asiatica* reduces Kim-1 mRNA expression. The phenolic compound responsible as an antioxidant agent in *C. asiatica* as it can donate a hydrogen atom to free radicals (13). The asiatic acid component in *C. asiatica* may improve tubule-interstitial fibrosis by reducing tubular injury, fibroblast activation and extracellular matrix (ECM) accumulation mediated by TGF-β1 signaling. It is also stated that the attenuation of tubulo-interstitial fibrosis by Asiatic acid is a dose-dependent that explain the lowest expression of KIM-1 in DMD3 group (14).

The result of this experiment clearly shows that eNOS expression is reduced by administration of captopril. This happened because angiotensin activates NADPH oxidase through angiotensin-1 receptors stimulation. NADPH oxidase will then produce superoxide ($O2^{\bullet-}$) that will bind with NO and creates eNOS encoupling ($ONOO^-$), which act as oxidative stress. The drugs that interfere with RAA system will reduce NADPH oxidase activation an eventually reduce eNOS encoupling (15). In a study done by Benzie and Tomlinson, out of all drugs tested in ACE inhibitor class, captopril has the highest antioxidant power (16).

There is statistically significant difference between DMD3 and DM group in term of eNOS mRNA expression, which suggest that there was decreasing of eNOS expression after treatment of C.asiatica in dose of 1000 mg / kg BW / day. eNOS is a gene that is associated with diabetic nephropathy. An in vivo study showed an increase in the number of eNOS in preglomelural vessels in diabetic rat glomeruli and the addition of NOS inhibitors prevented an increase in glomerolus filtration rate. This shows that NOS contributes to glomerular damage resulting in diabetic nephropathy (17).

In nephropathy, there are abnormal oxidative stress conditions. Oxidative stress is conditions which result from imbalance redox state in which pro-oxidant are exceed antioxidant capacity, which one of them is GPx. Hyperglycaemia state which is the main cause of diabetic nephropathy, is found to provoke the increase of Reactive Oxidative Stress (ROS). It was found that animal with nephropathy has lower GPx activities than it was at normal control animal (7,8). *C asiatica* is shown to prevent further diabetes pathogenesis. It also may prevent complication in type 2 diabetes patients such as diabetic nephropathy (11). The antioxidant effect of *C asiatica* (L.) Urban is shown to be as good as α-Tocopherol. The antioxidant effect is suggested that it is come from the phenolic compound within the extract, specifically within the leaf and roots (13). GPX is known to have a protective function from oxidative damage (9), this may explain the increase of GPX expression level in *C asiatica* group treatment.

CONCLUSION

C asiatica has the potential to prevent kidney damage in DM rat model by improving Kim-1, GPx1 and eNOS kidney mRNA expression.

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