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Original Article

Renoprotective effects of EGb761 in spontaneous hypertensive rats with diabetes

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Abstract: The major objectives of this study were to investigate whether EGb761 would exhibit renoprotective effects in spontaneous hypertensive rats (SHR) with coexisted diabetes mellitus (DM), and whether this effect might be associated with attenuation of the inflammatory response and oxidative stress. Streptozocin (STZ) was injected to SHR rats to establish SHR-DM animal model. EGb761 (50 mg, 100 mg and 150 mg /kg/day) was orally administrated to SHR-DM rats for 12 weeks. Blood pressure and glucose were monitored during the treatment. Renal damage biochemical markers (urinary osteopontin, KIM-1 and albumin), oxidative stress/antioxidant markers (MDA, H_2O_2, SOD and GSH-Px), NF-κB, and inflammatory cytokines (IL-1β, IL-6, TNF-α, VCAM-1 and hsCRP) were evaluated. Besides, Rat glomerular mesangial cells (GMCs) were cultured in medium with high-glucose and treated with EGb761. Contents of oxidative stress/antioxidant markers and inflammatory cytokines in medium as well as NF-κB in the supernatant the cell lysate were also measured. Results showed that rats in SHR-DM group had higher blood pressure and glucose, increased levels of osteopontin, KIM-1, albumin, MDA, H_2O_2, IL-1β, IL-6, TNF-α, VCAM-1 and hsCRP as well as decreased levels of SOD and GSH-Px if compared to the Wistar Kyoto (WKY) rats. However, EGb761 significantly reversed the changes of the markers in the EGb761 100 mg group and the EGb761 150 mg group compared to the SHR-DM group. The cell culture investigation also showed anti-inflammatory anti-oxidant activity of EGb761 in GMCs. Collectively, EGb761 has renoprotective, anti-inflammatory and anti-oxidant effects in SHR rats with coexisted DM.

Keywords: EGb761, hypertension with coexisted DM, renoprotective effect, anti-inflammation, anti-oxidative stress, GMCs

Introduction

In clinic, hypertension and diabetes mellitus frequently coexist, and this combination leads to much increased incidences of complications including renal damage than hypertension or DM alone [1]. Proper managements of blood pressure and blood glucose contribute to the improvement of renal damage. However, many patients with well-controlled blood pressure and blood glucose still suffer from various cardiovascular complications such as hypertensive nephropathy (HN) and diabetic nephropathy (DN). So, more management in addition to controlling blood pressure and blood glucose is necessary for such patients.

Recent studies have revealed the relationships between inflammation and HN/DN [2]. Both hypertension and DM are inflammatory disorders. Inflammation plays important roles in the development and/or progression of hypertension and diabetes as well as their complications [3]. Increased levels of some inflammatory cytokines, such as IL-1β, IL-6, TNF-α, VCAM-1 and hsCRP, were found in subjects with HN or DN in pre-clinical and clinical studies [4, 5]. Controlling of the over-expression of these inflammatory cytokines showed improvement of the renal damage in subjects with HN or DN [6]. NF-κB is a key factor that regulates inflammatory response. The activated NF-κB can translocate from the cytoplasm to the nucleus to promote the production of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. There is evidence that NF-κB is involved in the pathogenesis of HN and DN [7]. Besides inflammation, it is increasingly recognized that oxidative stress is another major contributing factor in pathogenesis of HN and DN [7-9]. In

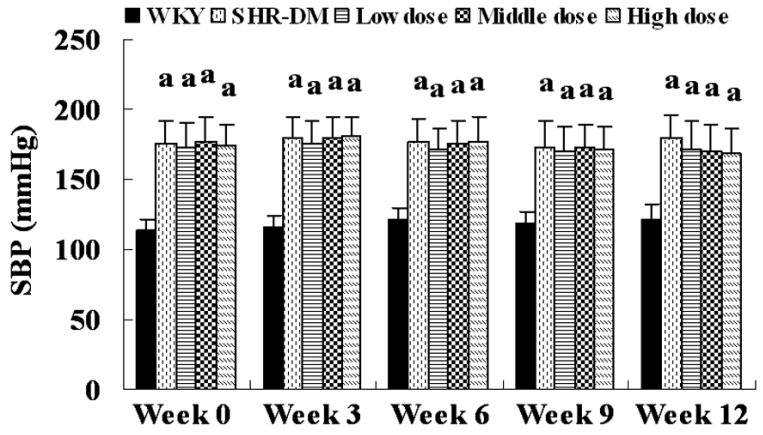

Figure 1. Effects of EGb761 on SBP of the rats. Each bar is the mean ± SD of the SBP of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

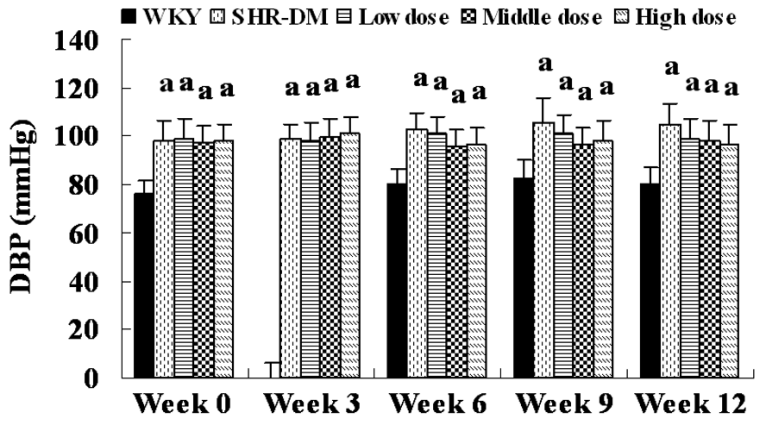

Figure 2. Effects of EGb761 on DBP of the rats. Each bar is the mean ± SD of the DBP of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

HN and DN, the oxidative stress activity is enhanced, for example excessive production of MDA and H_2O_2. However, the antioxidant activity usually decreases in HN and DN, for example reduced production of SOD and GPx as well as decreased total antioxidative capacity (T-AOC). According to literature, excessive oxidative stress, for example over-production of H_2O_2, can induce the activation of NF-κB, ultimately lead to inflammatory response. Some antioxidant agents have showed potential therapeutic effects on DN and HN [10, 11].

Some herbs have been used to manage DN and HN in pre-clinical and clinical studies [12-14]. EGb761, an extract from *Ginkgo biloba*, has anti-inflammatory, antioxidative, and neuroprotective activities as well as some other pharmacological effects [15, 16]. Recently, EGb761 has been used to treat some disorders, such arteriosclerosis, Parkinson's disease, lipopolysaccharide-induced acute lung injury and so on in animal studies [17, 18]. Besides, EGb761 been recently reported to confer renoprotective effects in renal ischemia-reperfusion injury in rats [19]. But its potential renoprotective activity in hypertension with coexisted diabetes remains unknown.

In the current study, we employed SHR-DM rats and rat GMCs, assessed the effects of EGb761 on renal function in the animals and investigated its effects on production of inflammatory cytokines and NF-κB as well as on the oxidative stress activity and antioxidant activity in SHR-DM rats and rat GMCs.

Material and methods

Animals

All experiments and procedures used in the current study were approved by the Ethics Committee of Affiliated Hospital of Weifang Medical College. Twelve months old male SHR rats and WKY rats (250-300 g) were obtained from the SLAC Lab Animal Center (Shanghai, China). Animals were housed in a room with controlled temperature at 23°C and kept on a 12:12 hour light/dark cycle. Rats were supplied with a standard diet and water *ad libitum*.

Induction of diabetes in SHR rats

Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, St. Louis, MO, USA) (freshly diluted with 0.01 M citrate buffer, pH 4.5) at the dose of 55 mg/kg body weight. The WKY rats only received i.p. injection of the

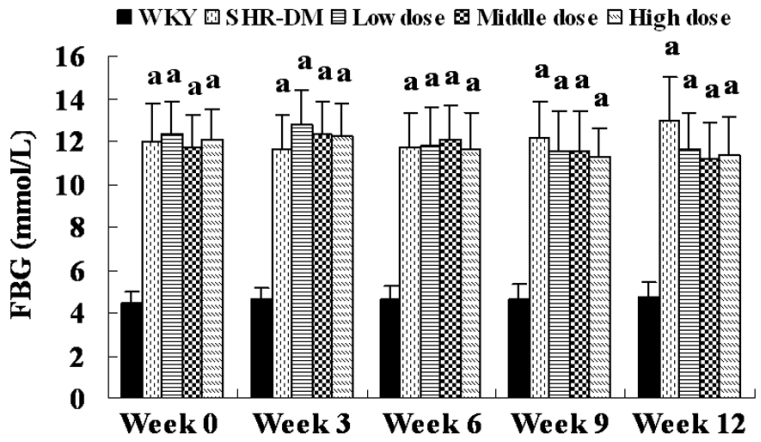

Figure 3. Effects of EGb761 on the FBG. Each bar is the mean ± SD of the FBG of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

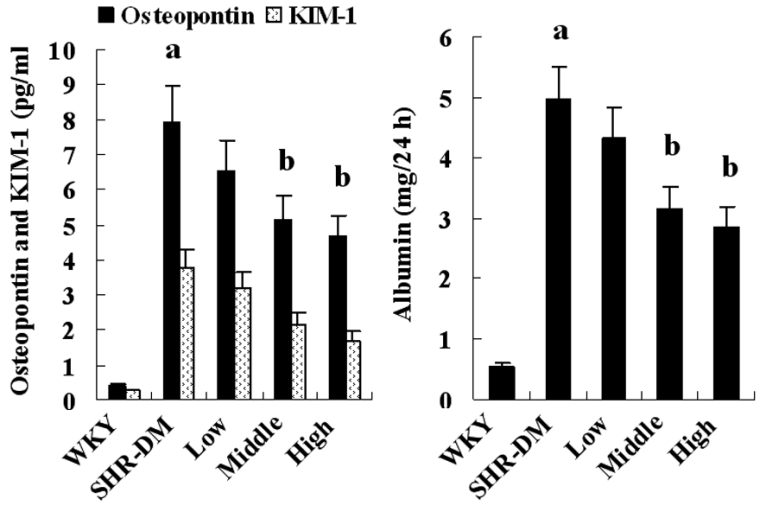

Figure 4. Effects of EGb761 on renal damage markers. Each bar is the mean ± SD of renal damage markers of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

same volume of 0.01 M citrate buffer. Blood glucose was measured using OneTouch®Ultra machine 72 h after STZ injection. Rats with hyperglycemia (glycemia>220 mg/dL) were included for further study.

Experimental groups and treatment

Rats were randomly divided into the following groups: WKY group, SHR-DM group, EGb761 50 mg group (low dose group), EGb761 100 mg group (middle dose group) and EGb761 150 mg group (high dose group). Rats in low dose group, middle dose group and high dose group were orally administrated with EGb761 (Dr. Willmar Schwabe Gmbh & CO. KG) (dissolved in saline), respectively at doses of 50, 100 or 150 mg/kg/day for consecutive 12 weeks after grouping; animals in the WKY group and SHR-DM group were orally administrated with the same volume of saline.

Preparation of renal homogenate and urine

After sacrifice, the kidney was removed and renal samples were homogenized in ice-cold isotonic saline (100 mg renal tissue/1 ml saline). The homogenates and the blood samples were centrifuged at 10000 × g for 15 min at 4°C and their supernatant was collected. Urine was collected by metabolic cages. All the collected samples were stored at -80°C for further analysis.

Cell culture and treatment

Rat GMCs (HBZY-1) were purchased from Chinese Center for Typical Culture Collection (Wuhan, Hubei, China). Cells were grown in Dulbecco's modified Eagles medium (DMEM, Wisent) containing fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% CO_2: 95% air at 37°C. Prior each experiment, cells were maintained in serum-free media for 24 h. GMCs were distributed into three groups: Normal group (cultured in normal-glucose medium, i.e. 5 mM glucose plus 25 mM mannitol), model group (cultured in high-glucose medium, i.e. 30 mM glucose), EGb761 group (cultured in high-glucose medium containing 10 mM EGb761). Cells were seeded in 24-well plates at about the density of 1 × 10^4 per well. EGb761 was added to the

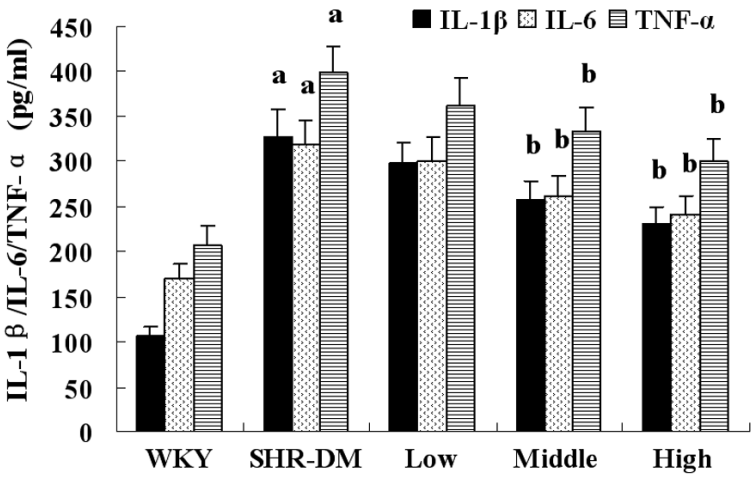

Figure 5. Effects of EGb761 on IL-1β, IL-6, TNF-α in kidney. Each bar is the mean ± SD of IL-1β/IL-6/TNF-α of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

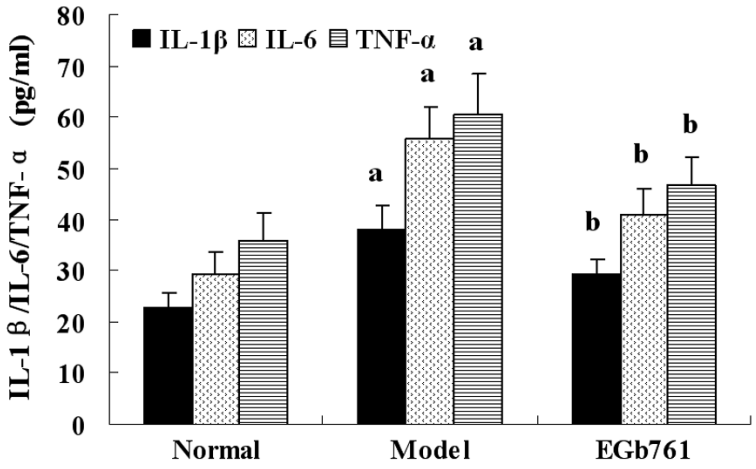

Figure 6. Effects of EGb761 on IL-1β, IL-6, TNF-α in medium. Each bar is the mean ± SD of IL-1β/IL-6/TNF-α of each group in rat GMCs cell culture medium. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. normal group; [b]*P*<0.05, vs. model group.

wells of the EGb761 group and cultured for 48 h after cells were allowed to attach. Then the cell culture supernatant (medium) was collected for further determination.

Cell lysis

After removing the medium, we lysed the cells in cell lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 0.1% Triton X-100, protease inhibitor, and 1% phosphatase inhibitor cocktail) and the cell lysates were centrifuged at 10000 × g for 10 min at 4°C. The supernatants were removed and stored at minus 80°C for the measurement of NF-κB.

Measurement of blood pressure

From the start of the EGb761 administration, SBP and DBP of the rats were measured using a non-invasive tail-cuff blood pressure measurement system (Taimeng Technology Company, Chengdu, China) once every 3 weeks.

Measurement of blood glucose

Blood glucose was measured by an OneTouch®Ultra machine using the blood freshly collected from the tail vein once every 3 weeks, starting from the administration of EGb761.

Measurements of renal damage biochemical markers

Levels of renal damage biomarkers urinary osteopontin, KIM-1 (Boster Company, Wuhan, China) and albumin (RayBiotech, Inc., Norcross, GA, USA) were determined using ELISA assay kits according to the instructions.

Evaluation of oxidative stress activity

The contents of MDA and H_2O_2 in the kidney homogenate and rat GMCs cell culture supernatant were measured using kits according to the manufacturer's instruction (Jiancheng Bioengineering Institute, Nanjing, China).

Evaluation of antioxidant activity

Activities of SOD and GSH-Px in the kidney homogenate and rat GMCs cell culture supernatant were also determined using kits according to the manufacturer's instruction (Jiancheng Bioengineering Institute, Nanjing, China).

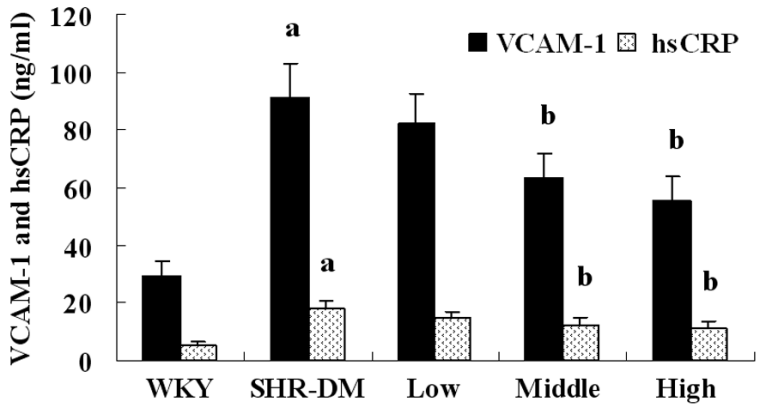

Figure 7. VCAM-1 and hsCRP in kidney. Each bar is the mean ± SD of VCAM-1/hsCRP of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

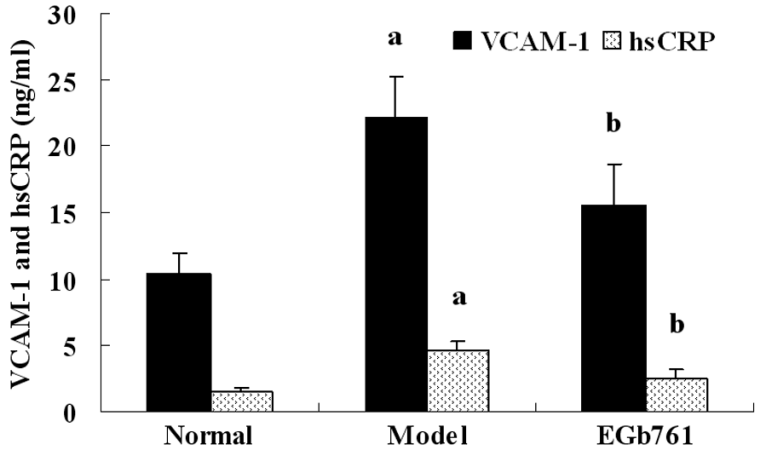

Figure 8. VCAM-1 and hsCRP in medium. Each bar is the mean ± SD of VCAM-1/hsCRP of each group in rat GMCs cell culture medium. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. normal group; [b]*P*<0.05, vs. model group.

Measurements of NF-κB

Levels of NF-κB in the kidney homogenate and the supernatant the cell lysate were measured using ELISA assay kits according to the manufacturer's instruction (Cusabio Company, Wuhan, China).

Measurements of inflammatory cytokines

Some inflammatory cytokines involved in the pathogenesis of HN and DN in the kidney homogenate and rat GMCs cell culture supernatant were investigated. The levels of inflammatory cytokines IL-1β, IL-6, TNF-α (Cusabio Company, Wuhan, China) and vascular cell adhesion molecule 1 (VCAM-1) (Bender MedSystems GmbH, Vienna, Austria) were measured using Elisa assay kits. High-sensitivity C-reactive protein (hsCRP) was measured using an automatic biochemical analyzer (Bio-Rad, Benicia, USA).

Statistical analysis

Data were presented as mean ± SD and analyzed using SPSS 13.0. For multiple comparisons, analysis was performed using one-way analysis of variance (ANOVA) with Students-Newman-Keuls (SNK) test. *P* value<0.05 was used as a criterion for statistical significance.

Results

Effects of EGb761 on blood pressure

The SBP and the DBP of rats in the SHR-DM group, low dose group, middle dose group and high dose group were markedly higher than those of the WKY group on week 0, 3, 6, 9, 12 (all *P*< 0.05). However, there were no significant differences in levels of the SBP and the DBP among the SHR-DM group, low dose group, middle dose group and high dose group on week 0, 3, 6, 9, 12 (all *P*>0.05). The results suggested that EGb761 had no hypotensive effect in SHR-DM rats. (Shown in **Figures 1**, **2**).

Effects of EGb761 on blood glucose

The fasting blood glucose (FBP) of rats in the SHR-DM group, low dose group, middle dose group and high dose group were markedly higher than those of the WKY group on week 0, 3, 6, 9, 12 (all *P*<0.05). However, there were no significant differences in levels of FBP among the SHR-DM group, low dose group, middle dose

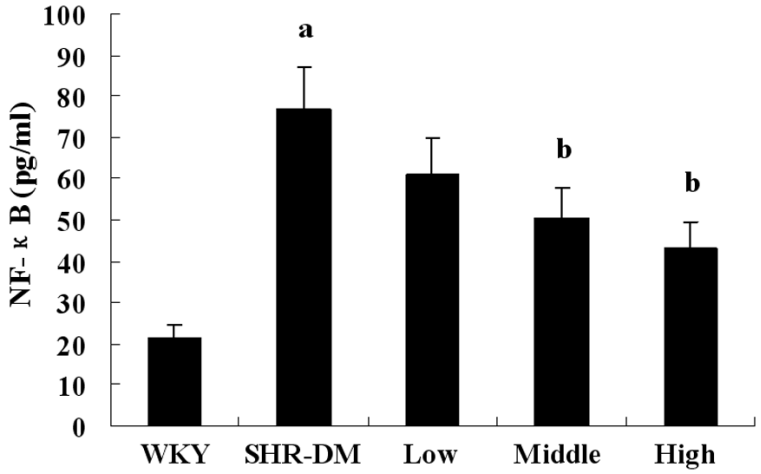

Figure 9. Effects of EGb761 on NF-KB in kidney. Each bar is the mean ± SD of NF-κB of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

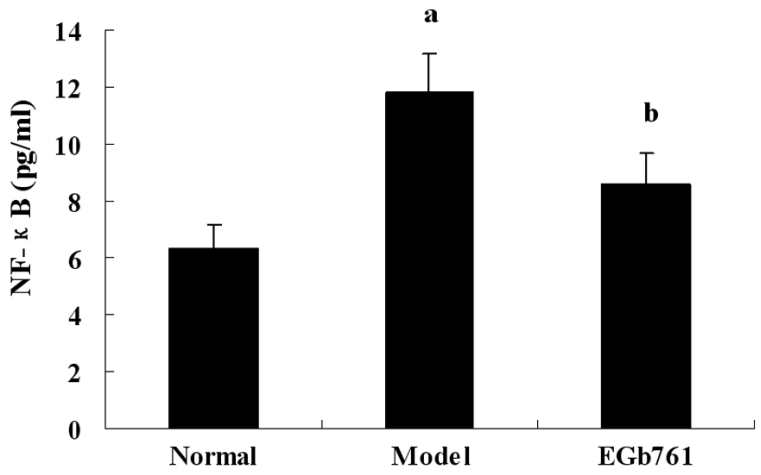

Figure 10. Effects of EGb761 on NF-KB GMCs cell. Each bar is the mean ± SD of NF-κB of each group in rat GMCs cell lysate. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. normal group; [b]*P*<0.05, vs. model group.

group and high dose group on week 0, 3, 6, 9, 12 (all *P*>0.05). The results suggested that EGb761 had no hypoglycemic effect in SHR-DM rats. (Shown in **Figure 3**).

Effects of EGb761 on renal damage biochemical markers

Levels of urinary osteopontin, KIM-1 and albumin were measured as the renal damage biochemical markers. The results showed rats in the SHR-DM group had much increased levels of urinary osteopontin, KIM-1 and albumin if compared to the WKY rats (all *P*<0.05). The middle dose and the high dose of EGb-761 significantly inhibited the increases of urinary osteopontin, KIM-1 and albumin levels if compared to the SHR-DM group (all *P*<0.05), and the effects of the two doses were similar (all *P*> 0.05); but the low dose had no marked effects on their levels (all *P*>0.05). (Shown in **Figure 4**).

Effects of EGb761 on inflammatory cytokines

IL-1β, IL-6, TNF-α, VCAM-1 and hsCRP were detected to evaluate the inflammatory response. Higher levels of these cytokines in kidney were found in rats from the SHR-DM group than the WKY rats (all *P*<0.05). But the middle dose and high dose of EGb-761 markedly reversed the elevations if compared to the SHR-DM group (all *P*< 0.05), but the low dose did not (all *P*>0.05). The cell experiment showed high-glucose increased production of these cytokines in medium if compared to the normal group (*P*<0.05), however, EGb761 could markedly inhibit the over-production (*P*<0.05). (Shown in **Figures 5**-**8**).

Effects of EGb761 on NF-κB

Rats in the SHR-DM group had much elevated levels of NF-κB in kidney compared to the WKY rats (*P*<0.05). But the elevation was markedly reversed by middle dose and high dose of EGb761 (*P*<0.05), but not by low dose (both *P*>0.05), if compared to the SHR-DM group. The cell experiment showed high-glucose increased the contents of NF-κB the supernatant the cell lysate if compared to the normal group (*P*<0.05), however, EGb761 could markedly inhibit the increase (*P*<0.05). (Shown in **Figures 9**, **10**).

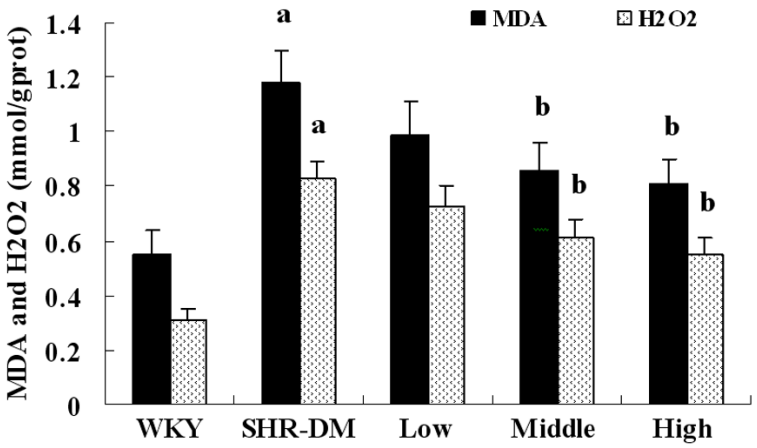

Figure 11. Levels of oxidative markers in kidney. Each bar is the mean ± SD of MDA/H_2O_2 of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

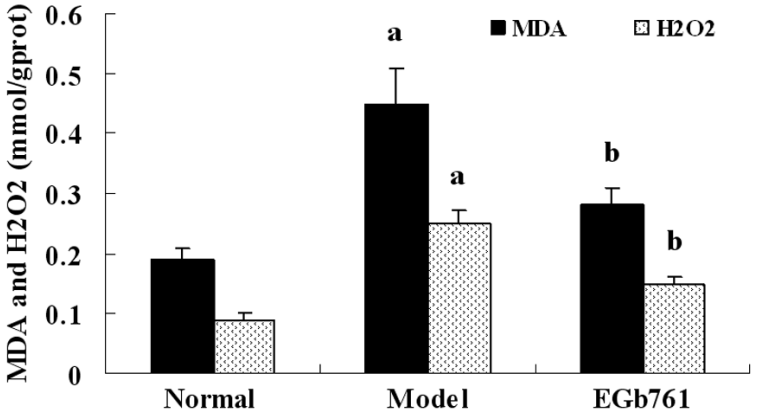

Figure 12. Levels of oxidative markers in medium. Each bar is the mean ± SD of MDA/H_2O_2 of each group in rat GMCs cell culture medium. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. normal group; [b]*P*<0.05, vs. model group.

Effects of EGb761 on oxidative stress and antioxidant markers

Rats in the SHR-DM group had much elevated levels of MDA and H_2O_2 as well as reduced levels of SOD and GSH-PX in kidney compared to the WKY rats (both *P*<0.05). But the changes were markedly reversed by middle dose and high dose of EGb761 (both *P*<0.05), but not by low dose (both *P*>0.05), if compared to the SHR-DM group. The cell experiment showed high-glucose increased the contents of MDA and H_2O_2 as well as reduced levels of SOD and GSH-Px in medium if compared to the normal group (*P*<0.05), however, EGb761 could markedly inhibit the changes induced by high-glucose (*P*<0.05). (Shown in **Figures 11-14**).

Discussion

In the present study, we found 12 weeks of EGb761 treatment at the dose of 100 and 150 mg/kg/day had significant renoprotective effects of in SHR rats with coexisted diabetes. EGb761 also inhibited oxidative stress and inflammatory response in the SHR-DM animals and rat GMCs.

The prevalence of hypertension is approximately 20% of the general adult population in China, and even higher in some other countries; DM is also a serious disease affecting millions of people worldwide. Both of the two diseases are well-known risk factors for cardiovascular (CV) disease including renal damage. However, hypertension coexisted with DM will lead to increased incidences of CV complications than hypertension or DM alone [1]. Although with well-managed blood pressure and blood glucose, patients may still suffer from the CV complications. Agents aiming at controlling pathogenesis beyond blood pressure and blood glucose may be helpful. Herbs have long been used to manage DN and HN [12-14, 20]. In order to observe the potential renoprotective effects of EGb761 (an extract from herb *Ginkgo biloba*) in hypertensive animals with coexisted DM, we firstly established the animal model by injecting STZ to SHR rats, which successfully induced hyperglycemia in the SHR rats. After 12 weeks of EGb761 treatment, we measured levels of urinary osteopontin and KIM-1, specific biomarkers of the early-stage renal damage [21], as well as urinary albumin. We found SHR-DM rats had significantly increased levels of these renal damage biochemical markers,

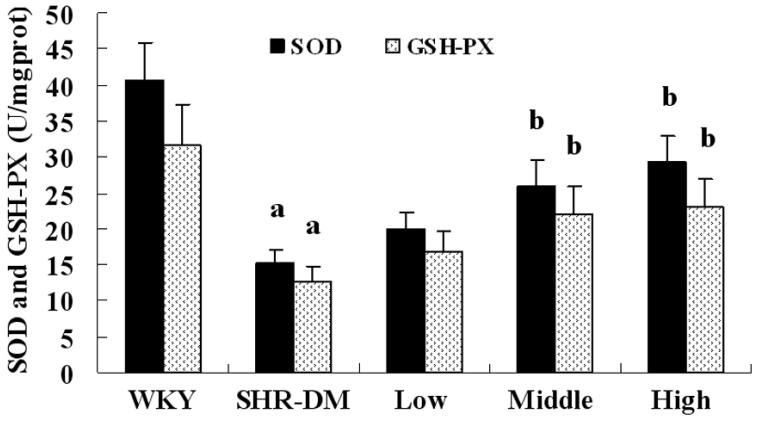

Figure 13. Levels of antioxidant markers in kidney. Each bar is the mean ± SD of SOD/GSH-PX of each group. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. WKY group; [b]*P*<0.05, vs. SHR-DM group.

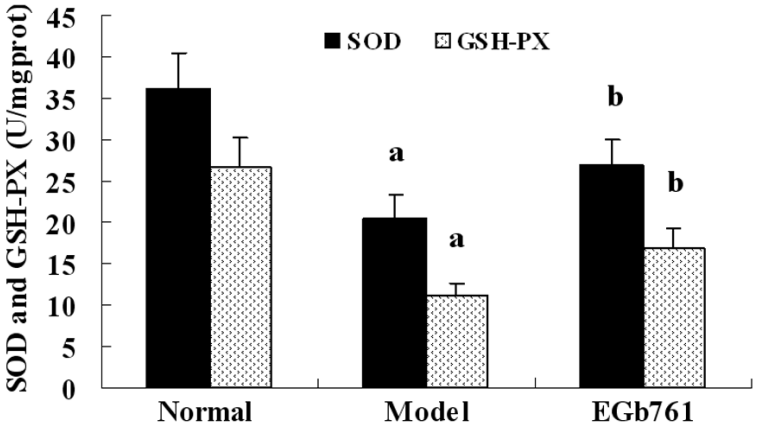

Figure 14. Levels of antioxidant markers in medium. Each bar is the mean ± SD of SOD/GSH-PX of each group in rat GMCs cell culture medium. Differences between groups were determined by ANOVA procedure and SNK test. [a]*P*<0.05, vs. normal group; [b]*P*<0.05, vs. model group.

however, EGb761 at the doses of 100 and 150 mg mg/kg/day markedly inhibited the increases in levels of these markers. The results indicated that EGb761 had renoprotective effects in animals with coexisted hypertension and DM.

In order to detect the possible mechanisms of the renoprotective effects, we firstly monitored the blood pressure levels in rats. We found rats in SHR-DM group, low dose group, middle dose group and high dose group had similar SBP and DBP during the course of the study, which were higher than the WHY rats, indicating that EGb761 had no marked effects of blood pressure of the rats. We also monitored the blood glucose levels of the rats, similarly, no marked effects on blood glucose were observed. The data strongly indicated that the renoprotective effects of EGb761 were not benefit from the controlling of blood pressure and blood glucose.

Recently, both hypertension and DM are considered as inflammatory state [22-24], and inflammation was well demonstrated to be involved in the development and progression of DN and HN [3]. Increased levels of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, VCAM-1 and hsCRP were observed in HN and DN [4, 5]. Moreover, the blockade of inflammatory response in subjects with HN or/and DN contributed to the improvement of renal damage [6]. So, anti-inflammation seems to be a potential method in treating HN and DN [25, 26]. NF-κB is a key factor that regulates inflammatory response, which can promote the production of IL-1β, IL-6 and TNF-α. The activation of NF-κB pathway was reported to have a role in HN and DN. EGb761 has been reported for its anti-inflammatory activity in human chondrocytes [27]. However, its action on inflammatory response in hypertension with coexisted DM has not been investigated. In the present study, we found SHR-DM rats had significantly increased levels of NF-κB, IL-1β, IL-6, TNF-α, VCAM-1 and hsCRP in the kidney homogenate, while EGb761 at the doses of 100 and 150 mg mg/kg/day markedly inhibited the increases in their levels. The results suggested that EGb761 could inhibit the local inflammatory response in kidney of the SHR-DM rats. Our cell culture investigation also further confirmed the anti-inflammatory activity of EGb761 in rat GMCs. Theoretically, the anti-inflammatory activity of EGb761 should contribute to the renoprotective effects.

Increasing evidence from studies has suggested that oxidative stress is involved in hypertension, DM and their CV complications [7-9]. Enhanced oxidative stress activity and decreased antioxidant activity were reported in rodents with HN or DN. Some antioxidant agents, including herbs and extracts from them, have showed potential therapeutic effects on DN and HN [28, 29]. EGb761 was reported to have antioxidant activity in rodents in several recent studies. In this study, we found elevated levels of MDA and H_2O_2 as well as reduced levels of SOD and GSH-Px in kidney of the SHR-DM rats compared to the WKY rats, suggesting the increased oxidative stress in the SHR-DM rats. According to literature, excessive oxidative stress, for example over-production of H_2O_2, can induce the activation of NF-κB, ultimately lead to inflammatory response. This was consistent with the inflammatory cytokine investigations in the present study. But the alternations of the oxidative stress and anti-antioxidant markers in the SHR-DM rats were partially reversed by EGb761 at the dose of 100 and 150 mg mg/kg/day. In addition, we cultured the rat GMCs with high-glucose which could induce oxidative stress according to literature. Our cell culture investigation also further confirmed the antioxidant activity of EGb761 in rat GMCs. We supposed that the inhibition of the oxidative stress should contribute to the improvement of the renal damage.

Taken together, EGb761 has renoprotective effects of in SHR rats with coexisted diabetes. EGb761 also suppress the inflammatory response and oxidative stress in the SHR-DM rats and rat GMCs which are involved in the initiation and progression of DN and HN. The findings suggest a renoprotective potential of EGb761 in patients with hypertension and diabetes.

Disclosure of conflict of interest

None.

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