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Protective effect of the standardized leaf extract of *Ginkgo biloba* (EGb761) against hypertension-induced renal injury in rats

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ABSTRACT

**Background:** *Ginkgo biloba* leaves extract has been widely used worldwide to protect against oxidative stress-induced cell damage and improves blood circulation. **Methods:** The potential protective role of the standardized leaf extract of *Ginkgo biloba* (EGb761) on hypertension-induced renal injury was investigated in rats. Hypertension was induced in rats by L-NAME. **Result:** Repeated treatment with EGb761 produced progressive reductions in the systolic, diastolic and mean arterial blood pressure. Also, EGb761 increased the progressive reductions in blood pressure induced by losartan. Hypertension-induced marked elevation of renal malondialdehyde (MDA) and nitrite levels and reduction of reduced glutathione (GSH) level were inhibited by EGb761. In addition, hypertension-induced increases in tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) levels in renal tissues were inhibited by EGb761. Also, treatment with EGb761 inhibited hypertension-induced decrease in endothelial nitric oxide synthase (eNOS) protein expression and increase in the protein expressions of inducible NO synthase (iNOS), TNF-α, IL-6 and IL-1β in the kidney tissues. EGb761 enhanced losartan effects on renal tissues oxidative stress, nitrite, and inflammatory markers levels and on protein expressions of eNOS, iNOS, TNF-α, IL-6 and IL-1β. **Conclusions:** These results indicate that EGb761 has the ability to protect against hypertension-induced renal injury.

**Introduction**

Hypertension is a major worldwide public-health challenge, which can lead to target organ damage and severe complications (1). Data has been accumulated to indicate that oxidative stress is an essential contributing factor in hypertension and plays a significant role in hypertensive renal damage (2). It has been found in hypertensive rats the kidney injury is due to increased renal oxidative stress (3,4).

Reduced endothelial nitric oxide synthase (eNOS) activity and low bioavailability of nitric oxide (NO) is associated with the pathogenesis of hypertension (5). However, NO overproduction by activation of inducible NO synthase (iNOS) plays a critical role in the pathogenesis of hypertension (6). In the kidney, NO resulting from eNOS usually plays a renoprotective effect (7), while overproduction of NO due to activation of iNOS may provoke injurious effects (8). In hypertensive rats, there is renal damage and an increase in the expression of kidney iNOS (4).

In clinical and experimental studies, there is increased activity of tissue and/or circulating levels of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin -1β (IL-1β) (9–11). Other study reported that both arterial and tubulointerstitial inflammation should be considered as the hallmark of essential hypertension (12). Inflammatory cytokines expression was upregulated in the hypertensive rat’s kidney (13). Increased inflammatory cytokine levels induce membranous glomerulonephritis in rats (14). An inflammatory infiltration by macrophages and lymphocytes takes place in the kidney of hypertensive animals (15,16).

The standardized leaf extract of *Ginkgo biloba* (EGb761) has been commonly used worldwide as an herbal remedy to produce neuroprotective effects (17). It increases antioxidant enzyme activities and non-enzymatic antioxidant levels in different tissues of experimental animals (18) and patients (19). Also, EGb761 was found to be a potent free radical scavenger and Chang et al. (20) reported that through increasing the activity and expression of eNOS and release of NO protected the functions of blood vessels. However, EGb761 was found to reduce NO production by inhibiting gene and protein expressions and enzymatic activity of iNOS (21,22). Furthermore, EGb761 pretreatment inhibited lipopolysaccharide-induced neutrophil infiltration and inflammatory responses in mice (23). EGb761 inhibits the production and expression of TNF-α, IL-6, and IL-1β in the atherosclerotic rat brain (24), in lipopolysaccharide-stimulated macrophage cells (25) and in metabolic syndrome patients (19).

In light of these observations, we investigated the effect of EGb761 on hypertension-induced renal damage in rats. The role of oxidative stress, NO, NO synthase isoforms, inflammatory cytokines and infiltration of inflammatory cells in mediating these effects was also monitored in this study.
Materials and methods

Chemicals
Malondialdehyde- bis(dimethyl acetal) was obtained from Merck (Germany). Thiobarbituric acid was purchased from MP Biomedicals INC. (France). Losartan was obtained from Sigma-Aldrich Co. (USA). Ginkgo biloba extract (EGb761) powder was obtained from Amriya Pharm. Ind. (Egypt). All other chemicals were of analytical grade.

Animals
Male adult Wistar rats weighing 120–160 g, obtained from animal house of Faculty of Medicine, Assiut University were used in all experiments. The animals were housed in stainless steel cages under a 12-hour light/dark cycle at 25°C. Rats were allowed water and food (laboratory chow) ad libitum. This study was conducted in accordance with the internationally accepted principles for Guide for the Care and Use of Laboratory Animals. The experiments reported here were approved by our institutional ethics committee.

Induction of hypertension
To induce hypertension, animals were treated daily with L-N (G)-nitroarginine methyl ester (L-NAME) at a dose level of 10 mg/kg (1% solution in saline) intraperitoneally (ip) for 12 weeks (26,27).

Non – invasive blood pressure measurement
Blood pressure was measured by the tail-cuff method in all groups of rats using LE5001 Non Invasive Blood Pressure Meter (Panlab Harvard Apparatus, Barcelona, Spain). The rat was placed in a restrainer of appropriate size and its tail was placed inside a tail-cuff at room temperature and allowed to equilibrate for a few minutes. When the monitor gives a ready to measure, the start button was pressed to inflate cuff for systolic, diastolic and mean arterial BP measurement. Each animal was adapted for the restrainer and blood BP measurements for 3 days before taking BP readings. The systolic, diastolic and mean arterial BP of each rat was measured before and weekly after starting of the treatment. The average of 3 consecutive measurements was taken for presentation.

Experimental protocol
The animals were divided into 4 groups, 8 rats each. Animals of group-I were treated ip with 10 mg/kg/day L-NAME for 12 weeks. Group-II rats were received orally 100 mg/kg/day EGb761 (4% suspension in 0.25% carboxymethylcellulose in saline) starting from the 9thweek of continued daily treatment with 10 mg/kg L-NAME ip to the end of the 12 weeks treatment duration. Animals of group-III and IV received orally, starting from the 9thto the 12thweek of continued daily treatment with 10 mg/kg L-NAME ip, 1 mg/kg/day losartan (0.1% solution in saline) and 1 mg/kg/day losartan in combination with 100 mg/kg/day EGb761, respectively. Control animals were treated likewise with the pure vehicles. Doses and duration of treatments with EGb761 and losartan were selected depending on our preliminary evaluation and in accordance with previous studies (26,28).

Biochemical measurements
Blood samples were collected for biochemical measurements from the orbital sinus of the overnight fasted rats before the experiment. At the end of experimental duration and after recording the BP changes, the overnight fasted rats were sacrificed by decapitation. Blood and kidney tissues were obtained from each animal for biochemical measurements.

- After centrifugation of blood samples for 10 min, the serum was collected for estimation the levels of urea and creatinine. The samples could be used directly or stored at −20°C until assay. Briefly, the serum level of urea was measured by the urease-colorimetric method using a commercially available SPECTRUM Diagnostics Urea/BUN-Liquizyme Kit (Egyptian Company for Biotechnology, Cairo, Egypt) according to the manufacturer’s instructions. The serum level of creatinine was measured by buffered kinetic Jaffe reaction without deproteinization method using a commercially available SPECTRUM Creatinine-Jaffe kit (Egyptian Company for Biotechnology, Cairo, Egypt) according to the manufacturer’s instructions.
- One kidney was rinsed in ice-cold saline, dissected and cleaned from fat and other tissues, then cut into small pieces, blotted carefully and weighed. A suitable weight of kidney tissue was homogenized in 10% w/v phosphate buffer (pH 7.4) or saline by using a motor-driven Teflon pestle. The homogenate was divided into 2 portions. The first portion was centrifuged for 10 min at 10,000 rpm and the supernatant was used for estimation of malondialdehyde (MDA) as a marker of lipid peroxidation, nitrite, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) levels directly or stored at −20°C until assay. To the second part of homogenate, an equal volume of perchloric acid (1 mol/l) was added and mixed by vortexing. The mixture was allowed to stand for 5 min at room temperature. After centrifugation for 10 min, the supernatant was collected carefully and used for estimation of intracellular reduced glutathione (GSH) directly or stored at −20°C until assay.
- MDA is an end product of lipid peroxidation and its level was determined spectrophotometrically by use of thiobarbituric acid reactive substances method previously described by Ohkawa et al. (29). A standard curve was run simultaneously with each set of samples by using 1, 1, 3, 3-tetramethoxypropane as an external standard.
- The intracellular GSH content of the neutralized supernatant was assayed using Ellman’s reagent [5, 5-dithio-bis-2-nitrobenzoic acid (DTNB solution)] according to the method of Ellman (30). A standard reference curve was prepared for each assay.

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- The intracellular GSH content of the neutralized supernatant was assayed using Ellman’s reagent [5, 5-dithio-bis-2-nitrobenzoic acid (DTNB solution)] according to the method of Ellman (30). A standard reference curve was prepared for each assay.
Nitric oxide (NO) formation was measured in the supernatant by assaying nitrite, one of the stable end-products of NO oxidation. Serum nitrite concentration was assayed spectrophotometrically using Griess reagents [1% sulfanilamide in 5% phosphoric acid (sulfanilamide solution) and 0.1% N-1-naphthylethylenediamine dihydrochloride in bidistilled H2O (NED solution)] as described by Miranda et al. (31). A standard curve was run simultaneously with each set of samples.

The kidney levels of TNF-α, IL-6 and IL-1β were determined in tissue homogenate by enzyme-linked immunosorbent assay using the commercially available Rat TNF-α, IL-6 and IL-1β ELISA kits, respectively (KOMA BIOTECH INC., Seoul, South Korea) according to the manufacturer’s instructions.

**Histopathological examination**

One kidney was obtained from each animal after scarification at the end of the experiment. It was immediately fixed in 10% neutral buffered formalin. Specimens of kidney tissues were embedded in paraffin and cut into sections of 4–5 μm thickness and stained with hematoxylin and eosin stain (H&E. stain). These sections were examined under the light microscope to assess glomerular sclerosis, cloudy swelling of the tubules, interstitial inflammation, and interstitial fibrosis as previously described (32). The features of histopathological changes were scored on a semiquantitative scale as follows: 0: no change; +: less than 25% of tissues were affected; ++: 25–50% of tissues were affected; +++: >50% of tissues were affected.

**Immunohistochemical analysis**

Other sections of 3–5 μm thickness were cut from the previous paraffin blocks and mounted on aminopropyltriethoxysilane (APES) coated slides. Once mounted, the slides were dried at 60°C oven overnight to remove any water that may be trapped under the section.

Endothelial NO synthase, inducible NO synthase (iNOS), TNF-α, IL-6, and IL-1β were examined immunohistochemically by using the standardized commercially available UltraVision Detection System anti-polyvalent HRP/DAB kit (Thermo Fisher Scientific, USA) according to manufacturer’s instructions.

Negative control slides were done by omitting the primary antibody. Sections from lung, breast carcinoma, rat spleen and rat colon were stained as a positive control for eNOS. Frozen sections containing vascular endothelium were stained as a positive control for eNOS.

**Statistical analysis**

The results were expressed as the mean ± standard error of the mean (X ± SEM). Statistical analysis of the difference between groups was done using the one-way analysis of variance (ANOVA) and two-way ANOVA followed by Newman-Keuls test as a post hoc analysis for the one-way method and Bonferroni’s test as a post hoc analysis for the two-way method. All statistics were carried out using GraphPad Prism software (GraphPad; San Diego CA, USA).

**Results**

**Induction of hypertension in rats**

Daily treatment of rats with 10 mg/kg L-NAME ip produced a progressive increase in the systolic, diastolic and mean arterial BP. The systolic and mean arterial BP increased significantly after 3 weeks of treatment, while the diastolic BP of rats increased significantly after 4 weeks of daily treatment with 10 mg/kg L-NAME ip. After 12 weeks of treatment, the systolic BP increased progressively from the control value of 123.30 ± 2.95 to 215.50 ± 5.42 mmHg (F(12,182) = 26.88, p < 0.01, Figure 1 A), the diastolic BP increased from the control value of 80.88 ± 4.04 to 158.10 ± 4.87 mmHg (F(12,182) = 27.69, p < 0.01, Figure 1B) and the mean arterial BP increased from the control value of 97.75 ± 3.36–178.40 ± 5.79 mmHg (F(12,182) = 29.28, p < 0.01, Figure 1C).

**Effect of EGB761 on the systolic, diastolic and mean arterial blood pressure of hypertensive (L-NAME) rats**

Starting from the 9th week of continued treatment of animals with 10 mg/kg/day L-NAME, concurrent daily
administration of 100 mg/kg EGb761 orally to the end of the experimental duration produced a progressive reduction in the systolic, diastolic and mean arterial BP. The systolic BP started to decrease significantly after the 11th week and reached the maximal reduction after the 12th week of treatment ($F_{(12,182)} = 86.75$, $p < 0.01$, Figure 1A). The diastolic BP was reduced significantly after the 12th week of treatment ($F_{(12,182)} = 66.02$, $p < 0.01$, Figure 1B). The mean arterial BP started to decrease significantly after the 11th week of treatment and reached the maximal reduction after the 12th week of treatment ($F_{(12,182)} = 61.00$, $p < 0.01$, Figure 1C).

**Effect of EGb761 on losartan-induced reduction in the systolic, diastolic and mean arterial blood pressure of hypertensive (L-NAME) rats**

Daily administration of 1 mg/kg/day losartan orally starting from the 9th week of continued daily treatment of rats with 10 mg/kg L-NAME to the end of the experimental duration produced a progressive reduction in the systolic, diastolic and mean arterial BP. The systolic BP significantly reduced after the 9th week of treatment. This reduction reached its maximal value after the 12th week of treatment ($F_{(12,182)} = 44.21$, $p < 0.01$, Figure 2A). The diastolic and mean arterial BP significantly reduced after the 10th week of treatment. After the 12th week of treatment, the maximal reduction in diastolic ($F_{(12,182)} = 43.43$, $p < 0.01$, Figure 2B) and mean arterial ($F_{(12,182)} = 40.10$, $p < 0.01$, Figure 2C) BP was observed.

Starting from the 9th week of treatment of rats with 10 mg/kg/day L-NAME ip, administration of 100 mg/kg/day EGb761 and 1 mg/kg/day losartan orally to the end of the experimental duration reduced progressively the systolic, diastolic and mean arterial BP. The reduction in the systolic, diastolic and mean arterial BP was significant after the 9th week of treatment. EGb761 increased significantly the effect of losartan on the systolic BP after the 11th and 12th week of treatment. ($F_{(12,182)} = 37.72$, $p < 0.05$, Figure 6A). EGb761 increased the effect of losartan on the diastolic ($F_{(12,182)} = 32.65$, $p < 0.05$, Figure 2B) and on mean arterial ($F_{(12,182)} = 31.05$, $p < 0.05$, Figure 2C) BP after the 12th week of treatment.

**Effect of EGb761, losartan and their combination on serum urea and creatinine levels in hypertensive (L-NAME) rats**

In preliminary experiments, daily administration of 10 mg/kg L-NAME ip to rats for 8 weeks produced a significant increase in the systolic, diastolic and mean arterial BP. This treatment produced insignificant changes in the serum urea and creatinine levels and insignificant histopathological changes as compared to the control animals. Marked changes in these parameters were detected after 12 weeks of treatment with L-NAME. Figure 3 shows that daily administration of 10 mg/kg L-NAME for 12 weeks ip to rats increased the serum urea ($F_{(2,15)} = 147.71$, $p < 0.01$) and creatinine ($F_{(2,15)} = 94.71$, $p < 0.01$) levels. EGb761 increased the effect of losartan on the serum urea and creatinine levels. (Figures 3A, 3B)
The histopathological examination of the kidney tissue obtained from rats treated daily with 10 mg/kg L-NAME for 12 weeks showed glomerulosclerosis, arterial wall thickening, cloudy swelling of tubules, interstitial inflammation and fibrosis (Figure 4B and C) compared to control rats (Figure 4A).

Treatment of rats with 100 mg/kg/day EGb761 or 1 mg/kg/day losartan orally starting from the 9th week of continued treatment with 10 mg/kg L-NAME ip to the end of the 12 weeks experimental duration ameliorated glomerulosclerosis, cloudy swelling of tubules, interstitial inflammation and fibrosis (Figure 4D and E).

The addition of EGb761 to losartan treatment of hypertensive rats nearly showed no histopathological changes (Figure 4F). The renoprotective effects of EGb761, losartan and their combination were significant in comparison to hypertensive rats.

**Effect of EGb761, losartan and their combination on the renal tissue malondialdehyde (MDA), intracellular reduced glutathione (GSH) and nitrite levels of hypertensive (L-NAME) rats**

As shown in Figure 5 administration of 10 mg/kg/day L-NAME ip for 12 weeks to rats resulted in a significant increase in the renal tissue MDA (F(2, 21) = 77.47, p < 0.01) level and a significant decrease in the renal tissue intracellular GSH level (F(2, 21) = 41.46, p < 0.01). The renal tissue nitrite level was increased by this treatment (F(2, 21) = 51.30, p < 0.01).
Administration of 100 mg/kg/day EGb761, 1 mg/kg/day losartan or their combination orally starting from the 9th to the 12th week of continued treatment of rats with 10 mg/kg/day L-NAME decreased renal tissue MDA (F(3, 28) = 44.01, p < 0.01, Figure 5A) level and increased the renal tissue intracellular GSH level (F(3, 28) = 11.29, p < 0.05, Figure 5B). The renal tissue nitrite level was decreased by this treatment (F(3, 28) = 13.14, p < 0.05, Figure 5C). EGb761 increased the effect of losartan on the renal tissue MDA, intracellular GSH and nitrite levels.

Effect of EGb761, losartan and their combination on the renal tissue tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) levels of hypertensive (L-NAME) rats

Data presented in Figure 6 show that administration of 10 mg/kg/day L-NAME ip to rats for 12 weeks resulted in a significant increase in the renal tissue TNF-α (F (2, 15) = 86.35, p < 0.01), IL-6 (F (2,15) = 41.40, p < 0.01) and IL-1β (F (2,15) = 72.85, p < 0.01) levels.

Oral administration of 100 mg/kg/day EGb761, 1 mg/kg/day losartan or their combination starting from the 9th to the 12th week of continued daily treatment of rats with 10 mg/kg L-NAME decreased the renal tissue TNF-α (F (3,20) = 50.42, p < 0.01, Figure 6A), IL-6 (F (3,20) = 28.11, p < 0.01, 6B) and IL-1β (F (3,20) = 25.61, p < 0.01, Figure 6C) levels as compared to L-NAME-treated group. EGb761 increased the effect of losartan on the renal tissue TNF-α, IL-6, and IL-1β levels.

Immunohistochemical analysis

In this study, the immunohistochemical analysis of the kidney tissues obtained from rats after 12 weeks of administration of 10 mg/kg/day L-NAME ip to rats showed a decrease in eNOS protein expression (Figure 7 IC) and decreased the protein expression of iNOS (Figure 7 IIC) as compared to control animals (Figure 7 I and IIA).

Oral administration of 100 mg/kg/day EGb761 starting from the 9th week of continued treatment of rats with 10 mg/kg L-NAME ip to the end of the experiment increased the protein expression of eNOS (Figure 7 IC) and decreased the protein expression of iNOS (Figure 7 IIC). Similar treatment with 1 mg/kg/day losartan increased the protein expression of eNOS (Figure 7 ID) and decreased the protein expression of iNOS (Figure 7 IID). EGb761 enhanced the stimulatory effect of losartan on the protein expression of eNOS (Figure 7 IE) and enhanced the inhibitory effect of losartan on the protein expression of iNOS (Figure 7 IIE).

In hypertensive (L-NAME) rats, EGb761, losartan, and their combination produced significant effects on the protein expressions of eNOS (Figure 7 IF) and iNOS (Figure 7 IIF). EGb761 enhanced the effects of losartan. After 12 weeks of administration of 10 mg/kg/day L-NAME ip to rats, the protein expressions of inflammatory cytokines, TNF-α (Figure 8 A), IL-6 (Figure 8 B) and IL-1β (Figure 8 C) were increased compared to control rats (Figure 8 I, II and III).

Oral administration of 100 mg/kg/day EGb761 starting from the 9th week of continued treatment of rats with 10

Figure 4. Photomicrographs of representative kidney sections from: (A) control rats showing normal glomeruli and tubules, (B) hypertensive (L-NAME) rats showing glomerular sclerosis (thick arrow) and arterial wall thickening (thin arrow), (C) hypertensive (L-NAME) rats showing interstitial inflammation with fibrosis (arrow), (D) hypertensive (L-NAME) rats treated with EGb761 showing normal and some sclerosed glomeruli (arrow) (E) hypertensive (L-NAME) rats treated with losartan showing normal and some sclerosed glomeruli (arrow) and (F) hypertensive (L-NAME) rats treated with EGb761 and losartan showing nearly no pathological changes (H&E stain x 400).
mg/kg L-NAME ip to the end of the experiment decreased the protein expression of TNF-α (Figure 8 IC), IL-6 (Figure 8 IIC) and IL-1β (Figure 8 IIC). Similar treatment with 1 mg/kg/day losartan decreased the protein expression of TNF-α (Figure 8 ID), IL-6 (Figure 8 IID) and IL-1β (Figure 8 IIID). EGB761 enhanced the inhibitory effect of losartan on the protein expression of TNF-α (Figure 8 IE), IL-6 (Figure 8 IIE) and IL-1β (Figure 8 IIE).

In hypertensive (L-NAME) rats, EGB761, losartan and their combination produced significant effects on the protein expressions of TNF-α (Figure 8 IF), IL-6 (Figure 8 IIF) and IL-1β (Figure 8 IIF). EGB761 enhanced the effects of losartan.

Discussion
The antihypertensive effect of EGB761 in several hypertensive animal models (28,33) has been reported after several weeks of treatment. However, the results of the antihypertensive...
The effect of extended treatment with EGb761 in humans are contradictory (34). Our results indicate that repeated administration of L-NAME ip to rats resulted in a progressive increase in the systolic, diastolic and mean arterial BP. Treatment of these hypertensive rats with EGb761 for several weeks reduced the systolic, diastolic and mean arterial BP.

In the rat model of L-NAME hypertension, renin-angiotensin-aldosterone system is activated (35). Thus, to verify the antihypertensive activity of EGb761, we studied its effect on the antihypertensive effect of losartan, an angiotensin II (Ang II) type 1 receptor blocker. The present study revealed that repeated treatment of hypertensive rats with losartan for several weeks decreased the systolic, diastolic and mean arterial BP. Co-administration of EGb761 enhanced the effect of losartan.

Hypertension was found to be a principal cause of hypertensive nephropathy and one of the main factors responsible for the development of glomerular, tubulointerstitial diseases and impaired kidney function (36). Moreover, it has been found that chronic inhibition of NOS by L-NAME increases the arterial BP and the levels of renal function markers in the serum. Histopathological analysis showed glomerulosclerosis, interstitial fibrosis, and macrophage infiltration in rats (37).
Figure 8. I - Immunohistochemistry of tumor necrosis factor-α (TNF-α) in kidney sections from: (A) control rats showing weak protein expression in the tubules, (B) hypertensive (L-NAME) rats showing strong protein expression in glomeruli and tubules, (C) hypertensive rats treated with EGb761 showing moderate protein expression in glomeruli and tubules, (D) hypertensive rats treated with losartan showing moderate protein expression in glomeruli and tubules and (E) hypertensive rats treated with EGb761 and losartan showing weak expression TNF-α in glomeruli and tubules. (IHC x 400). (F) Protein expression of TNF-α in kidney tissue of hypertensive rats treated with EGb761, losartan or their combination rats. Each value represents the mean ± SEM of 5 observations. ** p < 0.01 vs. control values; ## p < 0.01 vs. L-NAME values; ++ p < 0.01 vs. L-NAME + LOS values.

II - Immunohistochemistry of interleukin-6 (IL-6) in kidney sections from: (A) control rats showing weak protein expression in the tubules (B) hypertensive (L-NAME) rats showing strong protein expression in the tubules, (C) hypertensive rats treated with EGb761 showing moderate protein expression in the tubules (D) hypertensive rats treated with losartan showing moderate protein expression in the tubules and (E) hypertensive rats treated with EGb761 and losartan showing weak protein expression in the tubules (IHC x 400). (F) Protein expression of IL-6 in kidney tissue of hypertensive rats treated with EGb761, losartan or their combination. Each value represents the mean ± SEM of 5 observations. ** p < 0.01 vs. control values; ## p < 0.01 vs. L-NAME values; ++ p < 0.01 vs. L-NAME + LOS values.

III - Immunohistochemistry of interleukin-1β (IL-1β) in kidney sections from: (A) control rats showing weak protein expression in the tubules, (B) hypertensive (L-NAME) rats showing strong protein expression in the tubules, (C) hypertensive rats treated with EGb761 showing moderate protein expression in the tubules, (D) hypertensive rats treated with losartan showing moderate protein expression in the tubules and (E) hypertensive rats treated with EGb761 and losartan showing weak protein expression in the tubules. (IHC x 400). (F) Protein expression of IL-1β in kidney tissue of hypertensive rats treated with EGb761, losartan or their combination. Each value represents the mean ± SEM of 5 observations. ** p < 0.01 vs. control values; ## p < 0.01 vs. L-NAME values; ++ p < 0.01 vs. L-NAME + LOS values.
Data of the current study coincided with these findings. The serum levels of urea and creatinine were increased in hypertensive rats. Histopathological examination of the kidney tissue of hypertensive rats showed the presence of glomerulosclerosis, arterial wall thickening, cloudy swelling of tubules, interstitial inflammation and fibrosis. Our results also showed that the elevated levels of serum urea and creatinine and histopathological changes were reduced in hypertensive rats by repeated EGb761 treatment for several weeks. Similar results were obtained by repeated treatment of hypertensive rats with losartan. EGb761 enhanced the protective effect of losartan on the kidney function and structure in hypertensive rats.

Yet, the mechanism(s) by which EGb761 produces its renoprotective effect in hypertensive rats needs to be clarified. It has been shown by experimental (38) and by clinical studies (4) that oxidative stress plays an essential role in the pathogenesis of hypertension-related kidney damage. In agreement with these observations, our results indicate that renal injury induced by hypertension in rats was associated with oxidative stress in kidney tissue. This oxidative stress was indicated by increasing the renal MDA level and lowering the intracellular GSH level. Since, EGb761 has a potent antioxidant activity (18,19) therefore; we assessed the role of this activity in its protective effect against hypertension-induced oxidative renal tissue injury. Data obtained in the present study show that the protective effect of repeated treatment with EGb761 for several weeks against renal injury induced by hypertension in rats was associated with a reduction of the renal tissue oxidative stress. This was evidenced by decreasing the renal MDA level and increasing the intracellular GSH level. Furthermore, losartan was found to reduce renal oxidative stress and to produce a renoprotective effect in hypertensive animals (39,40). Also, the results of our study indicate that the protective effect of repeated treatment with losartan for several weeks against renal deleterious effects induced by hypertension in rats was correlated with its ability to reduce renal tissue oxidative stress. EGb761 enhanced the effect of losartan. This may support the role of antioxidant activity of EGb761 in its renoprotective effect.

Under normal physiological conditions, NO is released from eNOS, which is a protective NO-generating enzyme (7). Nevertheless, in hypertension eNOS activity and NO bioavailability were reduced (5). On the other hand, in hypertension, iNOS activity and NO production were increased (6), which might be implicated in renal injury (8). Our results indicate that in hypertensive rats, the renal injury was associated with a significant elevation of the renal nitrite level. Immunostaining of the renal tissue revealed an increase in the protein expression of iNOS and a decrease in protein expression of eNOS. In support of these findings, it has been found that in the damaged kidney of hypertensive rats, the expression and activity of iNOS and tissue nitrite levels were increased (4,41) and the expression of eNOS was decreased (42). However, on the contrary, it has been found that chronic L-NAME administration to rats resulted in a significant depletion of serum NO and renal tubular damage (43,44).

In light of these observations, the most likely explanation of our results is that chronic inhibition of NOS isoforms by L-NAME particularly in vascular endothelium results in hypertension. Chronic hypertension leads to inhibition of eNOS and activation of iNOS. Activation of iNOS induces NO overproduction in various organs such as the kidney resulting in tissue damage.

It has been demonstrated that application of therapeutically feasible doses of EGb761 caused endothelial NO production by increasing eNOS activity and eNOS expression in blood vessels (45). On the other hand, Yang et al. (46). concluded that the beneficial effects of EGb761 in cerebral ischemia/reperfusion injury may result from the reduction of NO production. EGb761 can decrease NO production by inhibiting gene and protein expression and enzymatic activity of iNOS.

In light of these contradictory observations, the role of NO and NOS isoforms in the renoprotective effects of EGb761 was investigated in this study. Our results indicate that in hypertensive rats, the renoprotective effect of EGb761 was associated with a decrease in the renal level of nitrite. Immunostaining of renal tissue showed that EGb761 decreased the protein expression of iNOS and increased the protein expression of eNOS. Consequently, the renoprotective effect of EGb761 is due to inhibition of renal NO overproduction and inhibition of the protein expression of the cytotoxic iNOS and induction of protein expression of the protective eNOS. Frequent administration of losartan for several weeks to hypertensive rats produced similar effects. EGb761 enhanced the effect of losartan. This may confirm the role of NO in the renoprotective effect of EGb761.

Inflammation exerts a critical pathogenic role in the development of hypertension. Renal inflammation has been identified as a characteristic of hypertension, and there is an evidence that inflammatory cytokines contribute to the development of renal injury (47). The renal injury in spontaneously hypertensive rats was associated with an increase in the expression of renal inflammatory cytokines, including TNF-α, IL-6, and IL-1β (13). Also, in patients with glomerulonephritis, the level of IL-1β is increased in blood (48). Moreover, hypertensive renal damage is predominantly caused by inflammatory cells such as macrophages and lymphocytes infiltrating target organ (15). Our results confirm this correlation between inflammatory responses and renal damage in hypertensive rats. In the kidney tissue of hypertensive rats, there was a significant increase in the levels of TNF-α, IL-6, and IL-1β. The protein expression of TNF-α, IL-6, and IL-1β was also increased. Histopathological examination of the kidney tissue showed an increase in inflammatory cell infiltration.

Ginkgo biloba extract was found to inhibit the expression and production of inflammatory cytokines and neutrophil infiltration in experimental animals and lipopolysaccharide-stimulated macrophages (23,25) and in metabolic syndrome patients (19). In addition, the renoprotective effect of losartan was attributed to its anti-inflammatory effect(49). Data of the current study show that repeated administration of EGb761 to hypertensive rats for several weeks led to a reduction in the renal tissue levels of TNF-α, IL-6, and IL1β. Immunohistochemical analysis showed that EGb761 decreased the protein expression of renal TNF-α, IL-6, and
IL-1β. Infiltration of inflammatory cells was inhibited by EGb761 treatment. Repeated treatment of hypertensive rats with losartan for several weeks produced similar effects. EGb761 enhanced the effect of losartan. This may support the role of anti-inflammatory activity of EGb761 in its renoprotective effect.

Taken together, our results indicate that EGb761 has the ability to protect against hypertension-induced renal injury. The ability of EGb761 to provide this renoprotective effect may positively correlate, besides its antihypertensive effect, to its ability to suppress renal oxidative stress, nitrosative stress and inflammation. Similarly, EGb761 has the ability to potentiate the antihypertensive effect of losartan and to potentiate its renoprotective effects. EGb761 may potentiate this effect through enhancement of its ability to attenuate oxidative and nitrosative stresses and suppression of inflammatory responses in the kidney. Thus, EGb761 appears to have a therapeutic potential as an adjuvant agent for protection against kidney injury in hypertensive cases.

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References


