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The Potential Protective Effect of the Standardized *Ginkgo biloba* Leaves Extract EGb761 against Contrast-Induced Acute Kidney Toxicity in Rats via Mitigating Renal Tissue Redox Imbalance, Inflammation, Cell Apoptosis and Mitochondrial Damage

Mahmoud M. Farag [1,*], Mariam A. Wagdy [1], Heba E. Ahmed [1], and Amani H. Kazem [2]

[1] Departments of Pharmacology, Medical Research Institute, Alexandria University, Alexandria 21561 Egypt
[2] Departments of Pathology, Medical Research Institute, Alexandria University, Alexandria 21561 Egypt
* Correspondence: mahmoudfarag2012@hotmail.com; Tel.: +20-1006632014
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Abstract: In clinical medical practice, the applications of diagnostic and interventional procedures requiring iodinated contrast media (CM) administration have recently markedly increased. However, the intrinsic CM toxicity may lead to contrast-induced acute kidney injury (CI-AKI), particularly in patients with renal disease or diabetes. As successful therapy of CI-AKI is rather limited, effective strategies to prevent CI-AKI have become an insistent demand. The aim of this study was to evaluate the potential protective effects of the standardized extract of Ginkgo biloba leaves EGb761 against the pathophysiology of CI-AKI in a rat model. In this study, CI-AKI in rats was evaluated histopathologically and biochemically by measuring serum biomarkers of kidney function and tissue markers of oxidative stress, inflammation, tubular cell apoptosis and mitochondrial injury. Our results showed that CM administration led to several kidney morphological changes with alterations in serum and renal tissue parameters indicative of acute renal toxicity. These changes were moved to normality upon EGb761 treatment before CM exposure via integrated suppression of CM-induced renal tissue redox imbalance, inflammatory response, cell apoptosis activation and tubular cell mitochondrial damage. These findings demonstrated the nephroprotective effectiveness of EGb761 in alleviating CI-AKI pathophysiology through multiple effects. In conclusion, our study suggests a new therapeutic strategy for attenuating CI-AKI via administering EGb761 before CM use and may serve as an experimental basis for further studies to elucidate the promising clinical impact of EGb761 as a nephroprotective agent in patients at the risk of developing CI-AKI.

Keywords: EGb761; CI-AKI; oxidative stress; inflammation; apoptosis; mitochondrial damage

1. Introduction

Because of its role as the primary eliminator of hydrophilic drugs and metabolites besides its relatively large blood flow, the kidney is particularly prone to develop various forms of injury because of the accumulation of excreted drugs and/or their metabolites in renal tubular cells during the processes of tubular reabsorption and secretion [1]. Iodinated contrast-enhanced X-ray, computed tomography, or angiography imaging examinations are done frequently, in clinical practice, for diagnostic or interventional purposes [2]. To achieve these goals, iodine-based contrast media (CM) are administered intravenously or intra-arterially to increase tissue conspicuity and to improve diagnostic and therapeutic accuracy and ability. Despite these benefits, one potential adverse effect that occurs with intravascularly administered CM is contrast-induced acute kidney injury (CI-AKI) with rapid deterioration of renal function within 48 to 72 h after CM administration resulting in serious complications such as acute renal failure and pulmonary edema [3]. CI-AKI pathogenesis has been linked to several pathophysiological mechanisms including: (1) reactive oxygen species (ROS) overproduction in the renal tissue leading to oxidative stress and inflammation with subsequent cell injury [4], (2) direct renal tubular cell injury leading to mitochondrial damage and cell apoptosis [5], and (3) altered renal hemodynamics leading to renal medullary ischemia and hypoxia with subsequent ischemic kidney injury [6]. The latter mechanism was confirmed by the studies reported by Agmon et al., 1994 [7] and Lee et al., 2006 [8] who found that nitric oxide and

prostaglandins protected the outer medulla in rat kidneys from CM-induced nephrotoxicity via increasing regional blood flow. These authors also reported that pretreatment of rats and mice with the inhibitors of nitric oxide and prostaglandin synthesis increased radiocontrast nephrotoxicity as in these experimental models, like healthy humans, CM administration does not induce acute renal injury in animals not bearing any risk factors [6].

Due to the steady increase in the use of radiological procedures that utilize CM for better medical imaging, CI-AKI has become the third most common cause of hospital-acquired AKI after impaired kidney perfusion and medication with nephrotoxic drugs [9]. Since the effective way to treat CI-AKI remains an unmet medical necessity, it is imperative to find an effective strategy to ameliorate or prevent the development of CI-AKI after CM intravascular administration [10]. In the past few years, this issue attracted considerable scientific interest and became an existing topic of several experimental studies and clinical trials to evaluate the nephroprotective efficacy of some therapeutic procedures and agents including intravenous sodium bicarbonate, oral N-acetylcysteine, statins, ascorbic acid and calcium-channel blockers. There is a big controversy surrounding the use of most of these strategies to prevent CI-AKI as many studies have been published since the last three decades with conflicting results [11–13].

EGb761 is a standardized extract of a well-defined mixture of active constituents extracted from *Ginkgo biloba* leaves [14]. This extract contains two main groups of active compounds, flavonoid glycosides (24%) and terpene lactones (6%), which act synergistically on various targets to exert numerous pharmacological effects including antioxidant, anti-inflammatory, anti-apoptotic, and vasodilatory effects, and protection against mitochondrial dysfunction [14,15]. Clinically, EGb761 has been reported to have ameliorative effects in several disorders including cardiovascular diseases and neurodegenerative conditions [16–18]. Furthermore, EGb761 showed beneficial effects in some studies on nephrotoxicity induced by some drugs, e.g., adriamycin and cisplatin [19,20]. To the best of our knowledge, the impact of EGb761 has not been evaluated on CI-AKI despite being an interesting condition with several mechanisms involved in its pathogenesis. Therefore, the present study was designed to evaluate the effects of EGb761 on renal tissue oxidative stress, inflammation, apoptosis and mitochondrial damage, the events that play a crucial role in the development of kidney injury in a model of CI-AKI in rats.

2. Materials and Methods

2.1. Drugs and Chemicals

Ginkgo biloba leaves extract EGb761 powder (Medizen Pharma Co., Alexandria, Egypt) was used as a 4% suspension that was freshly prepared daily in 1% solution of carboxymethyl cellulose (CMC, El-Gomhouria Co. for Drugs, Cairo, Egypt). N(ω)-nitro-L-arginine methyl ester (L-NAME) powder (Thermo Fisher (Kandel) GmbH, Erlenbachweg, Germany) was freshly prepared as a 1% solution in saline. Indomethacin (INDO) ampoules were purchased from El-Nile Pharma Co. Cairo, Egypt. Urografin 76% (ampoules, Bayer Zydus Pharma, Kundaim, Goa, India), containing a mixture of sodium amidotrizoate and amidotrizoate meglumine, is an injectable iodinated ionic monomer, high osmolar radiographic contrast medium (CM) in an aqueous solution. This CM solution was used, in the present study, to induce AKI in rats.

2.2. Animals

This study was performed on adult male albino rats 8-to 10-week-old weighing 200–250 g. Animals were acclimatized for 2 weeks, housed in cages, two per cage, kept under standard conditions of temperature (20–22 °C), humidity (60%) and light (a 12-h light-dark cycle). The rats were maintained on a standard pellet diet and water *ad libitum.* All experimental procedures complied with the ethical Guidelines of the Animal Care Committee of the Medical Research Institute, Alexandria University.

2.3. CI-AKI induction and Animal Grouping

CI-AKI model in rats was conducted as previously described with minor modifications [12]. Briefly, a predisposing effect on CI-AKI induction was provided by dehydration for 24 h, followed by treatment with 10 mg/kg L-NAME, as a nitric oxide synthase inhibitor, and 10 mg/kg INDO, as a prostaglandin synthase inhibitor [12]. After that, CI-AKI was induced using a 12.5 mL/kg injection of CM. The doses of L-NAME, INDO and CM were administered intraperitoneally with 30-min time intervals. Following the completion of these injections, water restriction was continued for a further 6 h to make the kidneys more susceptible to CM nephrotoxicity. Thereafter, water was allowed for 18 h till the end of the study (24 h after CI-AKI induction). Four groups of rats (5–7 rats per group) were included in this study:

- Control group received the vehicles only throughout the study.
- EGb group received EGb761 at a dose of 100 mg/kg orally once daily [21] for 5 days.
- CM group received vehicles for 5 days. On the 4th day of the study, the rats were exposed to dehydration for 24 h. L-NAME, INDO, and CM were given after dehydration as mentioned above.
- EGb + CM group received EGb761 at a dose of 100 mg/kg orally once daily for 5 days. On the 4th day, the rats were exposed to dehydration for 24 h followed by giving L-NAME, INDO, and CM as mentioned in the CM group.

Twenty-four hours after CI-AKI induction, all rats were scarified, and blood samples were collected. Both kidneys were rapidly isolated, rinsed with ice-cold saline, blotted dry, and weighed. Blood samples were left to clot for 30 min at room temperature. Serum was separated and kept at −20 °C for later measuring of serum parameters. The right kidney was used for the histopathologic examination. The left kidney was immediately washed in ice-cold phosphate-buffered saline (0.1 M at pH 7.4), cut into small pieces, homogenized, and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was separated and stored at −80 °C until assayed.

2.4. Serum Biochemical Assays

Serum creatinine and urea concentrations were determined calorimetrically using commercially available diagnostic kits. Serum neutrophil gelatinase-associated lipocalin (NGAL) levels were determined using a rat NGAL ELISA kit (Chongqing Biospes Co., Chongqing, China).

2.5. Kidney Tissue Biochemical Assays

2.5.1. Oxidative Stress and Antioxidant Markers

Tissue malondialdehyde (MDA) content was determined using the method of Draper and Hadly [22]. The enzymatic method described by Griffith, OW [23] was used to measure glutathione and glutathione disulfide (GSSG) contents and then to calculate the content of reduced glutathione (GSH). For superoxide dismutase (SOD) assay, the activity of the enzyme was the determined by the pyrogallol method of Marklund and Marklund [24].

2.5.2. Proinflammatory Cytokines

Tissue levels of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α) were determined using rat ELISA kits (My BioSource Co., San Diego, CA, USA) according to the instructions of the manufacturer.

2.5.3. Caspase-3 Activity

Tissue caspase-3 activity was determined using a rat caspase-3 assay kit (Elabscience Co., Houston, TX, USA) following the manufacturer's instructions.

2.5.4. Mitochondrial DNA (mtDNA) Copy Number per Cell

A quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay has been developed to estimate levels of mtDNA copy numbers in samples [25,26]. This approach measures the mtDNA copy number by determining the ratio of PCR amplicons of mitochondrial sequence to that of a single nuclear gene in experimental samples. After total genomic DNA isolation, a specific primer pair for mtDNA and a primer pair for nuclear peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α, Table 1) were used to perform the same number of PCR cycles and calculate the relative mtDNA signal to the nuclear DNA (nDNA) signal. Total DNA was isolated from the rat kidney tissue using DNeasy mini kit (Qiagen Co., Germantown, MD, USA) according to the manufacturer's instructions (The procedure of this assay was described in Supplementary Materials).

Table 1. Primers for PGC-1α and mtDNA for real time-PCR.

Gene Name		Primer Sequence
PGC-1α	F	5′-ATGAATGCAGCGGTCTTAGC-3′
	R	5′-AACAATGGCAGGGTTTGTTC-3′
mtDNA	F	5′-ACACCAAAAGGACGAACCTG-3′
	R	5′-ATGGGGAAGAAGCCCTAGAA-3′

PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1α; mtDNA, mitochondrial DNA; F, forward; R, reverse.

2.5.5. Total Protein Content

The protein content of kidney tissue samples was determined according to the method described by Lowry et al., using bovine serum albumin as a standard [27].

2.6. Kidney Histopathology

Representative portions of the excised right kidney were fixed in 10% neutral buffered formalin for 24 h and, thereafter, dehydrated in graded alcohol concentrations and embedded in paraffin. Four micron-thick sections were cut and stained with hematoxylin and eosin (H&E) for light microscopic examination.

2.7. Statistical Analysis

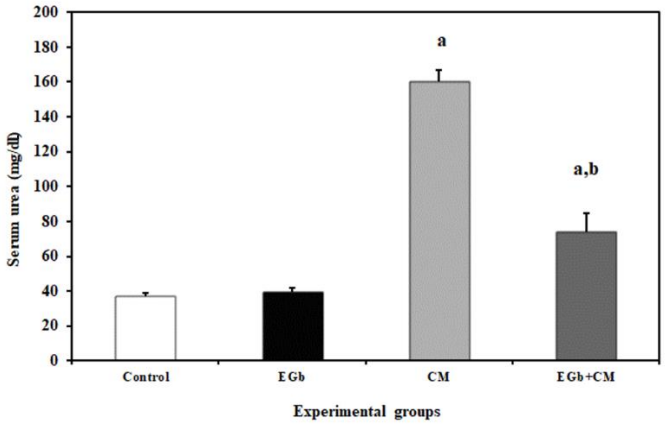
The computer package SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for the analysis of the present study data. Shapiro-Wilk test was performed on all data sets to ensure normal distribution [28]. The results of this test are presented in a table form in Supplementary Materials. The data were expressed as mean ± SEM. For comparisons among groups, the analysis of variance (one-way ANOVA) method was applied, followed by Tukey's multiple comparison procedure. The correlation between variables was tested by computing the correlation coefficient (r, Pearson's test). Values of $p < 0.05$ were considered significant.

3. Results

Two out of nine of rats in the CM group died in this study. No mortalities were recorded in rats of other experimental groups.

3.1. Influence of Treatment on Serum Biochemical Parameters of Kidney Function

In the current study, kidney function was evaluated by measuring serum levels of urea, creatinine and NGAL. No significant changes in the levels of these parameters were observed in the EGb group, as compared to the control group. Figure 1 shows the levels of serum urea, creatinine and NGAL in all groups. In CM-treated rats, serum urea, creatinine and NGAL levels increased by 3.3, 2.5, and 4.6 folds, respectively, compared to control rats ($p < 0.05$). These notable rises in serum levels of urea, creatinine and NAGL were significantly reduced by 53.9, 61.6 and 67.3%, respectively, in rats of the EGb + CM group, as compared to the CM group.



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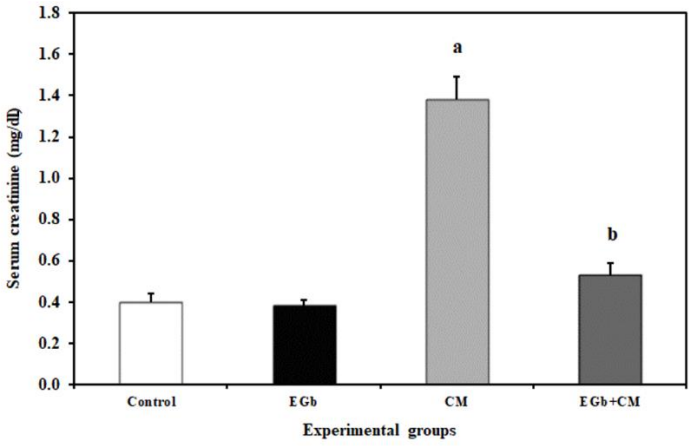

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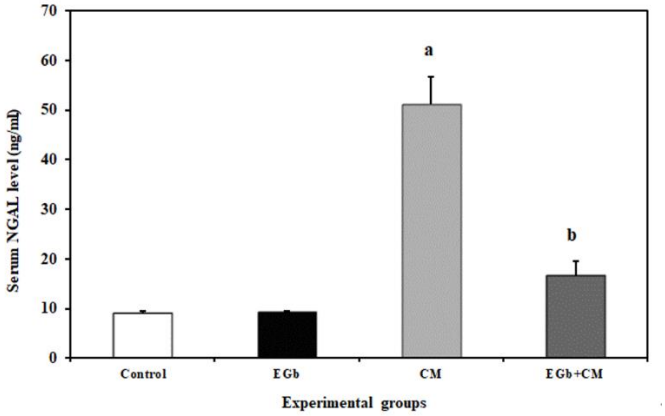

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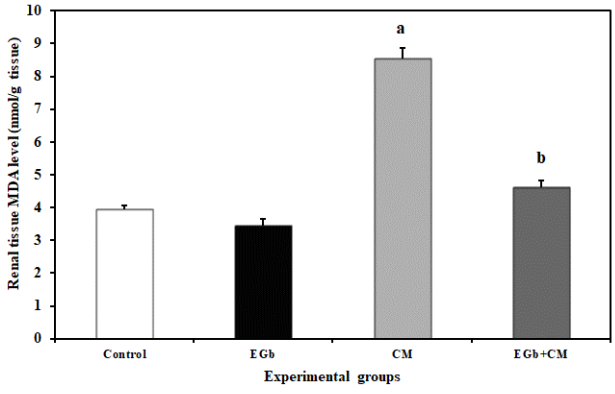
Figure 1. Effects of EGb761 on CM-induced changes in serum urea (**A**), creatinine (**B**) and neutrophil gelatinase-associated lipocalin (NGAL, **C**) levels. Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

3.2. Influence of Treatment on Kidney Tissue Oxidant/Antioxidant Markers

3.2.1. Tissue Levels of MDA as a Marker of Lipid Peroxidation

As shown in Figure 2A, CM administration resulted in a significant increase by 1.2-fold in renal tissue MDA level, compared to the control level. A significant reduction of this elevated MDA level by 46% was observed in the EGb + CM group, compared to the CM group. In fact, the renal tissue MDA level in the EGb + CM group was very close to the corresponding level in control rats.



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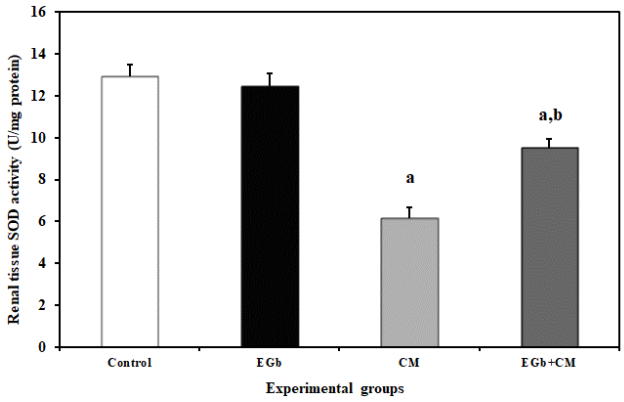

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Figure 2. Ameliorative effects of EGb761 on CM-induced changes in renal tissue levels of malondialdehyde (MDA) content (**A**) and superoxide dismutase (SOD) activity (**B**). Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

3.2.2. Tissue Levels of the Antioxidant Enzyme SOD

Renal tissue SOD activity was found to be significantly reduced by 52.4% in the CM group, compared to the control group (Figure 2B). Treatment of rats with EGb761, before CM exposure, significantly elevated the CM-induced reduction in renal tissue SOD activity by 54.4% in the EGb + CM group, as compared to the CM group.

3.2.3. Tissue Changes in GSH and GSSG Levels

As shown in Table 2, the levels of the nonenzymatic antioxidant GSH and GSH/GSSG ratio, in renal tissue significantly decreased by 40.3 and 70.6%, respectively, whereas the levels of the oxidant disulfide GSSG significantly increased by 100% in the CM group, as compared to the control group. Pretreatment of CM-treated rats with EGb761 significantly elevated the CM-induced reduced renal tissue levels of both GSH content and GSH/GSSG ratios by 41.8 and 100.5%, respectively, and significantly reduced the CM-induced elevated GSSG tissue levels by 29.5% in the EGb + CM group, as compared to the CM group.

Table 2. Effects of EGb761 on CM-induced changes in renal tissue reduced glutathione (GSH) and oxidized glutathione (GSSG) contents, and GSH to GSSG ratio in rats.

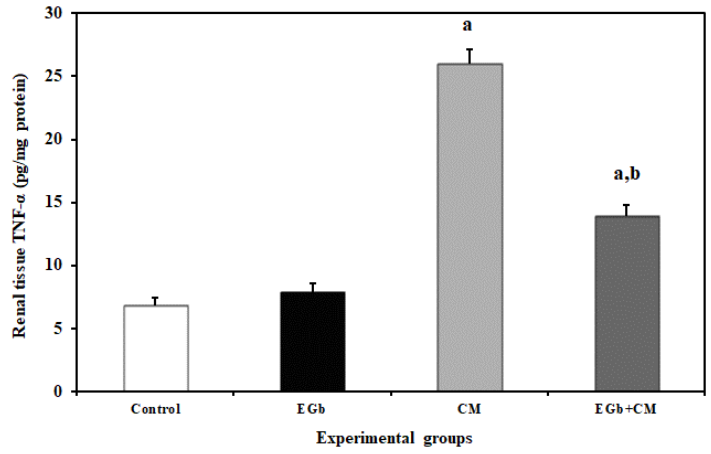
	Rat Groups	GSH (nmol/mg Protein)	GSSG (nmol/mg Protein)	GSH/GSSG Ratio
-	Control	9.94 ± 0.40	0.53 ± 0.03	18.95 ± 0.74
-	EGb	9.98 ± 0.35	0.47 ± 0.03	21.48 ± 1.35
-	CM	5.93 ± 0.26 [a]	1.06 ± 0.04 [a]	5.58 ± 0.18 [a]
-	EGb + CM	8.41 ± 0.26 [a,b]	0.75 ± 0.03 [a,b]	11.19 ± 0.30 [a,b]

Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract (EGb 761); CM, contrast medium. a: $p < 0.05$, as compared to the control group. b: $p < 0.05$, as compared to the CM group.

3.3. Influence of Treatment on Kidney Tissue Pro-Inflammatory Cytokines

In CM-treated rats, a significant elevation in the renal tissue TNF-α level by 2.8-fold, was observed, as compared to the control group (Figure 3A). On the other hand, pretreatment of rats with EGb761 reduced this CM-induced elevation in tissue TNF-α content by 46.6% in the EGb + CM group, as compared to the CM group ($p < 0.05$).

Also, CM administration led to a marked increase by 3.6-fold in the tissue IL-1β content compared to the control group. This elevation was found to be significantly decreased by 45.1% in the EGb + CM group, compared to the CM group (Figure 3B). In addition, CM administration resulted in a notable rise in the renal tissue IL-6 content by 2.2-fold, compared to the control group, and this CM-induced elevation in the tissue level of this parameter was significantly reduced by 42.2% in the EGb + CM group, as compared to the CM group (Figure 3C).



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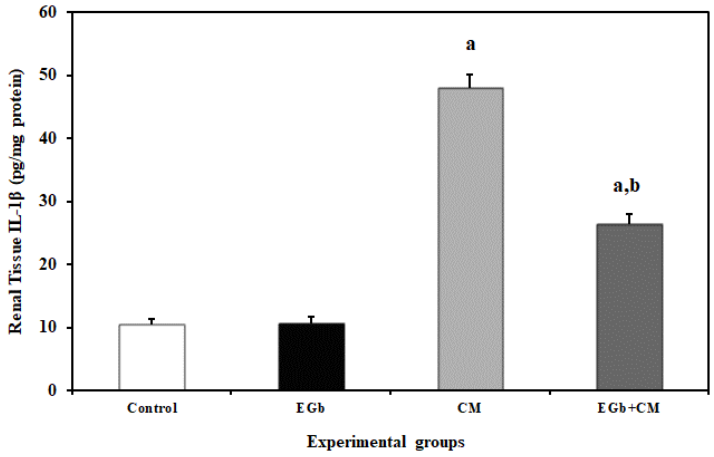

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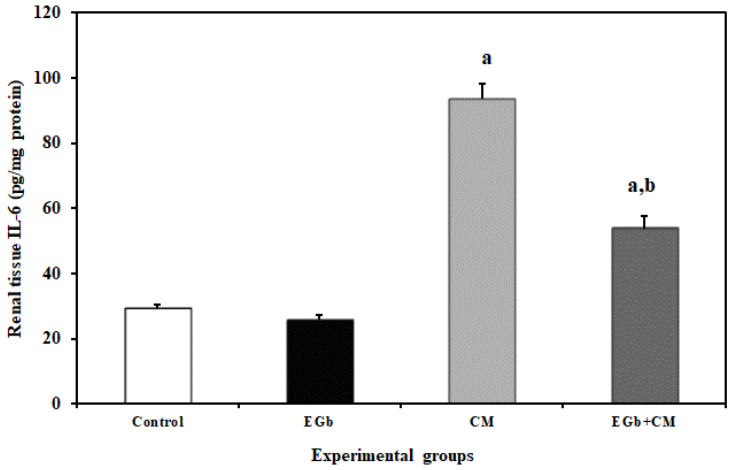


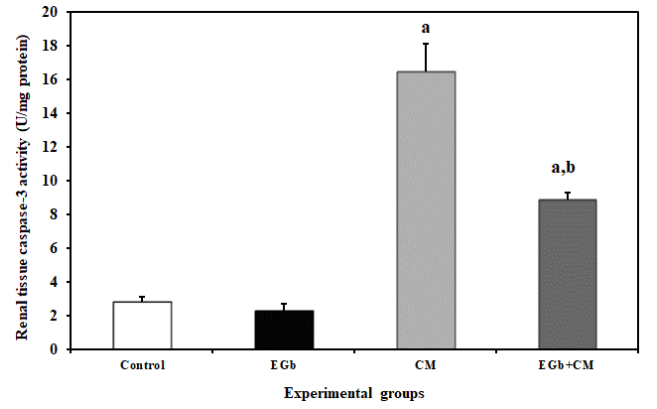
(**C**)

Figure 3. Ameliorative effects of EGb761 on CM-induced changes in renal tissue tumor necrosis factor-alpha (TNF-α, **A**), interleukin-1 beta (IL-1β, **B**) and IL-6 (**C**) levels. Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

3.4. Influence of Treatment on Kidney Tissue Apoptosis and mtDNA

3.4.1. Tissue Caspase-3 Activity Changes

As shown in Figure 4A, a marked increase in tissue caspase-3 activity by 4.9-fold was noticed in CM-treated rats, compared to control rats. Pretreatments of rats with EGb761 significantly reduced the CM-induced elevations in caspase-3 activity by 46.2%, compared to the CM group (Figure 4A).



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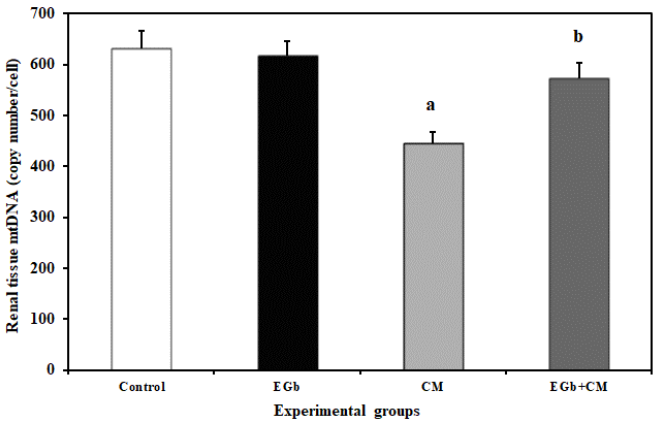

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Figure 4. Ameliorative effects of EGb761 on CM-induced changes in renal tissue caspase-3 activity levels (**A**) and mitochondrial DNA (mtDNA) contents (**B**). Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

3.4.2. Tissue mtDNA Content Changes

The involvement of mitochondrial malfunction and damage in kidney cell death was evaluated, in this study, by measuring the renal tissue mtDNA content (Figure 4B). CM administration resulted in a significant decrease in renal tissue mtDNA copy number/cell by 29.4%, compared to the control group. Treatment of rats with EGb761, before CM exposure, normalized the level of mtDNA copy number/cell as this pretreatment prevented the CM-induced decline in this parameter (Figure 4B).

3.5. Tissue and Serum Data Correlation Assessment

Putting together results from all experimental groups, highly significant positive correlations were found between renal tissue levels of the apoptotic marker caspase-3 activity and both renal tissue levels of MDA, as a biomarker of lipid peroxidation, and serum levels of NGAL, as a biomarker of kidney dysfunction (Table 3). Conversely, the renal tissue levels of mtDNA content correlated negatively with the renal tissue levels of both caspase-3activity and MDA and, also, with serum levels of NGAL (Table 3).

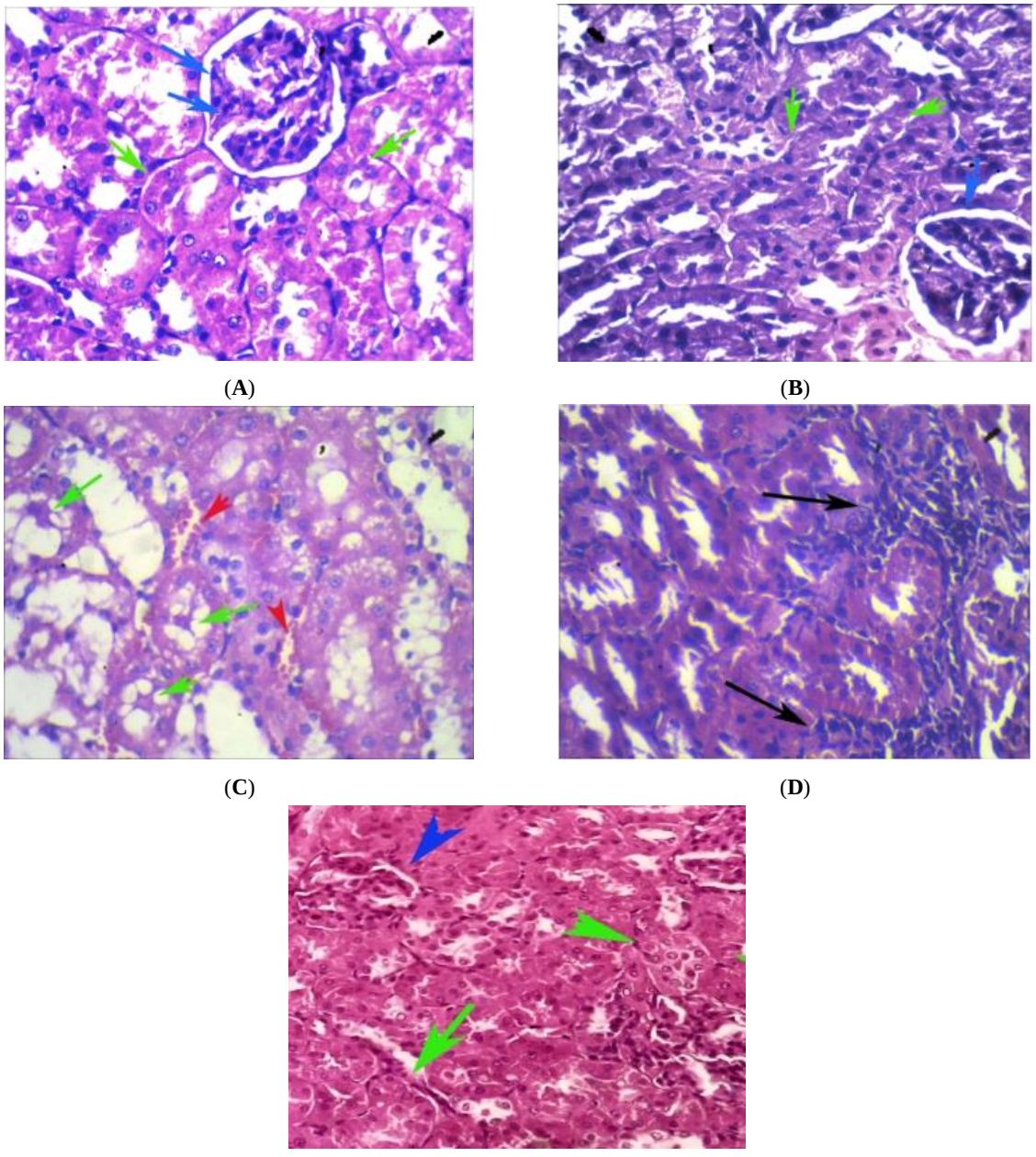
Table 3. Correlation coefficients (r values, Pearson's test) between markers of apoptosis, mitochondrial injury, oxidative stress and kidney function using the data of rats in all experimental groups at the end of the study.

Renal Tissue Markers	Serum NGAL	Renal Tissue	
		MDA	Caspase-3 Activity
Caspase-3 activity	0.707 [a]	0.827 [a]	-
mtDNA content	−0.667 [a]	−0.736 [a]	−0.637 [a]

[a] $p < 0.001$, n = 24. Caspase-3 activity, a marker of apoptosis; mtDNA content, a marker of mitochondrial injury; NGAL, neutrophil gelatinase-associated lipocalin, a marker of kidney function; MDA, malondialdehyde as a marker of oxidative stress.

3.6. Influence of Treatment on Kidney Histopathology

Photomicrographs of kidney tissue sections from rats of control and EGb groups (Figure 5 A, B, respectively) were similar and showed a normal kidney histological morphology with intact normal well-defined glomeruli and tubules. In the CM group rats, the kidney tissue sections displayed several major histopathological abnormalities including congestion of the intertubular blood vessels, interstitial mononuclear cells (mainly lymphocytes) infiltration and evidence of tubular injury in the form of vacuolar degenerative changes of renal tubular cells, tubular dilatation, and dilated tubules filled with hyaline casts (Figure 5C,D). Examination of kidney tissue sections from the EGb + CM group showed marked improvement of the CM-induced abnormalities as the glomeruli and renal tubules appeared somewhat normal (Figure 5E), having the same histological features of kidneys of control rats (Figure 5A, B).



(**A**) (**B**)

(**C**) (**D**)

(**E**)

Figure 5. Protective effects of EGb 761 on CM-induced renal histopathological changes. Photomicrographs of kidney tissue sections (H&E, ×400) from: *Control (**A**) and EGb-treated rats (**B**) showing renal cortex with intact normal glomeruli (blue arrows) and normal tubules lined with columnar epithelial cells (green arrows), *CM-treated rats (**C**,**D**) showing dilated tubules filled with hyaline casts with vacuolar degenerative changes of renal tubular cells (green arrows), congested intertubular blood capillaries (red arrows), multiple areas of atrophic cystic tubules (**C**), and heavy interstitial mononuclear cells infiltration mainly lymphocytes **(**black arrows, **D**), and *EGb + CM group (**E**) showing nearly normal kidney histoarchitecture with intact tubules lined by columnar epithelial cells (green arrows) and normal glomeruli (blue arrows).

4. Discussion

As far as we know, no previous research work studying the potential effects of EGb761 on CI-AKI has been reported. The current study was designed to investigate the potential protective effect of EGb761 against CI-AKI in rats. The results of the present study demonstrated that CM-treated rats showed the features of AKI and treatment of rats with EGb761 before CM exposure provided nephroprotection as evidenced by several major findings in the EGb + CM group. First, as compared to the nonpretreated CM group, the results showed significant decreases of the CM-induced elevated serum levels of urea, creatinine and NGAL (as indices of kidney function), and of the renal tissue levels of MDA (an index of lipid peroxidation), TNF-α, IL-1β and IL-6 (as indices of inflammation), and caspase-3 activity (an apoptotic marker). Second, significant increases of the CM-induced reduced renal tissue levels of SOD, GSH and GSH/GSSG ratio (as indices of tissue antioxidant defenses) and mtDNA copy number/cell (a biomarker of mitochondrial function). Third, the histological architecture of kidneys from the EGb + CM group rats appeared normal having features like those of control rats.

In the current study, the rats of the CM group showed marked impairment of kidney function with elevated serum creatinine and urea levels. Based only on these parameters, the assessment of renal function may not always be satisfactory as their serum levels may be affected by extra-renal factors and may not change until a significant fraction (>50%) of kidney function has already been lost [29,30]. Therefore, serum NGAL level was measured as it fulfills many criteria for a sensitive and specific biomarker for AKI as described by Bolignano et al. [31]. The CM group rats, in the current study, showed a marked rise in serum NGAL level, as compared to control rats. This finding is in agreement with the results of several studies that have shown that serum NGAL level can be used as a diagnostic biomarker for AKI as it increases proportionally to the extent of kidney damage [31–33].

There is growing evidence that CM administration may lead to imbalance between oxidative and antioxidative factors in the kidney tissue resulting in excessive accumulation of ROS, especially hydroxyl and superoxide radicals [5,34]. In our study, CM-induced oxidative stress was manifested by a reduction in renal tissue GSH content and SOD activity with a marked elevation in the tissue level of MDA. The histopathological changes observed in kidney sections from CM-treated rats confirmed the induction of AKI and provided an explanation for the observed changes in kidney function. In addition, it has been reported that increased ROS production in response to kidney injury may lead to increased synthesis and release of inflammatory mediators that can initiate and amplify inflammation and exacerbate apoptosis in renal tubular cells [4,35]. In parallel, increased ROS at the site of inflammation may cause endothelial dysfunction leading to opening of the interendothelial junctions with subsequent increased migration of inflammatory cells across the endothelial barrier into the renal tissue [34,36]. In accordance with these reports, our results showed marked elevation in renal tissue levels of proinflammatory cytokines and the apoptotic marker besides the finding of interstitial mononuclear cell infiltrations in kidney sections from CM group rats.

Noteworthily, the results of the studies on mitochondria reported by Pello et al. [37] and Vakifahmetoglu et al. [38] suggest that there is a direct link between oxidative stress-induced mitochondrial dysfunction and cell death. Mitochondrial dysfunction is a hallmark in a variety of diseases including CI-AKI as it results in inefficient cellular energy production, enhanced ROS generation and increased cellular apoptosis [37,38]. In fact, mitochondria are the main source of intracellular ROS production and, at the same time, the main target for the ROS unfavorable effects including mitochondrial damage [38]. Within the mitochondrion, mtDNA seems to be the main target for ROS-induced oxidative damage due to its lack of protective histones and its closeness to the electron transport chain, the principal site for the generation of ROS [39]. Recently, mtDNA copy number has been suggested as a promising biomarker of mitochondrial dysfunction especially in conditions associated with oxidative stress as the increased ROS production may cause damage to cellular components including mtDNA [40]. Therefore, assessment of mtDNA copy number changes could help in understanding the pathogenesis of AKI [41]. Our results indicated that renal tissue levels of mtDNA copy number, in CM group rats, were significantly lower than the corresponding values in control rats, a change that could be attributed to the CM-induced oxidative damage to mtDNA. This interpretation is supported by the negative correlation, observed in the current study, between the renal tissue levels of both mtDNA copy number and the lipid peroxidation marker MDA.

Apart from its involvement in many of physiological processes, renal cell apoptosis seems to play a pivotal role in drug-induced nephrotoxicity including CI-AKI [42]. In this regard, excessive ROS production has been suggested as an initiator of apoptotic cell death through the activation of apoptosis signaling pathways [43]. Recently, mitochondria have also gained great importance as a primary player in cellular apoptosis. It has been reported that mitochondrial damage results in the release of several proteins into the cytosol including pro-caspases and cytochrome C that activate catabolic caspases including caspase-3, a critical factor in the apoptotic execution

stage [44]. In agreement with these findings, our results showed that renal tissue caspase-3 levels correlated negatively with renal tissue mtDNA levels but positively correlated with renal tissue MDA levels.

As no available pharmaceutical agents have been proven to effectively treat CI-AKI, many researchers have evaluated a variety of synthetic drugs and natural products for the prevention of CI-AKI [9,45]. Although some of the tested agents have been proven to be effective, there is still room for improvement via targeting the pathophysiologic mechanisms involved in CI-AKI development. In recent years, herbal medicines are increasingly gaining greater acceptance from the public and medical profession due to good therapeutic efficacy, low side effects and lower cost than synthetic drugs [46]. In this regard, EGb761 is one of the most common herbal medicines that has multiple uses associated with several health claims, mostly in relation to central nervous system disorders, cardiovascular problems and pulmonary diseases [17,18,47]. In the present study, rat treatment with EGb761, before CM administration, significantly reduced the CM-induced elevation in serum urea levels and normalized elevated serum creatinine and NGAL levels as compared to control rats. This improvement in kidney function was associated with marked amelioration of the CM-induced renal tissue histopathological changes with apparently normal glomeruli and renal tubules. Moreover, our results demonstrated that rat treatment with EGb761, before CM administration, improved the endogenous renal antioxidant defense status as indicated by the observed increases in renal tissue levels of GSH, GSH/GSSG ratio and SOD activity, compared to CM group rats, leading to normalization of the elevated renal tissue MDA levels. The EGb761-induced increase in intracellular GSH content could serve to detoxify ROS by directly scavenging them as well as indirectly by acting as a co-substrate in the glutathione peroxidase-catalyzed reduction of H_2O_2 and lipid peroxides [16,48]. Also, the increased SOD activity induced by EGb761 could lead to dismutation of superoxide radicals and prevents further production of free radicals such as peroxynitrite and hydroxyl radicals [49,50].

Apart from oxidative stress, inflammation is a defensive host reaction, in response to pathogenic stimuli, that includes recruitment and proliferation of inflammatory cells in addition to the secretion of cytokines that play a crucial role in inflammation regulation [51,52]. Accordingly, inhibition of excessive production of these mediators could be a strategy to prevent the occurrence and/or progression of inflammation associated with CI-AKI. The present study showed that treatment of rats with EGb761, before CM administration, significantly reduced CM-induced elevations in renal tissue levels of the proinflammatory cytokines with marked amelioration of kidney inflammatory histopathological features, as compared to the CM group. These results agree with several studies which revealed that EGb761 exerted a marked anti-inflammatory effect in different models of acute tissue injury [21,53,54]. Several mechanisms have been suggested for the anti-inflammatory effect of EGb761 including inhibition of excessive mRNA and protein expression levels of both inducible nitric oxide synthase and cyclooxygenase-2, suppression of nuclear factor kappa-B and inhibition of secretion of pro-inflammatory cytokines [53,54]. In addition, our results demonstrated that pretreatment of rats with EGb761 displayed an antiapoptotic effect through ameliorating the CM-induced elevation of renal tissue caspase-3 activity in the EGb + CM group, as compared to the CM group. Previous studies had related the antiapoptotic effect of EGb761 to its ability to cause downregulation of tissue Bax mRNA and p53 mRNA expressions in different rat models [55,56]. Regarding its role in apoptosis, p53 expression was reported to be upregulated following mtDNA damage leading to activation of caspase-3 enzyme and initiation of mitochondrial apoptosis [55,57].

Because of the well-established role of mitochondria in cellular energy production, these organelles are especially abundant in renal proximal tubular cells and are of critical importance in kidney function as kidneys require a great amount of energy to perform their tasks [58]. As a key indicator of mitochondrial function, mtDNA copy number abnormalities have been observed during the development of AKI [41]. Mitochondrial exposure to excessive ROS might cause damage to DNA replication enzymes and thereby aggravate the reduction of the mtDNA copy number [59]. In the current study, the reduced levels of renal tissue mtDNA, observed in the CM group, were reversed by EGb761 pretreatment in the EGb + CM group. The mechanism of the EGb761 protective effect on renal tissue mitochondria may be related to both mild uncoupling of mitochondria with subsequent reduction of ROS production and direct scavenging of ROS by the flavonoids present in EGb761 [60,61].

5. Conclusions

The present study, to the best of our knowledge, is the first research work demonstrating that treatment with EGb761 before CM exposure in rats had a significant nephroprotective effect against CI-AKI development with preservation of kidney function. This beneficial effect of EGb761 could be attributed to interrelated suppressions of CM-induced increases in renal tissue oxidative stress, inflammation, apoptosis and tubular cell mitochondrial damage. Taken together, our results are potentially of clinical significance and suggest a new therapeutic strategy for attenuating CI-AKI via using EGb761, as a preventive agent, before CM administration. Thus, our findings

may serve as an experimental basis for further studies to elucidate the clinical implications of EGb761 as a nephroprotective agent in patients at the risk of developing CI-AKI.

Supplementary Materials: The supporting information can be downloaded at: https://www.sciltp.com/journals/jmnp/articles/2505000593/s1.

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