



Chlorogenic acid combats cyclophosphamide-induced nephrotoxicity; The role of the antioxidant defenses, inflammatory signals, and apoptosis

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ABSTRACT

Introduction: Cyclophosphamide (CPA) is an effective antineoplastic agent whose clinical utility is often limited by its potential to induce marked renal injury. This nephrotoxicity has been attributed to disrupted oxidative balance, exaggerated inflammatory signaling, activation of apoptotic pathways, and impairment of autophagic processes.

Objectives: This experimental study explored the possible reno-protective effects of chlorogenic acid (CGA) against CPA-induced nephrotoxicity.

Materials and Methods: In a rodent model of CPA-induced nephrotoxicity, CGA was administered orally at 2 dose levels for ten days, with a single CPA injection (150 mg/kg) given on the 4th day.

Results: In our study, CGA treatment dose-dependently attenuated CPA-induced elevations in renal damage biomarkers and reduced renal malondialdehyde levels in a dose-dependent manner and enhanced antioxidant defense systems in kidney tissue. In addition, CGA dose-dependently suppressed inflammatory mediators, including nuclear factor kappa B (NF-κB), interleukin-1 beta (IL-1β), and transforming growth factor beta 1 (TGF-β1). Moreover, it downregulated the apoptotic marker cleaved caspase-3 while upregulating the autophagy-related protein beclin-1 in a dose-dependent fashion. The reno-protective effects of CGA were further supported by histopathological studies.

Conclusion: All these findings suggest that CGA dose-dependently alleviates CPA-induced nephrotoxicity via suppression of redox imbalance, inflammation, apoptosis and promotion of autophagic activity.

Implication for health policy/practice/research/medical education:

This study emphasizes the importance of chlorogenic acid (CGA) for amelioration of cyclophosphamide (CPA)-induced nephrotoxicity in rats. It highlights the underlying mechanistic pathways, emphasizing oxidative stress, inflammatory cascade, and markers of cell death/autophagy.

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Introduction

Chemotherapy continues to be a pillar of cancer treatment, yet it is frequently accompanied by considerable side effects because its toxic effects often extend beyond tumor cells and can damage healthy rapidly dividing cells, cause complications across multiple organ systems, and substantially impair quality of life across physical, emotional, and social domains (1-3). Cyclophosphamide (CPA) is used widely for management of various neoplasms, including breast, ovarian, lung, and hematologic cancers (4). Nephrotoxicity is one of the main concerns because the kidney is responsible for the

elimination of antineoplastic drugs and their metabolic byproducts, rendering renal tissue vulnerable to damage (3). Thus, CPA administration has been associated with a wide variety of toxic effects, and renal damage is one of the most noticeable complications (5).

Hepatic metabolic activation of CPA leads to the formation of 4-hydroxycyclophosphamide, which is further converted to the toxic metabolites acrolein and phosphoramidate, central to CPA-induced kidney injury (6). These reactive metabolites can covalently bind to cellular proteins, disrupting essential cellular processes and eventually triggering cell death (7). The excretion of

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unchanged CPA in urine accounts for a small percentage and the considerable renal tubular reabsorption favors intrarenal retention of toxic metabolites (8). This accumulation seems to directly contribute to renal injury. Oxidative stress is now recognized as a major mechanism of chemically induced proximal tubular toxicity (9). Experimental data point to oxidative stress as an important factor in the pathogenesis of CPA nephrotoxicity, especially the overproduction of free radicals and reactive oxygen species (ROS) in the kidney (10). Increased ROS generation also worsens the intrarenal inflammatory responses through upregulating cytokine-mediated inflammation and the activation of transcriptional pathways, which in turn enhance the apoptotic signaling and induce remarkable nephron injury (11). Therefore, endogenous and exogenous antioxidants such as phytochemicals and other small molecules that can scavenge free radicals have been considered as potential nephroprotective interventions against the oxidative, inflammatory and apoptotic damage associated with CPA exposure (12).

Chlorogenic acid (CGA) is a dietary polyphenolic compound abundantly present in numerous plant-derived foods, including coffee, certain fruits (e.g., apples, blueberries), and vegetables such as aubergine and chicory, and is also a major constituent of several traditional Chinese herbal formulations (13). CGA is increasingly recognized as a pharmacologically active compound with protective effects in several organ systems. The relevance of CGA has been reported in experimental and clinical studies of metabolic diseases like obesity, type 2 diabetes, oncological diseases such as colorectal cancer, and neurodegenerative diseases (14). These wide biological actions are mainly due to their well-known antioxidant and anti-inflammatory effects, as well as their effects on redox-sensitive signaling pathways and on the regulation of autophagy-apoptosis crosstalk in different tissues (15). In models of metabolic or toxic injury of the kidney and other organs, CGA reduces oxidative stress, blocks inflammatory signaling, and reduces apoptotic and autophagic dysregulation, thus showing important cytoprotective effects (16). Collectively, these findings indicate that CGA might have a beneficial protective effect on CPA-induced renal injury.

Objectives

This experimental study evaluates the impact of CGA administration on CPA-induced renal toxicity in rats and characterizes the mechanistic pathways involved, with a particular emphasis on oxidative stress, inflammatory processes, and apoptosis- and autophagy-related markers.

Materials and Methods

Experimental drugs and chemicals

Cyclophosphamide (purity $\geq 98\%$; CAS No. 50-18-0) was obtained from Santa Cruz Biotechnology, Inc. (Dallas,

Texas, USA), whereas CGA in solid crystalline form was supplied by Cayman Chemical (Ann Arbor, Michigan, USA, Item No. 70930, purity $\geq 95\%$). Formaldehyde, which was used for the preparation of fixative solutions for histopathological tissue processing, was sourced from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

The animals used and the experimental protocol

Sixty male adult rats of the Wistar strain weighing 150–210 g were kept in wire-mesh cages under appropriate hygienic standards and standardized animal housing conditions. The study protocol underwent thorough revision and approval by the Research Ethics Committee of the Faculty of Pharmacy, Helwan University, Egypt (Approval code: 27A2024). These rats were left to acclimatize for two weeks before the start of the experiment.

The animals were randomly allocated into four groups of equal size, each group consisting of fifteen rats, which were treated over a 10-day experimental period. The control group received a single intraperitoneal (i.p.) injection of normal saline on day 4. The CPA group was administered a single i.p. of CPA (150 mg/kg) on day 4 (17). Groups III and IV were pretreated via oral administration of CGA at 2 dose levels, namely 100 and 150 mg/kg, dosed once each day for 10 days (18). On day 4, these groups also received a single i.p. injection of CPA (150 mg/kg), administered 1 hour following CGA treatment.

Upon completion of the experiment (day 10), the rats were placed individually in metabolic cages for 24-hour collection of urine. Urinary N-acetyl- β -D-glucosaminidase (NAG) was then measured according to the method described by Price RG previously (19). Afterward, the animals underwent overnight fasting, followed by blood sample collection the next morning by cardiac puncture under ether anesthesia. The blood was centrifuged at 3000 rpm for 15 minutes using a refrigerated centrifuge to separate the serum, which was then used to measure serum creatinine and blood urea. Following blood collection, the rats were sacrificed by cervical dislocation, the kidneys were excised, and the right kidneys were reserved for subsequent pathological investigations. The left kidneys were processed by homogenization using an ACCULAB USA homogenizer, after which the homogenates were centrifuged at 4000 rpm for 15 minutes at 4°C, and the collected supernatants were stored at -80°C for later biochemical analysis.

Biochemical evaluation of renal functional status

Colorimetric assay kits supplied by StressMarq Biosciences Inc., Victoria, BC V8T 2C1, Canada were employed to measure blood urea and serum and urinary creatinine (Catalog No. SKT-213 and SKT-217, respectively). Creatinine clearance was determined using the Cockcroft–Gault equation (20). Urinary NAG levels were quantified using kits purchased from Crystal Chem, Itasca, IL 60143, United States (Catalog No. 80390). All

assays were conducted following the manufacturers' recommended protocols.

Evaluation of biochemical parameters in renal tissue

Renal tissue supernatants obtained after homogenization were used for the assay of Malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity utilizing commercial assay kits obtained from SunLong Biotech Co., Ltd, Binjiang District, Hangzhou, Zhejiang, China (Catalog No. SL1189Ra and AK0583, respectively). Renal reduced glutathione (GSH) content was determined using kits sourced from AFG Bioscience, Northbrook, IL 60062, United States (Catalog No. EK720391). In addition, interleukin-1 beta (IL-1 β), transforming growth factor beta 1 (TGF- β 1), and nuclear factor- κ B (NF- κ B) concentrations were quantified using ELISA (enzyme-linked immunosorbent assay) kits acquired from Wuhan Fine Biotech Co., Wuhan, 430074, Hubei, China (Catalog numbers ER1094, ER1187, and ER1378, respectively). All assays were executed according to the manufacturers' recommended protocols, and the results were normalized to kidney tissue content.

Immunohistochemical assessment of cleaved caspase-3 in renal tissue samples

The extent and intensity of cleaved caspase-3 immunostaining in renal tissue were assessed in formalin-fixed, paraffin-embedded sections following the method outlined by Buyuklu et al (21). Kidney specimens were fixed in formalin, embedded in paraffin, and sectioned at a thickness of 5 μ m using a BioLAB rotatory microtome (BHTP-203, Biolab Scientific Ltd., Ontario L3R 0B8, Canada). Tissue processing, antigen retrieval, and blocking of endogenous peroxidase activity were performed as described by Buyuklu et al (21). Then, these sections were incubated with a rabbit monoclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA; catalog number 9664) for 24 hours at a temperature of 4 °C. After washing, a biotinylated secondary antibody (Proteintech Group, Inc., Rosemont, IL 60018, United States; catalog number RMX00003) was applied, and hematoxylin was used as the nuclear counterstain. Then, further tissue processing was performed and the intensity of staining was assessed in 10 randomly selected fields for each section under an Olympus light microscope (Olympus, Japan) at a magnification of $\times$ 400. Immunostaining was quantitatively graded as weak, moderate, or strong according to the study by Buyuklu et al (21). ImageJ software (version 1.54k, NIH, Bethesda, MD, USA) was conducted for quantitative analysis of cleaved caspase-3 immune expression in the renal tissues.

Immunohistochemical (IHC) profiling of Beclin-1 in renal tissue samples

Beclin-1 immunohistochemical staining was performed on formalin-fixed, paraffin-embedded renal tissue

sections following the protocol described by Wasfey et al (22). Kidney specimens were cut at a thickness of 5 μ m using a BioLAB rotatory microtome (BHTP-203, Biolab Scientific Ltd., Ontario L3R 0B8, Canada), mounted on glass slides, deparaffinized, and rehydrated through a graded ethanol series. Tissue processing, antigen retrieval, and blocking of endogenous peroxidase activity were performed as described by Wasfey et al (22). Then, the non-specific binding sites were saturated before incubation with a rabbit polyclonal anti-beclin-1 primary antibody (Elabscience, USA; catalog no. E-AB-53242). The sections were then treated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA; catalog no. sc-48381). Immunoreactivity was visualized with DAB chromogen. After counterstaining with hematoxylin, the intensity of staining was assessed in 10 randomly selected fields for each section under an Olympus light microscope (Olympus, Japan) at a magnification of $\times$ 400. Immunostaining was quantitatively graded as mild, moderate, or strong according to the study by Wasfey et al (22). Quantitative analysis of renal beclin-1 expression was employed using ImageJ software (version 1.54k, NIH, Bethesda, MD, United States).

Light microscopic histopathological evaluation of the collected renal specimens

Neutral-buffered formalin solution (10%) was used for preservation of the kidney tissue specimens for 48 hours. Then, these specimens were processed, embedded in paraffin, producing blocks that were cut using a microtome into sections of 4 μ m thickness, mounted on glass slides, and stained with hematoxylin and eosin (H&E) and examined under light microscopy. ImageJ software (version 1.54k, NIH, Bethesda, MD, United States) was the tool used for quantification of the severity of renal tissue injury, which was determined according to the endothelial/glomerular/tubular/interstitial (EGTI) score. In this score, the extent of renal tissue injury ranges from 0, which denotes no histopathological abnormality, to 14, which indicates severe renal damage. The total EGTI score was calculated as the sum of four component scores: endothelial and glomerular injury were scored on a 0-3 scale, while tubular and interstitial injury were graded on a 0-4 scale (23). The pathologist was blinded to the treatment groups during EGTI scoring.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8 (GraphPad Software, LLC, San Diego, CA, USA). The Shapiro-Wilk test was used to assess data distribution, while Bartlett's test was used for evaluation of homogeneity of variance. For datasets satisfying the equal-variance assumption, group differences and comparisons were carried out by one-way ANOVA followed by Tukey's post hoc test. Median and interquartile range were used

to present non-parametric data, which were analyzed using the Kruskal–Wallis's test, with Dunn's multiple comparison test subsequently applied for post hoc evaluation. A two-tailed P value ≤ 0.05 was regarded as statistically significant.

Results

Impacts of CPA monotherapy and CGA co-administration on urine NAG levels and renal function markers

The effects of CPA, either alone or in combination with CGA, on renal function measures and urine NAG levels are summarized in Figure 1. Exposure to CPA alone caused marked deterioration in renal function, as shown by elevations of 2.6-fold in blood urea and 4.8-fold in serum creatinine, relative to the control. In contrast, CGA pre-treatment with doses of 100 and 150 mg/kg/d significantly abrogated CPA-induced elevations in a dose-dependent manner. CPA administration also markedly increased urinary NAG levels by 4.4-fold compared with controls and reduced creatinine clearance by 72.2%. However, co-administration of CGA at 100mg/kg improved creatinine clearance by 82.7%, and 150 mg/kg, markedly improved it by 210%, and concurrently lowered NAG levels in urine by 41% and 60.3%, respectively, relative to the CPA-only group.

Impacts of CPA monotherapy and CGA co-administration on redox homeostasis biomarkers in renal tissues

As illustrated in Figure 2, CPA treatment markedly disrupted renal oxidative balance, producing a 3.5-fold elevation in renal tissue MDA levels relative to the control group, together with substantial declines in GSH levels and SOD activity of 58.2% and 57.2%, respectively. In contrast, CGA pre-treatment (100 and 150 mg/kg) significantly blunted the rise in MDA and dose-dependently restored antioxidant defenses (GSH and SOD) compared with CPA treatment alone.

Impacts of CPA monotherapy and CGA co-administration on renal tissue inflammation-related biomarkers

As observed in Figure 3, in comparison to the control group, the administration of CPA significantly increased the levels of the inflammatory mediators IL-1 β by around 2.5 times and NF- κ B by around 4.8 times. On the other hand, compared to the CPA-only group, CGA pre-treatment at daily doses of 100 mg/kg and 150 mg/kg dramatically decreased these inflammatory markers, in a dose-dependent manner.

Impacts of CPA monotherapy and CGA co-administration on renal tissue TGF- β 1 levels

Administration of CPA markedly elevated renal tissue

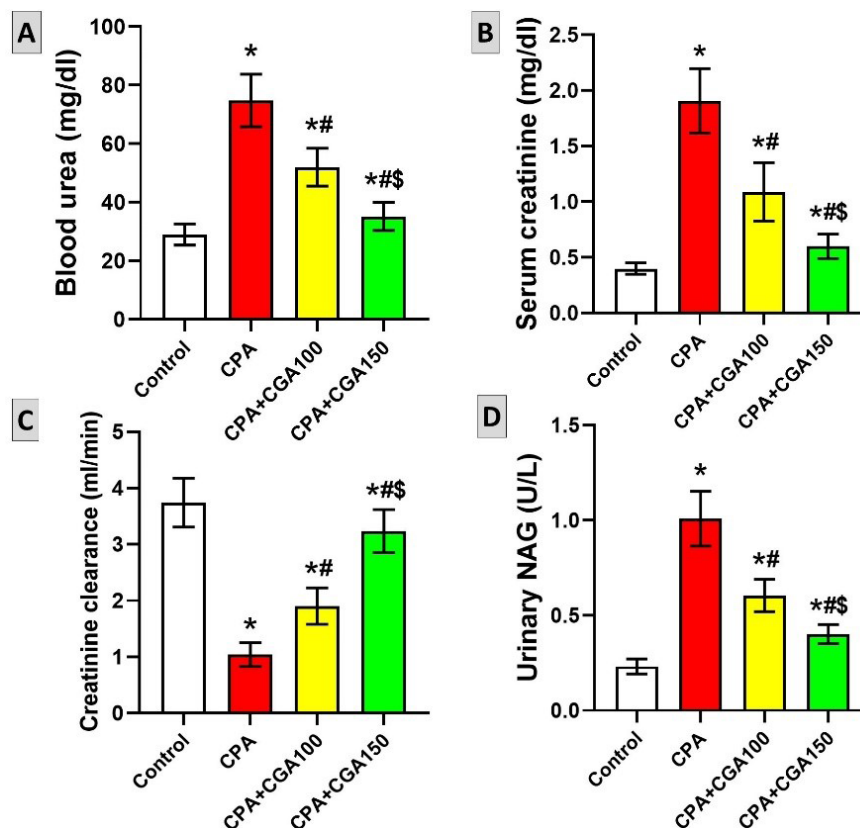


Figure 1. Impacts of cyclophosphamide (CPA) monotherapy and chlorogenic acid (CGA) co-administration on the renal function markers. (A) Blood urea, (B) serum creatinine, (C) creatinine clearance, and (D) urinary N-acetyl- β -D-glucosaminidase (NAG). Data are presented as mean $\pm$ standard deviation (SD), with 15 rats included in each experimental group. * versus control; * versus the untreated CPA; * versus CPA + CGA100.

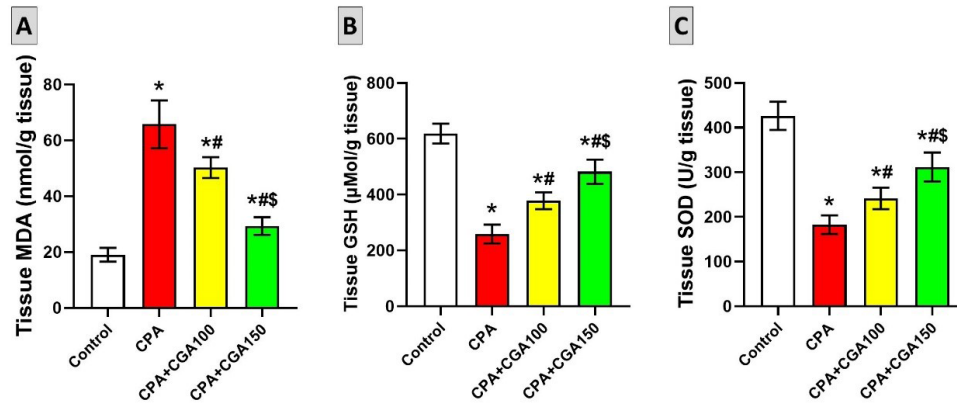


Figure 2. Impacts of cyclophosphamide (CPA) monotherapy and chlorogenic acid (CGA) co-administration on renal tissue oxidative stress biomarkers. (A) Malondialdehyde (MDA), (B) reduced glutathione (GSH), and (C) superoxide dismutase (SOD). Data are presented as mean $\pm$ standard deviation (SD), with 15 rats included in each experimental group. * versus control; # versus the untreated CPA; \$ versus CPA + CGA100.

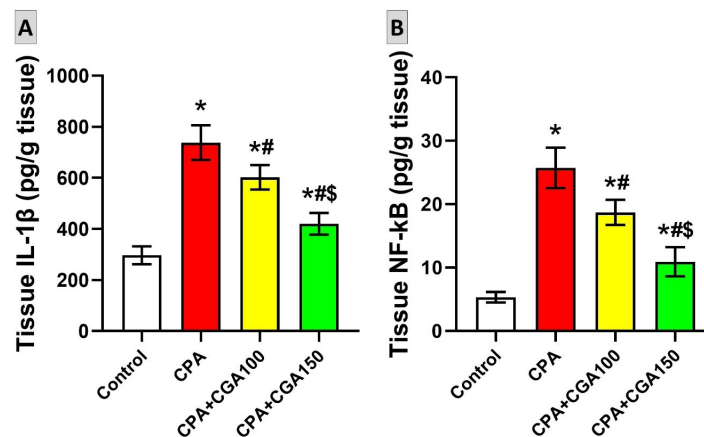


Figure 3. Impacts of cyclophosphamide (CPA) monotherapy and chlorogenic acid (CGA) co-administration on the inflammation-related biomarkers in the renal tissues. (A) Interleukin-1 β (IL-1 β) and (B) nuclear factor kappa B (NF- κ B). Data are presented as mean $\pm$ standard deviation (SD), with 15 rats included in each experimental group. * versus control; # versus the untreated CPA; \$ versus CPA + CGA100.

TGF- β 1 levels, producing a 4.4-fold increase relative to controls. In contrast, prophylactic treatment with CGA at 100mg/kg significantly diminished TGF- β 1 levels by 29.3% and at 150 mg/kg markedly reduced them by 58.6%, in a dose-dependent manner relative to the CPA-treated group (Figure 4).

Impacts of CPA monotherapy and CGA co-administration on the immuno-expression of cleaved caspase-3 and beclin-1 in renal tissue

Figures 5 and 6 illustrate that CPA administration markedly increased the renal cleaved caspase-3 immune-expression, while significantly decreasing the immune-expression of beclin-1 compared with controls. Conversely, daily co-treatment with CGA (100 mg/kg and 150 mg/kg) dose-dependently and significantly attenuated cleaved caspase-3 immune-expression while enhancing beclin-1 immune expression in renal tissues relative to the untreated CPA group.

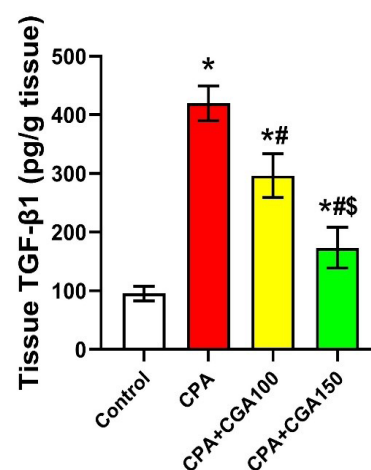


Figure 4. Impacts of cyclophosphamide (CPA) monotherapy and chlorogenic acid (CGA) co-administration on transforming growth factor beta 1 (TGF- β 1) levels within renal tissue. Data are presented as mean $\pm$ standard deviation (SD), with 15 rats included in each experimental group. * versus control; # versus the untreated CPA; \$ versus CPA + CGA100.

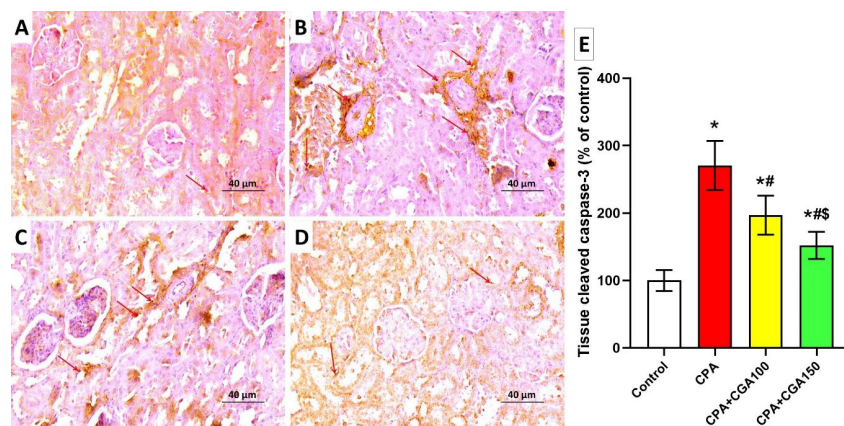


Figure 5. Representative photomicrographs of immunohistochemical staining for cleaved caspase-3 in renal tissue (original magnification $\times 200$; scale bar = 40 μm). (A) Control group showing minimal positive immunoreactivity (arrow); (B) cyclophosphamide (CPA)-treated group showing intense positive immunoreactivity (arrows); (C) CPA + chlorogenic acid (CGA) 100 mg/kg/day group exhibiting moderate positive immunoreactivity (arrows); (D) CPA + CGA 150 mg/kg/day group exhibiting mild positive immunoreactivity (arrows); and (E) Quantitative analysis of the percentage of cleaved caspase-3 immunoreactivity in renal tissues from CPA-treated rats receiving CGA (100 or 150 mg/kg/day, p.o.). Data are presented as mean $\pm$ standard deviation (SD), with 15 rats included in each experimental group. * versus control; # versus the untreated CPA; \$ versus CPA + CGA100.

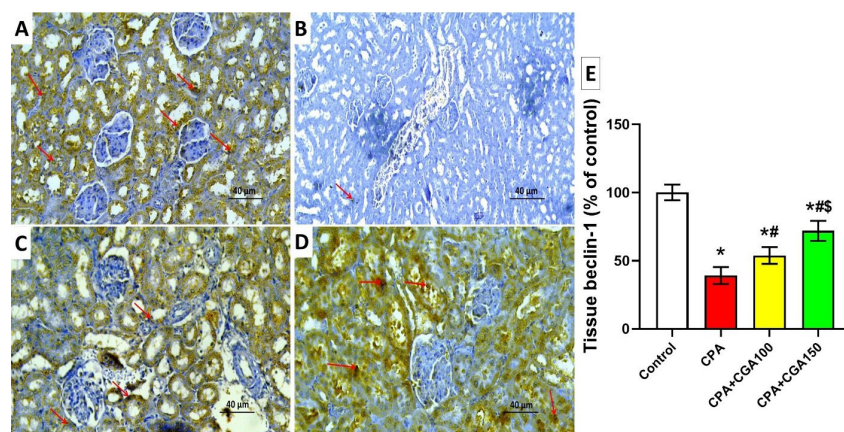


Figure 6. Representative photomicrographs of immunohistochemical staining for beclin-1 in renal tissue (original magnification $\times 200$; scale bar = 40 μm). (A) Control group showing strong positive beclin-1 immunoreactivity (arrows); (B) cyclophosphamide (CPA)-treated group showing minimal positive immunoreactivity; (C) CPA + chlorogenic acid (CGA) 100 mg/kg/day group exhibiting moderate beclin-1 immunoreactivity; (D) CPA + CGA 150 mg/kg/day group exhibiting strong beclin-1 immunoreactivity; and (E) Quantitative analysis of beclin-1 immuno-expression across all experimental groups. Data are presented as mean $\pm$ standard deviation (SD), with 15 rats included in each experimental group. * versus control; # versus the untreated CPA; \$ versus CPA + CGA100.

Impacts of CGA on CPA-induced renal histopathological changes

The kidneys from the control group showed maintained histological organization, with normal interstitial tissue, renal tubules, and glomeruli, as seen in Figure 7. On the other hand, exposure to CPA at a dose of 150 mg/kg induced severe histopathological alterations, including diffuse glomerular shrinkage, tubular epithelial vacuolation and degeneration, massive interstitial bleeding, and dense infiltration of inflammatory cells. These lesions were considerably and dose-dependently improved by co-treatment with CGA at doses of 100 and 150 mg/kg, which reduced vascular congestion and inflammatory infiltration while largely restoring the normal glomerular

and tubular architecture.

Discussion

Cyclophosphamide-associated renal dysfunction is a well-documented adverse effect of the treatment (24). The current study demonstrated the dose-dependent amelioration of CPA-induced nephrotoxicity by CGA, a phenolic molecule found in fruits and vegetables (25). This reno-protective effect was evidenced by reductions in the elevated levels of urea, creatinine, and NAG activity, together with restoration of creatinine clearance and improvement of the histopathological alterations induced by CPA.

Oxidative stress appears to be a central mechanism

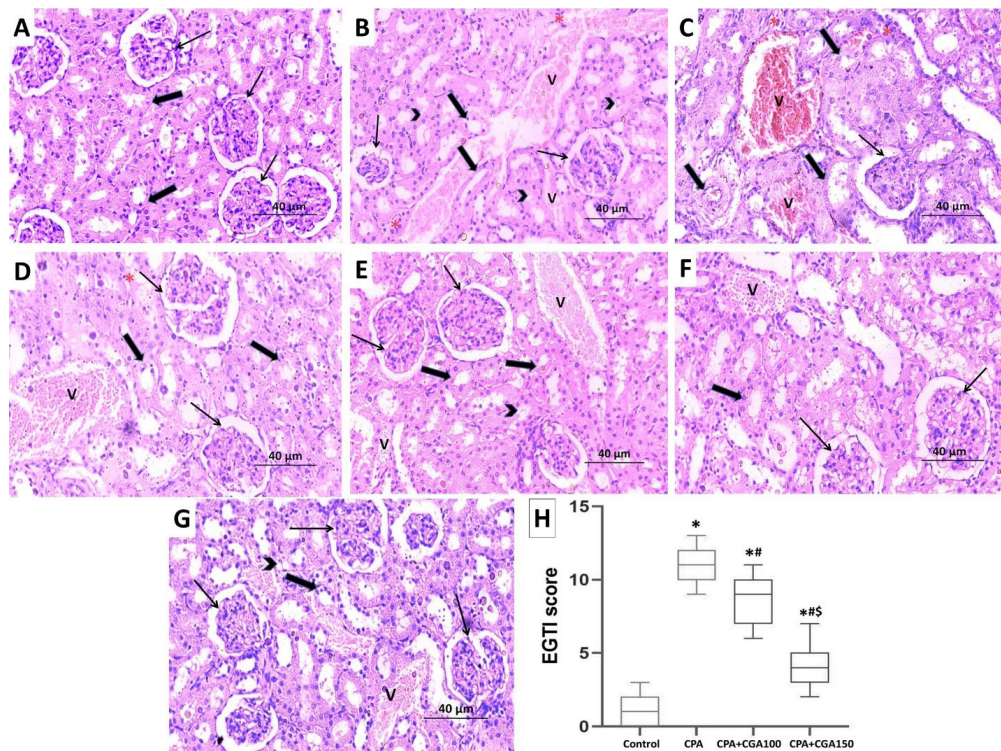


Figure 7. Representative hematoxylin and eosin (H&E)-stained renal sections (original magnification $\times 200$; scale bar = 40 μm). (A) Control group demonstrating preserved renal histoarchitecture with preserved renal tubules (thick arrows), glomeruli (thin arrows), and interstitial tissue. (B, C) cyclophosphamide (CPA)-treated group showing marked histopathological damage characterized by glomerular atrophy (thin arrows), tubular degeneration (thick arrows), extensive cytoplasmic vacuolation (arrowheads), diffuse vascular congestion (V), and dense inflammatory cell infiltration (asterisk). (D, E) CPA + chlorogenic acid (CGA) 100 mg/kg/day group exhibiting a marked reduction in inflammatory cell infiltration (asterisk), moderate vascular congestion (V), scattered intact glomeruli (thin arrows), occasional glomerular atrophy (arrowhead), and attenuated tubular degeneration (thick arrows). (F, G) CPA + CGA 150 mg/kg/day group demonstrating near-normal glomerular architecture (thin arrows) with only occasional glomerular atrophy (arrowhead), histologically normal-appearing renal tubules (thick arrows), and mild interstitial hemorrhage. (H) Quantitative assessment of the renal EGTI (endothelial/glomerular/tubular/interstitial) histopathological score in CPA-treated rats receiving CGA (100 or 150 mg/kg/day, p.o.). Data are presented as median (interquartile range, IQR) from 15 rats per group. Statistical comparisons were performed using the Kruskal–Wallis test followed by Dunn’s multiple-comparisons post hoc test. Statistical significance was defined as $p < 0.05$. * versus control; # versus the untreated CPA; \$ versus CPA + CGA100.

in CPA-mediated kidney injury, with ROS playing a major pathogenic role (8). As described previously, only a limited proportion of CPA is eliminated through the kidneys because of its marked tubular reabsorption (26), whereas hepatic metabolic activation generates acrolein and phosphoramidate mustard (27). Acrolein depletes GSH, thereby impairing antioxidant defenses and triggering lipid peroxidation in renal tubular cells (9). Consistent with this mechanism, CGA pre-treatment dose-dependently lowered the CPA-induced elevation in MDA while significantly restoring GSH content and SOD activity relative to the CPA-treated group. These findings agree with earlier reports attributing the nephroprotective effects of CGA to its antioxidant activity (25,28).

Inflammation also represents a major mechanism underlying CPA-induced renal toxicity (8,29,30). Notably, NF- κB , a transcription factor involved in the regulation of multiple inflammatory disorders (31), is activated by CPA-induced oxidative stress and subsequently promotes the secretion of pro-inflammatory mediators, including IL-6, IL-1 β , and TNF- α (tumour necrosis factor alpha),

culminating in renal tissue injury (32). In accordance with this mechanism, CGA pre-treatment significantly reduced the elevated renal levels of NF- κB and IL-1 β in a dose-dependent manner compared with rats receiving CPA alone. This finding is consistent with previous evidence showing that the anti-inflammatory activity of CGA mitigates renal injury induced by gentamicin (33), vancomycin (34), lead (35), and cisplatin (36).

Sustained renal injury caused by CPA can also trigger fibrotic pathways through increased expression of fibrogenic mediators (37). Among these, TGF- $\beta 1$ is widely recognized as a key profibrotic marker driving the progression of renal fibrosis (38). In the current study, CGA administration to CPA-treated rats markedly decreased TGF- $\beta 1$ levels in a dose-responsive manner relative to the CPA-only group. This observation is supported by previous work demonstrating that CGA counteracts cardiac fibrosis through suppression of galectin-3 (39).

This fibrotic remodeling naturally leads to apoptosis because programmed cell death is now recognized as a central mechanism linking tissue injury, inflammatory

signaling, fibroblast persistence, and scar progression. Consistent with this concept, apoptosis is also a well-established contributor to CPA-induced renal injury (8,40). In this study, CGA dose-dependently reduced the expression of cleaved caspase-3, a key apoptotic marker, compared with the CPA-treated animals. A comparable anti-apoptotic action of CGA has previously been reported in the frontal lobe of diabetic rats, where it improved memory impairment by reducing oxidative stress and apoptosis (41).

At the cellular level, this apoptotic response is closely intertwined with autophagic dysregulation, because the same oxidative and inflammatory stressors that activate caspase-dependent cell death also alter autophagy-related signaling, and persistent failure of these adaptive pathways can favor profibrotic remodeling in CPA-injured kidney tissue. In this context, autophagy appears to be an important pathophysiological regulator of CPA-induced kidney injury. This self-protective cellular process eliminates damaged or dysfunctional components (42), and can be activated by several stressors, including hypoxia, nutrient deprivation, and DNA damage (43). In the current study, CPA markedly suppressed the expression of beclin-1, a critical autophagy mediator. Supporting this observation, earlier work showed that CPA disrupts autophagic regulation in lung tissue by inhibiting the PI3K/AKT/FoxO1 signaling pathway (44). Notably, CGA significantly enhanced beclin-1 expression relative to the CPA-treated group. This result agrees with prior evidence indicating that CGA alleviates carbon tetrachloride-induced liver fibrosis through activation of PI3K/AKT/mTOR-mediated autophagy (45).

Although the findings demonstrate that CGA can mitigate CPA-induced renal toxicity in rats, several limitations should be considered. The study was conducted exclusively in an animal model, which does not fully capture the complexity and heterogeneity of chemotherapy-associated nephrotoxicity in humans, thereby limiting direct clinical translation. In addition, only a single sex and species were examined, which restricts the broader generalizability of the findings across different biological settings. The study also did not investigate whether CGA alters the antineoplastic efficacy of CPA. Also, our assessment of autophagy in the present study relied solely on determination of the immunohistochemical expression of beclin-1, which is known to reflect only the initiation phase of autophagy. Absence of additional markers such as microtubule-associated protein 1 light chain 3-II (LC3-II) and sequestosome 1 (p62/SQSTM1) represents a limitation of the current work, which necessitates incorporation of these markers and complementary techniques such as Western blotting in the future studies to validate and extend our findings. Although the findings of the present study suggest a role for modulation of NF- κ B signaling and apoptotic pathways in mitigation of CPA-induced renal toxicity, the reliance on ELISA

and immunohistochemistry without complementary molecular assays such as Western blotting or RT-qPCR represents a limitation to the present work. Future studies incorporating these techniques are warranted to provide more definitive mechanistic evidence to our findings. Another limitation of the present work is the absence of a CGA-only group, which would clarify whether CGA alone exerts any baseline effects on the renal functions in healthy rats. Future research should include such groups to confirm that the observed protective effects of CGA in the present study are specific to CPA-induced renal toxicity.

Conclusion

Overall, CGA significantly alleviated CPA-induced renal injury in a dose-dependent manner by restoring redox homeostasis, suppressing inflammatory and apoptotic pathways, reducing fibrotic changes, along with enhancement of autophagy. These findings justify further clinical evaluation of CGA as a promising adjunctive agent for protection against CPA-associated nephrotoxicity. Nevertheless, future studies including a CGA-only control group and using alternative models of chemotherapy-induced renal injury, larger animal cohorts, longer treatment periods, and molecular validation (e.g., Western blotting or RT-qPCR) are required to confirm its therapeutic value and establish its relevance to human disease. Future work should also address the influence of CGA on the antitumor activity of CPA in experimental cancer models.

Authors' contribution

Conceptualization: Abeer Elkhoely, Amany A.E. Ahmed.

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Formal analysis: Ahmad G.A. Alenazi, Abeer Elkhoely, Ahmed M. Kabel, Amany A.E. Ahmed.

Investigation: Ahmad G.A. Alenazi, Abeer Elkhoely, Ahmed M. Kabel, Amany A.E. Ahmed.

Methodology: Ahmad G.A. Alenazi, Abeer Elkhoely, Ahmed M. Kabel, Amany A.E. Ahmed.

Validation: Ahmad G.A. Alenazi, Abeer Elkhoely, Ahmed M. Kabel, Amany A.E. Ahmed.

Visualization: Ahmad G.A. Alenazi, Abeer Elkhoely, Ahmed M. Kabel, Amany A.E. Ahmed.

Writing—original draft: Ahmad G.A. Alenazi.

Writing—review & editing: Ahmad G.A. Alenazi, Abeer Elkhoely, Ahmed M. Kabel, Amany A.E. Ahmed.

Conflicts of interest

The authors declare that they have no competing interests.

Data availability statement

Data are available from the corresponding author upon reasonable request.

Ethical issues

All the experiments performed in the current study complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Animal handling and experimental procedures adhered to the rules established by the Research Ethics Committee of the Faculty of Pharmacy, Helwan University, Egypt (approval code 27A2024). Additionally, all animal experiments were conducted in accordance with protocols approved by the United States National Institutes of Health (NIH, 1978). Accordingly, ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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