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The protective effect of glycyrrhizin against chemotherapy-related toxicity in kidneys of rats: An experimental approach

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Abstract
Chemotherapy is the most important treatment of neoplastic diseases and many antineoplastic agents have been developed for cancer treatment. A commonly used anticancer agent in chemotherapy is cyclophosphamide (CP). Despite the protective effect of CP against tumors, its use is limited due to nephrotoxicity, hepatotoxicity, urotoxicity, neurotoxicity, cardiotoxicity, teratogenicity, and immunotoxicity. Glycyrrhizin (GLZ) being a triterpenoid saponin, exhibits anti-oxidant, anti-inflammatory, nephroprotective, hepatoprotective, and cardioprotective effects. In this experiment, the reformative effect of GLZ against chemotherapy-related toxicity in the kidneys of rats was assessed. Twenty-eight rats into four equal groups as control, CP, CP+GLZ100, and CP+GLZ200 were used. Histopathological examination showed tubular degeneration, luminal casts, tubular cystic dilatation, hemorrhages, mononuclear cells infiltration in cortical and medullary areas, periglomerular inflammation, and glomerular hypercellularity in the CP-treated group. The significantly improved results were evaluated in the groups that received GLZ, especially the CP+GLZ200 group. Specific immunopositivity with 8-OHdG and less specific immunopositivity with BCL-2 markers were recorded in the CP-treated group. GLZ groups revealed less immunopositivity of 8-OHdG and specific immunopositivity of Bcl-2. Both the histopathological findings and immunohisto chemical results of 8-OHdG and Bcl-2 in this study are strongly supporting the ameliorating effect of GLZ on chemotherapy-induced nephrotoxicity.

Keywords: Chemotherapy, glycyrrhizin, nephrotoxicity, 8-OHdG, Bcl-2, rat

1. Introduction
The kidney is the vital organ of the body and performs many functions including maintenance of homeostasis, excretion of metabolites and drugs, detoxification and erythropoietin production [1]. There are many chemotherapeutic drugs and exogenous toxicants that cause nephrotoxicity and disturb the normal functions of kidney [2]. These nephrotoxic drugs cause damage in glomeruli, proximal tubules and surrounding matrix in kidney [3]. Reactive oxygen species (ROS) production, mitochondrial damage of tubular cells, disturbance in tubular transport system are the main factors for developing nephrotoxicity [4-7]. The percentage of nephrotoxicity induced by drugs has been reported and that is 20% [8-9].
Chemotherapy is commonly used treatment of neoplasia and many antineoplastic agents have been registered for the treatment purpose [10]. These therapeutic agents have cytotoxic effect and they kill not only tumor cells but also cause damage to normal tissues [11]. Chemotherapeutic agent (CP) extensively used for the treatment of not only cancerous diseases (i.e. colon cancer, lymphomas, solid type cancers) but also non-cancerous diseases (i.e. rheumatoid arthritis, multiple sclerosis and systemic lupus erythematous) [12]. CP has protective effect against tumors but due to its fatal side effects and toxicity such as nephrotoxicity, hepatotoxicity, urotoxicity, neurotoxicity, cardiotoxicity, teratogenicity and immunotoxicity [13-15], its use is limited.
Mulethi (*Glycyrrhiza glabra*), has many health-related positive effects against bacteria, microbes, fungus, viruses, malaria, tumor, inflammation, depression, convulsions, oxidative stress, coronary heart diseases, hyperglycemia, cough, brain problems, hepatotoxicity and immunotoxicity [16-18]. Glycyrrhizin (GLZ) is released from *Glycyrrhiza glabra*, is a triterpenoid saponin, and has sweet taste.
8-OHdG is an apoptotic marker and Bcl-2 is an anti-apoptotic marker. The correlation between these two markers is reverse and has been reported previously [19]. In this experiment, the reformative effect of GLZ against CP-related toxicity in kidneys of rats was evaluated by histo pathological and immunohisto chemical examination by staining the kidney tissues with 8-OHdG, and Bcl-2 markers.

2. Materials and Methods

In this experimental study, 28 male Sprague Dawley rats having weight between 220-250 g were purchased. Animals were purchased from Experimental Animals Unit of Afyon Kocatepe University. Approval for this study was given by the Ethical Committee of Afyon Kocatepe University and the reference number is AKUHADYEK-03-22. During the study, animals were lived in polycarbonate special cages and rat food, and fresh water were given ad libitum, in 12 hours light/12 hours' dark period, at 22±0.5 °C and appropriate humidity. After two weeks adaptation period, the experiment was started. The rats were separated into 4 study groups. Every group contains 7 rats. Glycyrrhizin (Sigma-Aldrich, CAS Number: 53956-04-0) was purchased commercially in its pure form. The control group was given only isotonic solution by gastric gavage (gg). In the CP group, 150 mg/kg of CP was injected by intraperitoneal route (i.p) at the beginning of the study. In CP+GLZ100 group first GLZ 100 mg/kg was given by gg, after 20 minutes CP 150 mg/kg was injected by i.p and after that two doses of GLZ 100 mg/kg were given by gg with the time period of 4 and 8 hours. In CP+GLZ group, first GLZ 200 mg/kg was given by gg, after 20 minutes CP 150 mg/kg was injected by i.p and after that two doses of GLZ 200 mg/kg were given by gg with the time period of 4 and 8 hours. At the experiment, rats were anesthetized with the help of general anesthesia and kidneys were collected in 10% buffered formalin solution after dissection.

2.1 Histopathological processing

Formalin fixed tissues were cut and overnight processing of tissues was done in automatic processing machine. After paraffin embedding, tissues were blocked. The sections of 4 micrometers were taken on slides from paraffin blocks of tissues. The slides were put in the incubator (55 °C for 1 hour) for the melting of extra paraffin from the tissues. Hematoxylin and eosin (HE) staining was done. The tissues were examined by the help of light microscope and grading was as normal (-/0), mild lesions (+/1), moderate lesions (++/2) and severe lesions (+++/3) for histopathological findings. Picrosirius red stain was done to investigate the presence of collagen fibers.

2.2 Immunohistochemical processing

For immunohistochemical staining, deperaffinization of tissues was done with xylene and clearing of tissues with different percentages of alcohols. Quenching of enzymes was done with the help of 10% hydrogen peroxide solution for 10 minutes. Specified antigen retrieval with citrate buffer was done in steamer of 90 °C for 15 minutes. Overnight incubation with primary antibodies for 8-OHdG (SANTACRUZ, 15A3, sc-66036) and Bcl-2 (SANTACRUZ, N-19, sc492) were done. After application of secondary antibodies, slides were incubated in humidity chamber for 120 minutes at 37 °C. Phosphate buffer solution was used to wash the slides during the immunohistochemical process. ABC kit (TA-125-UDX/Thermo Scientific-USA) was used according to the instructions of manufacturers. IgG in the biotinylated form was used and was incubated at room temperature for 60 minutes. Finally, peroxidase avidin in the conjugated form was dropped on the slides and waited for reaction for 40 minutes at room temperature. AEC (TA-125-HA, Thermo Scientific) peroxidase substrate was used for visualization of reaction. After completion of reaction, the slides were taken into distilled water and counter stained with Mayer's hematoxylin. Cover slips were used to cover the slides with the help of special aqueous adhesive medium. Microscopic evaluation was done with the help of light microscope in Pathology lab. The kidney sections were graded as no immunopositivity (−/0), mild immunopositivity (+/1), moderate immunopositivity (++/2), and specific immunopositivity (+++/3) according to positivity of 8-OHdG and BCL-2 markers.

2.3 Statistical Analysis

The values showed as mean ± standard error of deviation. One-way ANOVA and post hoc Duncan's test evaluated the differences of the rats groups. SPSS/PC software with the version of 18.0 was used and $p<0.05$ was the significant value.

3. Results

3.1 Histopathological evaluation

The kidneys of CP treated rats showed tubular degeneration ($p<0.05$), luminal costs ($p<0.05$), tubular cystic dilatation ($p<0.05$), hemorrhages ($p<0.05$), mononuclear cells infiltration in cortical and medullary area ($p<0.05$), periglomerular inflammation ($p<0.05$) and glomerular hypercellularity ($p<0.05$). Less severity in the tubular degeneration ($p<0.05$), luminal costs ($p<0.05$), tubular cystic dilatation ($p<0.05$), hemorrhages ($p<0.05$), mononuclear cells infiltration in cortical and medullary area ($p<0.05$), periglomerular inflammation ($p<0.05$) and glomerular hypercellularity ($p<0.05$) was found in CP+GLZ100 group. Significant improvement in these pathological lesions was evaluated in the CP+GLZ200 group. Statistical evaluation of pathological lesions in all groups is given in table 1. Normal histological structure of kidneys was presented by control group (Fig 2) (A). Hyperemia/ hemorrhages, tubular epithelial degeneration, luminal casts formation, tubular cystic dilatation, mononuclear cells infiltration in cortical and medullary area, periglomerular inflammation and glomerular hypercellularity were showed in the CP group (B-C). Significant decrease in pathological lesions in the kidneys of CP+GLZ100 group (D). The group received GLZ 200 mg/kg showed more protective effect against CP-induced renal damage as compare to the group received 100 mg/kg (E). For the confirmation of proliferation of collagen fibers in kidneys, Picrosirius red stain was used and there was no proliferation of collagen fibers in kidneys of rats.

Table 1: The superscripts (a, b, c) in the table reveal significant difference ($p<0.05$) between groups.

Groups	Tubular degeneration	Luminal casts	Tubular cystic dilatation	Hemorrhages	Mononuclear cells infiltration	Periglomerular inflammation	Glomerular hypercellularity
Control	0.28 ± 0.48^{c}	0.28 ± 0.4^{b}	0.28 ± 0.48^{b}	0.28 ± 0.48^{b}	0.28 ± 0.4^{b}	0.28 ± 0.48^{b}	0.28 ± 0.48^{b}
CP	2.71 ± 0.48^{a}	2.57 ± 0.5^{a}	1.85 ± 0.69^{a}	2.28 ± 0.48^{a}	2.14 ± 0.6^{a}	1.85 ± 0.37^{a}	1.71 ± 0.48^{a}
CP+GLZ100	1.28 ± 0.75^{b}	0.85 ± 0.6^{b}	0.71 ± 0.48^{b}	0.71 ± 0.75^{b}	1.00 ± 0.8^{b}	0.71 ± 0.48^{b}	0.85 ± 0.69^{b}
CP+GLZ200	0.57 ± 0.53^{c}	0.42 ± 0.5^{b}	0.28 ± 0.48^{b}	0.57 ± 0.53^{b}	0.42 ± 0.5^{b}	0.28 ± 0.48^{b}	0.28 ± 0.48^{b}

Normal histological structure of kidneys was presented by control group (Fig 1) (A). Hyperemia/ hemorrhages, tubular epithelial degeneration, luminal casts formation, tubular cystic dilatation, mononuclear cells infiltration in cortical and medullary area, periglomerular inflammation and glomerular hypercellularity were showed in the CP group (B-C). Significant decrease in pathological lesions in the kidneys of CP+GLZ100 group (D). The group received GLZ 200 mg/kg showed more protective effect against CP-induced renal damage as compare to the group received 100 mg/kg (E). For the confirmation of proliferation of collagen fibers in kidneys, Picrosirius red stain was used and there was no proliferation of collagen fibers in kidneys of rats.

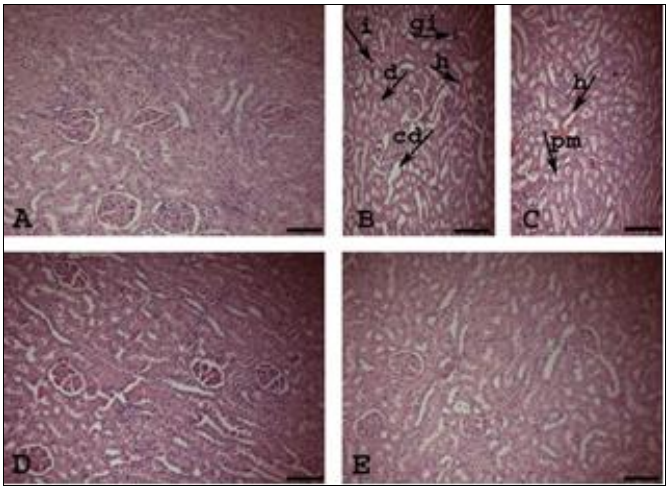

Fig 1: Histopathological evaluation of kidneys sections.

(A) Control group is showing normal structure of kidney section.
(B, C) CP group is showing tubular degeneration, luminal casts, tubular cystic dilatation, hemorrhages, mononuclear cells infiltration in cortical and medullary area, periglomerular inflammation and glomerular hypercellularity.
(D) CP+GLZ100 group is presenting less severity of pathological lesions.
(E) CP+GLZ200 group is presenting mild tubular changes and ameliorative effect on kidney section. Arrows pointing events; h: hemorrhage, d: degeneration, i: inflammatory cells infiltration, pm: proteinaceous material (luminal casts), cd: cystic dilatation, gi; periglomerular inflammation. (HE, x20, scale bar= 150 µm).

3.2 Immunohistochemical evaluation

Specific immunopositivity was not in the control group with 8-OhDG. CP group revealed specific immunopositivity with 8-OhDG marker. Both luminal and cytoplasmic immunopositivity of epithelial cells was found in cortex position of kidneys with 8-OhDG marker in the CP group. Cortical area of kidneys showed more immunopositivity in the form of islands. Glomeruli evaluated after staining with immunohistochemical stain but no immunopositivity was found in glomeruli with 8-OhDG marker. In the CP group cortical immunopositivity was found in the form of islands with 8-OhDG marker. The CP+GLZ100 group showed moderate immunopositivity in the form of island in cortical area with 8-OhDG marker. The CP+GLZ200 group not showed specific immunopositivity like control group with 8-OhDG marker. The CP+GLZ200 group showed more protective effect as compare to the CP+GLZ100 group. The findings in the CP+GLZ200 group were almost similar to control group with 8-OhDG marker (Fig 2 (A-D).
Fig 2: The significant difference (p<0.05) was assessed between groups. Immunohistochemical findings are presented in fig 3.

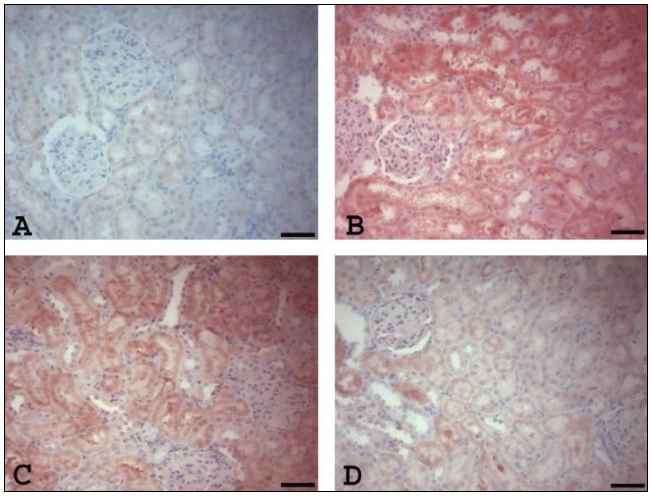

Fig 2: The expression of 8-OHdG marker in kidney sections of all groups. (A) No expression of 8-OHdG in untreated control group. (B) Specific luminal and cytoplasmic immunopositivity of epithelial cells in CP group with 8-OHdG. (C) Less specific expression of immunoreaction in CP+GLZ100 group with 8-OHdG. (D) No specific immunopositivity of 8-OHdG in CP+GLZ200 group. (Immunohistochemistry, x40, scale bar= 150 µm).

The significant difference (p<0.05) was assessed between all groups. Immunohistochemical findings are presented in Fig 3.

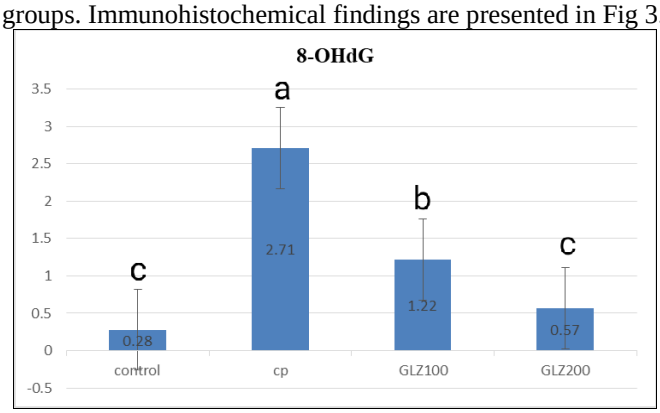

Fig 3: Graph showing immunopositivity of 8-OhDG in different groups; values presented as mean ± standard error f mean, superscripts (a, b, c) in the graph revealed significant difference (p<0.05) between groups.

Specific immunopositivity was revealed in the control group with Bcl-2 marker. The reaction was more intense and multifocal. The CP group not showed any specific immunopositivity with Bcl-2 marker. The CP+GLZ100 group showed moderate immunopositivity with Bcl-2 marker. CP+GLZ200 group showed specific (intense and multifocal) positivity like control group with Bcl-2 marker. The Bcl-2 marker revealed immunopositivity of cells at the border of cortex and medulla junction in control and CP+GLZ200 group. The positive cells were also in the form of islands on the cortex and medulla junction border in both of these groups. Immunopositivity was also found in glomeruli of kidneys with Bcl-2 marker. The Bcl-2 marker revealed different results in which CP group showed less positivity on the border of cortex and medulla junction as compare to control and CP+GLZ200 groups. The CP+GLZ200 group showed more protective effect as compare to the CP+GLZ100 group. The findings in the CP+GLZ200 group were almost similar to control group with Bcl-2 marker (Fig 4 (A-D).

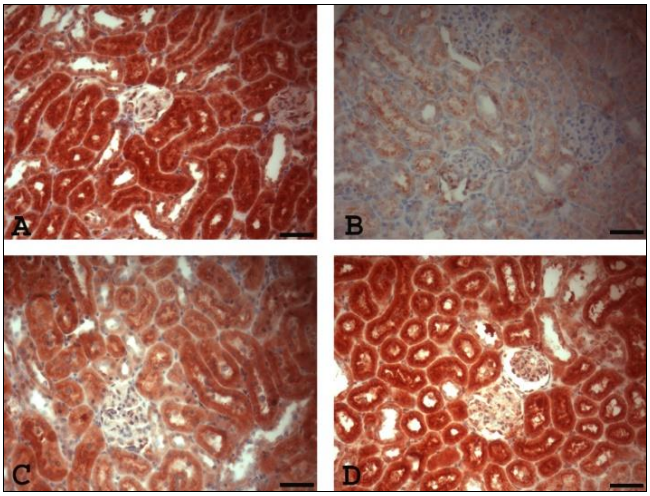

Fig 4: The expression of Bcl-2 marker in kidney sections of all groups. (A) Specific expression in tubular epithelial and glomerular cells with Bcl-2. (B) No specific expression of Bcl-2 in CP group. (C) Less specific expression of Bcl-2 in CP+GLZ100 group. (D) Specific expression of immunopositivity in CP+GLZ200 group with Bcl-2. (Immunohistochemistry, x40, scale bar= 150 µm)

The significant difference (p<0.05) was assessed between all groups. Immunohistochemical findings are presented in (Fig 5).

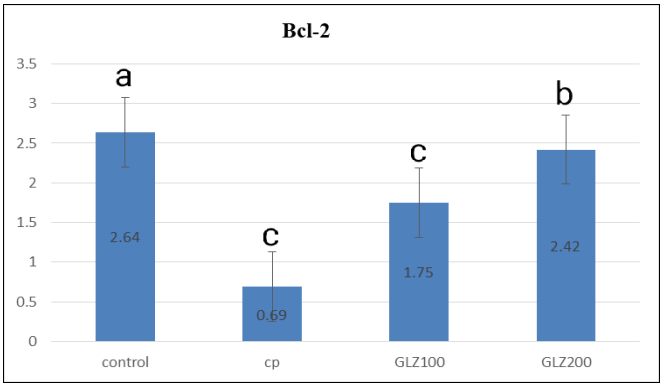

Fig 5: Graph showing the immunopositivity of Bcl-2 in different groups; values presented as mean ± standard error of mean, superscripts (a, b, c) in the graph revealed significant difference (p<0.05) between groups.

4. Discussion

Chemotherapy is one of the first line of treatment for neoplastic and some non-neoplastic diseases [12]. Many chemotherapeutic agents has been reported for the treatment purposes of these neoplastic and non-neoplastic conditions, and CP is one of the most important therapeutic agent for this purpose [10]. Although, CP has many positive effects against cancers but it has many adverse effects on kidney, liver, heart, brain, immune system and other organs of body [13-15]. Glycyrrhizin exhibits potential protective effect against bacterial [20], viral [21-22], malarial [23] and inflammatory [24] diseases. It also protects against toxicity of different organs including kidney [25-26], liver [27], and hurt [28].

CP-induced nephrotoxicity in rats [25, 29-30], and in mice [31-33] have been reported. In rats, tubular epithelial degeneration [34] collagen bundles around vessels, tubules and also in glomeruli [29], inflammatory cells infiltration, tubular necrosis [30] and glomerular necrosis [25] have been reported in kidneys of CP given animals. In mice, degeneration [31-33], swelling [31-32] necrosis and congestion [32], casts formation and atrophy [33] in tubuli and thickened basal membrane, widened Bowman's space [33], degeneration [31,33], atrophy [33]in glomeruli, and also inflammatory cells infiltration in cortical and medullary area [32-33] have been evaluated in CP-induced renal damage. Similar findings were found in our study but we have not found tubular and glomerular necrosis, and fibrotic changes because it may be due to short time of experiment and less quantity of dose of CP. On the other side we described CP-induced tubular cystic dilatation, periglomerular inflammation and hypercellularity in glomeruli which were different findings from the previous studies in mice and rats. In previous study of *Glycyrrhiza glabra* protective effect on CP-induced nephrotoxicity, kidney edema, glomerular necrosis, bleeding and shrinkage of glomeruli and fragments in glomeruli has been reported [25]. In contrast, we have not found these lesions in glomerulus. This difference in findings may be due to the long time usage of CP in that study.

Oxidative stress is the main source of oxidative damage of DNA, and one of the main marker for oxidative damage of DNA is 8-OHdG [35]. Activation of caspases are the reason for induction of apoptosis in cells and 8-OHdG as a mutagen and also participate in apoptosis process [36-37]. DNA damage shows response to cell cycle and causes the main factor of apoptosis activation of cells [38]. Expressions of 8-OHdG in kidney tissues of animals exposed to CP has been reported in many studies [39-42]. In all these studies control groups, not exposed to CP, showed negative immunopositivity of 8-OHdG [39-42]. 8-OHdG immunopositivity in CP-induced kidneys [42], nephrolithiasis condition [39] and in diabetic kidneys have been reported. In our study, findings are similar with these studies in which we found immunopositivity in kidneys of CP group and negative immunopositivity in control group.

Bcl-2 is an anti-apoptotic marker and has key role in the formation of kidneys tissues. Other members of Bcl-2 genes also has important role in non-neoplastic diseases of kidneys [43-44]. Expression of Bcl-2 in two studies of CP-induced nephrotoxicity has been reported [29, 45]. Specific immunopositivity has been reported in control groups and less specific immunopositivity in CP groups were recorded [29, 45]. The findings our study related to expression of Bcl-2 are similar with previous studies. The inverse correlation between 8-OHdG and Bcl-2 has been demonstrated in our study. This inverse correlation was also evaluated in previous study [19]. The histopathological findings and immunohistochemical results of 8-OHdG and Bcl-2 of previous studies are strongly supporting the results of our study. In future more apoptotic markers, anti-apoptotic markers and cell level studies are required to assess the reformative effect of glycyrrhizin on chemotherapy-related toxicity in kidneys of rats.

5. Conclusion

In conclusion, both the histopathological findings and immunohistochemical results of 8-OHdG and Bcl-2 in this study are strongly supporting the reformative effect of glycyrrhizin on chemotherapy induced toxicity in kidneys of rats.

6. Role of contributors

Study design, experimental work and histopathological analysis was done by Hikmet Keles and Ibrahim Keleş. Muhammad Nasir Bhaya performed the histopathological and immunohistochemical parts of the study and also wrote the manuscript. All the authors interpreted the data and approved the final version.

7. Conflict of interest

The authors declare no conflict of interests.

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