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The Protective Effect of Ellagic Acid on Cyclophosphamide Induced Renal Damage in Rats

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ABSTRACT

Cyclophosphamide (CP) is used alone or in combination with other therapeutic agents to treat different malignant tumors. Nephrotoxicity is one of the major side effects of it. Ellagic acid (EA) is a polyphenol, known for its antioxidant properties. In this study, the protective effect of EA on CP-induced renal damage was evaluated. For this purpose, 24 rats were divided into four groups, control, CP, CP+EA50, and CP+EA75. Histopathological examination of kidneys revealed hyperemia, epithelial degeneration, swelling, cystic dilatation and luminal proteinaceous material in tubuli, congested and hypercellular glomeruli and also inflammatory cells infiltration in the cortical and medullary area in CP group. Significant decrease of pathological lesions was found in CP+EA groups especially in the CP+EA75 group. In immunohistochemical sections, 8-hydroxy-2-deoxyguanosine (8-OHdG) and hypoxia-inducible factor-1 alpha (HIF-1α) revealed less positivity in EA received groups but B-cell lymphoma-2 (Bcl-2) revealed more positivity as compared to CP. Both histopathological and immunohistochemical findings revealed that EA has a protective effect against CP-induced renal damage in rats.

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HK contributed in study design, experimental work and histopathological analysis. MNB performed the histopathological and immunohistochemical parts of the study and also wrote the manuscript. Both authors interpreted the data and approved the final version.

Key words
Cyclophosphamide, Ellagic acid, 8-OHdG, Bcl-2 and rat

INTRODUCTION

Cyclophosphamide (CP) is a drug that upon bioactivation becomes an alkylating agent and after this bioactivation it has broad spectrum activity and high therapeutic index against human tumors (Sladek, 1988). There are some metabolites of CP that cause CP induced toxicity. Two major metabolites of CP are phosphoramide mustard and acrolein (Wahlang *et al*., 2015). CP induced toxicity in different organs is mainly due to mechanism of free radicals formation and oxidative stress (Wahlang *et al*., 2015). There are also many side effects of CP including nephrotoxicity (Lim *et al*., 2017). Significant degenerative tubular changes and casts in the lumen were observed in the rats treated with CP (Lim *et al*., 2017). CP induced toxicity has been reported in many studies of rats (El-Shabrawy *et al*., 2020; Ayza *et al*., 2020), mice (Sharma *et al*., 2017; Rehman *et al*., 2012; Hamzeh *et al*., 2018) and humans (Ruggiero *et al*., 2017).

Ellagic acid (EA) is a natural phenol that has been reported in different fruits and nuts, such as raspberries, strawberries, walnuts, longan seed, mango kernel (Soong and Barlow, 2004, 2006) and pomegranate (Wang *et al*., 2004). Antioxidant (Ateşşahín *et al*., 2007; Hassoun *et al*., 1997), antineoplastic (Whitley *et al*., 2003), antimutagenic (Loarca-Piña *et al*., 1998) and antiasthmatic (Rogerio *et al*., 2008) properties of EA have been reported. In some studies, exogenous antioxidants have been used for the alleviation of CP induced nephrotoxicity (Sharma *et al*., 2017; Goudarzi *et al*., 2016). The protective effect of EA on CP-induced genotoxicity and nephrotoxicity in mice has also been reported and EA may attenuate CP-induced oxidative stress and the subsequent DNA damage in mice was concluded in that study (Rehman *et al*., 2012).

Antigenotoxic and antinephrotoxic potential of EA on CP-treated mice has also been reported (Rehman *et al*., 2012) but according to literature research our study may be the first study in rats. In this study, we have made an attempt to evaluate the protective effect of EA on CP-induced renal damage in rats. 8-hydroxy-2-deoxyguanosine (8-OHdG) is one of the most widely studied oxidized metabolites and is used as a biomarker for oxidative damage of DNA (Kasai, 1997). Hypoxia-inducible factor-1 alpha (HIF-1α) is an oxygen sensitive

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subunit and its expression is induced under hypoxic conditions (Wang *et al*., 1995). Positive correlation of 8-OHdG and HIF-1α has been reported in one study in humans (Pialoux *et al*., 2009). B-cell lymphoma-2 (Bcl-2) proto-oncogene encodes an inner mitochondrial membrane protein that blocks programmed cell death (Hockenbery *et al*., 1991). Serum levels of p53 and 8-OHdG as well as p53, Bax, and Bcl-2 expression were examined in apoptotic condition in one study in humans and inverse correlation was reported between 8-OHdG and Bcl-2 (Najar *et al*., 2010). Negative correlation between HIF-1α and Bcl-2 has also been reported (Fan *et al*., 2002). In this study, the protective effect of EA on CP-induced kidney damage was evaluated with histopathological and immunohistochemical examination by staining the kidney tissues with 8-OHdG, HIF-1α and Bcl-2 markers.

MATERIALS AND METHODS

In this study, 24 male Sprague Dawley Rats, weighing 220-250 g were used. Rats were supplied from Afyon Kocatepe University Experimental Animals Unit. Approval for this study was obtained from Afyon Kocatepe University Animal Experiments Local Ethical Committee and the reference number is AKUHADYEK-115-21. During the experiment, animals were housed in polycarbonate cages and rat food and fresh water were given ad libitum, in 12 h light/12 h' dark period, at 22±0.5ºC and appropriate humidity. After a two weeks adaptation period, the experiment was started. The animals were divided into 4 study groups, 6 in each group, and one of the groups was determined as the control group and the third as the treatment group. The control group was given only isotonic solution by gastric gavage (gg). In the CP group, 150 mg/kg of CP was injected i.p. at the beginning of the study. For CPEA50 and CPEA75 groups, 150 mg/kg dose of CP i.p. and in addition, 50 mg/kg and 75 mg/kg ellagic acid were administered by gg route 20 min before, 4 h and 8 h after this application, respectively. At the end of second day rats were dissected under general anesthesia and kidneys were collected in 10% buffered formalin solution.

Histopathological method

Formalin fixed tissues were cut and processing of tissues was done in processing machine. After processing, tissues were blocked with paraffin and section of 4 micron were taken on slides for staining. Hematoxylin and eosin staining was done. The tissues were examined by light microscope (Zeiss Axio Lab. A1 Microscope - AxioCam ICc 5 Camera) and graded as normal (-), mild (+), moderate (++) and severe (+++) for histopathological findings. Picrosirius red stain was done to investigate the presence of collagen fibers.

Immunohistochemical staining

For immunohistochemical staining, tissues were deparaffinized with xylene and cleared with graded alcohol. Endogenous enzyme activity was quenched by treating the tissues with 10% hydrogen peroxide solution for 10 min. A specified antigen retrieval with citrate buffer was done in steamer of 90°C for 15 min. Overnight incubation with primary antibodies for 8-OHdG (SANTACRUZ, 15A3, sc-66036), HIF-1α (Abcam ab2185) and Bcl-2 (SANTACRUZ, N-19, sc492) were done. After application of secondary antibodies, slides were incubated in humidity chamber for 2 h at room temperature (37°C). After washing the slides with buffer solution, ABC kit (TA-125-UDX, Ultra Vision Polyvalent HRP Kit, LabVision/ThermoScientific-USA) application was started. Biotinylated IgG was used and was incubated at room temperature for 1 h. Finally, peroxidase conjugated avidin was dropped and allowed to react for 30 min at 37 °C. Slides were washed with buffer solution and tissues were treated with red colored AEC (TA-060-HA, AEC Substrate System, LabVision/ ThermoScientific-US) peroxidase substrate. After completion of reaction, the slides were taken into distilled water and counter stained with Mayer's hematoxylin. Slides were covered with coverslips using aqueous adhesive medium and examined under a light microscope (Zeiss Axio Lab. A1 Microscope - AxioCam ICc 5 Camera).

RESULTS

Histopathological findings

Normal histological architecture was achieved in control group (Fig. 1A). Hyperemia, tubular epithelial degeneration, intratubular proteinaceous material, cystic dilatated tubuli, and swollen, congested, and hypercellular glomeruli and inflammatory cells infiltration in cortical and medullary area were found in CP group (Fig. 1B, C). There was significant decrease in pathological lesions in the kidneys of the groups that were treated with EA (Fig. 1D). The group received 75 mg/kg EA showed more protective effect against CP-induced renal damage as compare to the group received 50 mg/kg EA (Fig. 1E). For the confirmation of proliferation of collagen fibers in kidneys, picrosirius red stain was used and there was no significant difference was found in all groups. The detail of histopathological findings in shown in Table I.

Table I. Grading of histopathological lesions in all groups.

Groups	Inflammatory cells infiltration	Tubular hyperemia	Intratubular proteinaceous material	Tubular epithelial degeneration	Cystic dilatation	Conges-tion	Swollen glomeruli	Glomerular hypercellu-larity
Control	_	_	_	_	_	_	_	_
CP	++	++/+++	++/+++	++/+++	++/+++	++/+++	++	++
CP+EA50	+	+/++	+/++	+/++	+/++	+/++	+	+
CP+EA75	_	_	_	_	+	+	_	_

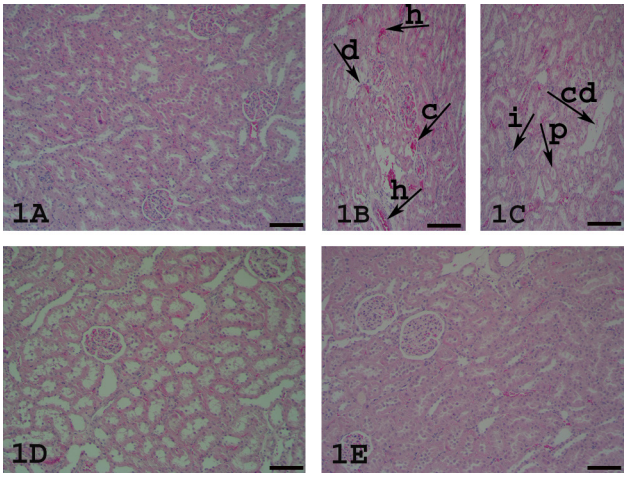

Fig. 1. Histopathological appearance of kidney tissues of all groups, HE staining, scale bar= 150 µm A, Control group: normal kidney morphology. B-C, CP group: hyperemia, epithelial degeneration, swelling, cystic dilatation, proteinaceous material in tubuli, congested and hypercellular glomeruli, inflammatory cells infiltration in cortical and medullary area. Arrows pointing events; h: hyperemia, c: congestion, d: degeneration, i: inflammation, p: proteinaceous material, cd: cystic dilatation. 1D, CP+EA50 group: significant decrease in pathological lesions. 1E, CP+EA75 group: more protective effect and normal architecture of kidney.

Immunohistochemical finding

8-OHdG revealed less positivity in EA groups as compare to CP group. Both luminal and cytoplasmic positivity of epithelial cells was found in cortex portion of kidneys with 8-OHdG marker. Cortical area of kidneys showed more positivity in the form of islands. Glomeruli were evaluated after staining with immunohistochemical stains but no positive stain was found in glomeruli with 8-OHdG marker. In the CP group cortical positivity was found in the form of islands with 8-OHdG. The group that received EA with the dose rate of 75 mg/kg showed less positivity as compare to the group that received EA with the dose rate of 50 mg/kg with 8-OHdG. The findings in the group received EA with the dose rate of 75 mg/kg were almost near to control group with 8-OHdG (Fig. 2A, D).

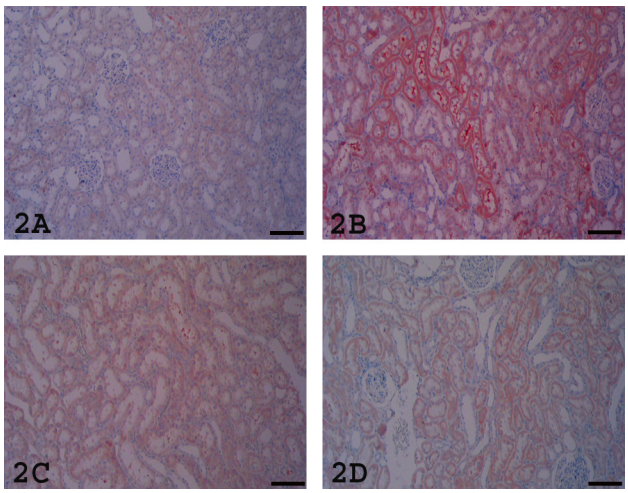

Fig. 2. 8-OHdG evaluation: kidneys stained with Streptavidin biotin peroxidase complex method with AEC chromogen and Mayer's Hematoxylin counterstain, scale bar=150 µm. A, Control group is not showing specific positivity. B, CP group is showing specific cytoplasmic and luminal positivity of tubuli. C, CP+EA50 group is showing mild positivity. D, CP+EA75 group is showing quite improved results of less specific positivity.

HIF-1α revealed less positivity in EA groups as compare to CP group. Medulla portion of kidneys revealed more positivity of cells with HIF-1α marker. All the area of medulla was not positive because HIF-1α also showed positivity in the form of islands. Glomeruli were also unstained with the HIF-1α. Positivity of medulla portion was found in the form of islands with HIF-1α. The group that received EA with the dose rate of 75 mg/kg showed less positivity as compare to the group that received EA with the dose rate of 50 mg/kg with HIF-1α. The findings in the group received EA with the dose rate of 75 mg/kg were almost near to control group with HIF-1α (Fig. 3A, D).

Bcl-2 revealed more positivity in EA groups as compare to CP groups during immunohistochemical

examination. Bcl-2 marker revealed positivity of cells at the border of cortex and medulla junction. The positive cells were also in the form of islands on the cortex and medulla junction border. There was no positivity in glomeruli of kidneys with Bcl-2 marker. Bcl-2 marker revealed different results in which CP group showed less positivity on the border of cortex and medulla junction as compare to EA groups. The group received EA with the dose rate of 75 mg/kg revealed more positivity of cells at the cortex and medulla junction and that positivity was almost like control group (Fig. 4A, D).

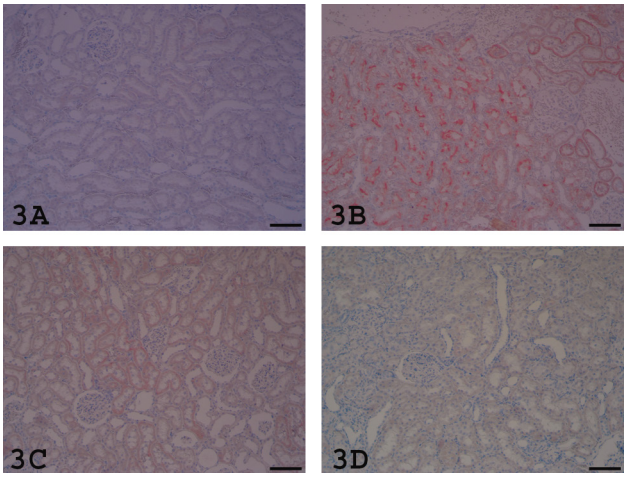

Fig. 3. HIF-1α evaluation: kidneys stained with Streptavidin biotin peroxidase complex method with AEC chromogen and Mayer's Hematoxylin counterstain, scale bar=150 µm. A, Control group revealed no positivity. B, CP group revealed specific positivity of tubuli. C, CP+EA50 group revealed mild positivity. D, CP+EA75 group revealed no positivity like control group.

DISCUSSION

CP is commonly used as antineoplastic agent and is effective against wide range of neoplastic and non-neoplastic pathological conditions. It also has some adverse effects including nephrotoxicity (Sinanoglu *et al*., 2012), hepatotoxicity (Mahmoud, 2014), immunotoxicity (Shirani *et al*., 2015), and peripheral neuropathy in experimental animals and humans. Several studies have been reported that chemotherapeutic agents can induce oxidative stress and this is responsible for damages in parankimateous organs including kidney (Ateşşahín *et al*., 2007), heart (Yilmaz *et al*., 2006), and liver (Pratibha *et al*., 2006). EA is a phenolic compound and naturally found in different fruits and nuts and has been reported to prevent oxidative stress induced by cisplatin in liver, heart and kidneys of rats (Yüce *et al*., 2007; Ateşşahín *et al*., 2007). EA has been reported to exert a potent scavenging action against both oxygen and hydroxyl radicals in vitro and also lipid peroxidation (Iino *et al*., 2001). EA being an antioxidant agent have many protective effects against CP-induced nephrotoxicity in mice (Rehman *et al*., 2012), cisplatin-induced oxidative stress in liver and heart of rats (Yüce *et al*., 2007), cisplatin-induced nephrotoxicity in rats (Ateşşahín *et al*., 2007), and cisplatin-induced injuries to sperm quality in rats (Türk *et al*., 2008).

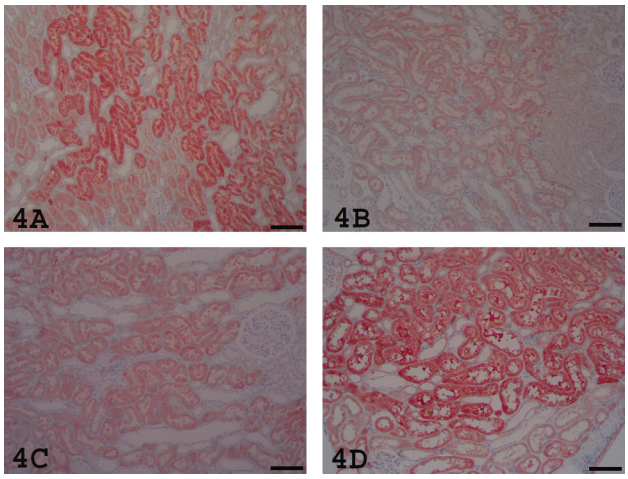

Fig. 4. Bcl-2 positivity evaluation kidneys stained with Streptavidin biotin peroxidase complex method with AEC chromogen and Mayer's Hematoxylin counterstain, scale bar=150 µm. A, Control group revealed specific cytoplasmic and luminal positivity of tubuli. B, CP group revealed less specific positivity. C. CP+EA50 group revealed mild positivity. D, CP+EA75 group revealed specific positivity like control group.

CP induced renal toxicity in rats (El-Shabrawy *et al*., 2020; Ayza *et al*., 2020) and in mice (Sharma *et al*., 2017; Rehman *et al*., 2012; Hamzeh *et al*., 2018) have been reported in previous studies. In rats CP-induced tubular epithelial degeneration (Lim *et al*., 2017), collagen bundles around vessels, tubules and also in glomeruli (El-Shabrawy *et al*., 2020), inflammatory cells infiltration and tubular necrosis (Ayza *et al*., 2020) have been reported in kidneys. In mice, degeneration (Sharma *et al*., 2017; Rehman *et al*., 2012; Hamzeh *et al*., 2018), swelling (Sharma *et al*., 2017; Rehman *et al*., 2012), congestion and necrosis (Rehman *et al*., 2012), casts formation (Hamzeh *et al*., 2018) and atrophy (Hamzeh *et al*., 2018) in tubuli and thickened basal membrane, widened Bowman's space (Hamzeh *et al*., 2018), degeneration (Sharma *et al*., 2017; Hamzeh *et al*., 2018) and atrophy (Hamzeh *et al*., 2018) in glomeruli, and also inflammatory cells infiltration in cortical and medullary area (Rehman *et al*., 2012; Hamzeh *et al*., 2018)

have been reported in CP-induced nephrotoxicity. Similar findings were found in our study but we have not found tubular 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 cystic dilation in tubuli and hypercellularity in glomeruli which was not found in previous studies of mice in rats.

According to literature research, we have not found any study about the protective effect of EA on CP-induced renal damage in rats. In a study conducted in mice, renal damage characterized with necrosis, desquamation of epithelial cells, and glomerular congestion and showed invasion of inflammatory cells in the cortical and medullary regions of kidneys. The nephrotoxicity was evaluated with widespread degeneration of tubular architecture, tubular congestion, swelling and necrosis. In comparison, renal sections obtained from mice that were pretreated with EA at a dose of 100 mg/kg body weight demonstrated marked reduction of the histological characters of renal damage (Rehman *et al*., 2012). In our study we gave EA with the dose rate of 50 mg/kg and 75 mg/kg in different groups and we also found significant decrease in pathological lesions especially in group received EA with the dose rate of 75 mg/kg in rats.

8-OHdG is one of the widely used biomarker of oxidative DNA damage in rats (Benzer *et al*., 2018). Reactive oxygen species (ROS) can be produced by free radical generating agents and it can oxidize the guanine residues of 8-OHdG, an oxidized nucleoside of DNA, and cause base modifications and thread breaks in DNA (Alak *et al*., 2017; Kandemir *et al*., 2017). The correlation between 8-OHdG and ROS has been reported and it indicates that ROS is main cause for the formation of 8-OHdG (Topal *et al*., 2017). Kandemir *et al*. (2017) found marked increase in 8-OHdG expression in the hepatic and renal tissues of rats in the CP-treated group, and pre-treatment with naringin (NG) significantly reduced this 8-OHdG over-expression. They described severe 8-OHdG expression in tubular epithelia and glomerular endothelial cells. In our study we also found overexpression of 8-OHdG in CP-induced as compare to control and EA groups. HIF-1 plays main role in the regulation of oxygen homeostasis (Brahimi-Horn and Pouysségur, 2009; Melillo, 2004). Disturbance in the HIF-1-dependent regulation of mitochondrial respiration led to high ROS level and apoptosis under conditions of prolonged hypoxia (Kim *et al*., 2006; Zhang *et al*., 2007). We have not found the direct expression of HIF-1α in CP-induced kidneys but Liu *et al*. (2015) have been reported that the expression of HIF-1α protein in the cisplatin-induced group was significantly increased when compared with the control group, which was increased by 1.13 times (Liu *et al*., 2015). In other study, they reported downregulation of TGFb1, IL-6, and caspase-3 and up-regulation of HIF-1α and VEGF in cisplatin group compared to the control group (Khedr *et al*., 2021). Cisplatin and CP both are therapeutic agents and both cause hepatotoxicity and nephrotoxicity in rats. In our study we found similar results of overexpression of HIF-1α in CP induced group as compare to control and EA groups. We evaluated positive correlation between 8-OHdG and HIF-1α that has also been reported in one study in humans (Pialoux *et al*., 2009).

El-Shabrawy *et al*. (2020) demonstrated CP-induced apoptosis and they evaluated apoptotic markers as caspase-3 and Bax and antiapoptotic marker as Bcl-2. They demonstrated the elevational level of apoptotic markers as caspase-3 and Bax but decreased expression of antiapoptotic Bcl-2 in renal tissue of CP group when compared with the control group. In our study we found the same result of Bcl-2 as CP group showed less positivity on the border of cortex and medulla junction as compare to control and EA groups (El-Shabrawy *et al*., 2020). We found negative correlation between 8-OHdG and Bcl-2, and HIF-1α and Bcl-2. This negative correlation 8-OHdG and Bcl-2, and HIF-1α and Bcl-2 has also been reported in humans (Najar *et al*., 2010; Fan *et al*, 2002). Both histopathological and immunohistochemical results of our study are strongly supporting the results of all the previous studies.

CONCLUSION

In conclusion, both the histopathological and immunohistochemical findings of this study revealed that EA has a protective effect against CP-induced renal damage in rats.

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Statement of conflict of interest

The authors have declared no conflict of interest

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