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Original article

Vitamin E partially ameliorates cyclophosphamide-induced nephrotoxicity in rats

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Summary. *Purpose:* Cyclophosphamide (CP) is a widely used anti chemotherapeutic drug, which causes nephrotoxicity due to its toxic metabolites. This study was carried out to assess the effects of vitamin E on cyclophosphamide induced renal toxicity in rats. *Model:* Twenty-eight Wistar albino rats were assigned to four groups, which were given 20 mg/kg CP, 20 mg/kg CP + 100 mg/kg vitamin E, 100 mg/kg vitamin E, or 20 mg/kg isotonic sodium chloride solution intraperitoneally each day for 7 days. Effects were assessed by histology of the kidney, TUNEL assay and measurement of serum uric acid and creatinine. *Results:* Cyclophosphamide significantly increased glomerular inflammation, edema, congestion and tubular degeneration, TUNEL positive cells, while addition of vitamin E significantly decreased glomerular inflammation, edema and TUNEL positive cells. Cyclophosphamide did not affect urea and creatinine levels, which may due to the absence of renal necrosis. *Conclusion:* Vitamin E application appears to partially ameliorate Cyclophosphamide induced renal toxicity.

Key words: kidney, cyclophosphamide, vitamin E, TUNEL

Introduction

Cyclophosphamide (CP), an alkylating agent, is widely used to treat breast cancer (1), multiple myeloma (2), Hodgkin's disease (3), acute (4) and chronic lymphocytic leukemias (5), and as an immunosuppressant in systemic lupus erythematosus (6), multiple sclerosis (7) and organ transplantation (8). Despite its wide spectrum of clinical uses, CP is known to cause several adverse effects (9).

CP is activated by hepatic microsomal cytochrome P450 to yield phosphoramide mustard and acrolein (10).

Acrolein is metabolized mainly by fast reaction with sulfhydryl groups of glutathione (GSG) to form mercapturic acid, which is eliminated in the urine. By this mechanism, acrolein directly induces cellular oxidative stress by oxidizing glutathione (11). Acrolein and its glutathione adduct, glutathionylpropionaldehyde, are metabolized by xanthine oxidase and aldehyde dehydrogenase to produce O_2 and HO. Xanthine oxidase oxidizes acrolein to produce acroleinyl radical and O_2. Aldehyde dehydrogenase metabolizes acrolein, but not acrolein radical, to form O_2. These oxygen radicals may play a role in the induction of lipid peroxidation by acrolein (12).

CP has been reported to increase lipid peroxidation (13) and to decrease antioxidant enzyme levels in the kidney (14).

Experimental studies suggest that CP causes kidney injury that is ameliorated by a variety of antioxidants (15-17).

Vitamin E is a fat soluble and acts as an antioxidants to prevent lipid peroxidation of polyunsaturated fatty acids (18, 19). Vitamin E is a major antioxidant in biological systems acting as a powerful chain breaking agent through the scavenging of peroxyl radicals (20). A number of studies have been reported to the protective effects of vitamin E in different biological models of tissues injury (19, 21-24). Researchers have been focused the role of vitamin E in protection of membranes lipids against oxidative stress (25, 26).

Therefore, we sought to assess the effect of vitamin E against the damage caused by repeated CP injection by histopathology, TUNEL staining, and serum indicators of kidney function.

Material and Methods

Chemicals

Cyclophosphamide was purchased from Endoksan as ampules containing 1000-mg drug in solution and administered following suspension in 50-mL isotonic sodium chloride, also obtained as ampules. Vitamin E was purchased as ampules from Evigen. Solutions were prepared based on prospectus information provided by the manufacturers.

Animals

The study protocol was approved by the Ethics Board of the Necmettin Erbakan University Kombassan Experimental Medical Research and Practice Center in decision number 2013/132. The project was supported by Necmettin Erbakan University Scientific Research Projects Coordination Center (BAP number 131318002).

Twenty-eight male Wistar albino rats (4 months old, 250-300 g) were obtained from NE University Kombassan Experimental Medical Research and Practice Center. The animals, having free access to food and water, were kept in a 12h dark-light cycle in a temparature and humidity controlled room.

Experimental protocol

The rats were divided into four groups. Drugs were injected by intraperitoneally daily once for 7 days. Drug or control administrations were as follows (second administration 30 min after the first):

- *Control:* isotonic sodium chloride solution (the CP solvent), then olive oil (the vitamin E solvent);
- *CP:* 20 mg/kg cyclophosphamide, then olive oil 30 min later;
- *CP + vitamin E:* 20 mg/kg cyclophosphamide, then 100 mg/kg vitamin E;
- *Vitamin E:* vitamin E 100 mg/kg, then isotonic sodium chloride.

Rats were sacrificed using ketamine (50 mg/kg)/xylazine (10 mg/kg).

Histopathology

The left kidneys were collected in formaldehyde solution and embedded in paraffin for histology and TUNEL staining. Three μm serial sections were assessed microscopically after staining with hematoxylin and eosin. Glomerular inflammation, edema, congestion, necrosis and tubular degeneration criteria were assessed histopathologically.

TUNEL metod

Apoptotic cells were labeled using an ApopTag In Situ Apoptosis Detection Kit (Millipore), in which DNA fragments are modified via the action of terminal deoxynucleotidyl transferase (TdT). All procedures followed the manufacturer's instructions. Twenty magnification areas (X400) were selected randomly and TUNEL-positive cells were counted in 100 tubule cells on each section (27).

Biochemical analysis

Plasma was obtained from blood samples that were centrifuged at 3500 rpm for 10 min and stored at −20°C. Creatinine and urea were measured photometrically using an Abbott Architect 16000c instrument.

Statistical assessment

Serum metabolites and TUNEL scores were compared using one-way analysis of variance (ANOVA) followed by Duncan's test. Histopathology results were compared using the Kruskal–Wallis test and differences between two groups were assessed using the Mann–Whitney U-test.

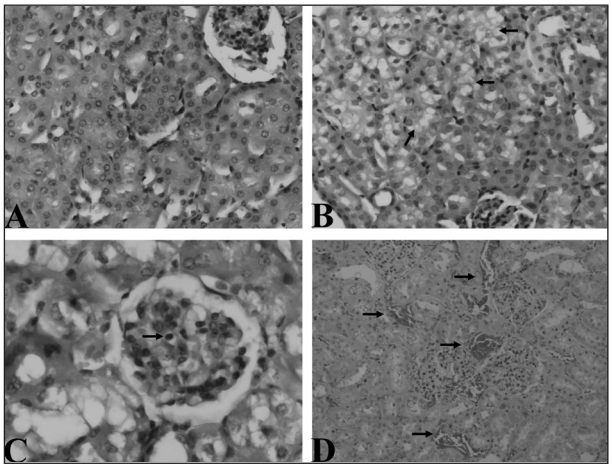

Figure 1. Representative hematoxylin-eosin (H&E)-stained kidney sections A:Control group, B: tubular degeneration, C: glomerular infiltration, D:Congestion.

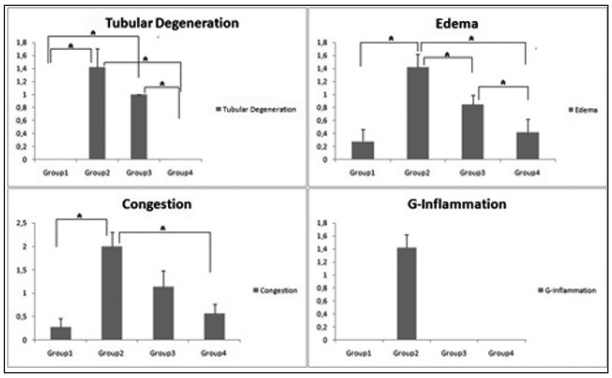

Figure 2. Quantification of histopathology, *:p < 0.05, mean ± SD

Results

Histopathology results

While the kidneys of rats receiving saline (Fig. 1A), or vitamin E appeared normal by histology, CP caused significant tubular degeneration (Fig. 1B), glomerular inflammation (Fig. 1C), edema, congestion (Fig. 1D) but no necrosis. Specifically, CP reduced the size of Bowman's space and caused the brush borders of the proximal tubular epithelium to fall into the tubule lumen.

Combining vitamin E with CP significantly decreased glomerular inflammation and edema compared to CP alone, but did not affect congestion or tubular degeneration (Fig. 2).

Biochemical results

CP did not significantly affect serum urea or creatinine (Tab. 1).

TUNEL results

While vitamin E alone had no effect on the number of TUNEL-positive cells relative to control (Fig. 3 A, 3D) in combination with CP (Fig 3C) it decreased the number of apoptotic cells (Fig. 4).

Discussion

Glomerular inflammation, oedema congestion and tubular degeneration were observed on kidney slices after CP induction. Administration of Vitamin E to CP induced rats was decreased the glomerular inflammation and oedema significantly but non effective to tubular degeneration and congestion. Absence of difference between groups according to urea and cre-

Table 1. Serum indicators of kidney function

	Group 1	Group 2	Group 3	Group 4
Creatinine	0.52 ± 0.007	0.48±0.13	0.49±0.09	0.49±0.014
Urea	46.57±1.79	44.77±3.21	43.11±2.54	43.54±1.27

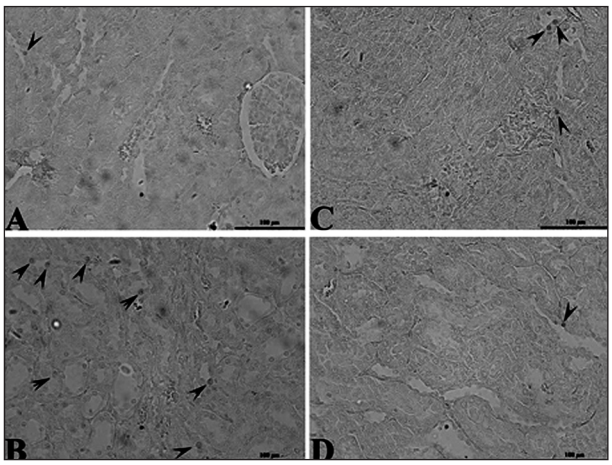

Figure 3. Representative liver sections stained in the TUNEL assay (arrows). A, control group; B, CP only; C, CP + vitamin E; D, vitamin E only

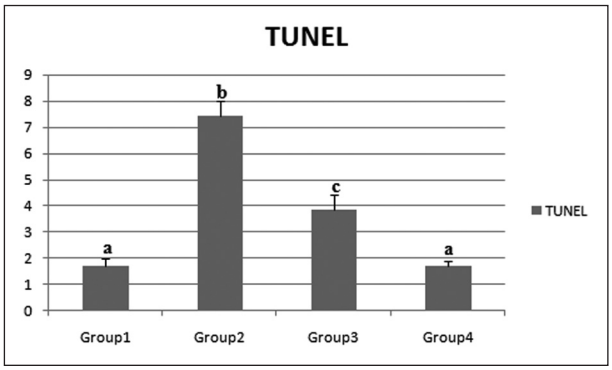

Figure 4. Number of TUNEL-positive cells in the kidney. Bold letters indicate significant differences ($p < 0.05$).

atinine levels was considered as a result of absence of necrosis. And again, Vitamin E was observed decreasing the count of TUNEL positive cells on CP group.

Acrolein and its glutathione adduct (glutathionylpropionaldehyde) are coactioned upon by xanthine oxydase and aldehyde dehydrogenase and O_2- and HO^- occurs (12). HO^- is a highly reactive compound and reported as possibly directly responsible from oxidative damage on biological systems (28). In cases of depression of detoxification systems and antioxidants or extremeness of reactive oxygenic species (ROS) production, DNA, protein and lipid oxidation occur (29).

Oxidative stress caused by CP induces renal apoptosis, increases expression of p53 and Bax mRNA and decreases expression of Bcl-2 (30). Apoptosis oxidizes cardiolipin to release cytochrome C from the mitochondrial inner membrane to the cytoplasm (29) accounting for the increased TUNEL-positive cell count in the kidneys of rats receiving CP in our study.

Biological membranes contain polyunsaturated fatty acids (PUFAs); OH^- ions remove hydrogen (H) atoms from PUFA, forming $PUFA^·$ radicals. O_2 attacks such radicals, creating peroxyl radicals ($PUFAOO^·$), which in turn extract H atoms from adjacent PUFAs, triggering a chain reaction. Vitamin E donates an H atom to $PUFAOO^·$, forming a stable lipid species, and is a non-reactive free radical in the H-lacking state. Vitamin E scavenges peroxyl radicals, thus preserving PUFAs and protecting low-density lipoproteins (LDLs) from lipid peroxidation (31). Thus, we concluded vitamin E ameliorates the CP-induced apoptosis by reducing oxidative stress.

Previous study; glutamine, was not found to affect the lipid peroxidation and kidney damage caused by a single injection of CP, though it did ameliorate glutathione exhaustion and neutrophil infiltration (32). The limited effect of vitamin E we observed against CP-induced kidney damage in our study may result from its known efficacy in reducing lipid peroxidation (13).

A single dose of CP has been shown to cause glomerular nephritis, tubular vacuolization, and edema and hemorrhage of the renal cortex in rats sacrificed at 6^{th}, 16^{th} and 24^{th} hours, as well as increased MDA and decreased glutathione levels. However, despite the histological damage, plasma creatinine was not significantly affected, causing the authors to suggest that other indicators of kidney damage should be monitored along with serum creatinine (33). In another study employing a single dose of CP, the drug was found to significantly increase serum urea and creatinine, as well as to cause tubular swelling, cellular vacuolization, pyknotic nuclei, and medullar congestion on histopathology of the kidney and to increase TUNEL-positive cell count. These authors also reported that vitamin E significantly reduces all damage caused by CP, including reducing serum urea and creatinine (34). In contrast to the findings of Sugumar et al (33), though the same dose of CP (150 mg/kg) was used in both studies. Despite the histological damage in our study, our results were in agreement with Sugumar et al (33), we observed no significant effect of CP on creatinine

and urea levels which may result from the absence of renal necrosis,

To minimize differences among studies of CP-induced renal toxicity, we suggest that a range of doses should be used in each. We observed that vitamin E partially ameliorates CP-induced kidney damage, suggesting that blocking this side effect requires stronger antioxidants.

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