

Vitamin E ameliorates cyclophosphamide induced nephrotoxicity

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Abstract: Cyclophosphamide (CP), is a cytotoxic alkylating agent and it is widely used in treatment of malignant diseases and autoimmune disorders. CP may be nephrotoxic, both in humans and animal models by generating reactive oxygen species. Vitamin E is a major antioxidant in biological systems acting as a powerful chain-breaking agent through the scavenging of peroxy radicals. The aim of this study was to evaluate the effect of vitamin E in CP induced nephrotoxicity. Forty rats were divided randomly into 4 groups (n=10). Group1: control group, receiving normal saline, group 2: CP group received a single intraperitoneal injection of CP in saline at the dose of 150 mg/kg. Group3: CP+Vit E: received CP plus Vit E (100mg/kg), group 4: received CP and olive oil. Twenty four hours after CP injection rats anesthetized and sampled for studying with light microscopy and biochemical studies. Data indicated that serum blood urea nitrogen (BUN) and creatinine (Cr), renal tissue injury index and renal tissue malondialdehyde (MDA) were higher in CP group comparing to CP+Vit E group significantly. Renal tissue glutathione peroxidase (GPx) and super oxide dismutase (SOD) activity were higher in CP+Vit E group than CP group significantly (P<0.05). Our results showed that one of the basic mechanisms involved in CP-induced nephrotoxicity might be oxidative stress and vitamin E pretreatment attenuated CP induced nephrotoxicity.

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1. Introduction

Cyclophosphamide (CP), a cytotoxic alkylating agent, is widely used in the treatment of acute and chronic lymphocytic leukemias, Hodgkin's disease, multiple myeloma, soft tissue sarcomas as well as it is an immunosuppressive agent for organ transplantation, multiple sclerosis, systemic lupus erythematosus, and other benign diseases (Dollery et al., 1999; West and Dollery et al., 1997). CP is nitrogen mustard which presents a cytotoxic action and interferes with normal mitosis and cell division in all rapidly proliferating tissues (Fraiser et al., 1991). Studies have shown that CP can be nephrotoxic, both in humans and animal models. Studies have shown that CP can result in glomerular dysfunction and tubular dysfunction, glomerular proteinuria, tubular proteinuria, reduction of glomerular filtration rate, and decrease in concentration function of kidney has been reported in children on chemotherapy (Sugumar et al., 2007; Ghosh et al., 1999; Senthilkumar et al., 2006). CP is a prodrug that is dependent on cytochrome P450 metabolism for its therapeutic effectiveness (Van et al., 2008). Recent studies have shown that reactive oxygen species (ROS) play an important role in CP-induced nephrotoxicity, these studies have shown that the indicator of oxidative stress, namely malondialdehyde (MDA) increases in the kidney after CP administration and CP reduces superoxide

dismutase (SOD) in which is an important cellular anti-oxidant (Haque et al., 2003; Abraham et al., 2009). The role of ROS in the pathogenesis of CP-induced nephrotoxicity is supported by the findings that antioxidants such as melatonin and ascorbic acid protect against CP-induced nephrotoxicity (Manda et al., 2003). Antioxidant defense mechanisms can effectively protect cells and tissues from free radical mediated deleterious effects. Vitamin E is a fat-soluble and acts as a free radical scavenger to prevent lipid peroxidation of polyunsaturated fatty acids and block nitrosamine formation (Valko et al., 2006; Poppel et al., 1997). Vitamin E is a major antioxidant in biological systems acting as a powerful chain-breaking agent through the scavenging of peroxy radicals (Beyer et al., 1994), so a number of studies have been carried out to determine the protective effects of vitamin E in different biological models of injury (Ernster et al., 1995). Currently, there is considerable interest in the role of vitamin E in protection of membranes lipids against oxidative stress (Kashif et al., 2004). Administration of Vit E alone or in combination with other vitamins, such as vitamin C, increases the activities of antioxidant enzymes such as SOD, catalase, glutathione-S-transferase in rats. In the present study, we aimed to investigate the effect of vitamin E on renal injury after CP administration.

2. Materials and methods

Male wistar rats weighing 235 ± 27 g were used in this study. The experimental protocol was approved by the medical ethics committee, and all animals received humane care in compliance with the guidelines of Tabriz medical university. Forty rats were divided randomly into 4 groups (n=10). Group1: control group, receiving normal saline, group 2: CP group, the rats in group 2 (n = 10) received a single intraperitoneal injection of CP in saline at the dose of 150 mg.kg^{-1} body weight [9] and also received olive oil since 3 days prior to CP injection. Group3: CP+Vit E: the rats in group 3 (n = 10) received a single intraperitoneal injection of CP in saline at the dose of 150 mg.kg^{-1} body weight, they also received Vit E (100 mg.kg^{-1}) dissolved in olive oil at dose of 100 mg.kg^{-1} since 3 days before CP administration, group 4: received Vit E (100 mg.kg^{-1}) dissolved in olive oil at dose of 100 mg.kg^{-1} to see if vitamin E have any effect on anti-oxidative and biochemical factors and renal tissue. Twenty four hours after CP injection rats anesthetized with Ketamin (50 mg.kg^{-1}) and xylazine (10 mg.kg^{-1}), blood serum obtained for measuring BUN, Cr and Tumor necrosis factor- α (TNF- α) from vena cava inferior. Kidney excised and one of them freezed at -70°C degree for measuring SOD, glutathion peroxidase (GPx) and MDA, and the other one fixed in 10% formalin for studying with light microscopy.

2.1. Histopathological analyzes

The specimens fixed in 10% formalin were embedded in paraffin. Sections of $4\mu\text{m}$ were prepared, stained with hematoxylin and eosin, and then examined by a pathologist under a light microscope. The histopathologic scoring analysis was performed according to previously described methods, the assessment was expressed as the sum of the individual score grades from 0 (no findings), 1 (mild), 2 (moderate), to 3 (severe) for each of the following 4 parameters from kidney sections: tubular cell swelling, cellular vacuolization, pyknotic nuclei and medullary congestion (Singh et al., 2004). The method for the terminal deoxynucleotidyl transferase-mediated nick-end labeling method (TUNEL) was performed as previously described; we used insitu cell death detection kit, POD, from Roche laboratories, Germany. Twenty high-power ($\times 400$) fields were randomly selected, and apoptotic cells were counted. The number of apoptotic cells was expressed per 100 of the tubular cells in each section (Chien et al., 2001).

2.2. Measurement of BUN and Cr levels

The plasma BUN (blood urea nitrogen) and creatinine (Cr) levels were determined

spectrophotometrically, as previously described (Gulmen et al., 2009).

2.3. Plasma cytokine concentrations

Plasma TNF- α , was determined using enzyme-linked immunosorbent assay (ELISA) technique, as previously described. The capture antibody was a polyclonal anti-mouse TNF- α , (Blender Systems, Austria). All plasma samples were tested in duplicate (Mosher et al., 2001).

2.4. Assay of antioxidant enzymes

2.4.1. GPx activity

One kidney of each animal was snap-frozen in liquid nitrogen and stored at -70°C degree until further preparation. In order to measure cytosolic enzyme activity, the kidney samples were homogenized in 1.15% KCL solution. GPx activity in kidney was measured using the method described by Paglia and Valentine. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to reduced form with a concomitant oxidation of NADPH to NADP^{+} . The decrease in absorbance at 340 nm is measured. (Ransod, Randox Laboratories Ltd. United Kingdom). Results obtained GPx Units/mg protein (Paglia et al., 1967).

2.4.2. SOD activity

SOD activity in kidney tissue was determined by using xanthine and xanthine oxidase to generate superoxide radicals which then react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-henyltetrazolium chloride to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction (Ransod, Randox Laboratories Ltd. United Kingdom). Results obtained SOD Unit/mg protein (Sun et al., 1988).

2.4.3. MDA level

Kidney MDA levels were studied using the thiobarbituric acid (TBA) abduction method as described previously. Briefly, $50 \mu\text{l}$ of sample was introduced into a tube containing 1 ml of distilled water. After addition of 1 ml of a solution containing 29 mmol/l TBA (Sigma Chemical Co.) in acetic acid (8.75 mmol/l, pH of the reaction mixture: 2.4–2.6) and mixing, the samples were placed in water bath and heated for 1 hour at $95\text{--}100^{\circ}\text{C}$. The samples were then cooled under running cold water. Twenty five μl of HCl (5 mol/l) was added and the reaction mixture was extracted for agitation with 3.5 ml of n-butanol (Sigma Chemical Co.) for 5 minutes. After centrifugation, the butanol phase was separated and the fluorescence of the butanol extract was measured

by spectrophotometry (Wasowicz et al., 1993; Kaya et al., 2004).

2.5. Renal tissue total protein

The protein content in the tissue homogenate was estimated by following the protocol of Lowry et al., (1951).

2.6. Statistical analysis

Values are presented as means \pm S.D. The one way ANOVA-test was used to compare the results obtained within the groups, Tukey post hoc test used for comparing the results between groups. A value of $p < 0.05$ was accepted as indicative of significant difference among groups.

3. Results

3.1. Effects of Vit E on histopathological changes of CP treated rat kidney

Kidney histological studies were used to determine the protective effect of Vit E on CP induced injury. As shown in table 1 the results of histopathological evaluation showed that Vit E exhibited renoprotective effect against CP induced kidney injury. CP treatment caused visible histology changes including structure damage in rat kidney,

whereas, Vit E alleviated the kidney damage in Vit E-treated rats. No appearance difference could be observed in the kidney between the rats treated with Vit E and control group ($P < 0.05$, table 1).

3.2. Effects of Vit E on serum TNF- α , BUN and Cr changes after CP treatment

For clarifying CP administration effect on serum TNF- α , BUN and Cr, we measured these factors. CP treatment increased TNF- α level significantly but didn't changes serum BUN and Cr level. Vit E decreased TNF- α production after CP administration significantly ($P < 0.05$, table 2).

3.3. Effects of Vit E on antioxidative status of CP-treated rat kidney

To determine whether Vit E can attenuate the increased oxidative damages in the kidney of CP-treated rat, we measured the activities of major antioxidant enzymes, including SOD, and GPx, in rat kidney. The results showed that Vit E could renew the activities of these antioxidant enzymes in the kidney of CP-treated rats (table 3). The result also showed that Vit E can reduce MDA production after CP administration significantly ($P < 0.05$).

Table 1: Renal tissue injury index

	Control	CP	CP+Vit E	Vit E
Renal injury index	0.77 \pm 0.44*	7.77 \pm 0.97**	4.87 \pm 1.24***	0.87 \pm 0.64*
Apoptic Index	1.22 \pm 0.44	11.66 \pm 1.58	7.62 \pm 1.92	1.37 \pm 0.51

Values =mean \pm S.D. Within a row, values with different superscripts (*, **, ***) are significantly different from each other ($P < 0.05$). CP: Cyclophosphamide, CP+Vit E: Cyclophosphamide +vitamin E, Vit E: Vitamin E.

Table 2: Clinical chemistry (kidney function tests and TNF- α) parameters of rats

	Control	CP	CP+Vit E	Vit E
TNF- α (pg/ml)	9.33 \pm 2.91*	77.22 \pm 7.98**	45.75 \pm 8.10***	11.25 \pm 3.15*
BUN(mg/dl)	15.66 \pm 1.58*	18.33 \pm 2.44**	16.00 \pm 1.51***	16.62 \pm 2.66*
Cr(mg/dl)	0.492 \pm 0.068*	0.590 \pm 0.072**	0.538 \pm 0.089***	0.520 \pm 0.086*

Values =mean \pm S.D. Within a row, values with different superscripts (*, **, ***) are significantly different from each other ($P < 0.05$). CP: Cyclophosphamide, CP+Vit E: Cyclophosphamide +vitamin E, Vit E: Vitamin E.

Table 3: Renal tissue biochemical parameters (GPX, SOD, MDA)

	Control	CP	CP+Vit E	Vit E
GPX(U/mg protein)	4.42 \pm 0.57*	1.65 \pm 0.50**	2.58 \pm 0.45***	4.21 \pm 0.46*
SOD(U/mg protein)	3.42 \pm 0.47*	0.78 \pm 0.36**	2.22 \pm 0.52***	3.19 \pm 0.30*
MDA(nmol/ mg protein)	0.51 \pm 0.14*	1.83 \pm 0.21**	1.38 \pm 0.20***	0.58 \pm 0.21*

Values =mean \pm S.D. Within a row, values with different superscripts (*, **, ***) are significantly different from each other ($P < 0.05$). CP: Cyclophosphamide, CP+Vit E: Cyclophosphamide +vitamin E, Vit E: Vitamin E.

4. Discussion

The findings showed that administration of Vit E significantly attenuated the release of TNF- α , and diminished the renal injury caused by CP in rat. The nephrotoxicity of CP is generally overlooked because

plasma Cr, an indicator of glomerular function of the kidney, is not altered significantly in patients on CP chemotherapy. However, in recent studies, it is demonstrated that, in rat model, CP induces renal damage histologically, but the plasma Cr, a reliable

biochemical marker of renal dysfunction, remains unaltered (Sugumar et al., 2007). In our study we observed that CP administration increased Cr and BUN levels significantly and there was significant histopathologic change in renal tissue. To protect cells from the damage caused by free radicals and related reactants, organism has evolved several defense mechanisms.

Oxidative stress and free radical-induced damages have been implicated in the etiology of several toxic effects caused by CP (Fadillio et al., 2003). Oxidative stress is induced by oxidant substances commonly known as ROS (Sheweita et al., 2005). Normally, the balance between ROS produced by pro-oxidant and that scavenged by antioxidant is maintained, and cellular damage arises when this equilibrium is disturbed (Sharma et al., 1996; Ilbey et al., 2009). The oxidants that are not scavenged by antioxidant defense system attack cellular components producing useless molecular debris and sometimes cell death. The antioxidant enzymes represent a first line of defense against toxic reactants by metabolizing them to innocuous byproducts (Eşrefoglu et al., 2007; Rodrigez et al., 2004). When cells are exposed to oxidative stress they increase the activity and expression of antioxidant enzymes as a compensatory mechanism to better protect them from the damage. Moderate levels of toxic reactants induce rises in antioxidant enzymes while very high levels of reactants reduce enzyme activities as a result of damage of the molecular machinery that is required to induce these enzymes (Pillai et al., 2005). In normal condition, per-oxidation injuries will promote anti-oxidation adaptation within human body. SOD and GPx are two different anti-oxidation enzymes major in the cytosol of living cells. We measured these two enzymes from renal tissue following the protocols of Randox, respectively. Function of intracellular GPx is degradation of H₂O₂ and hydroperoxides of free fatty acids, whereas in plasma GPx catalyses degradation of H₂O₂ and hydroperoxides of phospholipids. In addition GPx exerts a protective effect on membrane phospholipids by inhibiting their peroxidation processes (Rybus-Kalinowska et al., 2008). Changes in activity of antioxidant enzymes are accompanied by intensification of lipid peroxidation processes, which is confirmed by elevated MDA plasma levels that we observed in rats receiving CP. In quantitative terms, MDA is the most important component among reactive aldehydes originating from lipid peroxidation. For this reason, it is commonly considered as an index of oxidative stress severity (Niki et al., 1995). TNF- α is a pleiotropic cytokine that induces cellular responses such as proliferation, production of inflammatory

mediators, and cell. TNF- α is produced mainly by macrophages but also by a broad variety of other cell types including lymphoid cells, mast cells, endothelial cells, fibroblasts, and neuronal cells (Wajant et al., 2003). TNF- α induces cell death via apoptosis and necrosis pathways, so inhibiting TNF- α production decreases tissue injury. In this research we showed that Vit E may decrease TNF- α level and tissue injury induced by CP administration (Wu et al., 2009). Abraham et al., (2009) reported that, CP induced renal damage may be due to overproduction of peroxynitrite and resultant deficiency of NO, that plays an important role in renal physiology. Their results reveal that enhanced nitrosative stress may play a role in CP-induced renal damage. CP-induced renal damage may be a consequence of NO deficiency and or overproduction of peroxynitrite. Abraham et al., (2007) have observed decrease in the activities of lysosomal enzymes in the kidneys of CP treated rats. Lysosomes function as digestive system of the cell, serving both to degrade material taken up from the cell and to digest obsolete components of the cell. Normally, the proteolytic enzymes of the lysosomes degrade the proteins. Lysosomal dysfunction can result in lack of digestion of proteins that are regularly degraded by the lysosomes there by resulting in increased half life of proteins. These result in the accumulation of abnormal amount of proteins within the cell. Increase in protein content alone is being used as marker for hypertrophy, although increase in the ratio of protein to DNA is considered a better marker (Liu et al., 1997; Khandkar et al., 1996). Lysosomes are reported to play an important role in cell death and tissue damage due to drugs and toxins (Dell'Angelica et al., 2000). While lysosomes have been considered for many years as garbage compartments, it is now established that these organelles or their components can play an important role in numerous biological processes in eukaryotes such as programmed cell death and secretion of cytotoxic molecules (Mukherjee et al., 1997). Mukherjee et al., 1997 have reported that Vit E stabilizes lysosomal membrane against the damaging action of *Vipera russelli* venom phospholipase A₂, so Lysosomal membrane stabilization by Vit E against CP induced lysosomal damage may be one of the protective mechanisms of VitE administration against CP induced renal injury, although for approving this hypothesis other studies are needed.

In conclusion, our results show that one of the basic mechanisms involved in CP-induced nephrototoxicity might be oxidative stress. In our study the Vit E pretreatment was performed with the aim of raising the levels of both SOD and GPx thus strengthening the antioxidant defenses. Our results

showed that groups which received CP had lower levels of SOD and GPX when compared to control group. Although there is ongoing research focusing on renoprotection and therapy that may affect a common pathophysiological pathway of CP induced nephrotoxicity, thus minimizing renal damage, further studies taking measurements over different factors and different drugs are needed to finding better agents for renal protection against CP induced nephrotoxicity.

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