Investigation of the effect of sodium selenite on metallothioneine expression in the liver and kidney in experimental cyclophosphamide toxicity

Research Article

ABSTRACT

In this study, the effect of sodium selenite (SS) on metallothionine expression in liver and kidney tissues of rats administered cyclophosphamide (CP) at high dose intraperitoneally (i.p.) was investigated. In the study, a total of 24 Wistar Albino female rats with a weight of 200±10 g were used in 4 groups. Grouping in the study: Group I (Control group; daily 1 mL serum physiologique given as ip), Group II (CP group; 200 mg/kg/day CP administered as ip for 1 day), Group III (SS Group; 1 day ip 1 mg/kg/day SS was given as a treatment), Group IV (CP Group + SS Group; 200 mg/kg/day CP and 1 mg/kg/day SS was given as ip for 1 day) by forming 4 different groups. The effect of histopathological findings was found to be significantly lower in the group in which CP was given at high doses and SS was applied at the same time, compared to the group in which only CP was applied. When immunohistochemical findings related to MTs of liver and kidney tissues in this group were compared with histopathological findings, similar results were observed in terms of evaluation results. The study was concluded to be important in terms of animal and human health because SS is well tolerated in the organism and has no side effects at the appropriate dose.

Keywords: Cyclophosphamide, metallothionein, sodium selenite

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INTRODUCTION

Cyclophosphamide (CP) is one of the alkylating antineoplastic agents widely used in oncology (Poll et al., 1988). It is a drug in the oxazophosphorine structure, which is in the alkylating class of chemotherapeutic agents (Bernacki et al., 1987). For CP treatment purposes, it is used effectively in pediatric solid tumors, acute lymphocytic leukemia in children (Limandal, 2013), non-Hodgkin lymphomas (Glode et al., 1981), soft tissue sarcomas, rhabdomyosarcomas, thrombocytopenic purpura, systemic lupus erythematosus (Bertram, 2012) and Behcet's disease (Ozyazgan et al., 1992). CP that is metabolized in the liver is excreted by the kidneys within 48 hours. 3-25% of the dose that was taken into the organism; is excreted in the form of the parent compound. The liver cytochrome P-450 enzymes allow CP to be activated to 4-hydroxy-cyclophosphamide and isomerized to aldophosphamide. Aldophosphamide is converted to phosphoramide mustard (FAM) and to acrolein (ACR), which have a cytotoxic effect in the urinary bladder (Kawabata et al., 1990; Bertram, 2012).

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Selenium (Se) was accepted as a toxic element until the 1930s. The protection of Se in liver tissue degeneration was first reported by Schwarz and Folz in 1957 (Schwarz & Foltz, 1957). The main source of Se is soil, but the way it is found in animals and plants is different. It is generally found in animal tissues in the form of selenocysteine and selenomethionine, while in plants it is found in the form of Se-methyl-selenomethionine, selenocysteine, selenocysteine and selenomethionine (Cousin & Cairney, 1961; Ullery, 1992; Aksoy, 2000). Selenoproteins enable Se to perform its biological activity in tissues. Se, located in the active site of the glutathione peroxidase enzyme in cells is in the form of selenocysteine. The glutathione peroxidase enzyme is very important for erythrocytes, it is a detoxifier of hydrogen peroxide (Akkus et al., 1991). Diseases such as nutritional myopathy, mad chick disease, exudative diathesis, pancreatic fibrosis, decreased egg production, and decreased brood productivity occur in sheep when Se is deficient (Bildik et al., 1996; Göger, 1997). The discovery of metallothioneines (MT) dates back to 1957. Margoshes and Vallee reported that MTs bind cadmium in mammalian kidney cells (Margoshes & Vallee, 1957). MTs exist in different forms in animals and plants, prokaryotes and eukaryotic microorganisms (Klaassen et al., 1999). MTs are important in the homeostasis of basic metals such as Zn and Cu and in detoxifying heavy metals such as Cu, Cd, Hg and Ag. MTs are proteins with a high cysteine content, the ability to bind metals, being soluble and temperature-stable, consist of 61-68 amino acids, and having a molecular weight of 6000-7000 Da (Viarengo & Nott, 1993). Metals and other physical factors such as hunger, pesticide-induced oxidative stress, salinity, heat-cold trauma, exercise, UV rays, chemicals such as carbon tetrachloride-paraquat, alkylating agents, drugs applied in tumor treatment, inflammation, interleukin I-6, and TNF induce MT expression. It can also be induced by hormones such as cytokines, bacterial infections, glucocorticoids, angiotensin-II, and glucagon (Sato & Bremner, 1993; Viarengo et al., 1999; Mosleh et al., 2005). It has been reported that MTs show cytotoxic resistance to the drugs used in malignant tumor treatments, especially in cases with poor prognosis (Cherian et al., 2003; Gomulkiewwicz et al., 2010). Antioxidants, alpha tocopherols and glutathione play an active and important role in the protection of liver cells. MTs, which are similar in structure to glutathione, are potential antioxidant proteins and have a very important place in the protection of liver cells (Zhou et al., 2002).

MATERIALS AND METHODS

Antioxidant and drug used

In the study, i.p. SS given as Sigma Aldrich (Germany), and as CP; Endoxan® (Mccaroll et al., 2008) containing 1069.9 mg of CP monohydrate, equivalent to 1g CP, was supplied from Eczacıbaşı Pharmaceutical Company (Türkiye).

Experimental animals used

Experiment and study design were approved by Aydın Adnan Menderes University, Experimental Animals Ethics Committee with the decision numbered 64583101/2016/146.

A total of 24 adult female Wistar Albino rats weighing 200±10 grams were used in the study. Rats were kept in transparent polycarbonate cages throughout the study. The rats were given drinking water and standard feed ad libitum. They were kept in special cages in special rooms with a temperature of 22±2°C, a humidity of 50-55%, and a lighting of 12;12 hours light/dark.

Experiment design

The rats used in the study were randomly determined and grouped into 4 different groups, with 6 rats in each group (Sabık & El-Rahman, 2009). Group I (Control group): During the study, 1 ml 0.9% saline daily i.p. given as). Group II (CP group): Each rat was given 1 day i.p. 200 mg/kg/day CP was given. Group III (SS

group): Each rat was given i.p. for 1 day. 1 mg/kg SS was given. Group IV (SS Group + CP Group): Each rat was administered i.p. for 1 day. As a result, 1 mg/kg SS and 200 mg/kg/day CP were given.

Pathological examination

After the necropsy procedures applied to rats, kidney and liver tissue samples were taken into formalin solution (10%) for 48 hours and tissue fixation was made. Afterwards, the fixed tissues were trimmed. Then, after the trimmed samples were taken into tissue tracking cassettes and washed under tap water, they were placed in the tissue tracking device (Leica TP1020, Germany), tissue tracking was performed and the samples were brought to the sectioning stage by blocking in paraffin. Block tissue samples in paraffin (Leica RM 2135, Germany) were cut with a microtome at a thickness of 4-6 microns and stained with hematoxylin-eosin for histopathological examinations and examined under a light microscope. Sections deemed necessary were stained with Masson's Trichrome and oil red O dye, respectively, and examined under a light microscope to examine connective tissue and hepatic lipidozis (Culling et al., 1985). Findings found as a result of macroscopic and microscopic examinations were evaluated by semiquantitative method in terms of parameters such as dilatation, hyperemia, degeneration, necrosis, hepatic steatosis, glomerular congestion, bleeding, separation of tubular basement membranes (0; no finding, 1; mild, 2; moderate and 3; severe) (Chmielewska et al., 2015).

Immunohistochemical examination

MT expression in tissues was evaluated immunohistochemically by streptavidin-biotin immunoperoxidase method. For this purpose, monoclonal anti-metallothionine clone E9 antibody (Thermo Fisher, U.S.A) was used (Chmielewska et al., 2015). Sections of 6 µm thickness cut from paraffin blocks were taken on slides coated with poly-L-lysine. Sections taken on slides were placed in an oven at 40°C, where they were incubated for 10 minutes and then serially exposed to xylol and alcohol, then washed with phosphate buffered solution (PBS; pH 7.2) for 3x5 minutes. Endogenase peroxidase activity in the tissues was removed by incubation for 30 minutes in absolute methanol containing 3% hydrogen peroxide (H_2O_2) at room temperature. Tissues on the slides were washed with phosphate buffered solution for 3x5 minutes and then kept in a 0.1% proteinase K solution at 37°C in a humid chamber for 10 minutes. In order to prevent non-specific antigenic binding, 1% bovine serum albumin was kept in a humid chamber for 20 minutes. Tissues were then coated with the primary antibody and incubated overnight at +4°C. Following this, the tissues were coated with biotinylated secondary antibody and incubated for 15 minutes at room temperature. The Histostain Plus IHC Kit (Thermo Fisher, U.S.A) was used for staining. Afterwards, after the sections were incubated with horseradish streptavidin peroxidase conjugate for 15 minutes at room temperature, 3,3'-diaminobenzidine tetrahydrochloride-H_2O_2 (DAB) (Invitrogen DAB-Plus Substrate Kit, U.S.A) substrate was applied. After using Harris hematoxylin for counterstaining, the sections were dehydrated in alcohol series. After the sections were cleared in xylol, they were mounted with adhesive (Entellan). After staining the sections under the same conditions and procedures, histopathological findings and MT expression intensities in tissues were evaluated semiquantitatively under light microscopy.

Statistical analysis

The data of the study were analyzed using SPSS 22 (Inc., Chicago, II, USA) software. The conformity of the data obtained in the study to the normal distribution was evaluated using the

Kolmogorov-Smirnov test. One-way analysis of variance was performed for the data that were suitable for normal distribution. Kruskal-Wallis test was used for the data not suitable for normal distribution. Post hoc multiple comparisons were performed using the Mann-Whitney U test with Bonferroni corrected. Within the scope of one-way analysis of variance, the homogeneity of the available data was determined by Levene's test. Statistical differences between groups according to the homogeneity of the obtained data were determined by Tamhane or Tukey test (Conover, 1980). Statistical differences between study groups were determined by the post-hoc Tukey test from GLM procedures. If the P value was less than 0.05, it was considered significant.

RESULTS

Macroscopic and microscopic findings

In the study, macroscopic findings were detected only in experimental animals in Group II. The cross-sectional surfaces of the liver and kidneys were edematous. Macroscopically, barely visible hyperemia and congestions were detected in both organs. The severity of hyperemias and congestions was slightly milder in Group IV. No macroscopic finding was observed in the other groups in the study. The severity and distribution of the microscopic findings of the liver and kidney tissues of Group II and Group IV are given in Table 1 and Table 2.

Table 1. Statistical evaluation of semiquantitative results of microscopic findings of livers of rats given high dose of cyclophosphamide (CP)

Liver					
Groups	Histopathological Findings				
	Sinusoidal dilation	Hyperemia	Degeneration	Necrosis	Steatosis
Group I	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Group II	1.75 ± 0.39^{b}	1.18 ± 0.30^{b}	3.00 ± 0.00^{c}	2.46 ± 0.08^{c}	0.08 ± 0.04^{b}
Group III	0.01 ± 0.04^{a}	0.10 ± 0.06^{a}	0.15 ± 0.16^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Group IV	1.70 ± 0.20^{b}	1.08 ± 0.17^{b}	1.76 ± 0.22^{b}	1.40 ± 0.25^{b}	0.00 ± 0.00^{a}
P value	<0.05				

Group I (Control group), Group II (CP; 200 mg/kg/day), Group III (SS; 1mg/kg/day), Group IV (CP; 200 mg/kg/day + SS; 1mg/ kg/day). $^{a\text{-}c}$: statistical difference in the same column, SS: Sodium selenite

Table 2. Statistical evaluation of semi-quantitative results of microscopic findings of kidneys of rats given high dose of cyclophosphamide (CP)

Kidney						
Groups	Histopathological Findings					
	Dilatation of tubules	Glomerular congestion	Hemorrhage	Degeneration	Necrosis	Separation of tubular basement membranes
Group I	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Group II	0.35 ± 0.50^{a}	0.50 ± 0.08^{c}	0.56 ± 0.45^{b}	2.83 ± 0.24^{c}	2.75 ± 0.29^{c}	2.10 ± 0.55^{c}
Group III	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Group IV	0.16 ± 0.26^{a}	0.30 ± 0.10^{b}	0.26 ± 0.29^{ab}	1.68 ± 0.14^{b}	1.81 ± 0.38^{b}	1.51 ± 0.24^{b}
P value	<0.05					

Group I (Control group), Group II (CP; 200 mg/kg/day), Group III (SS; 1mg/kg/day), Group IV (CP; 200 mg/kg/day + SS; 1mg/ kg/day). $^{a\text{-}c}$: statistical difference in the same column, SS: Sodium selenite

The most common and severe microscopic findings in the livers of Group II and Group IV animals, in which CP was given at high doses, were found in Group II. Microscopic findings were milder in Group IV, in which CP was administered simultaneously with sodium selenite. The detected lesions were listed as dilatation, congestion, disruption in the arrangement of the remark cords, hyperemia, degeneration (Figure 1) and single cell necrosis in hepatocytes. Dilatations in the sinusoids were mostly detected in the centrilobular areas. Single cell necroses and degenerations showed an irregular distribution in contrast to the dilatations. Degeneration, necrosis, sinusoidal dilatation, hyperemia and adiposity levels were found to be statistically significantly higher in experimental animals in Group II, where only CP was given as high dose, compared to the control group and the group given only selenium ($P<0.05$). Although the dilatation and hyperemia levels in the sinusoids were higher than Group IV in which CP was applied simultaneously with selenium, the difference was not statistically significant. Again, degeneration, necrosis and steatosis levels seen in hepatocytes were found to be lower in Group IV compared to Group II ($P<0.05$). In Group II and Group IV, where CP was given at high doses, the most severe findings in the kidneys were seen in Group II. The most common microscopic findings in this group are; tubular epithelial degenerations and necrosis (Figure 2). The hemorrhages were located cortical and medullary. Although the rate of dilatation in the renal tubules was higher in the CP group alone, it was not statistically significant compared to the other groups. Degeneration and necrosis rates were found to be lower in the group in which CP was applied together with SS compared to the group in which only CP was applied, but significantly higher than the control and only SS groups ($P<0.05$).

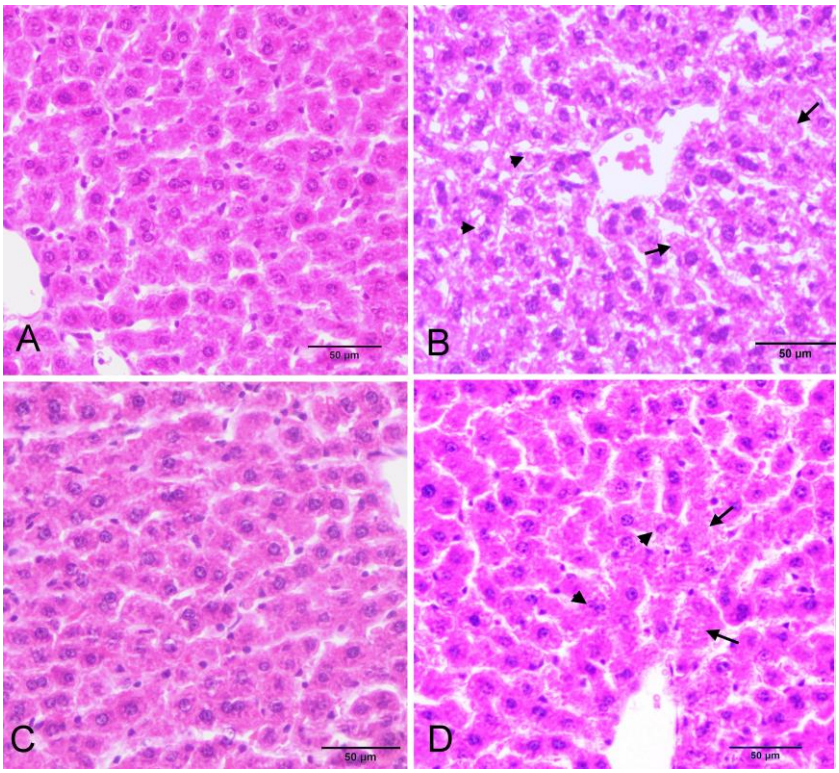

Figure 1. Liver. Degeneration (arrowheads) and necrosis (arrows) in hepatocytes. A: Group I (Control group), B: Group II (CP; 200 mg/kg/day), C: Group III (SS; 1 mg/kg/day), D: Group IV (CP; 200 mg/kg /day + SS; 1 mg/kg/day). HE. CP: Cyclophosphamide, SS: Sodium selenite

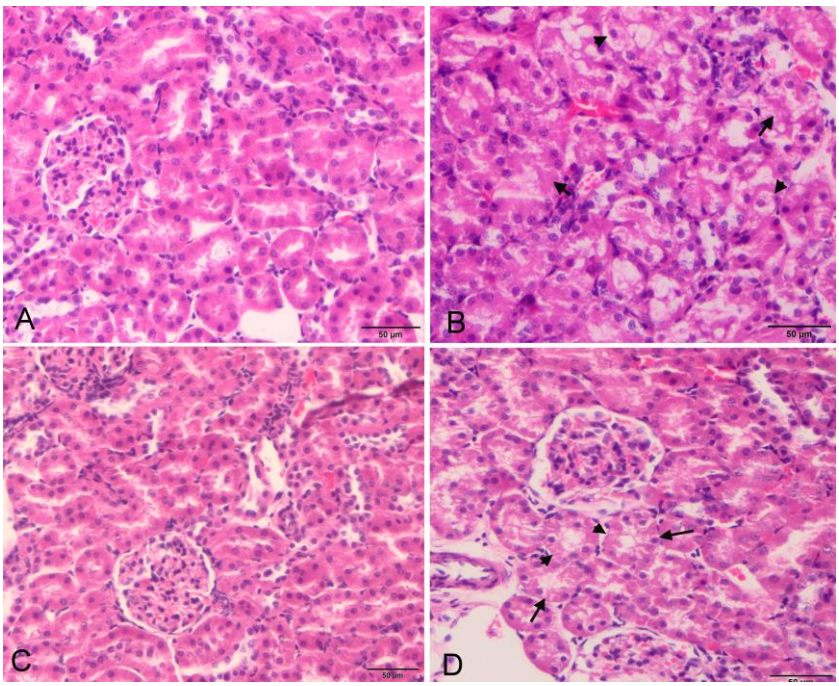

Figure 2. Kidney. Degeneration (arrows) and necrosis (arrowheads) in tubular epithelium. A: Group I (Control group), B: Group II (CP; 200 mg/kg/day), C: Group III (SS; 1 mg/kg/day), D: Group IV (CP; 200 mg/kg /day + SS; 1 mg/kg/day). HE. CP: Cyclophosphamide, SS: Sodium selenite

Immunohistochemical findings

The semi-quantitative evaluation results of the immunoistochemical findings of MT detected in the liver and kidney tissues of these groups in Groups II and Group IV, where CP was administered at high doses, are summarized in Table 3.

Table 3. Statistical evaluation of semiquantitative results of immunohistochemical findings of metallothionein antibody in which CP was given at high doses

Groups	Organs	
	Liver	Kidney
Group I	4.3 ± 0.2^{a}	5.0 ± 0.2^{a}
Group II	84.1 ± 3.2^{c}	85.7 ± 3.3^{c}
Group III	5.5 ± 0.3^{a}	6.2 ± 0.2^{a}
Group IV	41.8 ± 1.0^{b}	42.7 ± 0.9^{b}
P value	<0.05	

Group I (Control group), Group II (CP; 200 mg/kg/day), Group III (SS; 1 mg/kg/day), Group IV (CP; 200 mg/kg/day + SS; 1 mg/ kg/day). $^{a\text{-}c}$: statistical difference in the same column, CP: Cyclophosphamide, SS: Sodium selenite

In Group II and Group IV, where CP was administered at high doses, the most intense MT expression level in the liver (Figure 3) and kidneys (Figure 4) was seen only in Group II, where CP was administered. In Group IV, in which CP was applied together with SS, MT expression intensity was found to be statistically significantly low ($P<0.05$).

DISCUSSION

In cases where tumors show resistance to antineoplastic agents or the application doses of these agents are insufficient in human and veterinary medicine, high doses of many agents applied in chemotherapy, especially CP, are required (Cavalletti et al., 1986; Kaya et al., 2007). The most important factor limiting the increase of good therapeutic efficacy of CP, one of these chemical agents used in chemotherapy, is the toxic effects of the drug on liver and kidney tissues (Pool et al., 1988; Droller et al., 1982; Fraser et al., 1991; Schimmel et al., 2004).

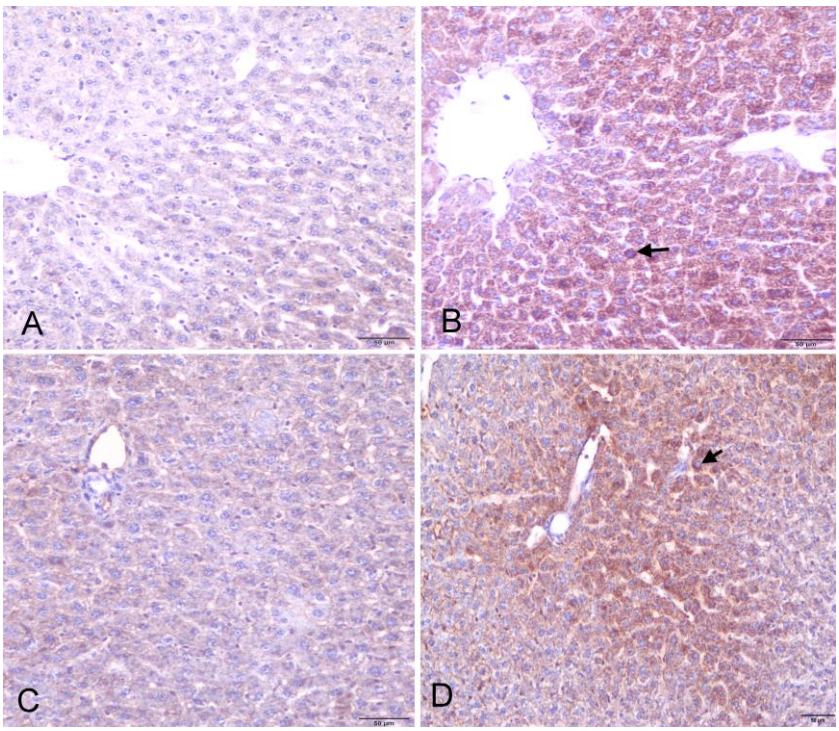

Figure 3. MT expressions in the livers of rats given high dose CP (arrows). A: Group I (Control group), B: Group II (CP; 200 mg/kg/day), C: Group III (SS; 1 mg/kg/day), D: Group IV (CP; 200 mg/kg /day + SS; 1 mg/kg/day). CP: Cyclophosphamide, SS: Sodium selenite

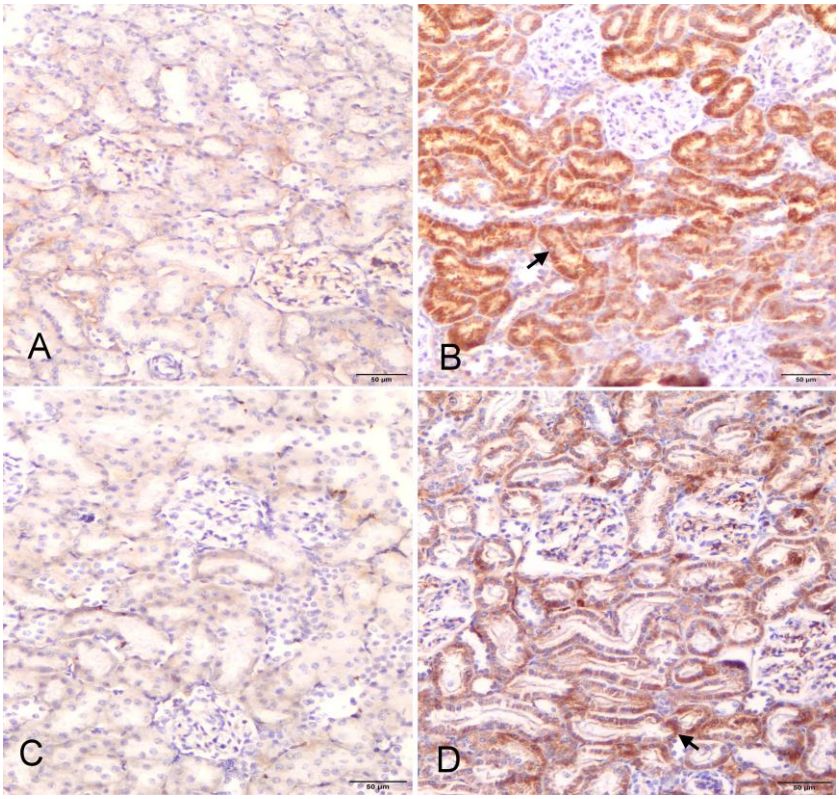

Figure 4. MT expressions in the kidneys of rats given high doses of CP (arrows). A: Group I (Control group), B: Group II (CP; 200 mg/kg/day), C: Group III (SS; 1 mg/kg/day), D: Group IV (CP; 200 mg/kg /day + SS; 1 mg/kg/day). CP: Cyclophosphamide, SS: Sodium selenite

The toxic effect of CP in tissues is related to its active metabolite, ACR. It has been stated that this toxic effect occurs by the destruction of the antioxidant defense systems of the organism by ACR, which is revealed by the metabolism of CP, and causes the formation of free radicals at high rates (Mccaroll et al., 2008). Although oxidative stress is effective in many pathological mechanisms in the organism, the excess free radicals that occur in these events are neutralized by the antioxidant system and a balance is provided in the organism. In case of disruption of this balance between the antioxidant system and free radicals, a number of pathological disorders occur (Bulkley, 1989; Valko et al., 2006). It has been reported that free radicals have a strong toxic effect and cause peroxidation and modification by oxidizing lipids, carbohydrates, proteins and DNA in cells (Hallıwell & Gutteridge, 1984). In the present study, it was observed that high dose CP administered to rats for 1 day caused hyperemia, degeneration, steatosis, necrosis and sinusoidal dilatation in hepatocytes in the liver, consistent with previous studies (Senthilkumar et al., 2006; Avcı et al., 2016). The histopathological findings we obtained in our study were associated with the toxic effect of CP caused by free radicals that started with FAM and emerged afterwards. In addition, high doses of CP were observed to cause glomerular congestion, hemorrhage, degeneration, separation and necrosis in the basal membranes of the tubules, in accordance with previous studies (Senthilkumar et al., 2006; Abraham et al., 2007). The histopathological findings in our study confirmed the knowledge that ACR, which is the metabolite of the toxic effect of CP, causes a high rate of free radical formation by destroying the antioxidant defense systems (Mccaroll et al., 2008). In this context, in our study, the use of SS, which is a strong antioxidant, was preferred in order to allow the use of CP at high doses. Se exerts its biological functions through the selenoproteins in the tissues. Selenoproteins modulate inflammation, eicozonoid synthesis, and prevent further progression of oxidative damage to biomolecules such as lipid, lipoprotein, and DNA (Rayman, 2000). Se is a necessary part of the glutathione peroxidase enzyme system; Glutathione is the coenzyme of glutathione peroxidase and plays an important role in scavenging free oxygen radicals. It also provides regeneration of antioxidant systems, reduction of nucleotides in DNA synthesis and intracellular redox state, which is important for cell proliferation. It protects endothelial cells against damage caused by peroxynitrite (Acar, 2015). There are studies showing that Se has a protective effect on the cell membrane by suppressing lipid peroxidation, as well as reducing the side effects of cytotoxic drugs such as cisplatin (Ilio et al., 1987; Yang et al., 2000). Regarding the protective efficacy of Se, in a study on CP-induced hepatotoxicity (Bhattacharjee et al., 2014), the protective efficacy of Se against CP-induced toxicity was confirmed histopathologically and reported. In another study (Acar, 2015) it was reported that Se given together with CP reduced the severity of histopathological findings such as liver degeneration and necrosis, and thus showed a protective effect against oxidative damage in the liver tissue. In the present study, it was observed that the severity of histopathological findings in the liver tissue in Group IV, which was administered high-dose CP+SS, was statistically significantly reduced compared to Group II, which was administered only high-dose CP. Again, this result was supported by the MT expression level in liver tissue. It was suggested that the decrease in the severity and distribution of histopathological findings in liver tissues may be related to the reduction of oxidative stress damage by sodium selenite. When the kidney tissues of rats belonging to all groups were examined, the most common and severe histopathological findings were observed only in Group II, where high-dose CP was given. These findings were similar to previous studies with CP in rats (Senthilkumar et al. 2006). In the study, the severity of the histopathological changes

observed in Group IV, in which CP+SS was applied, was found to be lower than in Group II, where only high-dose CP was applied. This result revealed that sodium selenite has a protective feature on kidney tissues at the histomorphological level in cases where CP is given at high doses. In addition, the low level of MT expression immunohistochemically, which confirmed the study, supported this view. Metallothionines are stress proteins. While they provide homeostasis of basic metals such as Zn and Cu, they also provide detoxification of heavy metals such as Cd and Cu (Viarengo & Nott, 1993). It has been stated that they participate in the process as natural anti-oxidative proteins, as they prevent cell damage against the oxidative effects of oxygen free radicals and hydroxyl radicals that arise for different reasons (Zhou et al., 2002). MTs have direct effects on cell proliferation by forming complexes with metals such as Cu and Zn. MTs have important effects in the prevention and treatment of inflammatory diseases in organs such as liver, kidney, pancreas, intestine and brain. Therefore, it is reported that the use of MTs for therapeutic purposes will be beneficial in maintaining the normal physiological activities of the organism (Simsek & Alabay, 2007). In this context, it was observed that sodium selenite, especially given together with high doses of CP, significantly reduced the severity of histopathological findings in liver and kidney tissue, and also significantly decreased the MT expression levels immunohistochemically in both tissues (Chmielewska et al., 2015). This result made us think that MTs can also be used as a stress protein marker in the liver and kidney when CP is given at high doses (Zambenedetti et al., 1998; Candan et al., 2017). In the present study, it was observed that sodium selenite was effective in terms of MT expression level against the toxic effects of CP in the liver and kidney tissues in cases requiring the use of CP in high doses. Therefore, it was concluded that sodium selenite can be used as an alternative supplement in cases where CP will be used in high doses.

CONCLUSION

In recent years, research on the development of methods that prevent the toxic effects of many drugs, especially CP, which is one of the chemotherapeutic drugs, and allow these drugs to be used at low doses for a long time or at high doses, continues. When these drugs are used in high doses and frequently, they cause toxic side effects as well as their intended therapeutic effects. In the study, the most severe histopathological findings were observed in Group II, in which only high-dose CP was used, and we concluded that CP had a highly toxic effect on liver and kidney tissues. The high level of MT expression in liver and kidney tissues immunohistochemically supported this view. The low severity of histopathological findings and immunohistochemical MT expression level in the liver and kidney tissues in Group IV, in which SS was applied together with CP, compared to Group II, which was given only CP, suggest that SS can be used in the treatment in cases that require the use of CP at high doses. conclusion was reached.

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Conflict of interest: The authors declared that there is no conflict of interest.

Ethical statement: This study was approved by Aydın Adnan Menderes University Veterinary Faculty Experimental Animals Ethics Committee with the decision numbered 64583101/2016/146.

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