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Pathobiochemistry

Hepato-preventive and anti-apoptotic role of boric acid against liver injury induced by cyclophosphamide

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ABSTRACT

This study aims to examine cyclophosphamide (CP) exsposure associated toxicity on rat livers and the likely defensive effects of boric acid (BA). The rats used in this study were divided into four groups: control group, CP group, BA group, and BA + CP group. The present study was carried out using routine histological H&E stain, immunohistochemical stain caspase-3 as apoptotic marker, serum biochemical analysis for liver function markers (alanine transaminase (ALT), aspartate transaminase (AST) and alkalen phosphatase (ALP)), oxidative stress markers (total oxidant status (TOS), oxidative stress index (OSI) and total antioxidant capacity marker (TAC)). In the CP group, the levels of ALT, AST, ALP, TOS, OSI and caspase-3 increased whereas TAC levels decreased compared with the control group. In the BA + CP group, the levels of ALT, AST, ALP, TOS, OSI and caspase-3 decreased whereas TAC levels increased compared with the CP group. The histopathological evaluation of light microscope images and immunohistochemical caspase-3 activity in the BA + CP group were found to be decrease compared with those in the CP group. In conclusion, BA was successful in defending the liver against apoptosis and histopathological changes that are attributable to CP.

1. Introduction

The therapeutic effectiveness of anticancer drugs is accompanied by cruel side effects due to their toxicity. Cyclophosphamide (CP), one of the most common anticancer drugs, has therapeutic effectiveness against various types of cancer and diseases such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis [1]. However, similar to other anticancer drugs, when used, CP is also known to cause toxicity in healthy tissues as well as in cancerous tissues. The use of CP in high doses has been reported in previous studies, which claim that it causes acute inflammation in the bladder, kidney damage and liver damage as well as apoptosis [2–4]. CP is converted into substances such as phosphoramide mustard and acrolein which cause oxidative stress by cytochrome P450 system [5,6]. Very few experimental studies note that oxidative stress is responsible for hepatotoxicity attributable to CP [7]. Studies have also suggested that certain antioxidant agents are effective in alleviating oxidative stress as far as the reduction of the toxic side effects due to anticancer drugs is concerned. A series of experimental studies showed that phytogenic compounds with antioxidant activity ensured protection against hepatotoxicity due to CP [4,8]. The cellular antioxidant status determines the susceptibility to oxidative damage and generally changes in response to oxidative stress. Accordingly, many antioxidants have recently been used to prevent oxidative damage caused by high oxidative stress attributable to CP. Antioxidants like vitamins (vitamins C and E), enzymes (catalase, glutathione peroxidase) and minerals (zinc, selenium, boron) have been reported to protect cells against lipid peroxidation and DNA damage, which is the first step in many pathological processes [9]. Many authors have reported that the antioxidant levels decrease as a result of the increased free radical production in the experimental study. Along with boric acid (BA) as a boron, which is a naturally occurring mineral, is widely used in industrial, agricultural and cosmetic applications as well as being traditionally used in medicine [10]. In many studies, BA has been shown to have antioxidant [11], hepatoprotective [10] and antigenotoxic effects [9]. Furthermore, BA has been suggested to increase glutathione deposits in the body and inhibit oxidative damage by inhibiting other reactive oxygen species. It has also been suggested that BA limits oxidative damage by enhancing the body's stores of

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glutathione and inhibiting other reactive oxygen species [11]. Taking all this into account, the present study aimed to investigate the protective effects of BA on CP-induced liver damage in rats.

2. Material and methods

2.1. Chemicals

99% pure BA, a boron compound, and CP were purchased from a commercial company (Sigma-Aldrich, Darmstadt, Germany). BA and CP were implemented as intraperitoneally (IP) and suspended in distilled water. The selection of the CP and BA doses was based on the previously published studies by Ince et al. [6], Cengiz [10], and Goudarzia et al. [12] respectively.

2.2. Treatment

All the animal studies were conducted in accordance with the approval obtained from Experimental Animals Ethics Committee of Eskişehir Osmangazi University (No: 2018/648-1), the rats were provided by the Public Health Center. The rats used in this study were fed in a standard environment with drinking water and standard food pellets. The rats were maintained under standard humidity (45–50%), temperature (22 ± 2 °C), and light (12 h light/12 h dark) conditions.

In the present study, rats were implemented with BA at 200 mg/kg (IP. This dose of BA is nontoxic for animals, because when BA was administrated orally, subcutaneously, and intravenously to animals for the short term, the LD50 (lethal dose, 50%) levels for BA in laboratory animals (e.g.) mice and rats) were in the range of approximately 1700–3450 mg/kg body weight [6,13,14].

The rats were divided into four groups with each group consisting of six rats and were treated as follows:

Group I (Control): The rats were given normal saline IP for 6 days.

Group II (CP): The rats were injected IP with a single dose of CP (200 mg/kg) on the fourth day.

Group III (BA): The rats were IP given BA (200 mg/kg) for 6 days.

Group IV (BA + CP): The rats were pretreated with an IP dose of BA (200 mg/kg) for 6 days followed by the administration of a single dose of CP (200 mg/kg) on the fourth day.

Twenty-four hours after the last BA administration, the rats were sacrificed under anesthesia. In the line with the ethical guidelines, at the end of the experiment, blood samples were collected in tubes without anticoagulant for the determination of serum liver enzymes (AST, ALT, ALP) and oxidative stress markers. The liver tissues of the rats were cultivated for biochemical, histological and immunohistochemical analyses [15].

2.3. Biochemical assays

2.3.1. Measurement of the total antioxidant capacity

The total antioxidant capacity (TAC) value of the supernatant fractions was determined using a new automated measurement method developed by Erel [16]. Hydroxyl radicals, the most powerful biological radicals, are produced by this method. In the assay, a ferric ion solution in Reagent 1 was mixed with the hydrogen peroxide present in Reagent 2. The radicals, which were produced such as brown dianisidinyl radical cations produced by hydroxyl radicals, are also strong radicals. By using this method, the anti-oxidative effect of the sample was measured against undue radical reactions induced by the hydroxyl radicals produced. The analysis has excellent sensitivity values of less than 3%. Results are expressed as nmol Trolox Equiv./mg protein.

2.3.2. Measurement of total oxidant status

The total oxidant status (TOS) of supernatant fractions was determined using a new automated measurement method developed by Erel [16]. The oxidants in the sample oxidized the iron ion-dianisidine complex to ferric ion. The oxidation reaction was enhanced by the glycerol molecules abundant in the reaction medium. The Iron ion makes a colored complex with the xylenol orange in an acidic environment. The color density, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in nmol H_2O_2 Equiv / mg protein.

2.3.3. Oxidative stress index

The percentage of the TOS level to the TAC level was accepted as the oxidative stress index (OSI). The OSI values were calculated according to the following formula [16]: OSI (arbitrary unit) ¼ TOS (nmol H_2O_2 Equiv / mg protein.) / TAC nmolTroloxEquiv / mg protein).

2.3.4. Evaluation of liver function markers

The serums were obtained from the blood samples of the rats by centrifuging for 10 min at 3000 rpm. The samples were then analyzed to determine serum alanine transaminase (ALT), aspartate transaminase (AST) and alkalen phosphatase (ALP) with the help of an automated biochemical auto-analyzer (HITACHI-917).

2.3.5. Histological analysis

The livers of the rats were fixed with a 10% formaldehyde solution and then were embedded in paraffin in accordance with routine histological preparations. These samples were derived into 5.0 µm thick serial sections and then stained with Haematoxylin-Eosin (H&E). The histological features of the sections were evaluated in due course [17]. The hematoxylin-eosin stained samples were observed under a light microscope (Leica DM6000 B).

2.3.6. Immunohistochemistry

The sections were deparaffinized and rehydrated. Antigen recovery with citrate buffer (pH 6.0) was performed by heating the sections in a microwave at 700 W for 10 min. After blocking with 3 mL/L H_2O_2 the sections were incubated. The primary antibodies directed against caspase-3 (Thermo, Waltham, MA, USA) in dilution of the ultra-vision quanta detection system (Thermo Scientific), respectively [18].

2.4. Statistical analysis

The data derived from the animal experiments were expressed as standard error of the mean (± SEM). The independent measurements and continuous data exhibiting a normal distribution were analyzed using One Way Anova. In addition, the Kruskal-Wallis test was applied to score the variants with an abnormal distribution. The differences observed in the experimental groups were accepted to be significant if the p value was < 0.001 *and* < 0.05.

3. Results

3.1. Histological evaluations

The structure of liver tissues collected from the control and 200 mg/kg BA groups were found to be normal (Fig. 1A and C). However, the liver structures of the rats that were given 200 mg/kg of CP were severely impaired. Among the features observed in these damaged liver tissues were turbidity in the cytoplasm of hepatocytes, fuzziness in the cytoplasm homogenity, as well as dark staining, shrinkage, irregularity in the nucleus boundaries and increase in eosinophilia in the cytoplasm. In addition, increases in congestion and erythrocytes accumulation in the vascular structures were noted (Fig. 1B1 and B2). In addition to minor regional changes in the examination of the liver sections of the rats given 200 mg/kg BA and 200 mg/kg CP, histological structures were well-preserved (Fig. 1D). In other words, the results from the BA + CP group were remarkably similar to those of the control group.

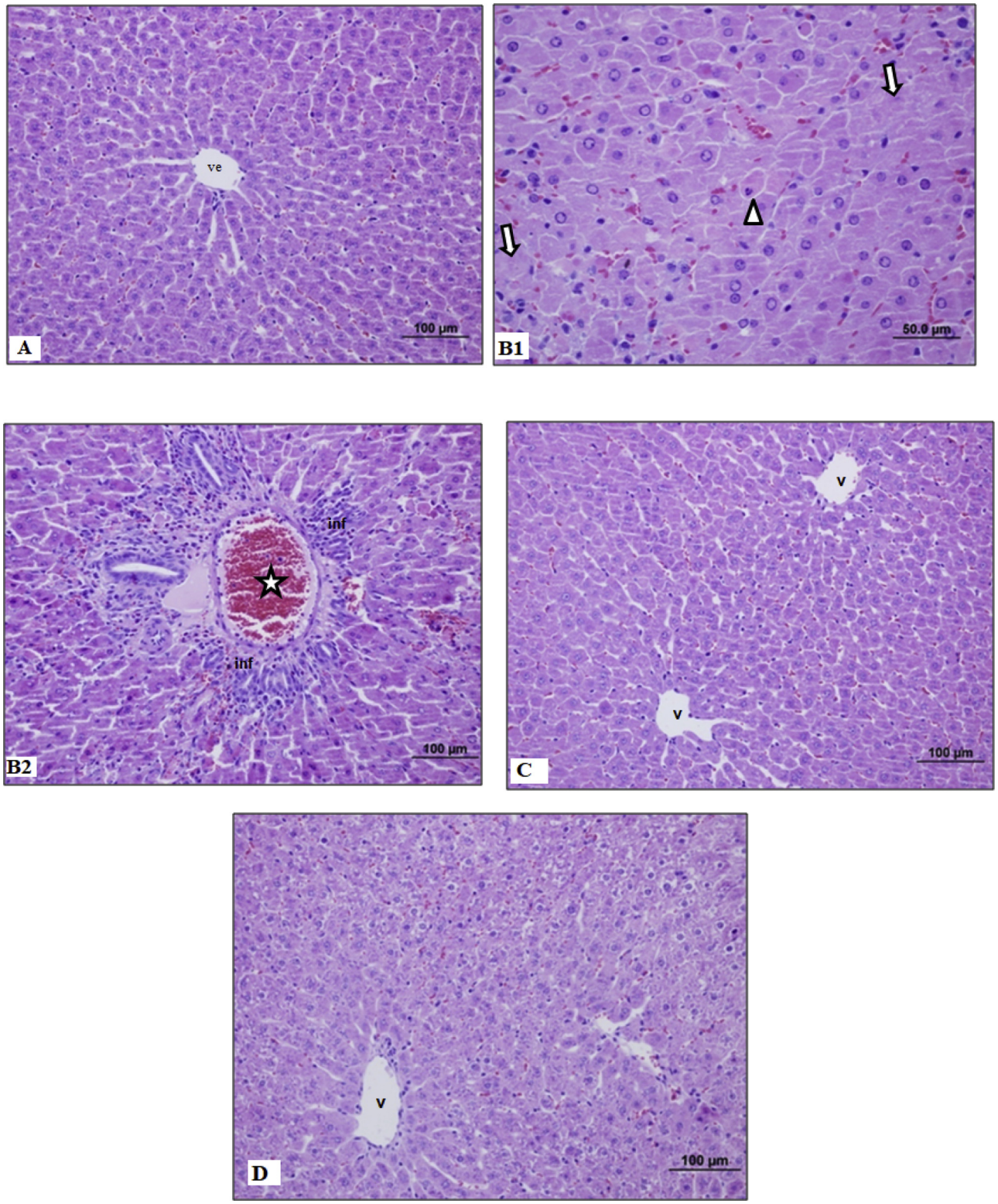

Fig. 1. In general, histological sections of the liver were observed in the images of the control and BA groups (Fig. 1A and C). In the sections of the CP group, congestion in the vessels, turbidity in the hepatocytes and cytoplasm, loss of homogeneity, dark eosinophilic staining of the cytoplasm in some hepatocytes and nuclei and dilatations (arrows) sinusoidal dilations were observed (Figures B1 and B2). In the CP group, a normal histological appearance was observed (D).

3.2. Apoptosis results

The liver sample of the study groups were immunohistochemically stained to determine the concentration and intensity of the caspase-3 antigen. In the CP group, the number of caspase-3 positive liver cells increased significantly compared to that of the control and BA groups. This increase was statistically significant ($p < 0.001$). In the CP + BA group, the number of caspase-3 positive liver cells was significantly reduced compared to the CP group, which was also of statistical significance ($p < 0.05$). The immunohistochemical findings showed that

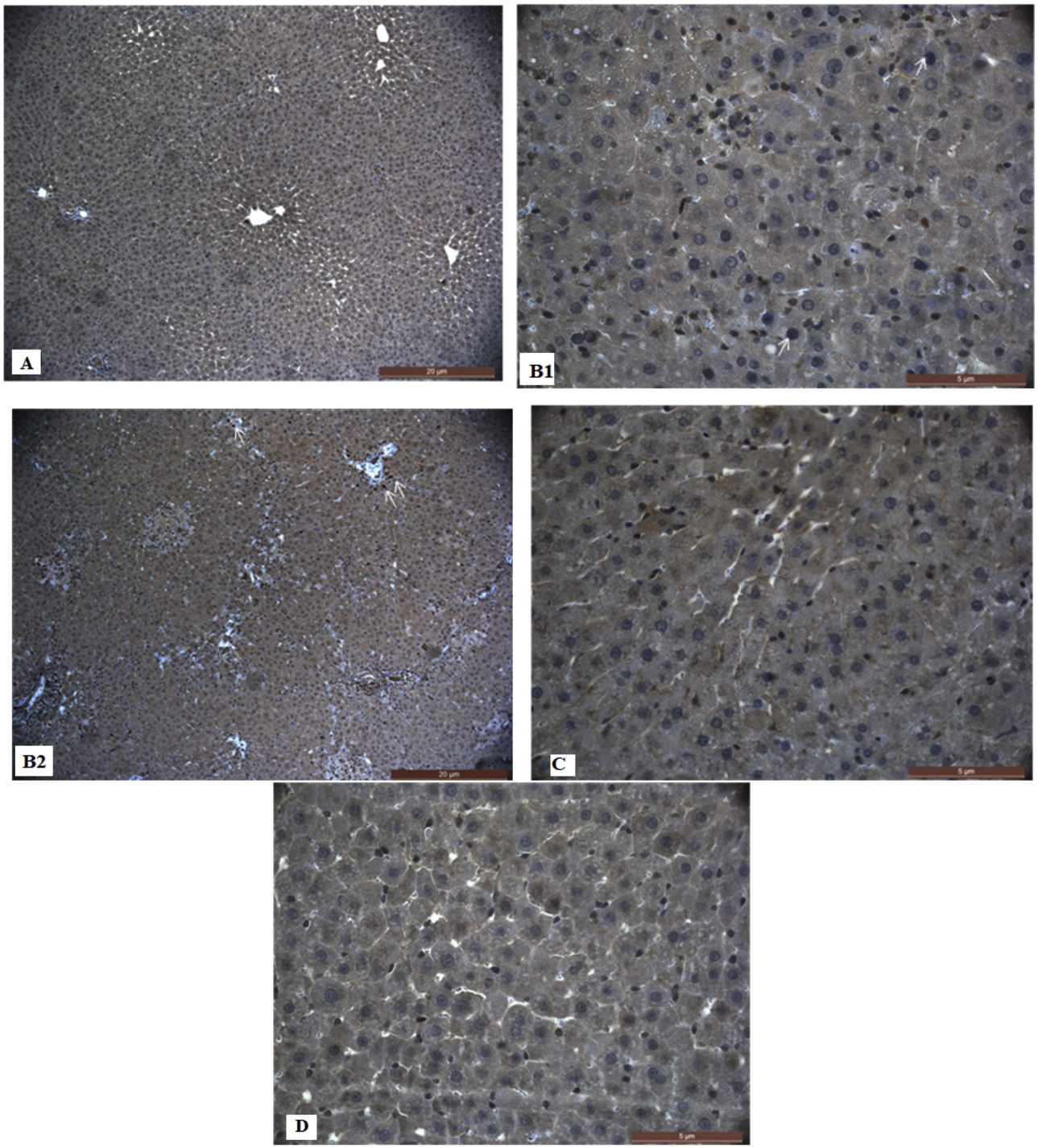

Fig. 2. The immunohistochemical staining of the liver samples of different study groups. Activated caspase-3 in the control (Image A) and BAgroups (Image C) showed no staining in normal liver cells. The caspase-3 protein expression in the CP group (Images B1 and B2) showed a more intense staining of liver cells and a diffused staining as shown with the arrows (Fig. 2). Almost no activated caspase-3 stained liver cells were seen in the livers treated with BA prior to CP in the BA + CP group (Image D) (bar: 20 μm-A, 5μm- 20 μm B1, B2, 5 μm-C, 5 μm-D HE).

BA decreased apoptosis due to CP (Figs. 2 and 3).

3.3. Biochemistry assessments

Statistical comparisons of the mean values of AST, ALT, ALP, TAC, TOS and OSI of all experimental groups and those of the control group given physiological saline solution are presented in Figs. 3–6. The biochemical data showed an increase in ALT, AST, ALP, TOS and OSI in the CP group, while TAC levels decreased compared to the control group, a result considered to be of statistical significance ($p < 0.001$). In contrast, the groups given CP after BA (200 mg/kg), a decrease was seen in ALT, AST, ALP, TOS and OSI levels, while TAC levels increased compared to the control group.

4. Discussion

In the present study, it was observed that CP-induced toxicity caused liver damage in rats. The experimental results proposed that pre-

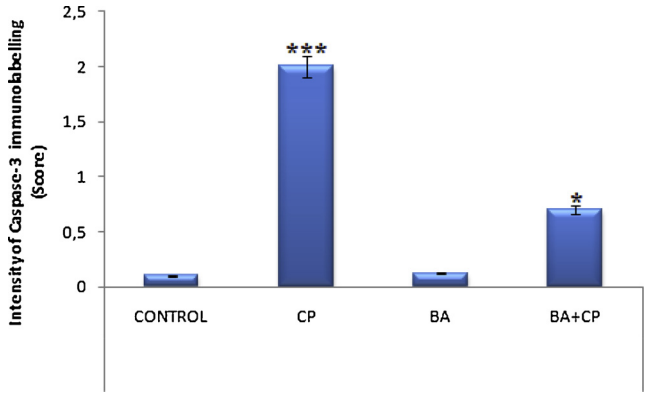

Fig. 3. Intensity of the immunolabeling score of the activated caspase-3 positive cells in the groups. ***$p < 0.001$ significant difference compared to control, *$p < 0.05$ different compared to control.

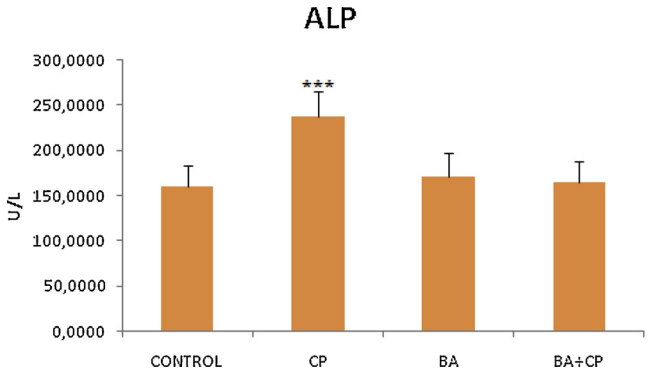

Fig. 4. Statistical comparison of mean values of ALP values of experimental groups with the control group. ***: p < 0.001 significant difference, *$p < 0.05$ different compared to control.

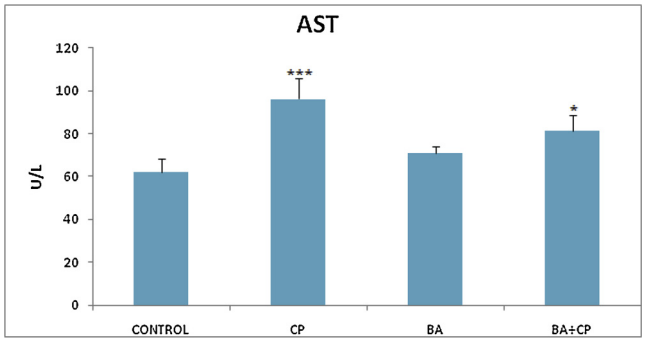

Fig. 5. Statistical comparison of mean values of AST values of experimental groups with the control group. ***: p < 0.001 significant difference, *$p < 0.05$ different compared to control.

treatment with BA could decrease the CP induced hepatic damage by modulating oxidative stress and apoptosis in the rats. In addition, the histopathological investigation confirmed that CP causes hepatic damage as evidenced by the turbidity in the cytoplasm of hepatocytes, fuzziness in the cytoplasm homogeneity, as well as dark staining, shrinkage, irregularity in the nucleus boundaries and increase in eosinophilia in the cytoplasm. The membrane-damaging effects of the CP metabolites may be the cause of these findings [20]. In parallel with the findings of this study, Senthilkumar et al. [21] reported a case of extensive swelling and sinusoidal narrowing of the liver tissues of CP treated rats. In their study, Ayhanci et al. [19] reported an increase in the serum ALT (145%), AST (226%), ALP (88%) and LDH (73%) levels in the 150 mg/kg CP group. In the histopathological analysis of their

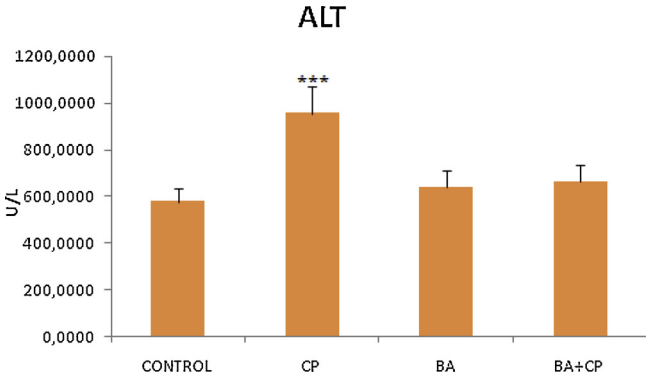

Fig. 6. Statistical comparison of mean values of ALT values of experimental groups with the control group. ***: p < 0.001 significant difference.

studiy, dark hepatitis and dark staining as a result of condensation of various hepatocyte nuclei, irregular nucleus boundaries and eosinophils in the cytoplasm were emphasized. Alqahtani and Mahmoud investigated CP-induced hepatocytes apoptosis [22] and determined immunohistochemically both gene and protein expression levels of the proapoptotic factors caspase-3 and Bax. In their study, the livers of the rats treated with CP for 2 weeks revealed that CP induced a significant increase in both caspase-3 expression and protein levels. Caglayan et al. [23] reported that CP (200 mg/kg) activated the apoptotic and autophagic pathway by increasing cysteine aspartate-specific protease-3 (caspase-3) expression and light chain 3B (LC3B) level and also increased the expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is the marker of oxidative DNA damage. In The present study obtained results that are similar to those of the studies mentioned above. In fact, in the CP group, the number of caspase-3 positive liver cells increased significantly compared to the control and BA groups (Figs. 2 and 3).

BA is a naturally occurring mineral that has antioxidant properties. In addition to being used traditionally in health services, BA is widely used in many areas such as industry and agriculture [6]. In the literature studies, two hypotheses emerged regarding the biochemical and physiological role of BA in living organisms. In principle, BA can act in response to hormone action and cell-membrane functions that affect both transmembrane signals and the movement of regulatory ions [6,10,11,24,25]. On the other hand, BA can play a role as a metabolic manager in some enzymatic systems. At the same time, BA increases the amount of reduced glutathione in the body, reduces the effects of oxidative damage and inhibits ROS production and apoptosis [9]. Moreover, BA treatment has been proved to improve the catalytic activity of cytochrome P450 [25]. In one study, an increase in ALT, AST, MDA and caspase-3 levels in only ethanol (1.5 g / kg) was reported and a decrease in SOD and CAT levels was reported. However, it was emphasized that ALT, AST, MDA and caspase-3 levels decreased and SOD and CAT levels increased in the 100 mg/kg boric acid + ethanol group [10]. Ince et al. [6] demonstrated that 200 mg/kg boric acid showed strong hepatoprotective effects on liver damage induced by Carbon tetra chloride (CCl_4) in mice, possibly as a result of both antioxidant defense system activity and inhibition of lipid peroxidation. In the CCl_4-treated group, there was a significant increase in serum AST, ALP and ALT activities, indicating CCl_4-induced damage to hepatic cells compared to the control group ($p < 0.05$). However, treatment with BA at a dose of 200 mg/kg prior to CCl_4 was found to reverse the CCI_4 -induced change of AST, ALP and ALT ($p < 0.05$). The results of these studies are similar to the findings of the present study. BA may have a possible indirectly preventive effect on apoptosis. BA may also increase anti-oxidant levels (probably by preventing GSH depletion) and limit inflammatory processes and reduce ROS levels preventing apoptotic cell death (Figs. 3 and 10). In conclusion, the present study further demonstrated that CP can induce oxidative stress while triggering apoptosis and liver injury in

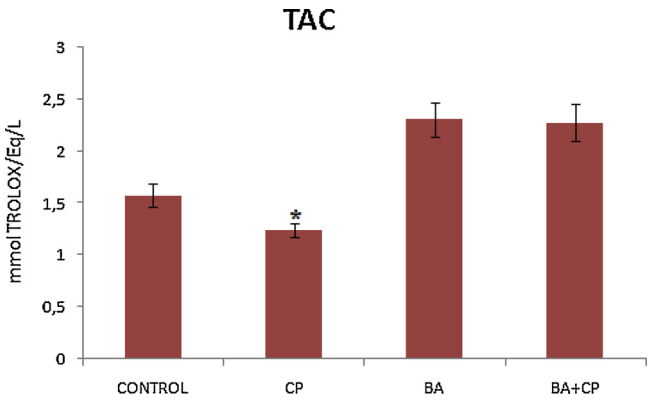

Fig. 7. Statistical comparison of mean values of TAC values of experimental groups with the control group. *: $p < 0.05$ significant difference.

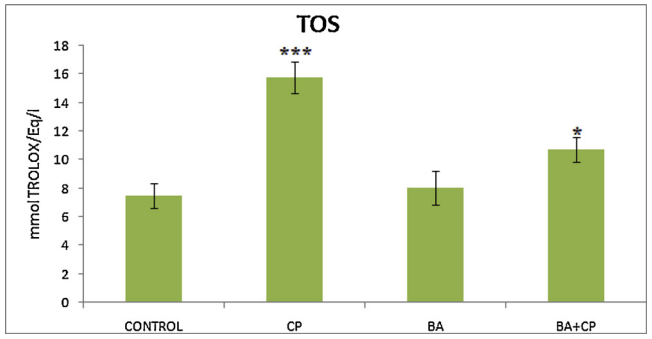

Fig. 8. Statistical comparison of mean values of TOS values of experimental groups with the control group. ***: $p < 0.001$ significant difference, *$p < 0.05$ different compared to control.

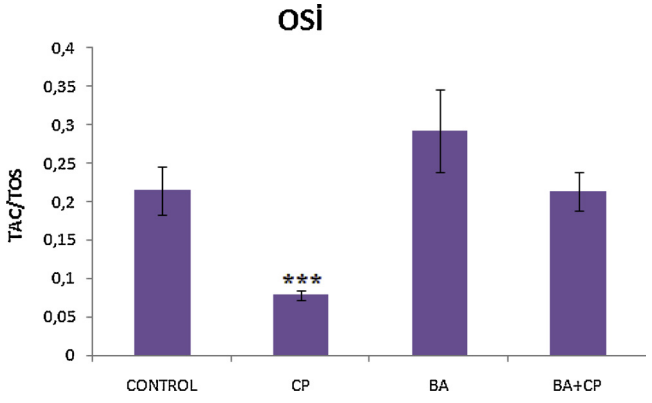

Fig. 9. Statistical comparison of mean values of OSİ values of experimental groups with the control group. ***: $p < 0.001$ significant difference.

rats. BA could prevent all these unwanted toxic effects of CP. The antioxidative stress effects of BA might be a part of its protective mechanism against CP-induced apoptosis and oxidative stress (Fig. 10). The results of this study may provide a potential BA application combined with CP to reduce the adverse effects of cancer treatment (Figs. 7–9).

In the present study, experimentally induced liver tissue damage was caused by damage to the membrane by CP metabolites. These pathological changes in the findings are consistent with the elevation of serum enzyme levels. A decrease in abnormal pathological findings such as tissue damage and necrosis at the BA dose given by CP suggests that liver tissue greatly improved.

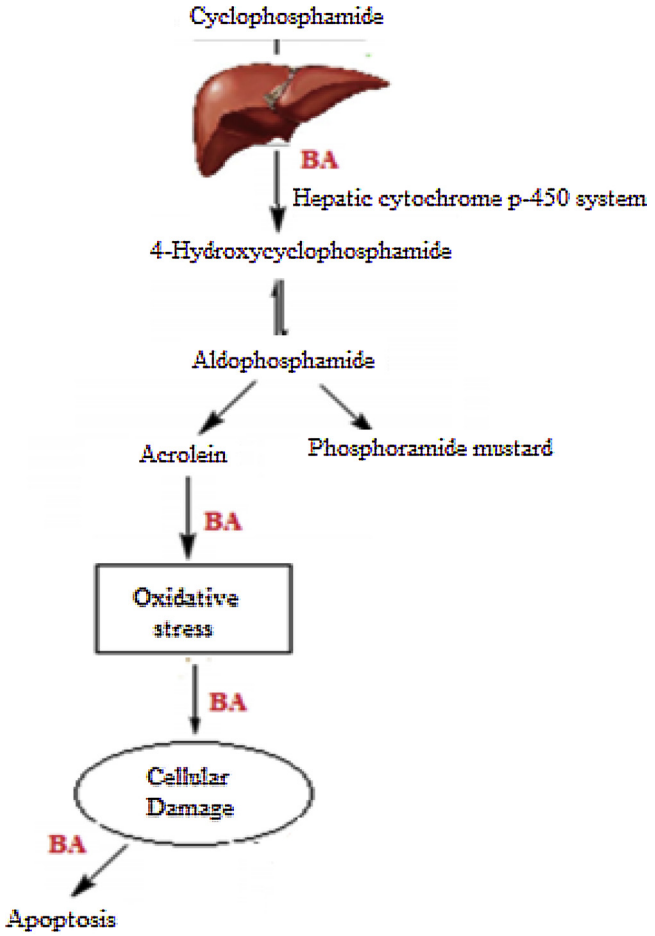

Fig. 10. The points to the possible effect of BA on the prevention of CP-induced apoptosis and oxidative stress.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

[1] M.A. Goldberg, J.H. Antin, E.C. Guinan, J.M. Rappeport, Cyclophosphamide cardiotoxicity: an analysis of doing as a risk factor, Blood 68 (1986) 1114–1118.
[2] H.M. Shulman, G.B. McDonald, D. Matthews, K.C. Doney, An analysis ofhepatic venocclusive disease and centrilobular hepatic degeneration following bone marrow transplantation, Gastroenterology 79 (1980) 1178–1191.
[3] A. Ayhanci, S. Yaman, S. Appak, S. Gunes, Hematoprotective effect of Seleno-L-methionine on cyclophosphamide toxicity in rats, Drug Chem. Toxicol. 32 (4) (2009) 424–428.
[4] N. Sharma, P. Trikha, M. Athar, S. Raisuddin, Inhibitory effect of Emblica officinalis on the in vivo clastogenicity of benzo[a]pyrene and cyclophosphamide in mice, Hum. Exp. Toxicol. 19 (2000) 377–384.
[5] N. Sladek, Metabolism of cyclophosphamide by rat hepatic microsomes, Cancer Res. 1 (1971) 901–908.
[6] S. Ince, H. Keles, M. Erdogan, O. Hazman, I. Kucukkurt, Protective effect of boric acid against carbon tetrachloride-induced hepatotoxicity in mice, Drug Chem. Toxicol. 35 (3) (2012) 285–292.
[7] K. Manda, A.L. Bhatia, Prophylactic action of melatonin against cyclophosphamide-induced oxidative stress in mice, Cell Biol. Toxicol. 19 (2003) 367–372.
[8] E. Selvakumar, C. Prahalathan, Y. Mythili, P. Varalakshmi, Mitigation of oxidative stress in cyclophosphamide-challenged hepatic tissue by DL-alphalipoic acid, Mol. Cell. Biochem. 272 (2005) 179–185.
[9] S. Ince, I. Kucukkurt, H.H. Demirel, D.A. Acaroz, E. Akbel, I.H. Cigerci, Protective effects of boron on cyclophosphamide induced lipid peroxidation and genotoxicity in rats, Chemosphere 108 (2014) 197–204.
[10] I. Sogut, S.O. Paltun, M. Tuncdemir, M. Ersoz, C. Hurdag, The antioxidant and antiapoptotic effect of boric acid on hepatoxicity in chronic alcohol-fed rats, Can. J. Physiol. Pharmacol. 96 (4) (2018) 404–411.
[11] M. Cengiz, Boric acid protects against cyclophosphamide-induced oxidative stress and renal damage in rats, Cell Mol. Biol. (Noisy le Grand). 64 (12) (2018) 11–14.

[12] M. Goudarzia, M.J. Khodayara, S.M.T.H. Tabatabaeib, H.F.I. Ghaznavic, S. Mehrzadi, Pretreatment with melatonin protects against cyclophosphamide-induced oxidative stress and renal damage in mice, Fundam. Clin. Pharmacol. (2017) 625–635.
[13] C.C. Pfeiffer, L.F. Hallman, I. Gersh, Boric acid ointment: a study of possible intoxication in the treatment of burns, J. Am. Med. Assoc. 128 (1945) 266–274.
[14] R.J. Weir, R.S. Fisher, Toxicologic studies on borax and boric acid, Toxicol. Appl. Pharmacol. 23 (1972) 351–364.
[15] M. Cengiz, A. Ayhancı, H.M. Kutlu, A. Musmul, Potential therapeutic effects of silymarin and silymarin-loaded solid lipid nanoparticles on experimental kidney damage in BALB/c mice: biochemical and histopathological evaluation, Turk. J. Biol. 40 (2016) 807–814.
[16] O. Erel, A new automated colorimetric method for measuring total oxidant status, Clin. Biochem. 38 (2005) 1103–1111.
[17] M. Cengiz, H.M. Kutlu, D.D. Burukoglu, A. Ayhanci, A comparative study on the therapeutic effects of silymarin and silymarin-loaded solid lipid nanoparticles on D-GaIN/TNF-α-induced liver damage in Balb/c mice, Food Chem. Toxicol. 77 (2015) 93-10.
[18] A. Ayhanci, M. Cengiz, H.M. Kutlu, D. Vejselova, Protective effects of ellagic acid in D-galactosamine-induced kidney damage in rats, Cytotechnology 68 (2016) 1763–1770.
[19] A. Ayhanci, Ö. Acar, V. Sahinturk, S. Gunes, S.İ. Kulcanay, A. Musmul, S. Uslu, Selenyum Siklofosfamid Nedenli Hepatotoksisiteyi İyileştirebilir, Osmangazi Tıp Dergisi 38 (3) (2016) 34–39.
[20] M. Cengiz, Y. Tekin, İ. Behcet, A. Ayhanci, Kekik Bitkisinin Temel Bileşeni Olan Karvakrolün Sıçanlarda Siklofosfamid Nedenli Üreme Sistemi Hasarı Üzerine Koruyucu Etkileri, Turk. J. Agric. Res. 4 (2) (2017) 171–175.
[21] S. Senthilkumar, T. Devaki, B.M. Manohar, M.S. Babu, Effect of squalene on cyclophosphamide-induced toxicity, Clin. Chim. Acta 364 (1-2) (2006) 335–342.
[22] S. Alqahtani, A. Mahmoud, Gamma-glutamylcysteine ethyl ester protects against cyclophosphamide-induced liver injury and hematologic alterations via upregulation of PPAR and attenuation of oxidative stress, inflammation, and apoptosis, Oxid. Med. Cell. Longev. (2016) 14 (4016209).
[23] C. Caglayan, Y. Temel, F.M. Kandemir, S. Yildirim, S. Kucukler, Naringin protects against cyclophosphamide-induced hepatotoxicity and nephrotoxicity through modulation of oxidative stress, inflammation, apoptosis, autophagy, and DNA damage, Environ. Sci. Pollut. Res. Int. 25 (2018) 20968–20984.
[24] H. Turkez, F. Geyikoglu, A. Tatar, S. Keles, A. Ozkanc, Effects of some boron compounds on peripheral human blood, Z. Naturforsch. 62 (2007) 889–896.
[25] S. Ince, I. Kucukkurt, I.H. Cigerci, F.A. Fatih, A. Eryavuz, The effects of dietary boric acid and borax supplementation on lipid peroxidation, antioxidant activity, and DNA damage in rats, J. Trace Elem. Med. Biol. 24 (3) (2010) 161–164.