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EXPERIMENTAL STUDY

Apoptosis induced by boric anhydrite (B_2O_3) after partial hepatectomy in rat liver

Ozen A, Canbek M

Department of Biology, Faculty of Science, Eskisehir Osmangazi University, 26480 Eskisehir, Turkey.
ahozen@ogu.edu.tr

ABSTRACT

Boron is one of the important elements that have a cell-growth suppressing effect. The apoptotic effects of B_2O_3 that were investigated in rats on liver regeneration following 70 % partial hepatectomy (PH). Wistar albino male rats were used and divided into 4 groups (n = 7). The Saline control groups were given only a single dose of saline; the B_2O_3-treated groups that were only given a single dose of 1800 $mg.kg^{-1}$ B_2O_3 by means of intraperitoneal injections following hepatectomy. Three and 6 hours after surgical procedures, all the groups were dissected and liver tissue samples were taken from the groups for NF-κB for caspase-3 gene and protein levels investigation by RT-PCR and TaqMan Protein Assay and histological analyses by TUNEL assay.

B_2O_3-treated animals were examined and it was observed that NF-κB levels were decreased; however, caspase-3 gene expression and protein levels were increased significantly. This study demonstrated that B_2O_3 induces caspase-3 and inhibits NF-κB at the early stage of liver regeneration *(Fig. 4, Ref. 26)*. Text in PDF *www.elis.sk.*

KEY WORDS: liver regeneration, RT-PCR, caspase-3, NF-κB, TaqMan Protein Assay, TUNEL assay.

Introduction

The liver is a unique organ with the ability to renew itself in the event of a factor (1). Damage can occur in liver due to numerous reasons. Such damage may lead to loss of function in hepatocyte and death (1). In general, the loss of hepatocytes in liver occurs by apoptosis. Apoptosis plays an important role in liver cell loss following partial hepatectomy (PH) (2).

Apoptosis is known to play an important role in the regulation of homeostasis (3). The most important elements of apoptosis are caspase-3 which acts by way of activating the apoptotic pathways and NF-κB which provides the continuity of cell life cycle (4, 5). The functionality of these factors affects the apoptotic processes. Hepatocytes after PH, which is called the priming phase within the first 6 hours before responding fully to growth factors are capable of proliferation (6, 7).

Boron is among the trace elements acting in cell membrane functions, enzyme reactions, and mineral and hormonal metabolism (8, 9). Boron has a structure that forms response against some damage and infections. In addition, it plays an important role in the suppression of cytokines related to inflammation response formation (10, 11). It was reported that different concentrations of boric acid and certain boron compounds have anti-proliferative effect on various cancer cells (9, 11, 12). Nevertheless, the regulation of apoptosis by boron is not clear.

Department of Biology, Faculty of Science, Eskisehir Osmangazi University, Eskisehir, Turkey

Address for correspondence: A. Ozen, Department of Biology, Faculty of Science, Eskisehir Osmangazi University, 26480 Eskisehir, Turkey.

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Liver regeneration after PH is the best known cell proliferation model. Our study was designated to investigate the apoptotic effects of B_2O_3 against liver regeneration after PH and the possible role of B_2O_3 in caspase3 activation and NF-κB inhibition during the early stage of liver regeneration *in vivo*. Our results show that B_2O_3 has an apoptotic effect on hepatocytes during regeneration.

Materials and methods

Animals and partial hepatectomy

The experimental protocols were approved by the Institutional Ethical Committee for Animal Care and Use at Eskişehir Osmangazi University, Turkey. Animals were obtained from the medical and surgical experimental research center of the institute and all experiments were carried out at the same center (protocol number: 271/2012).

Male *Wistar albino* rats, weighing between 200–250 g and aged 3–4 months were used in the study. The experiment was performed following a stabilization period in the laboratory. They were used after 2 weeks of adaptation. They were housed in polycarbonate cages in an air-conditioned room (12 Light/12 Dark, 22 ± 2 ºC, 50 ± 5 % humidity). They were fed laboratory pellet chows and water was given *ad libitum*.

PH procedure was carried out according to the technique of Higgings and Anderson (13).

Application of Boric Anhydride

Boric anhydride was obtained from Eti Mine Works General Management, Emet Boron Works Management. Boric anhydride was solved in saline solution.

Experimental protocol and B_2O_3 injection

The rats were randomly divided into four groups, each consisting of 7 animals.

Group I: PH+Saline-treated i.p. 3 h group.

Group II: PH+Saline-treated i.p. 6 h group.

Group III: PH+1800 mg.kg^{-1} B_2O_3-treated i.p. 3 h group.

Group IV: PH+1800 mg.kg^{-1} B_2O_3-treated i.p. 6 h group.

The entire surgical procedures were conducted under xylazine (10 mg.kg^{-1}) and ketamine (70 mg.kg^{-1}) anesthesia.

B_2O_3 was administered intraperitoneally after PH. The doses were prepared everyday afresh by being dissolved in saline.

RNA isolation and Real Time PCR (RT-PCR)

Total RNA was isolated from liver tissue by using PureLink RNA Mini Kit with Trizol reagent according to the manufacturer's instructions (Ambion, Cat. No: 12183018A). The cDNA was converted from RNA by using a High Capacity cDNA kit (Applied Biosystems, Cat. No: 4368814). All procedures were performed on a StepOne Plus RT-PCR System. Actin-β (Rn00667869_m1) was chosen as a housekeeping gene. Caspase-3 (Rn00563902_m1) and NF-κB (Rn01399583_m1) were compared with Actin-β for each sample.

Protein isolation and TaqMan Protein Assay (TPA)

TPA protein lysates were prepared by using a Paris kit (Applied Biosystems, Cat. No: AM1921). TPAwere performed on a StepOne Plus RT-PCR System. TPA procedure was performed by using the method of Swartzman et al (14). This method contains the steps of antibody-oligonucleotide pairs binding to target protein in tissue homogenates, combining oligonucleotides, and detection of the desired protein with TPA. Caspase-3 (LSBio – LS-C88630) and NF-κB (IMGENEX – IMG-150B) protein data were calculated relative to protein expression between untreated control and treated samples.

TUNEL assay

Following dissection, liver tissues were put in 10 % neutral formol for TUNEL analysis. Then, TUNEL marking was carried out within the scope of the protocol by using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Milipore, Cat. No: S7101).

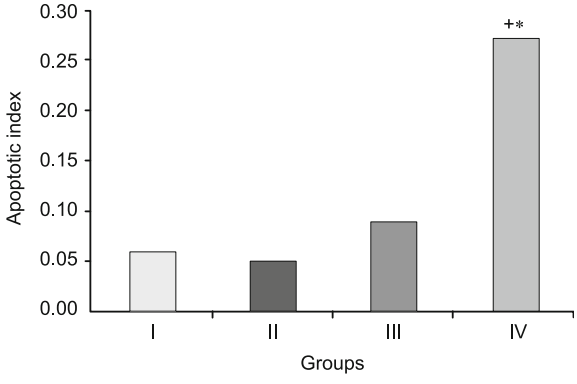

Fig. 1. Apoptotic index assay with TUNEL test. Results were expressed as median values. χ^2 = 50,834; standard deviation = 5; *+ p < 0.01 is different depending on the group and time (n = 7).

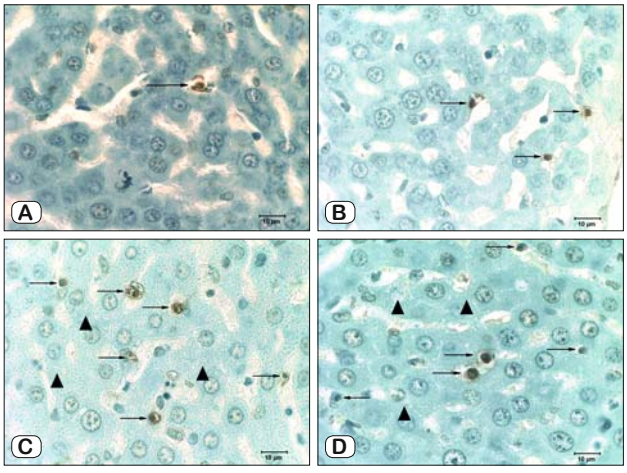

Fig. 2. TUNEL positive hepatocytes in TUNEL sections. Liver sections belonging to A) Group I, B) Group II, C) Group III, and D) Group IV animals. TUNEL-positive hepatocytes are shown with an arrow (↑) and vacuolization is shown with an arrowhead (▲).

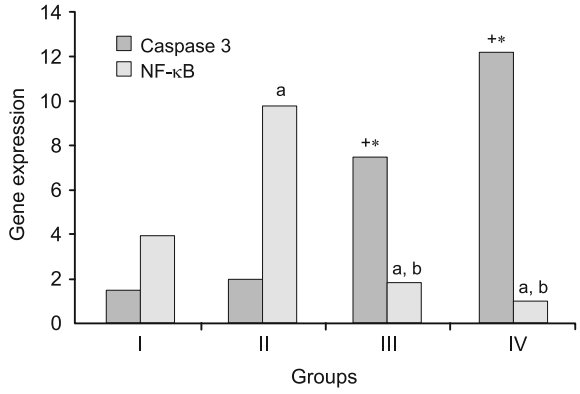

Fig. 3. Caspase-3 and NF-κB gene expression. Results were expressed as median values. Caspase-3; χ^2 = 11.733; standard deviation = 5; *+ p < 0.05 was significantly different per group and time. NF-κB; χ^2 = 15.69; standard deviation= 5; [a] p < 0.05 is significantly different per groups and [b] p < 0.05 is significantly different per time (n = 7).

Statistical analysis

All data were analyzed with Kolmogorov–Smirnov and Kruskall–Wallis tests by using IBM SPSS Statistics 20.0 software. When $p < 0.05$, differences were interpreted to be significant.

Results

Apoptotic index

When calculating apoptotic index, apoptotic cell count was estimated in each section and its ratio to other cells were calculated by using TUNEL assay.

The obtained findings revealed that the highest number of apoptotic bodies were in Group IV (Fig. 1). B_2O_3 administered following PH was observed to increase apoptosis in a time-de-

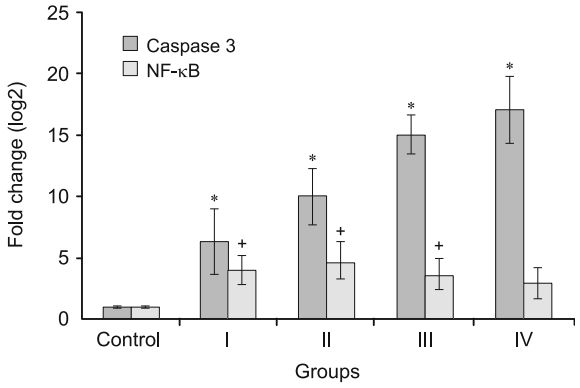

Fig. 4. Caspase-3 and NF-κB protein expression. Caspase-3 protein expressions were increased significantly in time-dependent manner. * p < 0.05 versus control. NF-κB protein expressions were decreased in boron-treated groups in time-dependent manner. + p < 0.05 versus control. Bars represent mean ± SD.

pendent manner in liver tissue. While normal liver morphology was observed in Saline groups, vacuolization was found in B_2O_3-treated groups (Fig. 2). When comparing the apoptotic indexes, apoptosis was observed to increase in 6h B_2O_3-treated groups (Fig. 1).

RT-PCR analysis

According to the obtained findings, caspase-3 gene expression was observed to increase in all groups in a time-dependent manner. However, caspase-3 gene expression level was high within the first 3 hours after B_2O_3 injections following the PH and highest level at the 6th hour (Fig. 3). It was found that in Saline control groups yielded partial increase in a time-dependent manner. However, when comparing the NF-κB gene expressions of groups in RT-PCR analysis, the Saline control groups showed a time-dependent expression increase, but the expression was observed to decrease in B_2O_3-treated groups (Fig. 3).

TPA protein analysis

In the TPA analysis, caspase-3 activation following PH was observed to be low in Group I. Furthermore, we observed the high value of activation in B_2O_3-treated groups in the first 3 hours, and it reached the highest level at the 6th hour (Fig. 4).

When examining the NF-κB activations of the groups, it was observed to be low in Group I and high in Group IV. While the NF-κB activation increased in a time-dependent manner in Saline control groups following PH, activity was observed to decrease in B_2O_3 treated groups (Fig. 4).

Discussion

In this selected model, apoptotic effects of B_2O_3 on hepatocytes were analyzed. According to our data, B_2O_3 induced apoptosis following PH. Also as a result of analyses, NF-κB activation was suppressed against liver regeneration and caspase-3 activation was provided to increase.

The apoptotic index was formed through apoptotic bodies established in TUNEL sections from which we analyzed apoptosis morphology in the liver in our study. When analyzing our apoptotic index data, the presence of apoptosis was established in all groups, but apoptosis was found to be greater in groups that received B_2O_3 following PH. Data of our study demonstrate resemblance with those found in literature. In previous studies conducted on boron and boron compounds, *in vitro* environments were generally used and apoptotic effects of boron compounds were analyzed in a dose- and time-dependent manner. Meijer et al (15) established that elemental boron in lower concentration induced apoptosis at the 72nd hour in melanoma cell lines. Their findings underlined that boron demonstrated its effect especially in the pre-mitotic phase. Similarly, Barranco and Eckhert (12, 16) examined in their study the anti-proliferative effects of boric acid in cell lines causing prostate cancer. According to their results, high doses of boric acid were observed to have apoptotic effects (12, 16). Scorei et al (17) noted that they induced apoptosis in cancer cell lines with low doses of calcium fructoborate. In addition, authors also suggested that proliferation with calcium fructoborate and boric acid was inhibited in a dose-dependent manner. Besides, authors also reported that pro-apoptotic effect and the inhibition in cell growth following the treatment were intensively observed. In their study, Cardoso et al (18) analyzed the effects of boron compounds on liver metastasis and liver regeneration induced with PH suggested that boron in high densities triggered apoptosis. Garabalino et al (19) noted that boron compounds could be used in the treatment of cancer in a concentration-dependent manner in liver metastasis.

We studied our gene and protein by using RT-PCR and TPA. TPA was successfully used by Pfister et al (20). They used TPA in apoptosis model to determine protein levels and demonstrated that TPA had a significant higher sensitivity rather than Western Blot or ELISA (20). It was observed in the findings of our study that NF-κB was activated in the liver following PH. NF-κB activation was observed to increase in a time-dependent manner in groups that received PH and saline. Sakuda et al (21) reported that NF-κB was activated immediately after PH, and that NF-κB activation could also be observed in control groups. The fact that NF-κB activation increased in a time-dependent manner was also emphasized in other studies (22, 23). Sánchez et al (22) analyzed NF-κB activation following PH and reported that the activity started 30 minutes after PH and increases were observed in activity for up to 2 hours. NF-κB inhibition after PH suggests that apoptosis takes place in liver regeneration (24). In our study, it was observed that B_2O_3 administered in rats that underwent PH suppressed the NF-κB activation time-dependently during liver regeneration. The NF-κB activation that remained low in groups that were administered B_2O_3 compared to saline groups. Based on our results, B_2O_3 was observed to suppress NF-κB transcription and translation in liver tissue following PH in a time-dependent manner. Despite the presence of studies conducted in this manner, there is no study with characteristics similar to our experimental model. However, in a study conducted on NF-κB inactivation of boron compounds, boric acid was noted to decrease NF-κB activation (10).

Caspase activity is highly important in the inhibition of cell proliferation. The most important factor in apoptosis pathways is

caspase-3 activation. Caspase-3 plays an important role in suppressing proliferation in uncontrolled cell growth. In the light of our results we obtained, caspase-3 activation was observed to demonstrate an increase depending on time. Especially following PH, caspase-3 activation was observed to occur. According to the results of our study, B_2O_3 stimulated caspase-3 transcription and translation. Caspase-3 amount demonstrated an increase in B_2O_3-treated groups compared to Saline control groups. B_2O_3 administered following PH was observed to increase caspase-3 activation in a time-dependent manner. Our results overlap with other studies. The previous studies demonstrate that caspase-3 activation may occur following PH (25, 26). There are studies on caspase-3 activation about boron. In their study, Scorei et al (17) examined the effects of boric acid and calcium fructoborate on breast cancer cell lines. Authors suggest that the dose increase was effective in caspase-3 activation. Predominantly, calcium fructoborate was noted to play an important role in caspase-3 activation (17).

Conclusion

Consequently, our results have shown that the boron-induced apoptosis in a normal cell proliferation as in the tumor cell lines. B_2O_3 was used at a high dose according to tumor cell lines or daily diet. This proves the apoptotic effects of boron in high doses. Our study has provided evidence that B_2O_3 induces caspase-3 activation and inhibits NF-κB in the priming phase of liver regeneration. The induction of apoptosis by B_2O_3 can contribute to the determination of antiproliferative effects of boron. But boron-induced apoptosis *in vivo* models on molecular studies is not enough. Further studies are needed to clarify the effects of boron on apoptotic pathways. Therefore, the investigation of the effects of boron compounds to apoptotic pathways in the liver or liver cancer is extremely important. In the light of the data obtained from our experimental model, B_2O_3 compound was observed to have an apoptotic effect by inducing caspase-3 and inhibiting NF-κB in sublethal doses in a time-dependent manner.

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