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Research Article

Brominated flame retardant BDE-99 promotes apoptosis by intrinsic mitochondrial pathway in rat liver

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Abstract: This study examined *in vivo* effects of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) on the liver of Wistar Albino rats (250-300 gr) in doses of 0.05 mg/kg and 0.1 mg/kg for ten days by gavage. Our objective was to investigate the effects of BDE-99 on the apoptotic process in the liver. Previous studies have shown that BDE-99 causes accumulation and oxidative damage in various tissues, especially the liver. Although the primary mechanism of BDE-99 toxicity is known to involve oxidative stress, limited information is available on its specific impact on apoptosis. Therefore, immunoreactivity *of Proliferating Cell Nuclear Antigen (*PCNA), Vimentin and Topoisomeraz2A (TOP2A) and Topoisomeraz2B (TOP2B) and Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) were determined in the liver. Superoxide dismutase (SOD), *Glutathione* peroxidase (GPX) and Catalase (CAT) activities were measured in the liver. qRT-PCR analyses for the p53, Bax, Bcl-2, PCNA and Vimentin genes were carried out from paraffin-embedded liver tissues. Cell membrane damage, hypertrophy, endothelial injury, mononuclear cell infiltration in the liver were determined by Hematoxylin & Eosin. Immunoreactivity of TUNEL, Vimentin, TOP2A and TOP2B increased in both doses, but immunoreactivity of PCNA significantly increased only 0.1 mg/kg BDE-99 dose ($p < 0.05$). SOD and GPX activities increased but CAT activity decreased significantly ($p < 0.05$) in the liver. Bax, Bcl-2, PCNA, Vimentin gene expressions increased in a dose-dependent manner and p53 expression increased only in 0.1 mg/kg BDE-99. In conclusion, our results point out BDE-99 inducing apoptosis of the intrinsic mitochondrial pathway in rat liver and indicate that exposure to BDE-99 is possible to be a potential risk factor for liver diseases.

Özet: Bu çalışmada 2,2',4,4',5-pentabromodifenil eter (BDE-99), 0,05 mg/kg ve 0,1 mg/kg dozlarıyla Wistar Albino (250-300 gr) sıçanlara on gün boyunca gavaj yoluyla uygulandı ve karaciğer üzerine *in vivo* etkileri araştırıldı. Amacımız BDE-99'un karaciğerdeki apoptotik süreç üzerindeki etkilerini araştırmaktı. Önceki çalışmalar BDE-99'un çeşitli dokularda birikime ve oksidatif hasara neden olduğunu göstermiştir. BDE-99 toksisitesinin birincil mekanizmasının oksidatif stresi içerdiği bilinse de apoptozis üzerindeki spesifik etkisi hakkında sınırlı bilgi mevcuttur. Bu nedenle, karaciğerde *Proliferating Cell Nuclear Antigen (*PCNA), Vimentin ve Topoizomeraz2A (TOP2A) ve Topoizomeraz2B (TOP2B)'nin ve Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) ile immünoreaktivitesi belirlendi. Karaciğerde Süperoksit dismutaz (SOD), Glutatyon peroksidaz (GPX) ve Katalaz (CAT) aktivitesi ölçüldü. Parafine gömülmüş karaciğer dokularından izole edilen total RNA kullanılarak sentezlenen cDNA örneklerinde, p53, Bax, Bcl-2, PCNA ve Vimentin genlerine yönelik qRT-PCR analizleri yapıldı. Karaciğerde hücre zarı hasarı, hipertrofi, endotel hasarı, mononükleer hücre infiltrasyonu Hematoksilen & Eozin boyama ile belirlendi. TUNEL, Vimentin, TOP2A ve TOP2B'nin immünreaktivitesi her iki dozda da arttı, ancak PCNA'nın immünreaktivitesi yalnızca 0,1 mg/kg BDE-99 dozunda anlamlı düzeyde arttı ($p < 0.05$). Karaciğerde SOD ve GPX aktiviteleri arttı ancak CAT aktivitesi anlamlı düzeyde azaldı ($p < 0.05$). Bax, Bcl-2, PCNA, Vimentin gen ekspresyonu doza bağlı olarak artarken, p53 ekspresyonu sadece 0,1 mg/kg BDE-99 dozunda arttı. Sonuç olarak, bulgularımız sıçan karaciğerinde BDE-99'un intrinsik mitokondriyal yolda apoptozu indüklediğine işaret etmekte ve BDE-99'a maruz kalmanın karaciğer hastalıkları için potansiyel bir risk faktörü olabileceğini göstermektedir.

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Introduction

Polybrominated diphenyl ethers (PBDEs) have been used commercially as a flame retardant on a global scale since 1970 (Alaee *et al.* 2003, Albina *et al.* 2010). While the main source of PBDEs is exposure to seafood, air, water, household waste and domestic dust, the most important way of intake is nutrition (Hooper & McDonald 2000, Bakker *et al.* 2008). Accumulation of 2.2',4.4',5-pentabromodiphenyl ether (BDE-99), a derivative of PBDE, is more common in liver and adipose tissues compared to other PBDEs (Guvenius *et al.* 2001). The deposition sites of BDE-99, a well-absorbed brominated flame retardant species and the second most abundant congener, were reported to be the lipophilic tissues (Birnbaum & Staskal 2004, Johnson-Restrepo *et al.* 2005). In a study with mouse and human preadipocyte, BDE-99 administration was shown to led to increased lipid accumulation. BDE-99 induced differentiation and lipid accumulation in undifferentiated preadipocytes (Armstrong *et al.* 2020). In a study on rats where a mixture of BDE-47 and BDE-99 was used, isoproterenol-induced lipolysis increased and altered insulin signaling (Hoppe & Carey 2007). Furthermore, tissue disposition and toxicokinetics of PBDEs, especially BDE-99 and BDE-47, were pointed out as risk factors for human health (Darnerud *et al.* 2001, Hakk *et al.* 2002, Staskal *et al.* 2006).

PBDE-induced hypertrophy and vacuolization in hepatocytes and increased liver weight were reported to occur in rats and mice after oral exposure (Dunnick & Nyska 2009). Histological examination of kidneys of adult mice showed that BDE-99 caused phagolysosomes in kidney (Chen *et al.* 2006, Albina *et al.* 2010). BDE-99 decreased Catalase (CAT) activity significantly but Superoxide dismutase (SOD) and *Glutathione* peroxidase (GPX) activities increased in liver of adult male mice after exposure for 45 days by gavage (Albina *et al.* 2010). BDE-99 decreased glutathione (GSH) and increased oxidized glutathione (GSSG) and GSSG/GSH levels in liver and radicalic damage was described as potential mechanism for hepatotoxicity by acute oral administration of BDE-99 (Albina *et al.* 2010). BDE-99 decreased testicular weight and sperm count at high doses in male rats (Kuriyama *et al.* 2005). Wang *et al.* (2015) reported, in their study investigating tumorigenesis, that BDE-99 may cause metastasis in cancer cell line via the PI3K/AKT/Snail pathway.

The consideration of results of the available studies shows that little is known about the mechanism of toxicity of BDE-99. One of the suggested toxicity mechanisms is the increase of reactive oxygen species (ROS) that can affect liver and kidney (Albina *et al.* 2010). There are limited published studies on the effect of BDE-99 on the apoptotic process, and all these studies are *in vitro* studies. Madia *et al.* (2004) defined the apoptotic properties of BDE-99 at immunohistochemical level through Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) Hoechst 33258 and p53. Souza *et al.* (2013) showed that a high dose (25 μM) of BDE-99 caused apoptosis in HepG2 cells. Wu *et al.* (2023) reported that BDE-99 induced spermatogenic cell apoptosis in GC-1 spg cell line. In the studies investigating the toxic effects of BDE-99, the main focus has been on the mechanism of this effect without sufficient explanation, especially on the triggering of the apoptotic process and the expression of related genes. The fact that the apoptotic properties of BDE-99 have not been demonstrated by *in vivo* studies also points to a gap in this regard. For this reason, this is the first *in vivo* study explaining the toxic effect mechanism of BDE-99 on apoptotic markers in rat liver. We aimed to reveal the effect of BDE-99 *in vivo* on the apoptotic process and expression of the related genes p53, Bax, Bcl-2, *Proliferating Cell Nuclear Antigen* (PCNA), Topoizomeraz2A (TOP2A), Topoizomeraz2B (TOP2B) and Vimentin in liver as a main detoxification organ.

Materials and Methods

Animals and the experimental protocol

Twenty-four adult male Wistar albino rats (250-300 g) obtained from the Trakya University Experimental Animal Research Unit were used in the study. The rats were kept under controlled conditions (22±10°C, 12 h light/dark cycle) during the experimental period and fed with drinking water and pellet feed containing 21% crude protein. Animals were divided into three random groups (*n=8 for* each). The animals of one of the two experimental groups were fed with 0.05 mg/kg body weight BDE-99 (Cas No: 60348-60-9 Sigma-Aldrich) dissolved in corn oil by gavage, and those of the other group was fed 0.1 mg/kg body weight BDE-99 dissolved in corn oil by gavage (1-1.5 ml dose volume). These doses are lower than the 0.6 and 1.2 mg/kg body weight doses used by Albina *et al.* (2010), which are the lowest *in vivo* doses previously tested. The control group was given only the same volume of corn oil orally. The design of the study and the experimental procedures were approved by the Ethical Committee of Trakya University (Protocol number: 2016/48).

Histopathological examination

After 10 days of the experiment, liver tissue samples were harvested by anaesthesia with Rompun (2%, Bayer) and Ketasol (10%, Richterpharma) for histopathological examination. The samples were fixed with formalin (10%) for 24 hours for light microscopy and embedded in paraffin. Sections (5 μm) were stained with Hematoxylin & Eosin (H&E).

Immunohistochemistry (IHC)

For avidin and biotinylated enzyme complex (ABC) method, firstly, liver sections (5μm) were kept in xylene for deparaffinization and rehydrated by series of ethanol. Then antigen retrieval was performed in citrate buffer (pH: 6.0) for 45 min using an IHC evaporating bath (IHC World, Woodstock, MD). After cooling to room temperature for 20 min, they were applied in 3% hydrogen peroxide (H_2O_2) and methanol for 5 min and treated twice

with phosphate-buffered saline (PBS) and once with PBS that contained 0.1% Tween-20 (PBS-T). Ultra V block (Thermo Scientific, Waltham, MA) was applied to each slide for 5 min in a humidified chamber (Bakar *et al.* 2015b). The sections were treated with anti-PCNA (1:500) (NB500-106/Novus), anti-Vimentin (1:100) (NBP1-31327/Novus), anti-TOP2A (1:100) (NBP2-67442/Novus) and anti-TOP2B (1:100) (NBP1-89527/Novus) as a primary antibody at room temperature for 1 hour and overnight at +4ºC. After washing three times for 5 min each time with PBS, they were treated with biotinylated goat anti-polyvalent (ab7481, Abcam) for 10 min and washed four times in buffer. Streptavidin peroxidase was then applied for 10 min at room temperature. The slides were washed with PBS and with PBS-T. After washing, the sections were stained with DAB (Vector SK-4100, Vector Laboratories, Peterborough, UK) and then Mayer's Hematoxylin (Sigma, Aldrich). Immunohistochemical analysis was performed by systematic random samplings in each group using ten cross-sectional areas and five slides. For the evaluation of immunoreactivity of PCNA, the proliferation index was calculated by counting the (+) cells in an average of 1000 cells in 5 different areas in the sections of 8 subjects belonging to each group. Positive cells of Vimentin, TOP2A and TOP2B were counted in 5 different fields and their immunoreactivity was scored between 1 (+) and 5 (+) at most. All scoring was done by a randomized selection of microscopic fields and a double-blind by two researchers, the mean of which was used for statistical analysis.

In situ detection of apoptotic cells (TUNEL)

Liver sections (5 µm) were taken on poly-L-lysine-coated slides (Sigma, PO425-72EA). Apoptotic liver cells were detected with the TUNEL method (ApopTaqPeroxidase In Situ Apoptosis Detection Kit; S7101-KIT, Millipore) following the manufacturer's instructions. Rodent mammary gland sections and distilled water were used as the positive and the negative controls, respectively. Ten randomly selected fields under the light microscope were evaluated for each animal of the groups and the apoptotic cells were determined by their intense brown nuclear staining. The average of apoptotic cell numbers per liver sample and standard deviation (SD) were expressed as the score of TUNEL (Bakar *et al.* 2015a).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Changes in expression levels of p53, Bax, Bcl-2, PCNA and Vimentin genes were determined by using qRT-PCR. For this purpose, firstly total RNA was isolated from paraffin embedded tissue samples using the High Pure FFPET RNA Isolation Kit (Roche, Cat No: 06 650775001) as recommended by the manufacturer. Complementary DNA (cDNA) synthesis was performed using the Transcriptor First Strand cDNA synthesis Kit (Roche, Cat No. 04896866001) using random hexamers according to the manufacturer's instructions. The obtained cDNA samples were stored at -20°C until the time to be used as a template in qRT-PCR. qRT-PCR was performed with ABI Step One Plus (Thermo Pico) thermal cycler. The sequences of the primers used in the study are shown in Table 1. Amplifications of the PCR products were monitored via SYBR Green I dye which is an intercalator-based method. qRT-PCR mix was prepared with 5 µl cDNA, 12.5 µl SYBR Green 2X master mix, 1 µl forward and reverse primers (10 pmol/µl) and 6.5 µl ddH_2O in 25 µl total reaction volume. The cycling program consisted of an initial denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 60°C for 1 min, 60°C for 1 min for all genes. The specificity of the primers used in PCR was confirmed by melting curve analysis during qRT-PCR as well as optimization studies. Expression levels of the target genes were normalized using the internal gene GAPDH and compared with the data obtained from the control group according to the 2-ΔΔCT method (Livak & Schmittgen 2001).

Antioxidant enzyme assay (SOD, GPX and CAT)

The liver samples were washed with saline and placed in deep freezer for storage at -80°C for the biochemical parameters by the time the assay is performed. The liver samples (0.5 g) were homogenized with phosphate buffer [0.05 M, pH: 7.0, containing 1% (g/mL) Triton X-100]. The extracted volume was centrifuged at 12,000 g at +4ºC for 20 min and the supernatant was assayed for SOD, GPX and CAT activities. The SOD and GPX activities were monitored by a spectrophotometer (Schimadzu UV-VIS) at 37ºC by the Ransod SD 125 kit (Randox) (Arthur & Boyne 1985, Bakar *et al.* 2015b) and Ransel RS 505 kit (Randox), respectively (Kraus & Ganther 1980, Topcu-Tarladacalisir *et al.* 2013, Bakar *et al.* 2015b). CAT activity was determined by measuring decomposed 1 µmol H_2O_2 per minute at 37ºC according to Aebi (1974) (Aebi 1974) One-unit SOD activity was defined as the amount of enzyme that causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(nitrophenol)-5-phenyltetrazolium chloride. One-unit GPX activity was defined as the amount of enzyme that oxidized 1 µmol NADPH to NADP in 1 min.

Table 1. Primary sequences of the target genes and the internal gene.

Target genes	Primary sequences (5' → 3')	References
p53	CACAGTCGGATATGAGCATC GTCGTCCAGATACTCAGCAT	(Zaragoza *et al.* 2003)
Bax	GACACCTGAGCTGACCTTGG GAGGAAGTCCAGTGTCCAGC	(Die *et al.* 2019)
Bcl2	GGGATGCCTTTGTGGAACTA CTCACTTGTGGCCCAGGTAT	(Die *et al.* 2019)
Vimentin	CGTACGTCAGCAATATGAAAG TGTGTCAGAGAGGTCAGCAAA CTTGGA	(Dong *et al.* 2014)
PCNA	GGTGCTTGGCGGGAGC ATCGCTTGAGCCCAGAAGT	(Moldovan *et al.* 2007)
GAPDH	GCATCTTCTTGTGCAGTGCC GATGGTGATGGGTTTCCCGT	(Potmesil *et al.* 1988)

One-unit CAT was defined as the amount of enzyme that decomposed 1 μmol H_2O_2 per minute at 37°C and pH: 7.0. The amount of total protein was measured according to Lowry (Lowry *et al.* 1951). All enzyme activities were presented as U/mg protein.

Statistical analysis

All data from body weight, immunohistochemical analysis, antioxidant enzyme activities, TUNEL assay and relative expression levels of target genes are expressed as the mean±standard deviation (SD). Non-normally distributed groups (body weights, all immunohistochemical scoring) were tested with the Kruskal-Wallis test and enzyme activities was tested Mann-Whitney U test for comparison between two groups. Student-t test was performed to determine changes in relative expression levels of target genes between the experimental groups and the control group. SPSS 16 for Windows (IBM SPSS Inc., Chicago, IL, USA) software was used for statistical analyses. The results were considered statistically significant when $p < 0.05$.

Results

The conditions of the animals were checked every day during the experiment period before the administration. No injuries to the bodies of the animals or any deterioration in their general condition were observed during the experiment. The initial number of subjects was maintained because there was no animal death. The body weights of the animals before and after the experiment are presented in Table 2 as mean± SD in all groups (*n=8*). Body weight increased significantly in both the 0.05 mg/kg and the 0.1 mg/kg BDE-99 groups compared to the control group.

Histophatology of the liver

The general histological structure of the tissue was preserved in the liver of the control group (Fig. 1a) and the degenerative changes consisted in the liver of the experimental groups (0.05 mg/kg BDE-99 and 0.1 mg/kg BDE-99).

Table 2. The body weights of the animals before and after the experiment are presented as mean±SD in all groups (*n=8*).

	Body weight (before) ± SD	Body weight (after) ± SD
Control group	265.20 ± 9.23	266.50 ± 12.10
0.05 mg/kg BDE-99	265.11 ± 16.22	283.12 ± 14.21 [a]
0.10 mg/kg BDE-99	266.50 ± 14.84	287.00 ± 10.12 [a]

[a] indicates statistical significance compared with the control group, $p < 0.05$.

Irregularity in hepatocyte cords was observed in the 0.05 mg/kg BDE-99 group. Loss of integrity as a result of endothelial damage in the veins and mononuclear cell infiltration around the veins in small areas were determined (Fig. 1b). Hypertrophic hepatocytes and cytoplasm loss in hepatocytes were observed throughout the liver tissue in the 0.1 mg/kg BDE-99 group. Damaged hepatocyte membranes and cytoplasm loss around the nucleus of hepatocytes occurred. In addition, mononuclear cell infiltration around the veins more than the low dose of BDE-99 was determined (Fig. 1c).

In situ detection of apoptotic cells (TUNEL)

TUNEL results showed that apoptosis increased significantly in a dose-dependent manner in both doses of BDE-99 in the liver (Fig. 2).

Immunohistochemistry of the liver (IHC)

IHC staining was performed and scored to determine PCNA, Vimentin, TOP2A and TOP2B for immunoreactivity in the liver (Figs 3-4). Immunoreactivity of PCNA and TOP2B increased significantly only 0.1 mg/kg BDE-99 group when compared with the control group. Vimentin and TOP2A increased significantly in a dose-dependent manner in both doses of BDE-99 (Fig. 4).

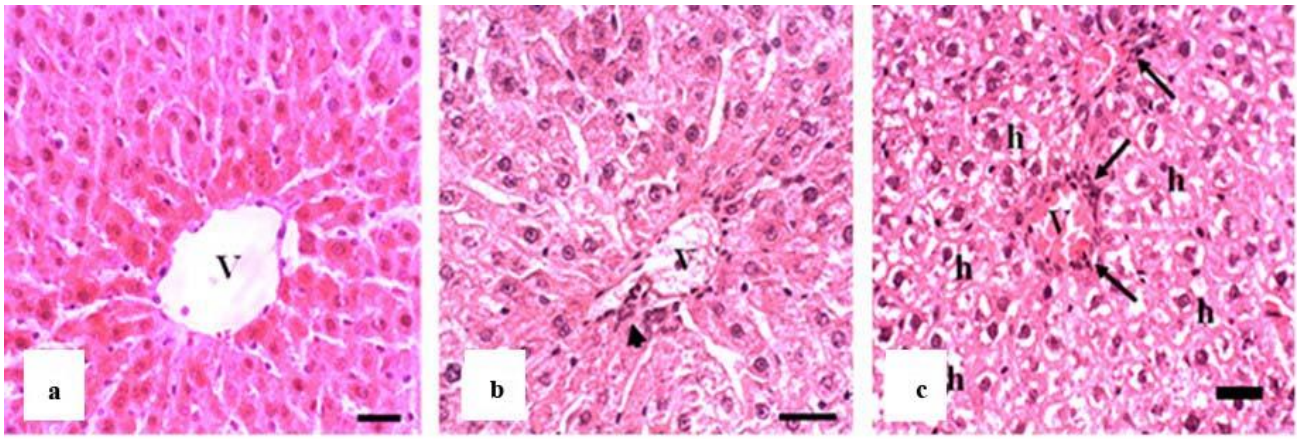

Fig. 1. Histopathology of liver (H&E staining) **a.** Control group; V: central vein, **b.** 0.05 mg/kg BDE-99 group; V: central vein, loss of integrity around central vein, arrow: irregularity in hepatocyte cords, and endothelial damage in the vein, **c.** 0.1 mg/kg BDE-99 group, h: hypertrophic hepatocytes and cytoplasm loss around the nucleus, arrows: mononuclear cell infiltration around the vein and loss of integrity in hepatocyte cords. Scale bar represents 40 μm.

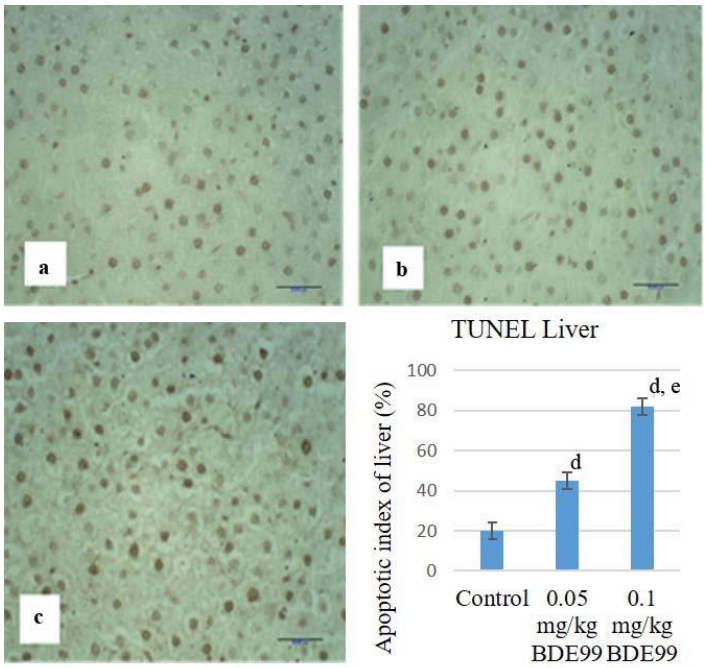

Fig. 2. TUNEL of liver. **a.** Control group, **b.** 0.05 mg/kg BDE-99 group, brown stained nuclei are indication of apoptotic cells, **c.** 0.1 mg/kg BDE-99 group, brown stained nuclei are indication of apoptotic cells. Scale bar represents 40 µm. Apoptotic index of liver (%) by TUNEL. Values are presented as the mean±SD, and *n=8* for all groups.

[d] indicates statistical significance compared with the control group. [e] indicates significance compared with the 0.5 mg/kg BDE-99 group, $p < 0.05$.

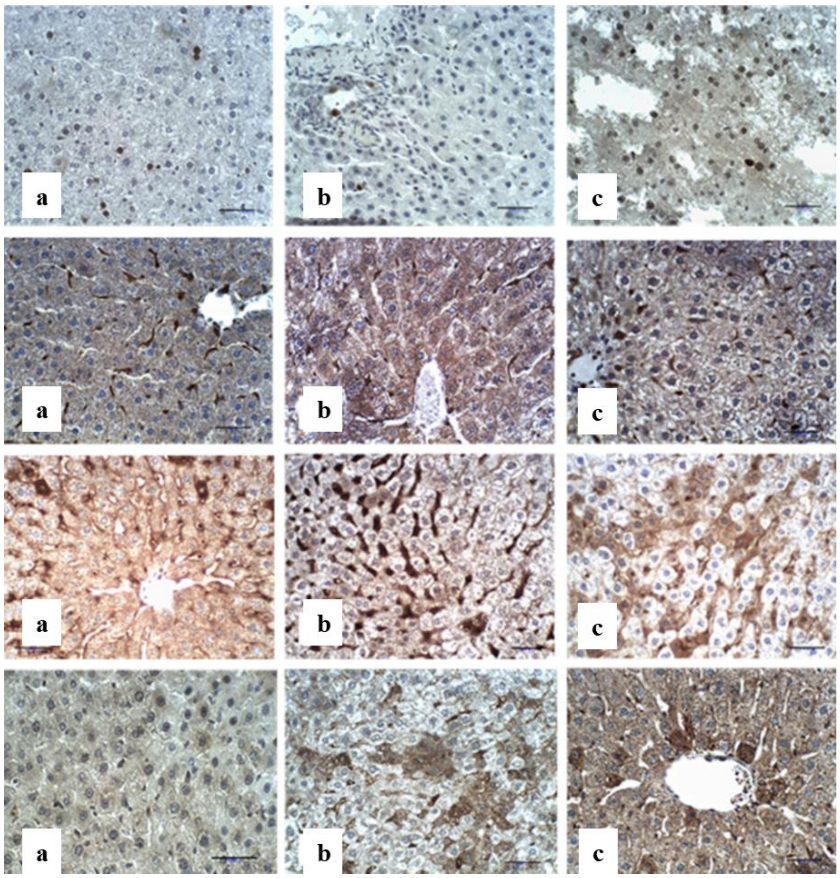

Fig. 3. Immunohistochemical (IHC) evaluation of liver. **a.** Control group, **b.** 0.05 mg/kg BDE-99 group, **c.** 0.1 mg/kg BDE-99 group. Line 1: PCNA, brown stained nuclei are indication of PCNA+ cells. Line 2: Vimentin, brown stained nuclei are indication of Vimentin+ cells. Line 3: TOP2A, brown stained nuclei are indication of TOP2A+ cells. Line 4: TOP2B, brown stained nuclei are indication of TOP2B+ cells. Scale bar represents 40 µm.

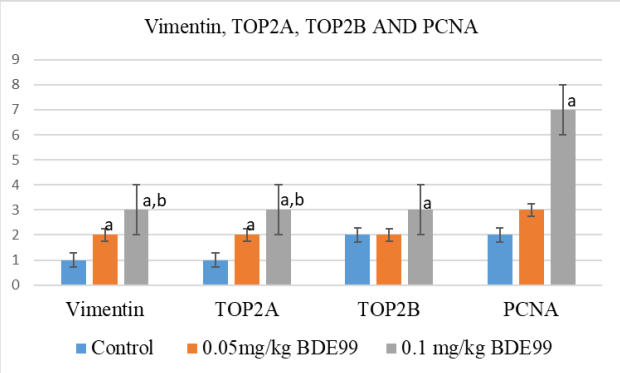

Fig. 4. IHC scores of Vimentin, TOP2A, TOP2B and PCNA in the liver. Values are presented as the mean±SD, and *n=8* for all groups.
[a] indicates statistical significance compared with the control group. [b] indicates significance compared with the 0.5 mg/kg BDE-99 group, $p < 0.05$.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Gene expressions of Bax (6.3 fold), Bcl2 (5.7 fold), PCNA (6.95 fold) and Vimentin (34.35 fold) significantly increased in 0.05 mg/kg and Bax (4.33 fold), Bcl2 (6.25 fold), PCNA (25 fold) and Vimentin (48.95 fold) in 0.1 mg/kg BDE-99 groups, but p53 gene expression significantly increased (4.74 fold) only in the 0.1 mg/kg group (Fig. 5).

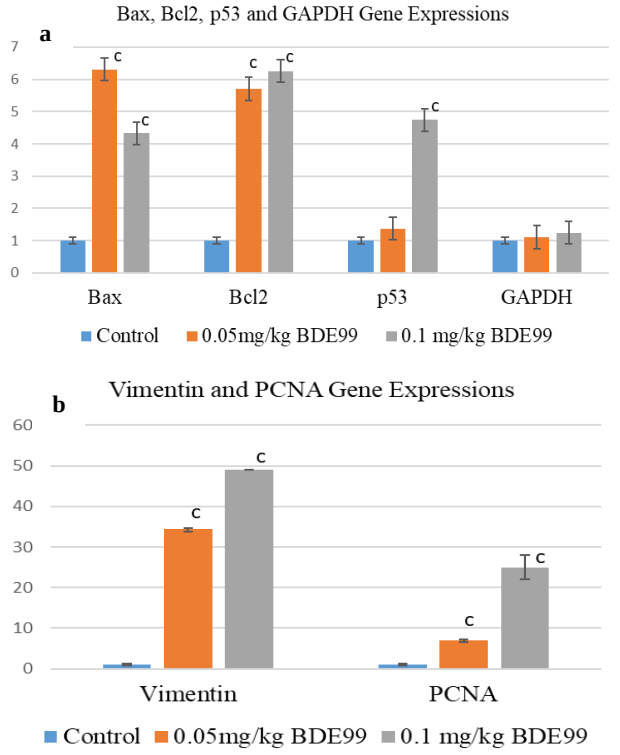

Fig. 5. Gene expressions in the liver. **a.** Bax, Bcl2, p53, GAPDH, **b.** vimentin and PCNA. Columns represent relative fold changes in mRNA expression of target genes normalized by GAPDH and mean±SD.
[c] represents statistically significant compared with the control group ($p < 0.05$).

Antioxidant enzyme assay (SOD, GPX and CAT)

SOD and GPX activity increased significantly in both the 0.05 mg/kg and the 0.1 mg/kg BDE-99 groups but CAT activity decreased significantly only in 0.1 mg/kg BDE-99 group compared to the control group (Fig. 6).

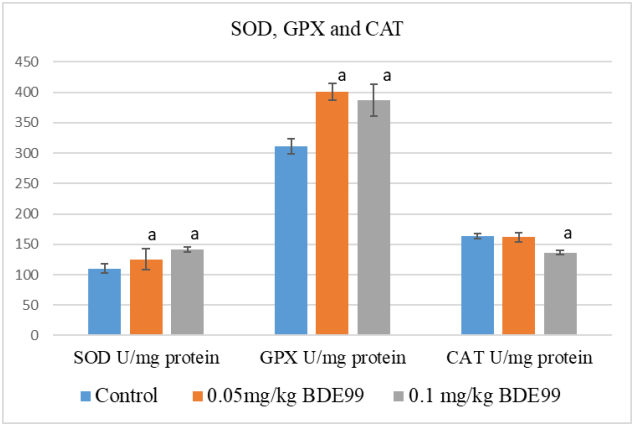

Fig. 6. SOD, GPX and CAT activities in the liver. Results were expressed as mean±SD.
[a] represents statistically significant compared with the control group ($p < 0.05$).

Discussion

BDE-99 toxicity is important in human health because of its widespread use in many areas. In a risk assessment study, it was stated that long-term exposure to BDE-99 is significant in terms of reproductive toxicity (Bakker *et al.* 2008). Die *et al.* (2019) reported that PBDE emissions and risks are lower in modern, recycling facilities but pointed out that the effectiveness of pollution controls in other areas needs to be investigated further (Die *et al.* 2019). Dong *et al.* (2014) showed that waste of electrical and electronic equipment recycling contributed 52% of the PBDEs concentration in the surrounding agricultural soils (Dong *et al.* 2014). Although significant accumulation of BDE in lipophilic tissues and toxic effects in many tissues such as liver, kidney and reproductive systems have been described (Kuriyama *et al.* 2005, Bakker *et al.* 2008, Albina *et al.* 2010, Armstrong *et al.* 2020), the knowledge about the toxicity mechanism of BDE-99 is limited. In previous studies, ROS formation and related cellular degenerations were defined as the main toxicity mechanism for BDE-99, but there are also recent studies showing that apoptosis is induced by BDE-99. However, these are all *in vitro* studies and there is no study investigating the effect of BDE-99 on apoptosis *in vivo* in rats. Therefore, in the present study, we aimed to reveal the effect of BDE-99 primarily on the apoptotic process in the liver in addition to its histopathological examination and the presence of ROS with changes in key antioxidant enzyme activities.

BDE-99 damaged the general integrity of the liver tissue of the experimental animals, and significant lacking of cytoplasm and hypertrophy were observed in hepatocytes in liver as revealed by staining with H&E (Fig. 1). Similarly, it was reported that histopathological

effects occurred in the liver in the acute oral administration of BDE-99 (Albina *et al.* 2010) increase liver weights and lesions were observed in the liver after treatment of BDE-99 (Dunnick & Nyska 2009). We believe that the increase in animal weights observed in this study is consistent with widespread hypertrophic change in the liver.

We determined that apoptosis increased in a dose-dependent manner in liver by TUNEL immunoreactivity (Fig. 2). Madia *et al.* (2004) defined the apoptotic properties of BDE-99 at immunohistochemical level via TUNEL, Hoechst 33258 and p53. Similarly, Souza *et al.* (2013) showed that a high dose (25 μM) of BDE-99 caused apoptosis in HepG2 cells. Wu *et al.* (2023) reported that BDE-99 induced spermatogenic cell apoptosis in GC-1 spg cell line by staining with Annexin V and count by flow cytometry. Our TUNEL results confirm that apoptosis is triggered in liver by the effect of BDE-99 in line with the *in vitro* studies mentioned above.

In the present study, PCNA immunoreactivity (Figs 3-4) and gene expression (Fig. 5) increased significantly in a dose-dependent manner in liver. PCNA, an important moderator of many functions in the DNA replication fork (Moldovan *et al.* 2007) indicates preparation for the increased mitotic activity for the repair of damaged liver tissue. We have not found any studies on how PCNA changes with the effect of BDE-99. Therefore, we think that this change is an adaptive response to the toxic effect of BDE-99.

Topoisomerase 2 is an enzyme that cuts two strands of DNA simultaneously and catalyses their reconnection, reducing the tension of the double strand of DNA. In this way, the effect of the rotation created by the helicase on DNA is prevented (Potmesil *et al.* 1988). Therefore, in cases where gene expression is increased, it is a normal physiological state to increase Topoisomeraz 2 expression. In our study, TOP2A immunoreactivity increased significantly in the liver at both doses, but TOP2B immunoreactivity increased only at the dose of 0.1 mg/kg (Figs 3-4). These changes observed in TOP2A and TOP2B immunoreactivity are also consistent with increased PCNA and TUNEL immunoreactivity in the liver. It is possible that the increased PCNA, TOP2A, and TOP2B immunoreactivity signifies an adaptive proliferative response to BDE-99-induced cellular damage. It seems that while cellular damage occurs by induced apoptosis, the markers of the restoration process are activated in the liver tissue.

Expression of p53 in the liver increased only at the dose of 0.1 mg/kg, and the expressions of Bax and Bcl-2 increased significantly at both doses (Fig. 5). p53 is a central protein that responds to numerous cellular stress signals (Vaseva *et al.* 2012) and can be activated by hypoxia and oxidative stress. Activation of p53 results in cell cycle arrest, followed by DNA repair or induced apoptosis in various ways (Gudkov & Komarova 2010, Pflaum *et al.* 2014). One of these pathways to apoptosis is the intrinsic mitochondrial pathway and proceeds via the Bcl-2 family, cytochrome c and caspases. In our study, we believe that the accumulation of p53 in the cytosol triggers the pro-apoptotic Bcl-2 proteins and the Bax protein directly, stimulating the cell to apoptosis. Consistent with the TUNEL results, the parallel increase in expressions of p53, Bax and Bcl-2 shows that the apoptotic process was triggered by the effect of BDE-99 in the liver (Fig. 5). Similarly, Wu *et al.* (2023) reported that PBDE-99 caused the formation of ROS, triggered autophagy and spermatogenic apoptosis in their studies examining the long-term effects of prenatal exposure on spermatogenic injuries. They showed that Bax, a pro-apoptotic protein, expression increased while Bcl-2 protein, anti-apoptotic protein, was slightly reduced due to BDE-99 toxicity with the other related genes. Our study determined that Bax gene expression and Bcl2 gene expression increased simultaneously. We observed that Bcl2 increased in a way contrary to Wu et al. (2023). When this increase in Bcl2 was evaluated with the increase in the p53 gene and TUNEL findings, we think that it occurs competitively with Bax and liver cells enter the intrinsic mitochondrial apoptotic pathway depending on the Bax/Bcl2 ratio. Yang *et al.* (2022) reported that induced oxidative stress, histopathological changes, DNA fragmentation, cell proliferation and apoptosis were observed in their study investigating the toxicity of BDE-47, the second most common type of PBDEs.

Wang *et al.* (2018) reported that apoptotic cells increased significantly in the brain of zebrafish (*Danio rerio*) embryos by early life exposure to BDE-47. Hou *et al.* (2019) showed that BDE-209, another toxic flame retardant, can induce apoptosis of vascular endothelial cells by increasing ROS production and induced ER stress. Consistently, the findings of our study are in line with other studies in which BDE-99 and/or some of its congeners have been reported to induce apoptosis.

Vimentin immunoreactivity and gene expression increased in the liver at both doses (Figs 4-5). The increase in both gene expression and the cytoplasmic amount of Vimentin protein in the liver, which is an essential component of the cytoskeleton, can be considered a sign of intracellular rearrangements. In light of these findings, it seems that this is accompanied by a rearrangement of the cytoskeleton, which is a cellular part of both the apoptotic process and the process of restoration by mitosis.

When we examine the changes in the activities of SOD, GPX and CAT, which are the key antioxidant enzymes (Fig. 6), we think that ROS-induced stress occurs in the liver, as indicated by the increase in SOD and GPX activities. The decrease in CAT activity also suggests that there may be a deficiency in the removal of H_2O_2 via peroxisomes, but a cellular response is effectuated with GPX to H_2O_2 in the cytosol. It has been stated in previous studies that BDE-99 causes oxidative damage (Albina *et al.* 2010) and it has been accepted as the main toxicity mechanism.

In conclusion, our findings display BDE-99 inducing apoptosis via the intrinsic mitochondrial pathway in the rat liver. We think that this study, in which we have shown *in vivo* that this pathway is triggered in the p53, Bax and Bcl-2 axis, will contribute to regarding studies on the subject in detail. We report that BDE-99, as a flame retardant species which humans are increasingly exposed to, is a potential risk factor for liver diseases.

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