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ANTIOXIDANT DEFENSE SYSTEM PARAMETERS IN ISOLATED FISH HEPATOCYTES EXPOSED TO BISPHENOL A – EFFECT OF VITAMIN C

Özlem Kaya and Burak Kaptaner*

Department of Biology, Faculty of Science, Yuzuncu Yil University, 65080 Van, Turkey

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In this study, isolated hepatocytes of pearl mullet *(Alburnus tarichi)* were exposed to bisphenol A (BPA) at concentrations of 25, 50, 100, and 200 μM for 24 h. Moreover, an *in vitro* antioxidant concentration of vitamin C (50 μM) was administrated to the culture medium along with the BPA exposures. Next, the antioxidant defense system parameters were analyzed. According to the results, the highest concentration of BPA (200 μM) proved to be severely toxic for the cells. The increased activities of superoxide dismutase (SOD) and glutathione-*S*-transferase (GST), the fluctuated activities of glutathione peroxidase (GPx), and the decreased content of reduced glutathione (GSH) were compared to the control group after the BPA exposures. Vitamin C co-administration was found to cause further increases in the SOD, GPx, and GST activities in some of the experimental groups and vitamin C could not restore the GSH content. Malondialdehyde (MDA) levels were observed to be unaffected in all exposure groups. These results show that BPA causes alterations in the antioxidant defenses of the isolated fish hepatocytes. In addition, vitamin C co-administration along with BPA was found to be non-protective against BPA-induced oxidative stress, consequently, aggravated a negative BPA impact.

Keywords: Alburnus tarichi – bisphenol A – vitamin C – isolated fish hepatocytes – antioxidant defenses

INTRODUCTION

Bisphenol A (BPA) is a well-known endocrine disrupter which is applied as a plastic monomer and plasticizer in many products used in daily life, such as plastic bottles, baby bottles, pipes, dental materials, personal care products, powder paints, automotive parts, compact discs, etc. [39]. It can reach aquatic systems because of its widespread usage and tons of production. The concentrations of the BPA in waste waters and surface waters have been reported to change between ng and μg/mL concentration ranges [2, 26]. In BPA-treated aquatic organisms, malformations in the embryonic and larval stages [8, 16], and endocrine disrupting effects, such as alterations in reproductive functions and the induction of hepatic vitellogenin, have been observed [7].

Many environmental pollutants have been reported to disrupt the prooxidant/antioxidant balance in cells, and, consequently, lead to oxidative stress [18]. Reactive

*Corresponding author; e-mail address: bkaptaner@yahoo.com, bkaptaner@yyu.edu.tr

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oxygen metabolites such as superoxide anion, hydroxyl radical, peroxyl radical, and hydrogen peroxide are cytotoxic agents that cause oxidative damage by attacking membrane lipids, proteins, and nucleic acids in cells. Free radicals and reactive oxygen species (ROS) formed in the cells are scavenged effectively by antioxidant defense system enzymatic and non-enzymatic antioxidants [27]. It has been reported in mammalian studies that BPA caused tissue damage, increasing ROS generation [5]. Studies in the past have mainly focused on the estrogenic potency and reproductive adverse effects of BPA in fish [29, 33−35, 41]. However studies on the effect of BPA on the hepatic antioxidant defenses are limited.

Vitamin C is one of the most important water-soluble antioxidants. Studies have shown that vitamin C has antioxidant and protective properties against oxidative damage formed by free radicals [14, 24]. On the contrary, it has been shown that supplementation of ascorbic acid into the culture medium induced apoptotic cell death by displaying prooxidant action [37].

Pearl mullet (*Alburnus tarichi* Güldenstädt 1814) is an endemic cyprinid fish living in Lake Van in Turkey [12]. It is biologically important because of its ability to inhabit the lake's highly alkaline water (pH: 9.80) and migrate into freshwater for spawning [9]. It also has an economic value due to an annual catch of approximately 10,000 tons [42]. Recent studies in the last decade reported gonad abnormalities that might arise from endocrine disrupting chemicals present in its ecological niche [43−44].

Fish hepatocytes are practical and advantageous tools used in toxicology and ecotoxicology and they are attractive models for the investigation of dose- and time-dependent chemically induced processes under certain conditions [38]. The aim of the present study was to investigate the effect of BPA on the antioxidant system parameters using isolated pearl mullet hepatocytes. In addition, co-administration of an in vitro antioxidant dose of vitamin C into the culture medium along with BPA was performed to determine whether it can prevent BPA-induced oxidative damage.

MATERIALS AND METHODS

Fish

Pearl mullet *(A. tarichi)* species (n = 5) were collected at the end of the reproductive season by electrofishing from the Karasu river, which drains into Lake Van. Fish were transported to the laboratory in aerated coolers filled with river water, and acclimated for 2 months in 50-L glass aquaria with dechlorinated tap water aerated by air pumps. During this time fish were maintained under natural photoperiod (12 h day–night cycle) at an average temperature of 22 °C, and were fed on commercial trout granule food. Ethical regulations were followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments. All of the procedures were approved by the Yuzuncu Yil University Animal Experiments Ethics Committee for the ethical concerns of the study.

Cell culture medium and chemicals

The cell culture medium, Leibovitz's L15 (L-15) with L-glutamine, was purchased from Sigma-Aldrich (St. Louis, MO). The antibiotic/antimycotic solution, collagenase (type IV) and Vitamin C [(+)-sodium L-ascorbate] were also purchased from Sigma-Aldrich. The BPA [(2,2-bis (4-hydroxyphenyl) propane), purity: ≥99%] was purchased from Sigma-Aldrich and dissolved in absolute ethanol (Merck, Germany). The kits for the antioxidant enzyme activity measurements were purchased from Randox Laboratories (Crumlin, UK). The thiobarbituric acid was purchased from Merck (Germany); butylated hydroxytoluene; trichloroacetic acid; ethylene diamine tetra acetic acid; reduced GSH; metaphosphoric acid; 5,5'dithiobis-(2-nitrobenzoic acid); potassium dihydrogenphosphate (KH_2PO_4) and sodium chloride used in this study were technical grade and supplied by Sigma-Aldrich. The ethylene glycol tetraacetic acid (EGTA); 2-phenoxyethanol and 1-chloro-2,4-dinitrobenzene (CDNB) were puchased from Fluka. All of the other chemicals used in this study were analytical grade and purchased from Merck (Germany).

Hepatocyte isolation

Hepatocytes were isolated in a 2-step perfusion method as described by Berry and Friend [3] and modified by Mortensen et al. [30]. Prior to the cell culture studies, all glassware and instruments were sterilized, and solutions were sterilized by passing through a 0.22-μm filter (Millipore Ireland, Cork, Ireland). Hepatocytes were prepared from 5 individual fish. Briefly, the liver of the anesthetized fish with 2-phenoxyethanol was carefully excised, transferred onto a glass petri dish, and rinsed with a cold calcium-free solution containing NaCl (7.14 g/L), KCl (0.36 g/L), $MgSO_4$ (0.15 g/L), Na_2HPO_4 (1.6 g/L), NaH_2HPO_4 (0.4 g/L), $NaHCO_3$ (0.31 g/L), and EGTA (20 mg/L) under sterile conditions. The liver was dissected into small pieces in this solution and agitated by pipetting. Next, tissue pieces were rinsed with a clear solution, and the process was repeated until all blood was flushed out. The whitened tissue pieces were then transferred into the same buffer containing 0.22 g/L $CaCl_2$, instead of EGTA, and collagenase (0.53 mg/mL). The tissue was digested for 15 min in this solution. The softened tissue was then triturated by pipetting with pipette tips of different size. At the final stage, the cell suspension was gently passed through the needle of a sterile injector for dissociation of the cells. The resulted cell suspension was transferred into sterilized centrifuge tubes and centrifuged at $60 \times g$ for 3 min. Cells were washed twice with Leibovitz's L15 (L-15) medium with L-glutamine containing antibiotic-antimycotic 1% (v/v) and $NaHCO_3$ (0.38 g/L). After the last wash, the cell pellet was resuspended in L-15. The cells were counted using a Thoma slide, and cell viability was assessed using a trypan blue exclusion test. It was found that the cells possessed more than 90% viability for cell culture studies.

Hepatocyte culture and exposure

The isolated hepatocytes were seeded at a density of 2×10^6/mL (1000 μL per well) in 48-well culture plates (Greiner Bio-One, Monroe, NC). The cells were allowed to attach for 24 h at 22 °C in a sterile incubator (Binder, Tuttlingen, Germany) without additional O_2/CO_2 prior to chemical exposure. BPA concentrations (25, 50, 100, and 200 μM) used in this study were selected based on previous in vitro studies conducted on fish [23, 33–35]. Stock solutions of BPA were prepared in absolute ethanol, and the final concentration of ethanol in the media never exceeded 0.1%. Control cells received only L-15 medium containing 0.1% absolute ethanol. Vitamin C [(+)-sodium L-ascorbate] was dissolved in sterile deionized water. In the experimental studies, the average antioxidant dose of vitamin C in vitro was determined to be 50 μM [20, 31]. Accordingly, this concentration of vitamin C was added along with BPA to the culture medium. The solutions of BPA and BPA + vitamin C were sterilized by passing through a 0.22 μm filter (Millipore Ireland, Cork, Ireland) before use. After 24 h of preculture, the old medium was removed, and the attached monolayer hepatocytes were exposed to BPA and BPA + vitamin C respectively, for 24 h at 22 °C. The cell cultures were routinely checked for morphological changes and contamination using an inverted microscope (Leica DM 6000, Germany), and photos were taken in brightfield. The experiments were repeated 5 times.

Biochemical analyses

After gentle pipetting of culture media from the wells, the cell suspensions were transferred to Eppendorf tubes. The tubes were centrifuged at $600 \times g$ for precipitation of the cells and then medium pipetted away. The cells were rinsed with ice-cold KH_2PO_4 (50 mM, 1 mL, pH 7.4) and cell suspensions (2×10^6/mL) were transferred into Eppendorf tubes. Next, the cells were lysed using a glass-porcelain ultrasonic homogenizer (Jencons Scientific Co., Herts, UK). The homogenate was centrifuged at $20{,}000 \times g$ for 15 min. All processes were carried out at 4 °C or on ice. Supernatant fractions were removed and stored at –20 °C, and used to determine the total protein content, lipid peroxidation and antioxidant defenses.

The total protein content in the supernatant fractions was assayed spectrophotometrically at 595 nm using the method of Bradford [6], with bovine serum albumin as a standard.

The superoxide dismutase (SOD) activity was measured using a commercial kit (Randox Lab., UK) at 505 nm and 37 °C, according to the manufacturer's instructions. The SOD activity was expressed as unit per milligram protein.

The glutathione peroxidase (GPx) activity was measured using a commercial kit (Randox Lab., UK) at 340 nm and 37 °C, according to the manufacturer's instructions. The GPx activity was calculated from the decrease in absorbance values. The GPx activity was expressed as unit per gram protein.

Glutathione-*S*-transferase (GST) activity was measured using CDNB as substrate [15]. GST activity was expressed as micromole of CDNB-glutathione conjugate per minute per milligram protein.

The glutathione (GSH) content was measured spectrophotometrically at 412 nm, using the method described by Beutler et al. [4]. The GSH levels were obtained from a standard curve derived from external GSH standards. Results were expressed as nanomole GSH per 10^6 cells.

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content, a product of lipid peroxidation, in the samples. The MDA concentration was measured spectrophotometrically at 532 nm, using the method described by Jain et al. [19], based on thiobarbituric acid reactivity. The results were expressed as nmol/10^6 cells.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software, version 16.0. Differences among the data were analyzed using a 1-way analysis of variance with a post-hoc Duncan's multiple comparison test. The results were expressed as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

RESULTS

After 24 h exposure to BPA, it was observed that BPA was severely toxic for cells at a concentration of 200 µM. Mostly dead and detached hepatocytes were observed at this concentration (Fig. 1). Accordingly, the antioxidant defense system parameters were investigated further at lower concentrations (≤100 µM).

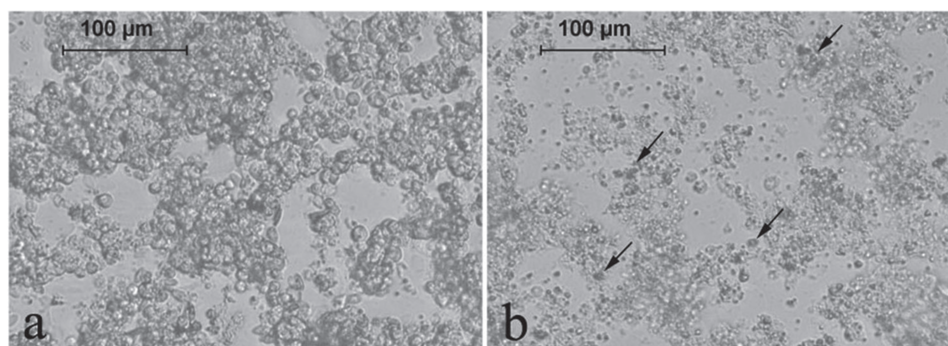

Fig. 1. Cytotoxic effect of BPA on isolated pearl mullet *(A. tarichi)* hepatocytes (a: control cells; b: 200 µM BPA exposed cells; arrows: dead cells)

Changes in the antioxidant defense system parameters are shown in Figure 2. Significant increases in the SOD activity in hepatocytes exposed to 50 and 100 µM BPA were observed. There was a further increase in the 50 µM BPA+Vitamin C-treated group compared to the BPA-treated groups. A statistically significant

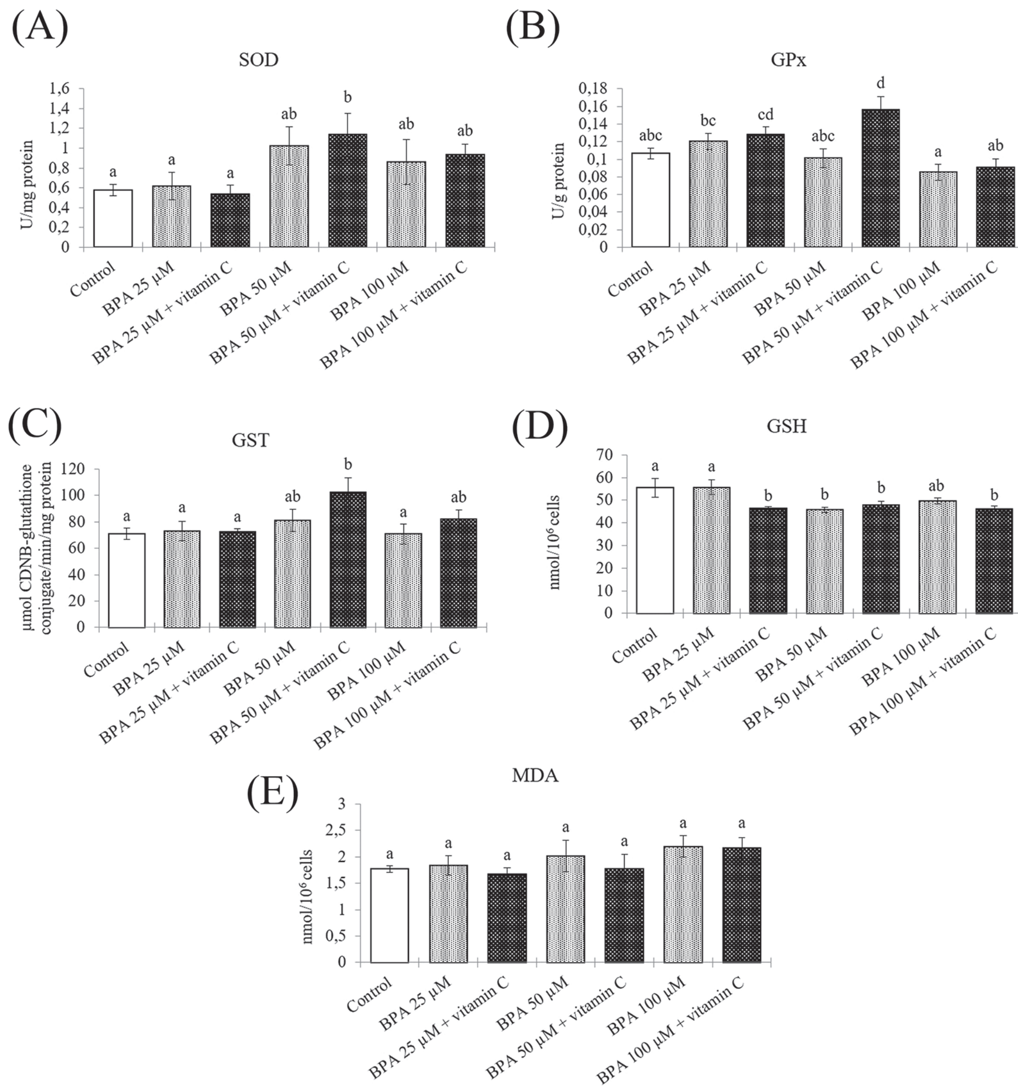

Fig. 2. Effect of BPA and co-administration of vitamin C along with BPA on the SOD activity (A), GPx activity (B), GST activity (C), GSH content (D) and MDA content of the isolated hepatocytes of pearl mullet *(A. tarichi)*. Values represent mean ± SEM. Different letters indicate significant differences between the groups using Duncan's multiple range test

increase was also observed in the 100 μM BPA+Vitamin C-treated group compared to the control (Fig. 2A).

The GPx activity significantly increased in the 25 μM BPA concentration while further increases were observed in the 25 μM BPA+Vitamin C- and 50 μM BPA+Vitamin C-treated groups. There was a significant decrease in the 100 μM BPA-treated cells. The same trend was also observed in the 25 μM BPA+Vitamin C-treated cells (Fig. 2B).

The GST activity was significantly increased with 50 μM BPA. Co-administration of 50 μM BPA and vitamin C showed a further increase in the GST activity. The GST activity was significantly induced by co-administration of vitamin C along with 100 μM BPA (Fig. 2C).

As shown in Figure 2D, there was a significant reduction in the GSH content of the 50- and 100 μM BPA-treated groups. On the other hand, significant decrease was also observed in all of the BPA+Vitamin C-treated groups.

Slight increases were also observed in the MDA level of the BPA- and BPA+Vitamin C-treated groups; however, those increases were not statistically significant (Fig. 2E).

DISCUSSION

In the present study, the effect of BPA on the hepatic antioxidant defense system was investigated using hepatocytes isolated from the pearl mullet *(A. tarichi)*. On the other hand, an in vitro antioxidant dose (50 μM) of vitamin C was applied to determine whether it prevents BPA-induced oxidative damage in the cells. Our results have shown that BPA could affect the antioxidant defense system after 24 h exposure. The SOD activity was significantly increased by 50 and 100 μM BPA. SOD generally converts the superoxide anion radicals into hydrogen peroxide and water, and is the primary step of the antioxidant defense system. Similar to our findings, Hulak et al. [17] reported increased SOD activities in the sterlet *(Acipencer ruthenus)* spermatozoa treated with BPA *in vitro*. The increases in the SOD activity could be due to superoxide anions [21]. GPx is the enzyme that reduces organic peroxides and hydroperoxides using GSH as a substrate [47]. The increased GPx activity may be attributed to the elevated levels of hydrogen peroxide and lipid peroxides [14, 46]. The reduction in the GPx activity could be due to the direct inhibitory effect of BPA on the enzyme. On the other hand, GPx activity is dependent on the GSH, and the decreased level of GPx activity might arise from the diminished availability of GSH sources, which are used for the elimination of free radical impact of the BPA [45]. GST is an enzyme that plays a role in the detoxification of xenobiotics by conjugating electrophilic metabolites into glutathione and protecting the cells from oxidative stress [11]. Elevated GST activity is possibly related to increased free radical production from the metabolism of BPA [22, 46]. GSH is a tripeptide that consists of *g*-glutamine, cysteine, and glycine, and is responsible for maintaining the redox status of the cells. It reacts directly with ROS and electrophilic compounds, and, protects

cells against toxicity [40]. The decreased GSH content, which indicates the oxidative stress that was observed in the present study, might be a result of ROS production [45]. On the other hand, utilization of GSH by GST and GPx might cause such decreases in the GSH pool [1]. Similar to our findings, it has been reported that BPA leads to decreased GSH levels [25].

MDA is a main product of lipid peroxidation and an increased MDA level is an important biomarker of oxidative injury [10]. It has been reported that BPA induces lipid peroxidation by the formation of hydroxyl radicals in fish [45]. In the present study, slight increase were observed in the MDA content after 24 h exposure to BPA; however, it was not statistically significant. Similar to our results, the increase in the MDA content in the tissues were not observed in mice, which were intraperitoneally injected with BPA (25 and 50 mg/kg/day for 5 days) despite a marked increase of ROS production [21]. On the other hand, in other studies, BPA has been reported to cause significant increases in lipid peroxidation in the liver of male rats orally exposed to BPA at doses of 0.2, 2.0, and 20 µg/kg/day for 30 days [5] and 25 mg/kg/day for 50 days [25]. The findings obtained in mammalian studies led us to conclude that the effect of BPA on lipid peroxidation might be changed depending on the experimental design and duration. Thus, the reason for the unchanged levels of the MDA content could be the short exposure time in the present study. Parallel to our findings, significant changes were not observed in the hepatocytes isolated from tilapia *(Oreochromis niloticus)* exposed to perfluorooctane sulfonate for 24 h, even with a significant induction of ROS generation [28].

Vitamin C is an important water-soluble antioxidant. Some *in vitro* studies have demonstrated that vitamin C protects cells against oxidative stress caused by different types of chemical compounds. It has been shown that vitamin C alleviated phenthoate-induced oxidative stress in freshly isolated mice hepatocytes [13]. Orta and Erkan [31] also reported the protective effect of vitamin C against oxidative damage by fluoride in TM4 Sertoli cells. Furthermore, vitamin C was reported to prevent BPA-induced ROS generation in the liver of male rats [5]. In the present study, an antioxidant concentration of vitamin C (50 µM) was administrated to the culture media, and a protective effect of vitamin C was not observed. Moreover, vitamin C was found to aggravate the negative BPA impact by leading to further increases in SOD, GPx, and GST activities. In addition, co-administration of vitamin C did not reverse the decreased GSH content in the hepatocytes. In accordance with our results, Korkmaz et al. [25] reported that vitamin C co-administration along with BPA aggravated oxidative damage in the liver of male rats. Such an effect of vitamin C in this study might be due to its prooxidant impact, where vitamin C converts Fe^{3+} into Fe^{2+}, which reacts with oxygen and hydrogen peroxide resulting in formation of hydroxyl radicals and superoxide anions [25, 36]. Supporting our results, Sakagami and Satoh [37] also reported that the addition of ascorbic acid to the culture medium increased dose-dependently the oxidation potential and acted as a prooxidant for the induction of apoptotic cell death. Thus, our findings may be the result of the prooxidant effect of vitamin C.

CONCLUSIONS

In conclusion, it was observed that BPA had toxic effects on isolated pearl mullet *(A. tarichi)* hepatocytes by disrupting the prooxidant/antioxidant balance. In addition, co-administration of vitamin C along with BPA was found to be ineffective against BPA-induced oxidative damage; conversely, it further aggravated the BPA impact. There is a need for more detailed studies to investigate the effect of vitamin C.

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