Benzo(a)pyrene-induced a mitochondria-independent apoptosis of liver in juvenile Chinese rare minnows (*Gobiocypris rarus*)[☆]

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ABSTRACT

To examine the effects of BaP on tissue apoptosis, laboratory studies were conducted using juvenile Chinese rare minnows (*Gobiocypris rarus*) exposed to 1, 5, 20, and 80 µg/L of BaP for 28 days. The post-treatment pathological findings in the liver were associated with hepatocyte swelling, karyopyknosis, and karyorrhexis. Moreover, an increase in the goblet cells in the intestine, epithelial hyperplasia of the gills and fusion of gill lamellae were observed. Significant increases in hepatocyte apoptosis using the TUNEL stain were observed in the liver tissue but not in the intestine and gills. In addition, BaP exposure significantly up-regulated the mRNA levels of *cyp1a1*, *p53*, *bax*, *bcl-2*, and *caspase-9* in the liver following the 5, 20, and 80 µg/L treatments, whereas the *apaf-1* was significantly down-regulated following all treatments. Moreover, the activities of caspase 3 and caspase 8 were markedly elevated, whereas the protein expression levels of Apaf-1 were down-regulated following the 20 and 80 µg/L treatments. Taken together, our results suggested that BaP strongly induces tissue-specific apoptosis *in vivo*, leading to significant pathological changes. The responsiveness of apoptotic-related genes demonstrates that BaP induced apoptosis in the liver may be through a mitochondria-independent pathway.

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1. Introduction

Apoptosis occurs normally during different development stages and as a homeostatic mechanism to maintain cell populations in tissues (Marino et al., 2014). The mechanisms of apoptosis are very complex and are mainly categorized into intrinsic and extrinsic pathways (Verbrugge et al., 2010). The intrinsic (mitochondrial)

pathway involves *bcl-2* pro-apoptotic proteins such as *bax*. This promotes the release of Cytochrome *c*, which is associated with the apoptotic protease activity factor-1 (*apaf1*) to form the apoptosome in the cytosol (Wu and Bratton, 2013). The apoptosome recruits Caspase-9, leading to its activation. Then, Caspase-3 initiates the caspase cascade and is cleaved by activated Caspase-9 (Chakraborty et al., 2012). Apoptosis also occurs as a defense mechanism, such as in immune reactions or when cells are damaged by disease or noxious agents (Aachoui et al., 2013; de Oliveira, 2016). Previously, inductions of apoptosis by PAHs and its metabolites *in vitro* had been reported (Chung et al., 2007; Souza et al., 2016).

Apoptosis was induced in the human lymphoblastic T-cell line L-MAT exposed to TCDD, and *caspase-3* activation via the Jun N-terminal kinase (JNK) was observed (Kikuchi et al., 2001). Furthermore, Kobayashi et al. (2009) reported that Ca²⁺/CaM signals might play an important role in the induction of apoptosis. In addition,

Abbreviations: BaP, benzo(a)pyrene; PAHs, polycyclic aromatic hydrocarbons; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; *apaf1*, apoptotic protease activity factor-1; AHR, aryl hydrocarbon receptor.

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PAH and its metabolites caused a rapid accumulation of the *p53* gene product in human and mouse cells (Hirano et al., 2013; Huang et al., 2012). However, the pathways by which *p53* leads to apoptosis are not well characterized, especially in fish.

Benzo(a)pyrene (BaP), a typical and extensively studied PAH, is widespread in our environment (Kim et al., 2013). The concentration of BaP in surface waters ranges from undetectable levels to 2.5 ng L^{-1} in the Henan Reach of the Yellow River (Sun et al., 2009). After a large-scale oil spill, nearly $20 \text{ } \mu\text{g mL}^{-1}$ ($79.37 \text{ } \mu\text{M}$) of BaP was detected in the near shore sea water environment of Mumbai (Ladwani et al., 2013). Importantly, carcinogenic and mutagenic effects of BaP in humans, animals and mammalian cell systems have been reported. In a previous study, serious pathological lesions were observed in liver sections of *Fundulus heteroclitus* after more than 8 months exposure to BaP (Wang et al., 2010; Wills et al., 2010). However, BaP itself is not genotoxic, its biotransformation by xenobiotic-metabolizing enzymes, including *cyp1a1* and *cyp1b1*, transforms it into a toxic form (Huang et al., 2015; Yuan et al., 2013). These enzymes can be mediated by binding the aryl hydrocarbon receptor (AHR) with its metabolic intermediates, which bind covalently to DNA and thus result in DNA damage (Huc et al., 2006; Souza et al., 2016). Moreover, in the HepG2 cell assay, BaP metabolites triggered the initiation of the expression of functional genes, including cytochrome *p450*, *mdm2*, and *p53* along with metabolic activation within the cells (Van Delft et al., 2012). Recently, significantly higher amounts of BaP metabolites were formed from the BaP-pretreated Trp53(−/−) mice (Krais et al., 2016). Therefore, this new role for *p53* in the metabolism of BaP found *in vivo* is very different from previous findings regarding its role in an *in vitro* setting (Hockley et al., 2008; Wohak et al., 2016).

Since there are obvious geographical features in the biological groups of water ecosystems in different regions, it is impossible to assess the effects of pollutants on specific areas of aquatic ecosystems by relying solely on one or several common patterns of organisms. Therefore, native species are more necessary to develop for environmental toxicological studies *in vivo* (Liang and Zha, 2016). Chinese rare minnows are Chinese native species and distributed mostly in the upstream region of the Yangtze River and in the Sichuan Province. This *Cypriniformes* species has been used extensively in recent years for aquatic toxicity testing (Chen et al., 2016; Yuan et al., 2013) and is considered an appropriate species for environmental toxicological studies due to its small size (30–80 mm total length), ease of culture, short life cycle, prolific egg production, and high fertilization and hatching rates.

In the present study, to investigate the underlying effects and mechanism of BaP-induced apoptosis on juvenile Chinese rare minnows at low concentrations, many toxicological endpoints, such as histology marking apoptosis of the liver, intestines, and gills, mRNA and protein expression changes, and enzyme activities of some key genes, were examined. We intend to investigate cellular apoptosis in different tissues of the rare minnow and thereby study the apoptotic pathway by which BaP exposure acts *in vivo*.

2. Materials and methods

All experimental protocols were approved by the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, and the methods were carried out in accordance with the approved guidelines.

2.1. Chemicals

BaP (purity > 98%) and acetone were purchased from AccuStandard Inc. (New Haven, CT, USA). A stock solution of BaP was

prepared by dilution in acetone. Final content of acetone in all of the exposure media was less than 0.01%.

2.2. Fish care

The rare minnow was kept in a flow-through system receiving charcoal-dechlorinated tap water (pH: 7.2–7.6; hardness: 44.0–61.0 mg CaCO_3) at a constant temperature of $25 \pm 1 \text{ } ^\circ\text{C}$, with a photoperiod of 16: 8 h (light: dark). All procedures for fish breeding were performed as described previously (Chen et al., 2016; Zha et al., 2007).

2.3. BaP exposure

Healthy juvenile rare minnows (3 months old) from the same pair of brood stock were used in this experiment. The average body weight and body length were $0.5 \pm 0.1 \text{ g}$ and $37.0 \pm 2.1 \text{ mm}$, respectively. Before exposure experiments were initiated, the fish were cultured for 2 weeks acclimation in 10 L glass tanks. Fish were exposed to BaP at different concentrations (0, 1, 5, 20, and $80 \text{ } \mu\text{g/L}$) with a solvent control. Each tank contained 15 fish, with 3 replicates were completed per treatment group. The exposure solutions were completely replaced once per day. Fish were fed twice a day with newly hatched brine shrimp. After 28 days exposure, the fish were removed from experimental tanks and anesthetized with MS-222 (Sigma, USA). Livers, intestines and gills were immediately sacrificed in each group and fixed in Bouin's solution (Sigma-Aldrich, USA), and the rest of the rare minnow samples were flash-frozen in liquid nitrogen and stored at $-80 \text{ } ^\circ\text{C}$ for further analysis.

2.4. Water concentrations of BaP

BaP concentrations in the exposure solutions were analyzed by gas chromatography-mass spectrometry (GC-MSD) (Wang et al., 2007). Briefly, water samples were filtered using Millipore glass microfiber filters ($0.7 \text{ } \mu\text{m}$, F142 mm, Millipore, USA) and enriched by SPE with C18 coupled with HLB cartridges for 24 h. The cartridges were eluted by DCM and concentrated under a stream of nitrogen. The samples were analyzed using an Agilent 6890 GC connected to an Agilent MSD 5975 mass selective detector (Agilent Technologies, USA). After 24 h of the test initiation, the concentrations (mean $\pm$ standard deviation; % analyzed/nominal) of BaP in the solutions were 0.8 ± 0.1 (80%), 3.8 ± 0.9 (76%), 14.5 ± 3 (72.5%), and 69.8 ± 8.8 (69.8%) $\mu\text{g/L}$. Nominal BaP values are used in the following text.

2.5. Histology

After 30 days exposure, the liver, intestine, and gill of rare minnows were dissected and fixed in Bouin's solution at $4 \text{ } ^\circ\text{C}$ overnight. Then the fixed tissues were dehydrated in gradient ethanol (50%, 70%, 75%, 85%, 90%, 95%, and 100%), hyalinized in xylene, and embedded in paraffin wax at $55 \text{ } ^\circ\text{C}$. Sections were cut at $5 \text{ } \mu\text{m}$, stained with an H&E Staining Kit purchased from Beyotime Institute of Biotechnology (Shanghai, China). An optical microscope (Zeiss, Germany) equipped with a digital camera, was used to observe the histologic lesions.

2.6. TUNEL assay, Hoechst 33342 staining, and quantitation of apoptosis

Tissues were immersed in 4% paraformaldehyde in a 0.1 mol/L phosphate buffer. Fixation was carried out at $4 \text{ } ^\circ\text{C}$ over night. Next, tissues were paraffin-embedded, cut into $5\text{-}\mu\text{m}$ -thick transverse sections and mounted on slides. Then, In Situ Cell Death Detection

Kit (Roche, Switzerland) was used to detect the single- and double-strand DNA breaks following the manufacturer's instructions (TUNEL assay). Finally, tissues were counterstained with Hoechst 33342 (Sigma, USA) for 5 min at room temperature and examined with a fluorescence microscope (OLYMPUS).

A quantitative method to assess TUNEL-positive cells was performed according to Soutschek et al. (Soutschek and Zupanc, 1996). The number of TUNEL-positive cells found in each section was corrected for split-cell errors through multiplication by a correction factor.

$$Ni = ni \times \frac{t}{t + d}$$

where N_i is the number of TUNEL positive cells present in section i , n_i is the number of labeled cells actually counted in section i , t is the section thickness, and d is the mean diameter of the TUNEL-positive nuclear fragment in a cell. Ten fields at 400 $\times$ magnification were counted per slide, the percentage of apoptotic cells in each field was calculated.

2.7. RT-qPCR

The expression levels of 7 genes (*cyp1a1*, *p53*, *bcl-2*, *bax*, *caspase-3*, *caspase-9*, and *apaf-1*) were measured by RT-qPCR. The amplification parameters were performed for 95 °C for 10 min, 40 cycles at 95 °C for 10s, 60 °C for 10s, and 72 °C for 10s. Each sample was run in triplicate. The primers in this study are shown in Table S1 (Yuan et al., 2016). The PCR efficiencies ranged from 93.2% to 103.0% in the present study. RT-qPCR experiments were performed on a Mx3005P real-time quantitative polymerase chain reaction system (Stratagene, USA). Differences were calculated according to the $2^{-\Delta\Delta Ct}$ method relative quantization method using β -actin as an internal control.

2.8. Caspase 3, caspase 8, and caspase 9 activity assays

The activity of caspase 3, caspase 8, and caspase 9 was determined using the caspase 3, caspase 8, and caspase 9 activity kit (Beyotime Institute of Biotechnology, China). The liver tissue sample lysates were prepared in the lysis buffer from the assay kits and were centrifuged at 12,000 rpm for 15 min at 4 °C. Then, the supernatants were collected and incubated at 37.8 °C for 2 h. The caspase activities were measured in a 96-well plate at an absorbance of 405 nm. The caspase activities were determined by a colorimetric assay based on the ability of caspase 3, caspase 8, and caspase 9 to change acetylAsp-Glu-Val-Aspp-nitroanilide (Ac-DEVD-pNA) and acetyl-Leu-Glu-His-Aspp-nitroanilide (Ac-LEHD-pNA) into a yellow formazan product (*p*-nitroaniline (pNA)). All results were expressed in nmol/h/prot.

2.9. Protein extraction and western blot analysis

Protein extraction was performed using 50 mg of frozen hepatic tissue per sample. The juvenile Chinese rare minnow livers were homogenized and sonicated intermittently in 500 μ L of ice-cold lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4% w/v CHAPS, 1% w/v DTT) containing a 10- μ L cocktail of protease inhibitors (M221, Amresco, USA) on ice for 5 min. Insoluble debris was removed by centrifugation at 12,000 $\times$ g for 20 min at 4 °C, and the supernatant was collected. The supernatant was stored at -80 °C, and the total protein concentration was determined by a Bradford assay using a Protein Assay Kit II with a BSA standard (Bio-Rad).

Proteins from the livers of Chinese rare minnows were extracted and separated by SDS-PAGE and transferred to PVDF membranes

(Millipore). These blots were incubated for 1 h at room temperature in TBST containing 5% skim milk. The anti-p53 polyclonal (#9282, diluted 1:1000, Cell Signaling Technology), anti-Apaf-1 polyclonal (sc-8339, diluted 1:200, Santa Cruz), and anti-actin polyclonal (#4967, diluted 1:1000, Cell Signaling Technology) primary antibodies were used. The blots were labeled with horseradish peroxidase-conjugated secondary antibody to rabbit IgG and visualized by ECL reagents (Pierce). Images were captured by ChemiDoc-It[®] 415 Imager (UVP, USA) and analyzed by Vision-WorksLS Image Acquisition and Analysis Software.

2.10. Statistical analysis

All data were analyzed with SPSS 16.0 software (SPSS, Chicago, IL, USA) and expressed as the mean $\pm$ standard error of the mean (S.E.M.). Significant differences between different treatment groups were identified on the basis of $P < 0.05$ using one-way analysis of variance (ANOVA) and *post hoc* least significant difference (LSD) test.

3. Results

3.1. Histopathology observation

3.1.1. Hepatic tissue

The normal hepatic tissues showed closely packed polygonal hepatocytes in a regular array with well-defined nuclei (Fig. 1A). After 28 days of BaP exposure, hypertrophy of the hepatocytes from all treatment groups was observed (Fig. 1C). In addition, karyopyknosis and karyorrhexis of the hepatocytes from the 20 and 80 μ g/L treatment groups were observed (Fig. 1B and C). These pathological changes in the liver cells following BaP treatments suggest that this compound seriously damages the liver tissues of Chinese rare minnow.

3.1.2. Intestinal tissue

Healthy intestinal tissue exhibits the structures of the intestinal wall and is composed of tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (Fig. 1D). Intestinal mucosa displays many villi and a simple columnar epithelium, which are associated with goblet cells and intra-epithelial lymphocytes (Fig. 1D). The goblet cells exhibited a supranuclear region characterized by a swollen distal region containing a translucent cytoplasm and a basal region with associated nuclei. Intra-epithelial lymphocytes were small oval to round cells with deeply stained nuclei. After 28 days of BaP exposure, the abnormal intestine tissue from treatments showed that the number of goblet cells and physaliphores in the intestinal mucosa significantly increased (Fig. 1E). Moreover, ecclasis of the striated border of the intestinal mucosa from the 20 and 80 μ g/L treatments was also observed (Fig. 1F). The pathological characteristics of the intestinal tissue suggested that BaP caused intestinal tissue toxicity in Chinese rare minnows.

3.1.3. Gill tissue

The structure of normal gill tissues exhibited well-shaped primary lamellae with cartilaginous tissue at the bottom and secondary lamellae. The gill's secondary lamellae are normally lined with squamous epithelial cells, which are one or two layers thick and contain a blood sinus spanned by pillar cells (Fig. 1G). After 28 days of exposure of BaP, no significant histopathological changes were observed between the gills between the 1 and 5 μ g/L treatments (data not shown). However, characteristic inflammatory and epithelial hyperplasia of the gill lamellae from the 20 and 80 μ g/L treatments were observed (Fig. 1H). Similarly, the histopathological results showed that BaP caused gill tissue lesions to occur at relatively higher concentrations.

Fig. 1. Photomicrographs H&E-stained paraffin sections showing liver, gill, and intestinal pathology in rare minnow juveniles 3 months after exposure to BaP ($\times 200$). (A) Normal hepatic tissue; (B) Abnormal hepatic tissues from the 20 $\mu\text{g/L}$ BaP treatment condition; the arrow indicates the karyopyknosis of the cell nuclei; (C) Abnormal hepatic tissues from the 80 $\mu\text{g/L}$ BaP treatment condition; the arrow indicates the turgescence of the hepatic cells; (D) Normal intestinal tissue; (E) Abnormal intestinal tissue from the 20 $\mu\text{g/L}$ BaP treatment condition; the arrow indicates the increase in the goblet cells of the intestinal mucosa; (F) Abnormal intestinal tissue from the 80 $\mu\text{g/L}$ BaP treatment condition; the arrow indicates ecclasis of the striated border of the intestinal mucosa; (G) Normal gill tissue; (H) Abnormal gill tissues from the 80 $\mu\text{g/L}$ BaP treatments; the arrow indicates the epithelial hyperplasia of the gills.

3.2. BaP-induced hepatocyte apoptosis can be detected by TUNEL/Hoechst 33342 double staining

TUNEL-positive cells were identified by their red-stained nuclei. No TUNEL-positive cells were observed in the Hoechst 33342 staining cells (Fig. 2 and Fig. S1). After 28 days of BaP exposure, TUNEL-positive cells in the livers were clearly observed in all treatments (Fig. 2). These results showed that BaP significantly induced hepatocyte apoptosis in Chinese rare minnows. Similarly, these results are consistent with the levels of karyopyknosis and karyorrhexis of hepatocytes observed using the HE stain. The rates of apoptosis of hepatocytes increased with the BaP exposure concentrations. Chinese rare minnows subjected to higher treatments had significantly higher numbers of abnormal hepatocytes than the

controls (Fig. 3). However, the rates of apoptosis in the gills and intestines from treatments did not show any significant differences with the controls (data not shown).

3.3. Measurement of apoptosis-related gene expression levels by RT-qPCR

The mRNA expression of apoptosis-related genes, including *cyp1a1*, *p53*, *bax*, *bcl-2*, *caspase-3*, *caspase-9*, and *apaf-1* in the livers of Chinese rare minnows, were determined after 28 days of BaP exposure (Figs. 4 and 5). No significant differences were observed between the mRNA expression levels of the vehicle control and the water control in any of the genes assessed (data not shown). A strong induction of *cyp1a1* following the 20 and 80 $\mu\text{g/L}$ treatments

Fig. 2. TUNEL and Hoechst 33242 staining revealed the BaP-induced apoptosis of hepatic tissues of rare minnows. The figure shows the control group and 80 µg/L BaP treatment group. More details about the remaining treatment groups in Fig. S1. The induction of apoptosis was determined by fluorescence microscopy; scale bars = 100 µm.

Fig. 3. Apoptotic index in the liver of Chinese rare minnows after exposure to benzo(a) pyrene for 28 d. The data are expressed as the mean $\pm$ S.E.M. of each treatment ($n = 6$). Significant differences between groups at $p < 0.05$ (ANOVA) are indicated by different letters.

(8.30-fold and 46.33-fold, respectively) was observed (Fig. 4). The p53 mRNA level significantly increased with treatments higher than 5 µg/L (Fig. 4).

The mRNA level of the pro-apoptotic protein *bax* (the *bcl2* associated *x* protein) was significantly up-regulated with treatment concentrations higher than 5 µg/L, with increases of 2.19 fold, 2.34 fold, and 4.02 fold (Fig. 4). However, the anti-apoptotic protein *bcl-2* mRNA levels in livers with 5, 20 and 80 µg/L BaP treatments increased by 1.97 fold, 1.52 fold, and 1.30 fold, respectively. In contrast, the mRNA levels of *apaf-1* were significantly down-regulated at all treatments, with decreases of 0.46, 0.42, 0.39 and 0.47 fold with 1, 5, 20, and 80 µg/L BaP treatments, respectively (Fig. 5).

The mRNA expression levels of the two key genes (*caspase-9* and *caspase-3*) of the caspase pathway were also determined. The mRNA level of *caspase-9* following 80 µg/L BaP treatments was significantly up-regulated compared to the control group (Fig. 5). However, the mRNA level of *caspase-3* in the treatments was not significantly different with that of the controls (Fig. 5).

3.4. Effects of BaP on the enzyme activity of caspase 3, caspase 8, and caspase 9

To ascertain whether BaP-induced hepatocyte apoptosis occurred via a mitochondria-dependent pathway, we analyzed the effects of BaP on the enzyme activity levels of caspase 3, caspase 8, and caspase 9. As shown in Fig. 6, the activity of caspase 3 in all treatment groups was induced in a dose-dependent manner after 28 days of exposure to BaP. Indeed, two of the high concentration groups (20.0 and 80.0 µg/L BaP) were significantly up-regulated (Fig. 6), which was consistent with the results observed in the TUNEL assay (Figs. 2 and 3).

The enzyme activity of caspase 9 and caspase 8 was also investigated, as these are the critical enzymes of the mitochondria-dependent and mitochondria-independent pathways of apoptosis, respectively. The obtained results demonstrate that BaP significantly increased the enzyme activity levels of caspase 8 in all treatments, yet caspase 9 was found to have a dramatic up-regulation in the low concentration group only (20 and 80 µg/L; Fig. 6).

3.5. Apoptosis-related protein expression

To further illustrate the apoptotic signaling after 28 days of BaP exposure, the protein expression levels of P53 and Apaf1 were investigated. As shown in Fig. 7, BaP induced a significant increase in P53 in the livers following the 5 µg/L BaP treatments. Apaf1 was effectively inhibited by the 20 and 80 µg/L BaP treatments.

4. Discussion

The mechanism of cell apoptosis has been demonstrated for most polycyclic aromatic hydrocarbons (PAHs), including benzo(a) pyrene, and more information is needed concerning the role of low concentrations and chronic exposure, especially *in vivo*. The present investigation suggests that exposure to high concentrations of BaP for 28 days caused severe tissue toxicity and significantly induced cell apoptosis in the liver through the high responsiveness of apoptosis-related genes, proteins, and enzymes in the livers of juvenile Chinese rare minnows.

In the present study, severe pathological changes in the livers, gills, and intestines of juvenile Chinese rare minnows were observed following exposure to 20 and 80 µg/L of BaP (Fig. 1), and these results generally concur with the results of studies conducted

Fig. 4. Relative expression levels of *cyp1a*, *bax*, *p53* and *bcl-2* in Chinese rare minnows after 28 days of exposure to BaP. The bars represent the relative mRNA expression in the exposed fish versus control fish. β -actin was used for normalization. Values are presented as the mean $\pm$ S.E.M. Significant differences between the groups at $p < 0.05$ (ANOVA) are indicated by different letters.

Fig. 5. Relative expression levels of *caspase-3*, *caspase-9*, and *apaf-1* in Chinese rare minnows after 28 days of exposure to BaP. The bars represent the relative mRNA expression in the exposed fish versus the control fish. β -actin was used for normalization. Values are presented as the mean $\pm$ S.E.M. Significant differences between groups at $p < 0.05$ (ANOVA) are indicated by different letters.

previously using higher BaP concentrations (Wang et al., 2010; Wills et al., 2010). Pathological changes in the liver cells of Chinese rare minnows exposed to more than 5 $\mu\text{g/L}$ BaP were associated with cell tumescence, karyopyknosis and karyorrhexis (Fig. 1). These changes are identical to those previously observed in Arctic charr (*Salvelinus alpinus*) (Ribeiro et al., 2007) and rainbow trout (*Oncorhynchus mykiss*) (Möller et al., 2014). In this study, goblet cells of the intestinal mucosa from Chinese rare minnows exposed to more than 20 $\mu\text{g/L}$ of BaP significantly increased. Similar pathological changes were also observed in *Anabas testudineus* exposed

to almix (Samanta et al., 2016) and *Cyprinus carpio* exposed to effluent (Patil and Ghole, 2010). In addition, histopathological observations of the gills from Chinese rare minnows exposed to 20 and 80 $\mu\text{g/L}$ of BaP revealed epithelial hyperplasia and amalgamation of secondary lamellae (Fig. 1). The changes were indicative of a diminished oxygen supply to the test fish, resulting in hypoxic respiratory responses (Abalaka et al., 2015; Das and Mukherjee, 2000). Therefore, our present findings provide evidence for hyperplasia as a protective mechanism against environmental irritants.

Fig. 6. The activity of caspase 3, caspase 8 and caspase 9 in the livers of Chinese rare minnows after exposure to BaP for 30 days. The data are expressed as the mean $\pm$ S.E.M. Significant differences between groups at $p < 0.05$ (ANOVA) are indicated by different letters.

Fig. 7. Protein levels of p53 and apaf-1 in Chinese rare minnows after 28 days of exposure to BaP. (a) Effects of BaP on the protein levels of p53 and apaf-1 were examined by Western blotting analysis using the corresponding antibodies. (b) Bands corresponding to the proteins were standardized by beta-actin, and densitometry data of three independent experiments are presented. The data are expressed as the mean $\pm$ S.E.M.

Apoptosis is critical for normal development and occurs in many developmental stages in invertebrates and vertebrates. In previous studies, BaP induced apoptosis in RL95-2 human endometrial cancer cells (Kim et al., 2007) and lung cancer A549 cells (Lin et al., 2015). Similarly, a significant increase in hepatocyte apoptosis was observed in the present study with juvenile Chinese rare minnows exposed to BaP (Figs. 2 and 3), although apoptosis was not increased in the gills and intestines. These different results are likely due to differences in the functionality of these various tissues. Additionally, BaP induced hepatocyte apoptosis after sole (*Solea senegalensis*) were injected with approximately 5 nmol BaP g⁻¹ fish wet mass for 24 h incubation (Costa et al., 2010). Moreover, the lower percentage of apoptotic cells observed when compared to our results (Fig. 3) could be due to the shorter exposure time and lower concentrations (Costa et al., 2010). In addition, our present results show that BaP induced hepatocyte apoptosis in juvenile Chinese rare minnows in a dose dependent manner, and this result agrees with that of a previous study *in vitro* (Yoo et al., 2003). The present finding confirms different responses in different tissues of Chinese rare minnows exposed to BaP.

The biological impact of BaP depends on the functional activation of AHR, which induces the transcriptional activation of genes involved in drug metabolism (Huc et al., 2006; Souza et al., 2016).

Cyp1a1, the major drug-metabolizing enzyme, is one of the genes known to be activated, and it acts on BaP to produce the ultimate carcinogen BPDE-1 (Schmidt and Bradfield, 1996; Yuan et al., 2013). Our study has demonstrated that BaP strongly induces increased mRNA levels of *cyp1a1* (Fig. 4). Similarly, *cyp1a1* has been shown to be activated in RL95-2 human endometrial cancer cells exposed to BaP (Kim et al., 2007). It was observed that BaP metabolites cause DNA damage, which in turn initiates the transcriptional activation of many genes, such as *p53* (Huc et al., 2006; Van Delft et al., 2012). In this study, the mRNA levels of *p53* were found to be significantly up-regulated, with an increase of 3.16 fold following exposure to the 80 µg/L BaP concentration (Fig. 4). Previous studies have indicated that the activation of the *p53*-related signaling pathway involves the induction of apoptosis *in vitro* (Lin et al., 2008). Protein levels of *p53* were not observed to be significantly up-regulated following the 20 and 80 µg/L BaP treatments (Fig. 7). Nevertheless, studies in human hepatoma HepG2 cells have shown that BaP exposure induced an obvious increase in phosphorylated P53 (Donauer et al., 2012; Park et al., 2006). In summary, our findings also reveal that the *p53*-related signaling pathway is activated *in vivo*.

The *bcl2* proteins are known to regulate the intrinsic pathway; moreover, the ratios between the *bcl-2* and *bax* proteins would change mitochondrial cytochrome *c* release. Cytochrome *c* associates

with *apaf-1* to form the apoptosome in the cytosol (Stenson-Cox et al., 2003). In the present study, the mRNA level of *bcl-2* increased at higher concentrations of BaP, whereas the *apaf-1* level decreased significantly with all concentrations (Figs. 4 and 5). Moreover, the protein levels of Apaf-1 were also down-regulated in the 20 and 80 µg/L BaP treatment conditions (Fig. 7) and no obvious changes were observed in the enzyme activity of caspase 9, except in the lowest concentration group (Fig. 6). Therefore, these results suggested that it could be difficult to form the apoptosome in the cytosol and could induce caspase-independent apoptosis through the mitochondrial pathway. Additionally, there is no significant differences in the expression of *caspase-3*, whereas a significant increase of *caspase-9* was observed only with 1 µg/L BaP (Fig. 5). Our findings suggest that caspase 9 may not contribute to the activation of the mitochondria-dependent signaling pathway leading to hepatocyte apoptosis induced by 20 and 80 µg/L of BaP. Similarly, Holme et al. (2007) reported that BaP induces the caspase-independent apoptosis of hepatic F258 cells by blocking the intracellular translocation of *bax* and the *cytochrome c* protein. In addition, previous studies have showed that B1-induced caspase-independent apoptosis in MCF-7 was mediated by the down-regulation of *Bcl-2* (Liang et al., 2011). Nevertheless, a recent study showed that BaP-induced apoptosis of mouse livers occurs via the activation of the intrinsic caspase cascade and mitochondrial dysfunction (Qin et al., 2015). Thus, it should be noted that the BaP-induced caspase independent apoptosis *in vivo* is very different *in vitro*. Accordingly, to further confirm the apoptosis pathway of BaP *in vivo*, the caspase 8 activity assay in our study was determined, and we found that it was significantly activated by exposure to BaP in all treatments (Fig. 6). This indicates that both intrinsic and extrinsic caspase-independent apoptosis pathways are activated by BaP in the low concentration groups. However, the intrinsic caspase-independent apoptosis pathway was weakened by the decrease of the Apaf-1 protein expression levels in high concentration groups. Briefly, the pathways of hepatocyte apoptosis induced by different concentrations of BaP were different. Despite this, responses to apoptosis-related genes would be useful in order to discover the apoptosis pathways that occur in response to environmental contaminants *in vivo*.

5. Conclusion

In summary, juvenile Chinese rare minnows exposed to BaP for 28 days show significant histopathological changes, induced apoptosis, and changes in the expression of apoptosis-related genes. Moreover, BaP significantly induced apoptosis in the liver but not in the gill or intestine. Our findings further elucidated the BaP-induced caspase dependent apoptosis pathway and found differences in the intrinsic caspase dependent pathways mediated by different concentrations of BaP. Despite this, more studies are warranted to study the gene expression and protein level changes that occur in cell apoptosis pathways *in vivo*.

Conflict of interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.08.005>.

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