

Research Article

A rapid approach to assess estrogenic transcriptional activity of bisphenol A in the liver of goldfish

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Abstract

Bisphenol A (BPA) is a synthetic compound widely used in types of consumer goods and medical tools. It has been shown that BPA acts as an endocrine disruptor chemical causing negative impact on reproductive functions. The present study used an *in vitro* approach to assess estrogenic transcriptional activity of BPA as a rapid and sensitive method. The cultured pieces of hepatic tissue of goldfish (*Carassius auratus*) were exposed to 1, 5, 25 $\mu\text{g L}^{-1}$ BPA and 1 $\mu\text{g L}^{-1}$ E₂ for comparison over 24 h. Compared to control, the mRNA transcript of *er β -I* showed a significant increase in the hepatic tissues exposed to all doses of BPA and E₂. The mRNA transcript of *er β -II* was significantly increased in hepatic tissues exposed to 1 and 5 $\mu\text{g L}^{-1}$ BPA and E₂. The mRNA transcripts of *vtg* in the hepatic tissues treated with 5 $\mu\text{g L}^{-1}$ BPA and E₂ were also increased, significantly. Finally, *cyp1a* mRNA transcript showed a significant increase in the hepatic tissues exposed to 5 and 25 $\mu\text{g L}^{-1}$ BPA and E₂. These results show an estrogenic activity of BPA similar to E₂, and suggest that *in vitro* approach can be used as an alternative to *in vivo* test to detect estrogenic effects of BPA.

Keywords: BPA, Esterogenic activity, Vitellogenin, mRNA transcript

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Introduction

Bisphenol A (BPA) is synthesized by catalyzed condensation of acetone and phenol (Prokop *et al.*, 2004). It is highly produced and used as a precursor of plastics for consumer goods (including food-packaging materials, surface coatings, and toys) and medical tools (including heart-lung machines, incubators, hemodialyzers, and dental sealants and fillers) (Liao and Kannan, 2011; Huang *et al.*, 2012; Liao *et al.*, 2012).

Exposure to BPA is linked to a wide range of health problems including reproductive impairments such as testicular dysgenesis syndrome, hormonal disorders, follicular loose, cryptorchidism, and diminished gamete quality in males and females, which may result in decreasing fertility (Richter *et al.*, 2007; Rochester 2013; Kundakovic *et al.*, 2013; Zhou *et al.*, 2016). In addition, BPA-caused reproductive impairments which could be transgenerationally transmitted by epigenetic mechanisms (Dolinoy *et al.*, 2007; Prins *et al.*, 2008; Abdel-Maksoud *et al.*, 2015; Kumar and Thakur, 2017; Bansal *et al.*, 2019).

In the aquatic environments, BPA originates from BPA-based products, effluents of landfill sites and wastewater treatment plants, or natural degradation. The environmental concentrations of BPA have been frequently measured which range from less than 1 $\mu\text{g/L}$ to higher than 21 $\mu\text{g/L}$ in hot spots (Belfroid *et al.*, 2002; Wintgens *et al.*, 2003; Gatidou *et al.*,

2007; Staples *et al.*, 2018; Xu *et al.*, 2018). In fish, BPA has been also detected, for instance its concentration in the body of fish caught around Taiwan, the Gulf of Naples in Italy, and local market of Hongkong were measured to be 0.2-25.2 (Lee *et al.*, 2015), 0.5-6.0 (Mita *et al.*, 2011) and 0.8-19.3 mg kg^{-1} (Wong *et al.*, 2017), respectively.

Similar to higher vertebrates, studies have shown that *in vivo* exposure of fish to BPA causes reproductive disorders. In males, BPA disrupts hormonal functions of the hypothalmo-pituitary-testis (HPT) axis (Lindholst *et al.*, 2000; Mandich *et al.*, 2007), which results in diminished sperm quality (Lahnsteiner *et al.*, 2005; Hatfeg *et al.*, 2012a, b). However there are still questions that remain unanswered to identify the modes of action of BPA on reproductive system.

In vertebrates, estrogens are key regulatory hormones in development and functions of the reproductive system, and their physiological functions are transmitted to target cells by intracellular estrogen receptors (ERs) (Nilsson *et al.*, 2001; Gustafsson, 2003; Heldring *et al.*, 2007). In fish, 17 β -estradiol (E_2) regulates vitellogenin (Vtg) synthesis in the liver, which is essential for the ovarian development in females (Hara *et al.*, 2016). It has been well demonstrated that exposure to or treatment with endogenous or exogenous estrogens induces Vtg production mediated by ERs in males, which may cause an intersex (the

presence of testis-ova) (Sumpter and Jobling, 1995; Wheeler *et al.*, 2005; Hutchinson *et al.*, 2006; Miyagawa *et al.*, 2014). Although, occurrence of intersex has been reported in fish exposed to BPA, *in vivo* (Metcalf *et al.*, 2001; Mandich *et al.*, 2007), however there are controversies in the estrogenic-like activity of BPA. *In vitro* studies have revealed a weak binding affinity of BPA to ERs (Gould *et al.*, 1998; Kuiper *et al.*, 1998).

To better understand BPA estrogenic-like activity using an *in vivo* protocol, it needs to manipulate duration of exposure, use a large number of sample size, examine various concentrations of BPA, and control the developmental stages. Therefore, as the Organization for Economic and Cooperation Development (OECD) suggested (Lee *et al.*, 2014), application of an *in vitro* protocol would be useful to control experimental condition, and run more tests with consideration to fish welfare. The aim of the present study was to investigate the estrogenic-like activity of BPA using an *in vitro* protocol. We examined the effects of different concentrations of BPA on transcriptional activity of *vtg*, *erβ-I*, and *erβ-II* as well as cytochrome p450 (*cyp1a*) in the cultured pieces of liver in goldfish (*Carassius auratus*). These genes are known as powerful biomarkers for estrogenic activity and toxicity of environmental contaminants (Hiramatsu *et al.*, 2006; Kim *et al.*, 2008; Søfteland *et al.*, 2010). This study provides a simple, sensitive and high

throughput screening method to investigate potential of hormonal-like activity of environmental contaminants.

Materials and methods

Exposure and experimental design

Experimental groups were composed of BPA (1, 5 and 25 $\mu\text{g L}^{-1}$), E₂ (1 $\mu\text{g L}^{-1}$), solvent control, and control. Bisphenol A and E₂ were dissolved in EtOH (Sigma Aldrich). Twenty male and female goldfish (mean body mass of 32 g and total length of 8-12 cm) imported to the laboratory and maintained in flow-through tanks to acclimize for around 5 days. Water temperature was kept approximately 20°C and photoperiod was 12 h light/12 h dark. This study was carried out in strict accordance with the recommendations in the Guide for the Care, Protection and Use of Laboratory Animals of the Canadian Council and the Ministry of Health of the Czech Republic approved by the Ethics Committee of the University of Calgary Animal Care Committees and by the Central Ethics Committee of the Ministry of Agriculture of the Czech Republic (§17, article 1 of the law 246/1992 Sb), respectively. All fish were anesthetized before being sacrificed to minimize suffering.

The liver was collected from male goldfish (12 individuals), washed in fresh media, and cut by scalpel into 1–3 mm³ pieces for culturing. Under a sterile hood, the pieces were washed again for several times with culture medium solution (M199 with Hanks

salts and containing 100 U ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin; Gibco). The liver pieces were added in equal amounts (approximately 50 mg) to each well of a 12-well plate and a total of 4 mL of prepared culture media was added containing BPA or E₂. All treatments were replicated in six separate experiments using different well preparations. Twenty-four h post-treatment, the media was discarded and the tissue pieces were collected in RNase free Eppendorf tubes containing 1 mL Trizol Reagent (Invitrogen, Cat. No. 15596-018), and frozen at -80°C until use. Data of control without EtOH did not show any significant difference with those of solvent control (data are not shown). Therefore, only the data for solvent control were used as reference for comparison with respective treatments.

RNA extraction and complementary DNA (cDNA) synthesis

Tissue was homogenized in Trizol, and chloroform was added. After mixing, samples were centrifuged (12,000 g for 15 min) and supernatants were transferred into the new tubes containing an equal volume of isopropanol. The mixture was centrifuged (12,000 g for 10 min), and the precipitated RNA was washed using 1 mL EtOH 75%. Total RNA concentration was estimated from absorbance at 260 nm (A₂₆₀ nm, Nanodrop, USA). The RNA quality was verified by A₂₆₀ nm/A₂₈₀ nm ratios

between 1.8 and 2 and A₂₃₀ nm/A₂₆₀ nm ratios higher than 2.

The cDNA was synthesized from 4 µg of total RNA of each sample using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Invitrogen, Cat No. 28025-013) and oligo (dT)₁₈ primer (Promega, Madison, WI, USA) following the manufacturer's instructions. Two µl of oligo (dT)₁₈ primer (500 µg ml⁻¹) was added to each sample and the reaction mixture was heated to 70 °C for 10 min, and then quickly chilled to 4 °C. After cooling, 4 µl of 5× first-strand buffer, 2 µl DTT (100 mM), 0.4 µl dNTP (100 mM) (Cat. No. dNTP-01, UBI Life Science, Canada) and 0.7 µl M-MLV (200 U µl⁻¹) and nuclease free water were added to a total volume of 18 µl. The reaction mixture was then incubated at 25 °C for 10 min and at 37 °C for 50 min using iQ cycler. The reaction was deactivated by heating at 70 °C for 15 min. A negative control was run for each sample. Each 18 µl reaction was diluted 3-fold in nuclease free water and used for quantitative real time PCR assay. iCycler iQ Real-time PCR Detection System (Bio-Rad Lab. Inc.) was used for studying mRNA levels with the following condition per reaction: 1 µl of diluted cDNA, 6.25 µM of each primer (0.26 µL of forward and reverse primers), 12.5 µl SYBR Green PCR Master Mix (Qiagen Mississauga, Canada) and ultrapure distilled water (Invitrogen) to a total volume of 25 µl. Primers for all genes were designed using NCBI tool Primer BLAST with

reference to the known goldfish sequences. Primer efficiencies on serial dilutions of cDNA were all between 90% and 100%. Table 1 shows the sequences of specific primers used in this study. PCR was run in the following cycling conditions: initial denaturation at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C, appropriate annealing temperatures for 31 s. The specificity of the amplified product in the quantitative PCR assay was determined by analyzing the melting curve to discriminate target amplicon from primer dimer or other non-specific

products. A single melt curve was observed for each primer set in all quantitative PCR reactions. No amplification product was also observed in non-template controls as well as no primer-dimer formations. Each sample was run in triplicate and the mean threshold cycles (determined by the linear portion of the fluorescence absorbance curve) were used for the final calculation. The mRNA levels were normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) using the standard $2^{-\Delta\Delta Ct}$ method.

Table 1: Primer sequences and annealing temperature applied for studying alternations in mRNA transcript of selected genes using qRT-PCR in the present study.

Genes	Forward Primer	Reverse Primer	Annealing temperature (°C)	GenBank Accession
<i>vtg</i>	GAAGTGCGCATGGTGGCTTGTATT	AGCTGCCATATCAGGAGCAGTGAT	55	DQ641252
<i>erβ - I</i>	GGCAGGATGAGAACAAGTGG	GTAAATCTCGGGTGGCTCTG	55	AF061269
<i>erβ - II</i>	GGATTATTCACCACCGCACG	TTCGGACACAGGAGGATGAG	55	AF177465
<i>cyp1a</i>	TGCCCTTGAGGAGCACATCAGC	CGTCGTCGTGGCTGTAGCG	58	DQ517445
<i>gapdh</i>	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57	AY641443

Genes: *vitellogenin (vtg)*, *estrogen receptor subunit β-I (erβ - I)*, *estrogen receptor subunit β-II (erβ - II)*, *cytochrome p450 1a (cyp1a)*.

House keeping gene: *glyceraldehyde 3-phosphate dehydrogenase (gapdh)*.

Statistical analyses

Homogeneity of variance and normal distribution of data were tested using Levene's and Kolmogorov–Smirnov's tests, respectively. As needed, data were transformed to meet assumptions of normality and homoscedasticity. Tukey–Kramer test was used in conjunction with an ANOVA to find which means are significantly different from another at each sampling time ($\alpha = 0.05$). In this context, data of

BPA treated groups were compared with solvent control. Similarly, E₂ treated group was compared with solvent control and with BPA treated groups. All data are presented as mean \pm standard error of mean (SEM).

Results

Estrogen receptor β I (erβ-I) mRNA transcript

The effects of BPA on *erβ-I* was evaluated in goldfish cultured liver

tissue by analysis of mRNA transcript of *erβ-I* after 24 h exposure to BPA and E₂. There was a significant increase in *erβ-I* mRNA level in cultured hepatic tissue treated with BPA concentrations,

which included 1 μg L⁻¹ (3.47-fold), 5 μg L⁻¹ (5.94-fold), 25 μg L⁻¹ (3.89-fold) and also E₂ (5.05-fold) compared to the control ($p < 0.05$, Fig. 1).

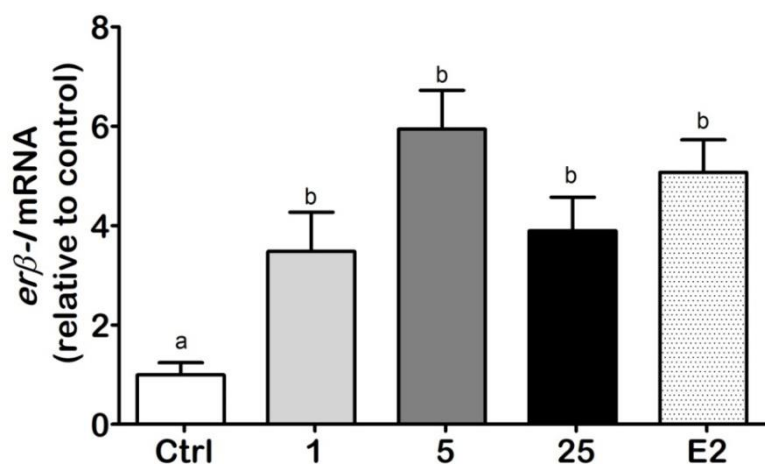


Figure 1: Alterations in the mRNA levels of estrogen receptor β -I (*erβ-I*) in the hepatic tissue of male goldfish exposed to 1, 5 or 25 μg L⁻¹ bisphenol A (BPA) and 1 μg L⁻¹ 17 β -estradiol (E₂), *in vitro*. Pieces of hepatic tissues were cultured in M119 culture media containing BPA, E₂, or ethanol as solvent. Alterations in transcriptional activity of *er* subtypes were studied using a quantitative real-time PCR following 24 h of exposure. Data are expressed as mean \pm SEM (n=6). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

*Estrogen receptor β -II subtypes (*erβ-II*) mRNA transcript*

Following 24 h exposure, expression of *erβ-II* was significantly higher with a 3.61-fold, 5.02-fold and 3.14-fold increase in the hepatic tissue exposed to 1 and 5 μg L⁻¹ of BPA and to E₂, respectively ($p < 0.05$, Fig. 2). However, the expression of *erβ-II* in hepatic tissue exposed to 25 μg L⁻¹ BPA remained unchanged compared to the control ($p > 0.05$, Fig. 2).

*Hepatic vitellogenin (*vtg*) mRNA*

There was a significant 3.62-fold increase in *vtg* mRNA expression when the cultured hepatic tissue treated with 5 μg L⁻¹ BPA compared to the control. Moreover, the mRNA levels of *vtg* in the cultured hepatic tissue treated E₂ had significant 3.18-fold increase after 24 h exposure ($p < 0.05$, Fig. 3). The mRNA level of *vtg* showed trends toward increases at 1 and 25 μg L⁻¹ BPA, however the differences were not significant compared to the control ($p > 0.05$, Fig. 3).

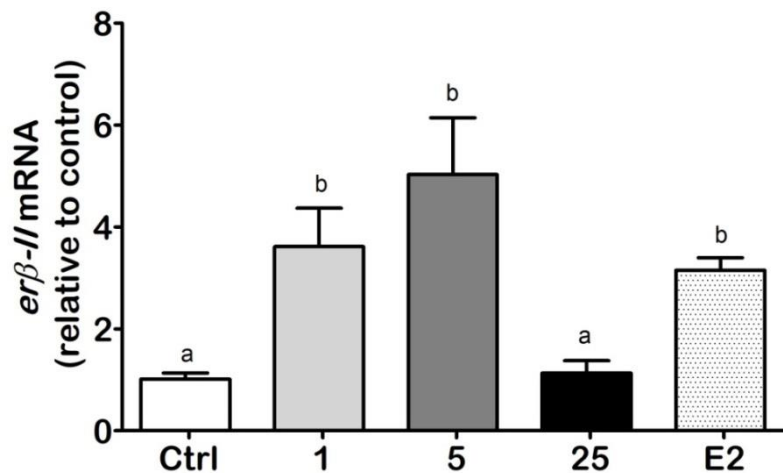


Figure 2: Alterations in the mRNA levels of estrogen receptor β -II (*erβ-II*) in the hepatic tissue of male goldfish exposed to 1, 5 or 25 $\mu\text{g L}^{-1}$ bisphenol A (BPA) and 1 $\mu\text{g L}^{-1}$ 17 β -estradiol (E_2), *in vitro*. Pieces of hepatic tissues were cultured in M119 culture media containing BPA, E_2 , or ethanol as solvent. Alterations in transcriptional activity of *er* subtypes were studied using a quantitative real-time PCR following 24 h of exposure. Data are expressed as mean \pm SEM (n=6). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

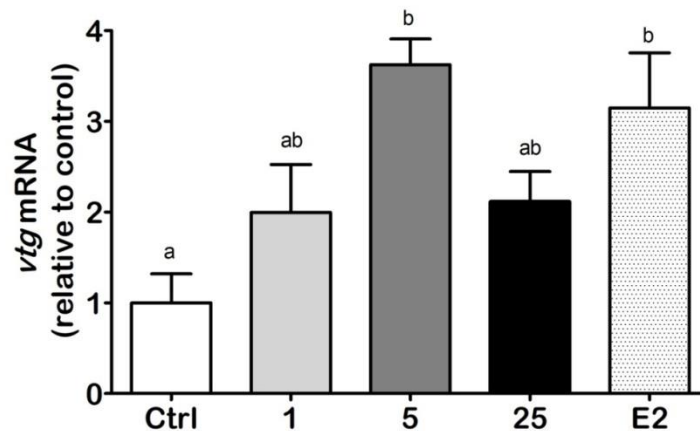


Figure 3: Alterations in the mRNA levels of vitellogenin (*vtg*) in the hepatic tissue of male goldfish exposed to 1, 5 or 25 $\mu\text{g L}^{-1}$ bisphenol A (BPA) and 1 $\mu\text{g L}^{-1}$ 17 β -estradiol (E_2), *in vitro*. Pieces of hepatic tissues were cultured in M119 culture media containing BPA, E_2 , or ethanol as solvent. Alterations in transcriptional activity of *vtg* were studied using a quantitative real-time PCR following 24 h of exposure. Data are expressed as mean \pm SEM (n=6). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

Cytochrome p450 1A (cyp1a) mRNA

Following 24 h exposure to BPA, *cyp1a* mRNA levels showed increases in a concentration manner, which were significant at 5 $\mu\text{g L}^{-1}$ (3.15-fold) and 25 $\mu\text{g L}^{-1}$ (3.57-fold) compared to the

control. Moreover, there was a significant 2.36-fold increase in *cyp1a* mRNA levels of hepatic tissue after 24 h exposure to E_2 ($p < 0.05$, Fig. 4).

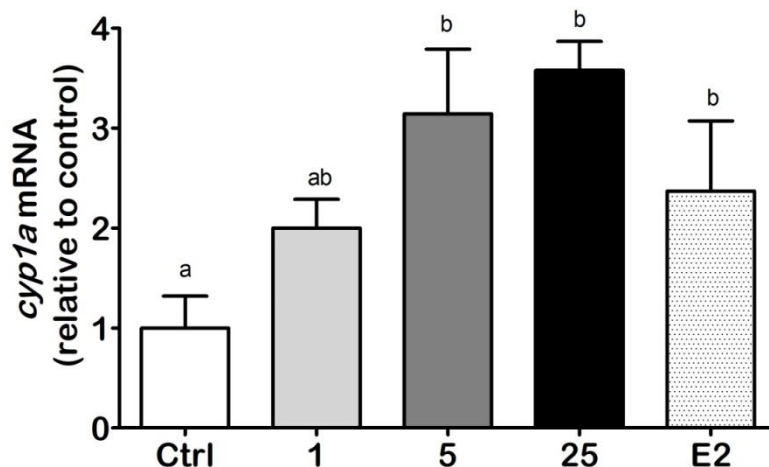


Figure 4: Alternations in the mRNA levels of cytochrome p450 1A (*cyp1a*) in the hepatic tissue of male goldfish exposed to 1, 5 or 25 $\mu\text{g L}^{-1}$ bisphenol A (BPA) and 1 $\mu\text{g/L}$ 17β -estradiol (E_2), *in vitro*. Pieces of hepatic tissues were cultured in M119 culture media containing BPA, E_2 , or ethanol as solvent. Alternations in transcriptional activity of *cyp1a* were studied using a quantitative real-time PCR following 24 h of exposure. Data are expressed as mean \pm SEM (n=6). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

Discussion

We have previously reported that BPA is capable of disrupting transcriptional activities of genes that are involved in reproduction of goldfish following 30-90 days of exposure, *in vivo* (Hatef *et al.*, 2012b). Briefly, our results have shown that BPA induces *vtg* mRNA in the liver mediated by stimulation in mRNA transcript of *er β* subtypes and P450 aromatase, which converts androgens to estrogen. In the present study, we used an alternative, rapid and highly sensitive detection method to

evaluate the estrogenic-like activity of BPA.

The present study showed that mRNA transcripts of *er β -I* were increased in BPA treated hepatic tissue, which is consistent with previous studies that have used an *in vivo* approach (Huang *et al.*, 2010; Hatef *et al.*, 2012b). However, mRNA transcript of *er β -II* was increased in hepatic tissue exposed to 1 and 5 $\mu\text{g L}^{-1}$ BPA and E_2 , but remained unchanged at 25 $\mu\text{g L}^{-1}$ BPA. These show that BPA effects on transcriptional activities of *er* subtypes

is different, and suggest that it might be contributed to affinity of BPA to *er* subtypes. Similarly, BPA-induced changes in transcriptional activity of *er* subtypes has been frequently reported when fish were exposed to BPA, *in vivo* (Seo *et al.*, 2006; Huang *et al.*, 2010; Hatef *et al.*, 2012b; Miyagawa *et al.*, 2014).

In E₂ treated hepatic tissue, either *erβ-I* or *erβ-II* mRNA levels were increased following 24 h of exposure, indicating estrogenic activity of E₂ is mediated by *er* subtypes (Kuiper *et al.*, 1998; Liu *et al.*, 2009; Nelson and Habibi, 2010). In BPA treated hepatic tissue, higher transcriptional activities of *er* subtypes were observed at 5 μg L⁻¹ BPA, represent inducing transcriptional activities of *er* subtypes required rather higher concentrations of BPA, compared to E₂. This might be addressed to higher estrogenic activity of E₂ (Gould *et al.*, 1998; Li *et al.*, 2015).

In males, the physiological Vtg level is very low and not properly detectable unless exposed to estrogen or xenoestrogens. Therefore, observation of high levels of Vtg has been used as a marker of estrogenic-like contaminants (Sohoni *et al.*, 2001; Mandich *et al.*, 2007; Amaninejad *et al.*, 2018). In the present study, we observed stimulation of *vtg* mRNA transcript of hepatic tissue exposed to 5 μg L⁻¹ and E₂, which is consistent with alternations in mRNA levels of estrogen receptor subtypes. The latter suggests that BPA-induced *vtg* transcription is particularly mediated by *erβ* subtypes. In the

present study, we did not assess the transcriptional activity of *era* as our previous study have shown that mRNA level of this gene remained unchanged in the liver of goldfish exposed to BPA, *in vivo* (Hatef *et al.*, 2012b). It is worth to note that several *in vivo* studies have reported the stimulation of Vtg synthesis in the liver of fish exposed to BPA including goldfish (Hatef *et al.*, 2012a), swordtail (Kwak *et al.*, 2001), fathead minnow (Sohoni *et al.*, 2001; Brian *et al.*, 2005) medaka (Kamata *et al.*, 2011), zebrafish (Muncke *et al.*, 2007), and rainbow trout (Lindholst *et al.*, 2000).

The *cyp1a* gene is one of the main members of the *cytochrome p450 1a* (*cyp1*) family, which is a detoxifying gene transcribed especially in the liver of fish (Olsvik *et al.*, 2007) and has suggested as a sensitive biomarker for investigating xenobiotics and water pollutants (Kim *et al.*, 2008; Søfteland *et al.*, 2010). In the present study, we observed the elevation in mRNA transcript of *cyp1a* in cultured pieces of hepatic tissue exposed to BPA was consistent with previous study where exposure to BPA increased mRNA transcript of *cyp1a* in fathead minnows, *in vivo* (Zare *et al.*, 2018). Modulation of hepatic *cyp1a* expression markedly affects the potential risks, which is important toxicologically (Williams *et al.*, 1998).

In conclusion, our results show an estrogenic-like activity of BPA, and support our previous study in which BPA-induced Vtg synthesis via *er*

subtypes has been reported in goldfish, *in vivo*. This study provides valuable information for biologists to establish and use *in vitro* approach as an alternative tool to *in vivo* test to detect hormonal like activity of endocrine disrupting chemicals.

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