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Effects of bisphenol A and light conditions on the circadian rhythm of the goldfish *Carassius auratus*

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ABSTRACT

We investigated how exposure to bisphenol A (BPA) under different photoperiodic conditions affected the expression of clock genes in the brain and liver of the goldfish, *Carassius auratus*. Three photoperiodic conditions were used: control, LD; continuous light, LL; and continuous dark, DD; the fish were exposed to three concentrations of BPA, namely 0, 10, or 100 μg/L. We measured changes in the expression of cryptochrome 1 (*Cry1*), period 2 (*Per2*), and melatonin receptor 1 (*MT-R1*). The levels of *Cry1*, *Per2*, and *MT-R1* mRNAs decreased with increasing BPA concentration and with increasing exposure time. Expression of *Cry1* and *Per2* increased more in the LL group than in the LD and DD groups. However, for *MT-R1*, the DD group showed increased expression compared to the LL and LD groups. Our analysis shows that circadian rhythms in goldfish can be disrupted by exposure to BPA and that the response can be modified by regulating the photoperiod.

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KEYWORDS
Bisphenol A; cryptochrome1; circadian rhythm; goldfish; period2

1. Introduction

As a consequence of the worldwide increase in the use of plastics, there is a growing interest in the possible toxicity on the aquatic environment of compounds used in the manufacture of plastics and generated by their disposal (Vermeirssen et al. 2017). One of the environmental toxicants associated with plastic generation and disposal is bisphenol A (BPA; CAS 85-05-7), a raw material for epoxy resin and plastic production, which has been reported to be toxic and induce oxidative stress in fish (Wu et al. 2013). In particular, BPA has estrogenic properties that can adversely affect the reproductive capacity of fish and can result in feminization of males (Wozniak and Murias 2008). BPA is also an endocrine disrupting chemical that can interfere with the function of fish endocrine systems to reduce immunity and growth rates (Segner et al. 2003; Wu et al. 2011).

In addition to toxins such as BPA, many environmental factors can influence the function of the endocrine system (Pierce et al. 2008; Jin et al. 2009). In particular, the duration and intensity of exposure to light affects fish endocrine systems (Bromage et al. 2001). Thus, light is an important environmental factor that controls the fish biological cycle, as it can not only

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induce or suppress physiological changes, but can also affect reproduction, growth, and behavior (Pierce et al. 2008; Shin et al. 2012).

In vivo, biological rhythms "circadian clocks" maintain physiological and behavioral homeostasis and regulate physiological changes due to environmental factors such as light (King and Takahashi 2000; Wu et al. 2016). A number of clock genes, termed "pace-makers", have been identified (Sugama et al. 2008; Nanako et al. 2012; Choi et al. 2016). Two representative clock genes that regulate the circadian rhythm are period2 (*Per2*) and cryptochrome1 (*Cry1*), which are rapidly induced when stimulated by light (Cermakian et al. 2002; Besharse et al. 2004). These genes show high levels of expression during daytime and low levels at night (Kim et al. 2012). In addition, they are found in most organisms and are known to be expressed in almost all tissues including the brain (Lin and Todo 2005; Albrecht et al. 2007).

Melatonin is also known to have an important role in the regulation of circadian rhythms. It is mainly produced in the pineal gland and retina (Jung et al. 2016) where it is synthesized from 5-hydroxytryptamine by the enzyme arylalkylamine N-acetyltransferase-1 whose activity is inhibited by light; the amount of melatonin secreted into plasma at night is increased compared to daytime (Klein et al. 2002). Melatonin functions by binding to melatonin receptors (MTs), which belong to the G-protein-coupled receptor superfamily, present in the membranes of target tissues; overall, MTs are preferentially distributed in the central nervous system and ganglia of vertebrate species, and play a role in controlling melatonin to perform physiological functions in these tissues (Park et al. 2013). Dubocovich et al. (2000) reported the presence of three MT subtypes, named MT1, MT2, and MT3. Of these, MT1 has a role in recognizing seasonal and environmental changes in the brain, particularly in the hypophysial pars tuberalis and hypothalamic suprachiasmatic nucleus (SCN), and controlling biological rhythms (Reppert et al. 1996).

Here, we investigated the effects of BPA and diurnal variations in light on biological rhythms in goldfish by exposing the fish to BPA and to different diurnal cycles and compared changes in the expression of *Cry1*, *Per2*, and *MT-R1* in brain and liver tissues.

2. Materials and methods

2.1. Experimental fish and conditions

For this experiment, common goldfish ($N = 540$, length, 6.4 ± 0.18 cm; mass, 12.4 ± 1.6 g) were purchased from a commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for 2 weeks in nine 300-L circulation filter tanks, consisting of four mini tanks, in the laboratory. Each filter tank (experimental group) contained 60 fish; each mini-tank contained 15 fish. Five fish were randomly selected from each mini-tank at each sampling interval. The goldfish were reared using an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Seoul, Korea). Water temperature was maintained at 22 °C.

We established a control group, which was not exposed to BPA, and two experimental groups, which were exposed to 10 or 100 µg/L BPA ($C_{15}H_{16}O_2$, Sigma, USA). No fish died as a result of the BPA exposure. Following BPA exposure, the control and experimental groups were exposed to light from a white fluorescent bulb placed 40 cm above the surface of water; for 2 days in 4 h intervals. Fish were first sampled 2 h after the beginning of BPA treatment; the sampling schedule was 2, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, and 46 h. The experiment started at 07:00. Photoperiods of 12 h L:12 h D (12L:12D), 24 h light (LL), and 24 h dark (DD)

were established. The 12L:12D treatment (lights on at 07:00 and lights off 19:00) is similar to a natural photoperiod.

For all experiments, the fish were given commercial feed daily until the day prior to sampling. The fish were anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, USA) to minimize stress prior to collection of blood and brain and liver tissues. Blood was collected from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 1000 × g, for 15 min) and stored at –80 °C until analysis. The tissues were removed from the fish, immediately frozen in liquid nitrogen, and stored at –80 °C until analysis.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using a TRIzol kit according to the manufacturer's instruction (Gibco/BRL, Gaithersburg, MD, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Total RNA (2 µg) was reverse transcribed in a total volume of 20 µL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at –20 °C for use in polymerase chain reaction (PCR) experiments.

2.3. Real-time quantitative PCR (RT-qPCR)

In this study, we followed the recommended guidelines for minimum information for publication of RT-qPCR experiments (Bustin et al. 2009). Total RNA was synthesized to cDNA by reverse transcription using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. RT-qPCR was performed using cDNA. RT-qPCR was conducted to determine the relative levels of *Cry1* (GenBank accession no. EF690700), *Per2* (EF690697), and *MT-R1* (AB481372) mRNA in the brain and liver. The primers used for the qPCR are presented in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database, to ensure the specificity of primers. The PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) following the manufacturer's instructions. The RT-qPCR was performed as follows: 95 °C for 5 min, followed by 50 cycles each of 95 °C for 20 s and 55 °C for 20 s. As an internal control, experiments were similarly performed to amplify β-actin, and all quantitative data are expressed relative to the corresponding β-actin (AB039726)

Table 1. Primers used for qPCR amplification.

Genes (accession no.)	Primer	Sequence
Cry1 (EF690700)	Forward	5′-CGG AGA CCT GTG GAT CAG-3′
	Reverse	5′-GTG GAA GAA TTG CTG GAA-3′
Per2 (EF690697)	Forward	5′- CTG GAG CCG CAA AGT TTC-3′
	Reverse	5′- CTG GAT GTC TGA GTC TAA-3
MT-R1 (AB481372)	Forward	5′-GGT TGG CAG TAG CGA TTT-3′
	Reverse	5′-CTC ACG ACG GAA GTT CTG-3′
β-actin (AB039726)	Forward	5′- TTC CAG CCA TCC TTC CTA –3′
	Reverse	5′- TAC CTC CAG ACA GCA CAG –3′

calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using the 2-ΔΔCt method [$\Delta\Delta Ct = 2\text{^}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.4. Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's *post-hoc* test was used to compare differences in the data ($p < 0.05$). The values are expressed as means ± standard error (SE).

3. Results

The relative levels of three biorhythm associated genes, *Per2, Cry1,* and *MT-R1*, were screened in the brains and livers of fish given different BPA and light treatments (Figures 1–6). The patterns of response of all three genes were similar in the brain and liver tissues and among different BPA treatments, although not for responses to light treatment. *Per2, Cry1*, and *MT-R1* mRNAs in fish exposed to BPA did not differ between the two tissues. Also, regardless of the concentration of BPA, the levels of *Per2* and *Cry1* expression were significantly higher in fish of the LL group, while the DD group had the lowest levels. By contrast, *MT-R1* expression was highest in the DD group. When compared to the BPA-untreated experimental group, the mRNA expression level of each of the three genes was not significantly different at 10 µg/L of BPA, but it was significantly decreased when exposed to 100 µg/L of BPA.

4. Discussion

BPA is an endocrine disrupting compound that acts *in vivo* by inducing toxic stress to decrease immunity and reproductive capacity, and disturbing biorhythms. However, the concentration of an endocrine disrupting compound required to induce an effect varies among target species (Rhee et al. 2014). Rhee et al. (2014) reported that expression of clock genes was disturbed when mangrove killifish, *Kryptolebias marmoratus*, were exposed to environmental toxins including BPA. Recent studies have used changes in expression of clock genes as an indicator of alteration of the circadian rhythm (Kim et al. 1996; Rhee et al. 2014). Therefore, in this study, we investigated the effect of BPA treatment on biorhythm changes in goldfish under various photoperiod conditions by comparing clock gene and melatonin gene expression patterns.

The relative levels of *Cry1* and *Per2* mRNAs in brain and liver of goldfish exposed to BPA showed no significant differences between the tissues. Regardless of the concentration of BPA, the mRNA level of each gene was significantly higher in the LL group and lowest in the DD group. Compared with the control group, no significant change in mRNA levels was seen after 10 µg/L BPA, but a significant decrease was seen after 100 µg/L BPA. The oscillation of expression was significantly reduced at each time.

In a similar study, Rhee et al. (2014) reported that the circadian pattern of clock gene mRNA expression decreased with time in mangrove killifish (*K. marmoratus*) and Japanese killifish (*Oryzias latipes*) exposed to 600 µg/L BPA, suggesting that the chemical directly inhibited expression of clock genes and induced asynchrony in expression of the gene. On the

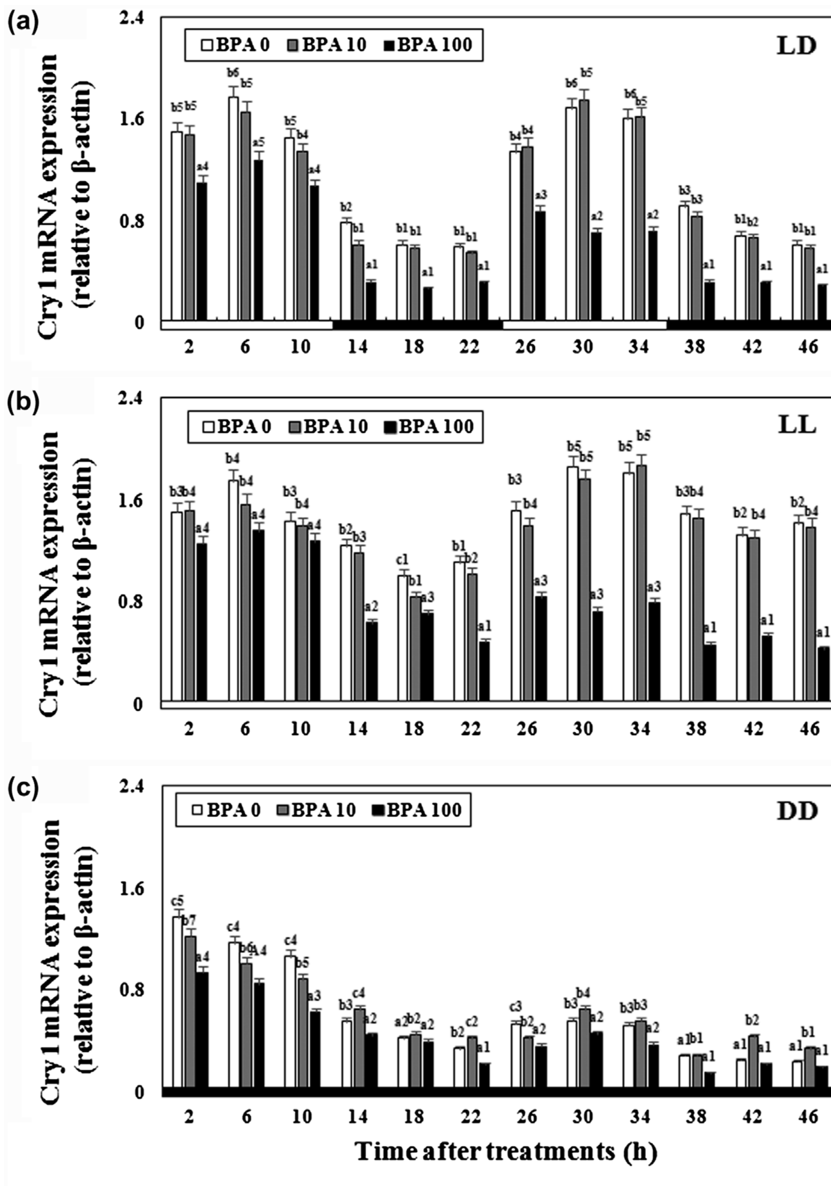

Figure 1. Changes in the levels of *Cry1* mRNA in the brain of goldfish exposed to BPA for different intervals under different photoperiods: (a) LD = 12 h light:12 h dark, (control); (b) LL = 24 h light; (c) DD = 24 h dark. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample.

Notes: The white bar represents the photophase and the black bar represents the scotophase; the three columns represent the different photoperiods in each group. The numbers indicate significantly different levels of mRNA within the same BPA concentration and photoperiod ($p < 0.05$). The lowercase letters indicate significant differences between each BPA concentration within the same photoperiod and time of BPA treatment ($p < 0.05$). All values are means ± SE ($n = 5$).

other hand, Swearingen (2016) reported that exposure to high-concentration BPA leads to oxidative stress, and that this stress in tissues of the peripheral nervous system can induce

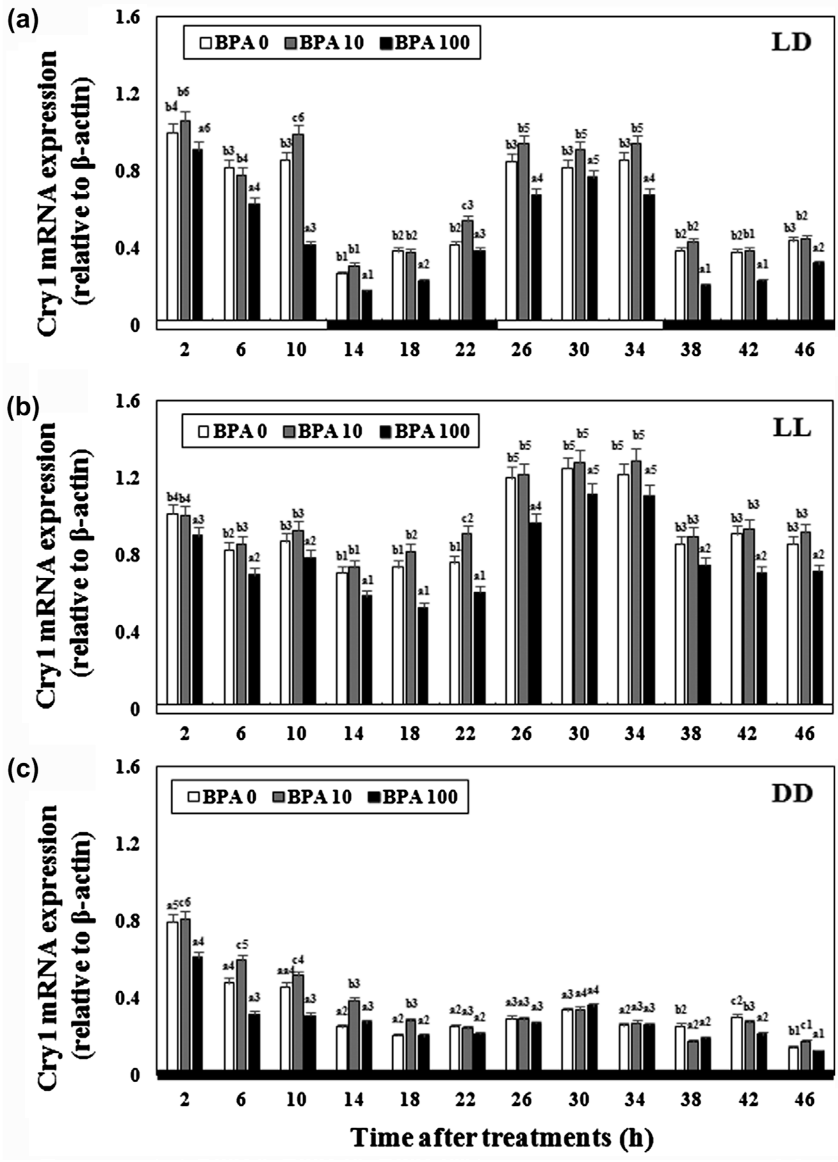

Figure 2. Changes in the levels of *Cry1* mRNA in the liver of goldfish exposed to BPA for different intervals under different photoperiods: (a) LD = 12 h light:12 h dark, (control); (b) LL = 24 h light; (c) DD = 24 h dark. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample.

Notes: The white bar represents the photophase and the black bar represents the scotophase. The numbers indicate significantly different levels of mRNA within the same BPA concentration and photoperiod ($p < 0.05$). The lowercase letters indicate significant differences between each BPA concentration within the same photoperiod and time of BPA treatment ($p < 0.05$). All values are means ± SE ($n = 5$).

or inhibit clock gene expression, resulting in disturbance of circadian rhythm through the action of glucocorticoid hormones.

We suggest that BPA acts directly on cells that express clock genes, not only by inhibiting mRNA synthesis, but also by directly or indirectly inducing the production of substances

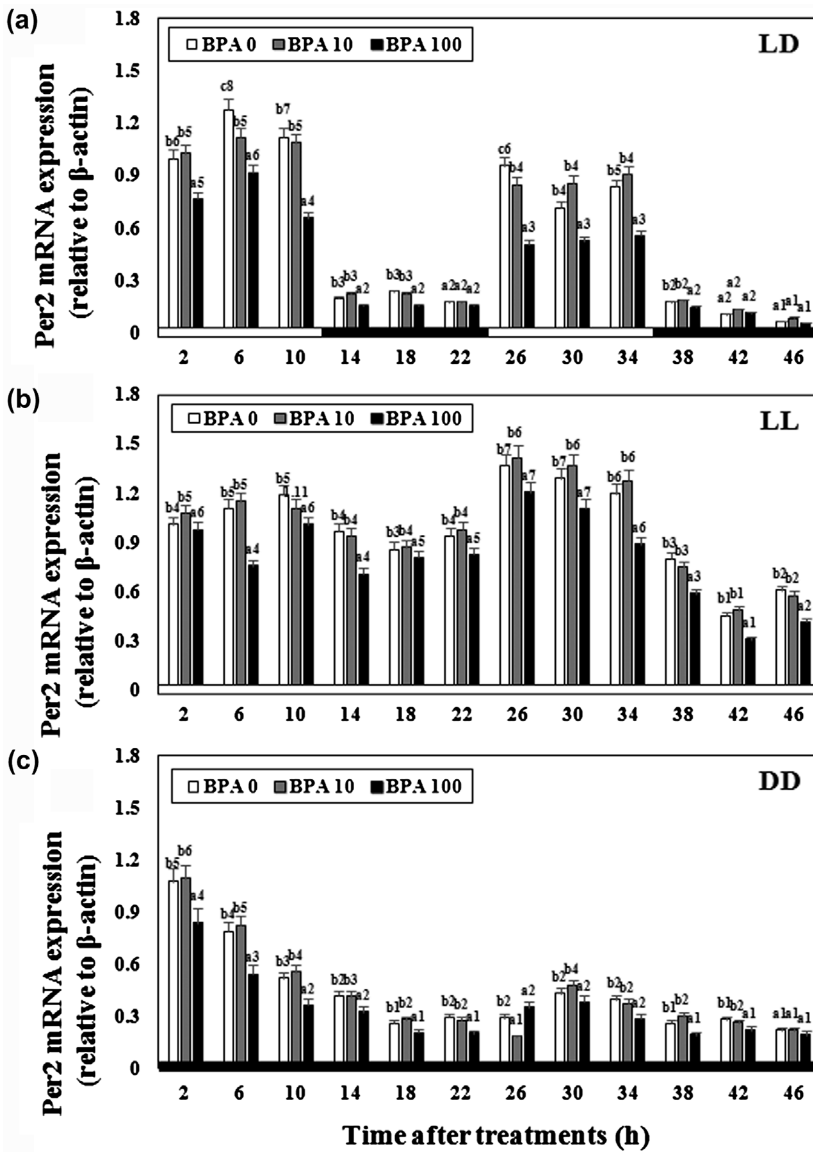

Figure 3. Changes in the levels of *Per2* mRNA in the brain of goldfish exposed to BPA for different intervals under different photoperiods: (a) LD = 12 h light:12 h dark, (control); (b) LL = 24 h light; (c) DD = 24 h dark. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample.

Notes: The white bar represents the photophase and the black bar represents the scotophase; the three columns represent different photoperiods in each group. The numbers indicate significantly different levels of mRNA within the same BPA concentration and photoperiod ($p < 0.05$). The lowercase letters indicate significant differences between each BPA concentration within the same photoperiod and time of BPA treatment ($p < 0.05$). All values are means ± SE ($n = 5$).

that inhibit the expression of clock genes, resulting in a significantly lower level of expression.

In the present study, consistent with previous studies, the levels of *Cry1* and *Per2* mRNAs in goldfish exposed to 10 μg/L BPA were not significantly different, but the expression levels

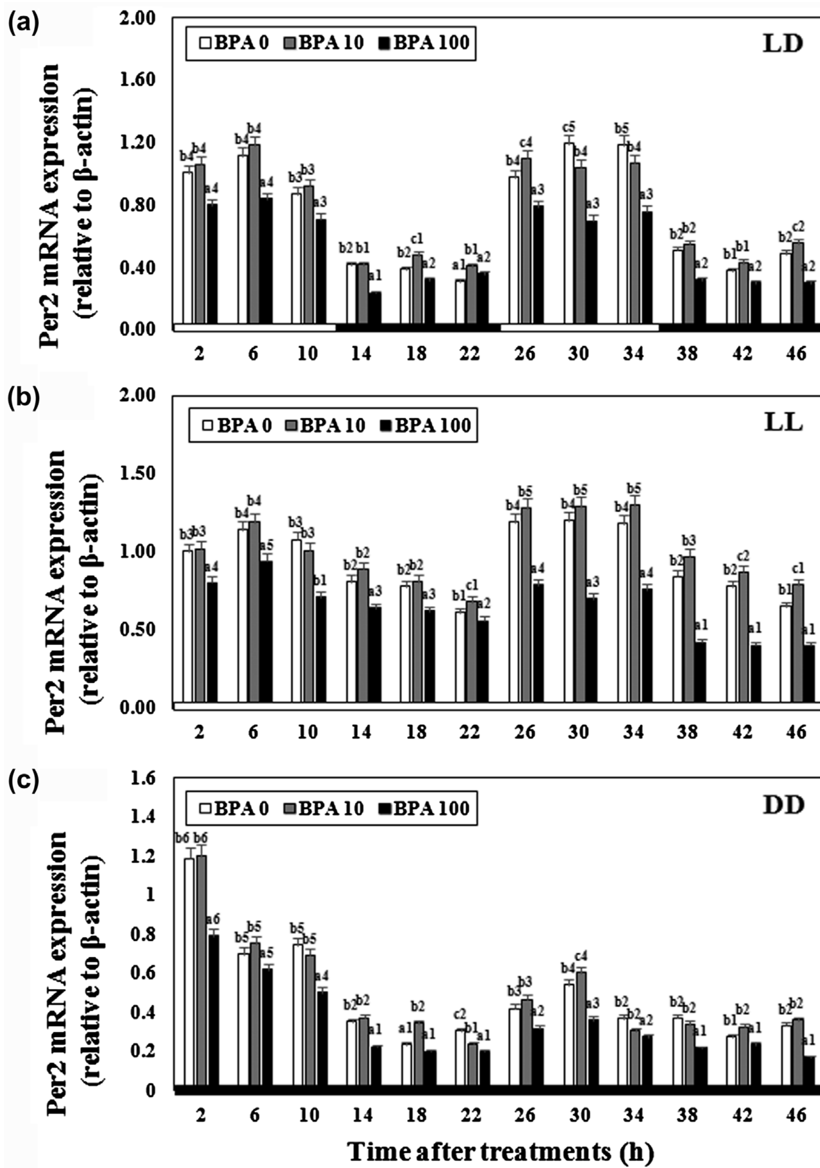

Figure 4. Changes in the levels of *Per2* mRNA in the liver of goldfish exposed to BPA for different intervals under different photoperiods: (a) LD = 12 h light:12 h dark, (control); (b) LL = 24 h light; (c) DD = 24 h dark. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample.

Notes: The white bar represents the photophase and the black bar represents the scotophase. The numbers indicate significantly different levels of mRNA within the same BPA concentration and photoperiod ($p < 0.05$). The lowercase letters indicate significant differences between each BPA concentration within the same photoperiod and time of BPA treatment ($p < 0.05$). All values are means ± SE ($n = 5$).

in fish exposed to 100 μg/L BPA were significantly decreased. In other words, BPA acted as an inhibitory factor in the biological rhythm of goldfish. Additionally, exposure to light seemed to be an important factor in the changes to biological rhythm regardless of BPA

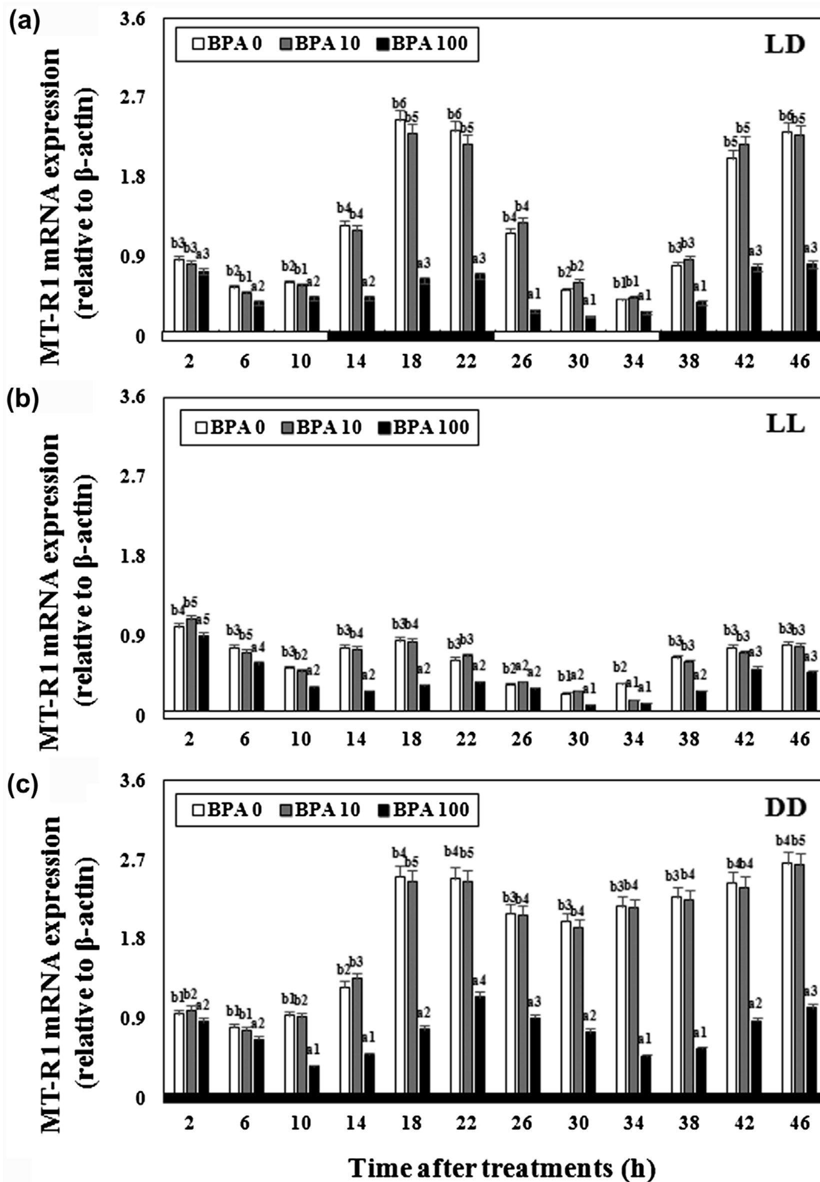

Figure 5. Changes in the levels of *MT-R1* mRNA in the brain of goldfish exposed to BPA for different intervals under different photoperiods: (a) LD = 12 h light:12 h dark, (control); (b) LL = 24 h light; (c) DD = 24 h dark. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample.

Notes: The white bar represents the photophase and the black bar represents the scotophase. The numbers indicate significantly different levels of mRNA within the same BPA concentration and photoperiod ($p < 0.05$). The lowercase letters indicate significant differences between each BPA concentration within the same photoperiods and time of BPA treatment ($p < 0.05$). All values are means ± SE ($n = 5$).

concentration. However, BPA can directly inhibit expression of biorhythm genes and also act as a toxic substance to induce oxidative stress *in vivo*, thereby suppressing expression of the clock gene and negatively affecting the biological rhythm.

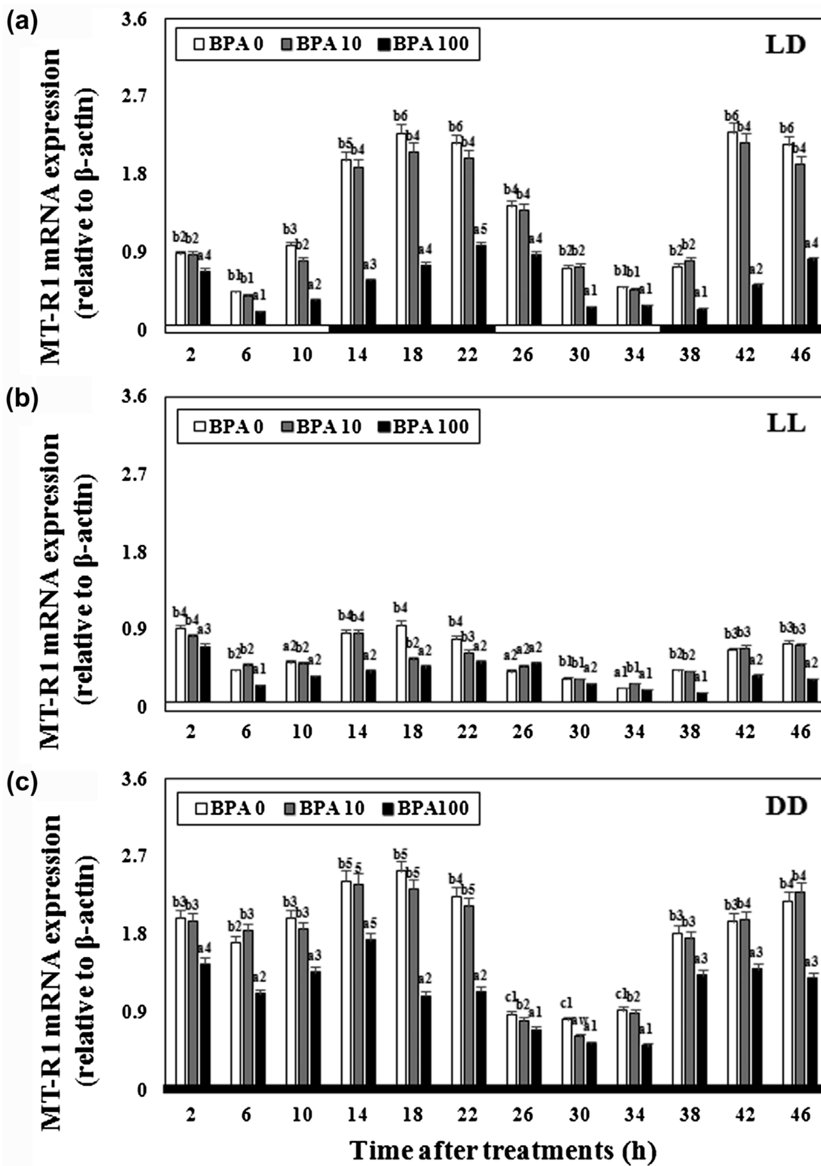

Figure 6. Changes in the levels of *MT-R1* mRNA in the liver of goldfish exposed to BPA for different intervals under different photoperiods: (a) LD = 12 h light:12 h dark, (control); (b) LL = 24 h light; (c) DD = 24 h dark. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample.

Notes: The white bar represents the photophase and the black bar represents the scotophase. The numbers indicate significantly different levels of mRNA within the same BPA concentration and photoperiod ($p < 0.05$). The lowercase letters indicate significant differences between each BPA concentration within the same photoperiod and time of BPA treatment ($p < 0.05$). All values are means ± SE ($n = 5$).

We also examined the effect of BPA treatments on expression of the melatonin gene *MT-R1*, which is an indicator for biological rhythms in fish. Melatonin plays a diverse role *in vivo* and is inhibited by light. It also functions to reduce oxidative stress caused by the external

environment and is important substance in terms of immunology and toxicology (Wu et al. 2013). We found here that the higher level of BPA significantly decreased the *MT-R1* mRNA level; we also found that the level of *MT-R1* mRNA decreased significantly with time. The level of *MT-R1* mRNA was significantly greater in the DD group than in the LD and LL groups.

Shin et al. (2011) reported that the nighttime circadian pattern of *MT-R* expression in the pineal gland of olive flounder, *Paralichthys olivaceus*, after exposure to LD or DD for 28 h was significantly higher than daytime expression. Melatonin concentrations in the plasma of rock bream, *Oplegnathus fasciatus*, are significantly decreased with increasing BPA concentration and exposure time (Choi et al. 2016).

Similar to previous studies, we found that *MT-R1* expression was significantly decreased with increasing BPA concentration in goldfish. *MT-R1* was mainly expressed at nighttime and significantly increased in the DD photoperiod group compared to the LD and LL groups. Therefore, we suggest that BPA acts as a toxic substance to goldfish and interferes with the circadian rhythm *in vivo*.

In conclusion, the results of this study suggest that (1) BPA acts directly on exposed tissue cells to inhibit the expression of clock genes and increase the disturbance of circadian rhythm and (2) the expression of circadian genes in daytime and nighttime time is reduced by the toxic effects of a high concentration (100 μg/L) of BPA, indicating that the biological rhythms of the goldfish were disturbed.

Disclosure statement

No potential conflict of interest was reported by the authors.

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