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Immunotoxicity of bisphenol A to *Carassius auratus* lymphocytes and macrophages following *in vitro* exposure

YIN Da-qiang[1,2,*], HU Shuang-qing[1], GU Ying[1], WEI Li[1], LIU Shu-shen[1], ZHANG Ai-qian[1]

1. State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, China. E-mail: yindq@nju.edu.cn

2. Key laboratory of Yangtze River Water Environment, Ministry of Education, Tongji University, Shanghai 200092, China.

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Abstract

Bisphenol A (BPA) is the monomer component of polycarbonate plastics and classified as an endocrine disrupting chemical (EDC). The reproductive toxicity of BPA has been extensively studied in mammals; however, relatively little information is available on the immunotoxic responses of fish to BPA. In this study, we investigated the effects of BPA on the immune functions of lymphocytes and macrophages in *Carassius auratus*. The effects of BPA were compared with those of two natural steroid hormones, estradiol and hydrocortisone. Proliferation of the two types of cells in response to PHA was measured using colorimetric MTT assay. Macrophage respiratory burst stimulated by Con A was measured using chemiluminescence assay. Results showed that BPA (0.054–5.4 mg/L), estradiol (0.0002–2.0 mg/L) and hydrocortisone (5–50 mg/L) significantly induced *Carassius auratus* lymphocyte proliferation while higher doses of hydrocortisone (500–5000 mg/L) appeared to be inhibitory. BPA (0.005–50 mg/L), estradiol (0.005–800 mg/L) and hydrocortisone (0.005–500 mg/L) markedly enhanced macrophage proliferation, whereas higher doses of BPA (500–1000 mg/L) appeared to inhibit cell proliferation. Furthermore, higher dosage of BPA (50 mg/L) and hydrocortisone (50 and 500 mg/L) suppressed the macrophages respiratory burst while estradiol is stimulative all the doses tested (0.05–500 mg/L). In conclusion, BPA could have immunotoxicity to *Carassius auratus* and functional changes of lymphocyte and macrophage in *Carassius auratus* may be different between low and high dosages.

Key words: bisphenol A; immunotoxicology; lymphocyte; macrophage; *Carassius auratus*

Introduction

There has been increasing public concern about potential adverse effects on human health and ecological safety of various environmental contaminants designated by some as endocrine disrupting chemicals (EDCs). It is now well known that natural estrogens such as 17-β-estradiol not only affect the reproductive system but also markedly influence the immune system. Recently, some of EDCs have also been shown to influence the immune system. Evaluating the effect of EDCs on immune system has become an emerging research field (Committee on Hormonally Active Agents in the Environment, 1999; Ahmed, 2000).

Bisphenol A (BPA) is the monomer component of polycarbonate plastics. Nearly 30 kt of BPA in China were manufactured in 2002 (Lu, 2002). Although sold almost entirely in polymerized form to consumers, the BPA monomer still contaminates foodstuffs and environment as a leachate of plastic packaging, BPA concentrations ranging from 0.02 to 0.50 μg/L in typical wastewater (Vilches *et al*., 2001). It acts like a weak estrogen in classic bioassays, including cell proliferation and cornification of the vaginal epithelium and pituitary prolactin secretion in the ovariectomized rat, and it produces estrogen-like effects in rat mammary gland and developing mouse prostate (Colerangle and Roy, 1997; Nagel *et al*., 1997; Steinmetz *et al*., 1997, 1998). Because of its endocrine disrupting activity, there is concern over human and animal exposure to BPA.

The immune system is essential to the health and survival of fish species in the presence of infectious diseases and tumors. In particular, fish lymphocytes can undergo proliferation in response to a variety of stimuli, produce antibodies and have the ability to recognize and lyse a number of tumour target cells (O'Halloran *et al*., 1998). Meanwhile, fish macrophages not only can proliferate in response to pathogens and initiate specific immune responses, but also serve as potent effector cells that ingest microbes directly. Fish macrophages secret a wide range of biologically active molecules including reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) etc., which are involved in the bactericidal activity, during a phenomenon termed the respiratory burst (Secombes

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1990). Previous studies have demonstrated response of fish immune cells to several EDCs such as lindane and cadmium (Albergoni and Viola, 1995; Betoulle *et al*., 2000). However, the response of fish immune cells to BPA is not clear.

In this study, we examined the proliferation of lymphocytes isolated from the peripheral blood and macrophages isolated from the head kidney of *Carassius auratus* in response to *in vitro* exposure to BPA. We also studied the respiratory burst of macrophages in this setting. The effects of BPA were compared with those of two natural steroid hormones (estradiol and hydrocortisone) in order to evaluate its immunotoxicity to fish.

1 Materials and methods

1.1 Test chemicals

Bisphenol A was purchased from Sigma Chemical Co. (USA). Hydrocortisone and estradiol were purchased from Nanjing Hormone Manufactory (Fig.1).

1.2 Test animals

Female *Carassius auratus* weighing 300 to 350 g, were purchased from Fisheries Research Institute of Jiangsu Province, and acclimated in laboratory for one week prior to use in experiments. All animals were maintained at 18±1.0°C, under a 12-h light and 12-h dark photoperiod in dechlorinated water (pH 6.8±0.2, DO 8.2±0.2 mg/L).

1.3 Preparation of peripheral blood lymphocytes

Lymphocytes were isolated as described by Rowley (1990). Blood samples were drawn into heparinized syringes from the caudal vein of fish, layered onto Histopaque-Ficoll 1077 (Sigma, USA) and centrifuged at 500×g for 30 min (17°C). Cells at the interface were collected and washed twice in 10 ml RPMI 1640 medium (Gibco/BRL, USA) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 15% (v/v) fetal calf serum at 500×g for 10 min (17°C). Then cells were resuspended in RPMI 1640 and counted. Viability was determined by 0.4% (w/v) trypan blue exclusion, and was greater than 95%. The cell suspension was diluted with RPMI 1640 medium to obtain 2×10^6 viable cells per ml.

1.4 Preparation of head kidney macrophages

Macrophages were isolated as described by Secombes (1990). The head kidney was aseptically dissected from the *Carassius auratus*, passed through a 100-μm nylon mesh and homogenized in Leibowitzs-15 medium (Pharmacia, Sweden) supplemented with heparin (10 IU/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The resulting cell suspension was layered onto a 34%/51% (v/v) Percoll density gradient (Pharmacia, Sweden). After centrifugation at 400×g for 25 min (17°C), the cells at the interphase were collected and washed twice with L-15 medium (centrifugation at 400 ×g, 10 min, 17°C). Then cells were resuspended in L-15 medium and incubated for 2 h at 18°C in round-bottomed 6-well plastic plates. The unattached cells were removed and monolayers of adherent cells washed twice with PBS, counted by 0.4% (w/v) trypan blue exclusion (95%). The cell suspension was diluted with L-15 medium to obtain 2×10^6 viable cells per ml.

1.5 Colorimetric MTT (tetrazolium) assay

Lymphocytes and macrophages proliferations were detected using colorimetric MTT assay, described by Mosmann (1983). Each culture in the 96-well culture plate comprised 2×10^5 cells suspended in 100 μl of RPMI supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 15% (v/v) fetal calf serum, and 50 μl of mitogen phytohaemoagglutinin (PHA). Cells were incubated for 24 h at 18°C, 5% CO_2, simultaneously with 50 μl of BPA, hydrocortisone and estradiol added into each well respectively. Controls were carried out with 50 μl of distilled water. After 24 h, 10 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma, USA) solution was added to each well of an assay, and plates were incubated at 18°C for 4 h. 100 μl of DMSO was added to all wells and mixed thoroughly to dissolved the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the optical density (OD) of plates was read on a Microplate reader (Bio-Tek, USA), using a test wavelength of 570 nm. All assays were expressed as mean of 8 determinations.

1.6 Chemiluminescence assay

Respiratory burst (ROS production) was detected by chemiluminescence (CL), using a modification of the method by Stave *et al.* (1983). Each assay vial was filled with a volume of 100 μl containing the cell suspension in PBS buffer (2×10^6 cell per ml), luminol (20 μl of 1×10^{-4} mol/L) and 30 μl stimulant Con A (Sigma, USA). Then 50 μl of BPA (0.005–50 mg/L), hydrocortisone (0.05–500

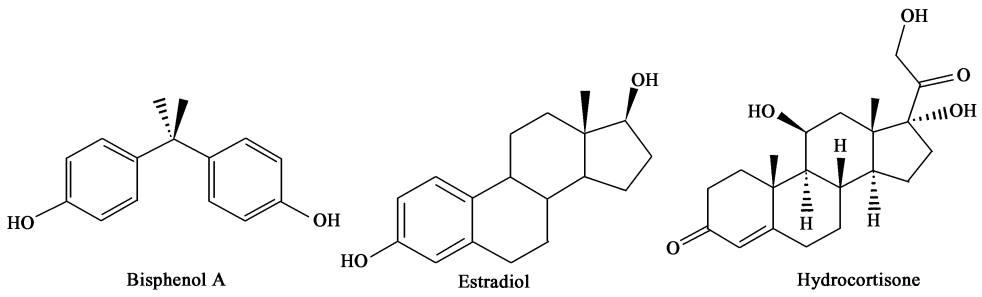

Fig. 1 Chemical structures of bisphenol A (BPA), estradiol, and hydrocortisone.

mg/L) and estradiol (0.05–500 mg/L) were added, respectively. Controls were carried out with 50 μl of distilled water. Chemiluminescence was determined at 17°C with a luminometer (Turner Designs TD-20/20, USA). Data were collected in terms of relative luminescence units (RLU) taken from readings every minute for 15 min and the maximal value was recorded. All assays were performed thrice.

1.7 Statistical analysis

All the data were checked for normality using normal probability plots. Differences among means were determined using Student's *t*-test. Differences were considered statistically significant at $P<0.05$.

2 Results

2.1 Effects of BPA, estradiol and hydrocortisone on lymphocyte proliferation

Fig.2 shows the effects of *in vitro* exposure of 0–5.4 mg/L BPA, 0–2 mg/L estradiol and 0–5000 mg/L hydrocortisone on *Carassius auratus* lymphocyte proliferation in response to stimulation by PHA. BPA (0.054–5.4 mg/L) significantly induced lymphocyte proliferation with maximal induction at 0.54 mg/L ($P<0.05$). There is a significant, concentration-dependent (from 0.0002 to 2 mg/L) activation of lymphocyte proliferation by estradiol. Lymphocyte proliferation was significantly induced by hydrocortisone at concentrations of 5 and 50 mg/L. The maximal induction occurred at 50 mg/L. The concentrations of hydrocortisone at 500 and 5000 mg/L led to inhibition of lymphocyte proliferation ($P<0.05$).

2.2 Effects of BPA, estradiol and hydrocortisone on macrophage proliferation

Fig.3 shows effects of BPA (0–1000 mg/L), estradiol (0–1000 mg/L) and hydrocortisone (0–500 mg/L) on *Carassius auratus* macrophage proliferation in response to stimulation by PHA. At concentrations of 0.005 to 50 mg/L, BPA induced a dose-dependent induction of macrophage proliferation with maximal induction at 50 mg/L ($P<0.05$). The pro-prolifertive effect of BPA reduced at 100 and 200 mg/L. At concentrations of 500, 800 and 1000 mg/L, BPA markedly inhibited proliferation of macrophage. At concentrations from 0.005 to 800 mg/L, estradiol significantly induced macrophage proliferation,

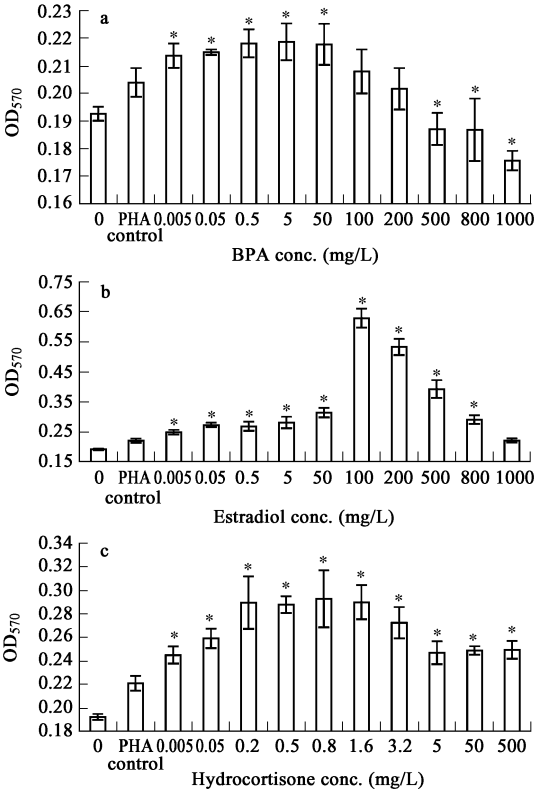

Fig. 3 Effects of BPA (a), estradiol (b) and hydrocortisone (c) *in vitro* exposures on *Carassius auratus* lymphocyte proliferation induced by PHA. Controls were carried out with distilled water. Each column represents means of OD_{570} obtained from eight experiments. The asterisk (*) indicates significant difference from the PHA control (*t*-test, $P<0.05$).

the maximal induction occurred at 100 mg/L ($P<0.05$). However, no significant effect was found at 1000 mg/L ($P>0.05$). At concentrations from 0.005 to 500 mg/L, hydrocortisone induced proliferation of macrophage significantly, with maximal induction occurring at 0.8 mg/L.

2.3 Effects of BPA, estradiol and hydrocortisone on macrophage respiratory burst

Table 1 shows effects of *in vitro* exposure of *Carassius auratus* to BPA, estradiol and hydrocortisone on macrophage respiratory burst in response to stimulation by Con A. At concentrations from 0.005 to 0.5 mg/L, BPA stimulated a dose-dependent respiratory burst of macrophage with the highest effect occurred at 5 mg/L, 1 min. The respiratory burst declined at 5 mg/L of BPA,

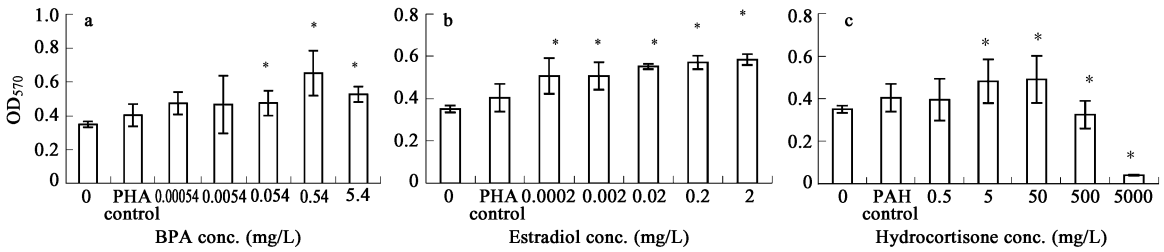

Fig. 2 Effects of BPA (a), estradiol (b) and hydrocortisone (c) *in vitro* exposures on *Carassius auratus* lymphocyte proliferation induced by PHA. Controls were carried out with distilled water. Each column represents means of OD_{570} obtained from eight experiments. The asterisk (*) indicates significant difference from the PHA control (*t*-test, $P<0.05$).

Table 1 Effects of BPA, estradiol and hydrocortisone on macrophage respiratory burst of *Carassius auratus*

Chemical	Concentration (mg/L)	Appearance time of RLU (min)	Maximal value of RLU	*P*-value
BPA	0	7	2.04±0.071	
	0.005	4	1.39±0.017	<0.01
	0.05	6	1.84±0.192	>0.05
	0.5	9	2.07±0.006	<0.01
	5	1	2.88±0.385	<0.05
	50	4	0.68±0.031	<0.001
Estradiol	0	3	0.08±0.040	
	0.05	2	0.14±0.037	>0.05
	0.5	3	0.28±0.010	<0.05
	5	14	0.65±0.100	<0.01
	50	1	0.48±0.004	<0.01
	500	1	0.49±0.010	<0.01
Hydrocortisone	0	7	2.04±0.071	
	0.05	8	1.05±0.001	<0.01
	0.5	10	3.11±0.449	<0.05
	5	2	2.91±0.842	>0.05
	50	1	1.42±0.180	<0.05
	500	1	1.39±0.459	<0.05

Values are mean±*SD* of three experiments, controls were carried out with distilled water, data were analysed by Student *t*-test of significance, values differ significantly from their respective control from $P<0.05$.

and then remained significantly higher than the control. Fifty mg/L of BPA markedly inhibited macrophage respiratory burst. Estradiol significantly inducted macrophage respiratory burst at concentrations from 0.5 to 500 mg/L ($P<0.05$). Five mg/L of estradiol induced highest respiratory burst (0.65±0.100 RLU), which reached the maximum at 14 min. Hydrocortisone significantly inhibited macrophage respiratory burst at concentrations of 0.05, 50 and 500 mg/L compared to the PHA control ($P<0.05$). Hydrocortisone markedly reduced macrophage respiratory burst at 0.5 mg/L, reaching maximal value at 10 min post-stimulation. However, no significant difference was found from the control at 5 mg/L of hydrocortisone ($P>0.05$).

3 Discussion

The present study describes the effects of *in vitro* exposure to BPA and two natural steroid hormones on cells involved in the immune response of *Carassius auratus*. Our results showed that BPA (0.054–5.4 mg/L), estradiol (0.0002–2.0 mg/L) and hydrocortisone (5–50 mg/L) facilitated lymphocyte proliferation of *Carassius auratus* in response to PHA, whereas higher doses of hydrocortisone (500–5000 mg/L) inhibited lymphocyte proliferation. In *in vitro* studies on Jurkat CD4+ T lymphocytes, Jenkins *et al*. (2001) showed that exposure of estradiol (10^{-4} mol/L) for 48 h significantly inhibited lymphocyte proliferation but slightly inhibited proliferation at 10^{-5} mol/L and 10^{-6} mol/L; additionally, there was no significant difference for 24 h exposure of estradiol compared with that of the control. Several other studies reported that estradiol and hydrocortisone can reduce mouse lymphocyte proliferation following *in vivo* exposure (Hoffman-Goetz, 1999; Gupta *et al*., 1999). The discrepancy between our results and other's could be explained by the *in vitro* assay used in our study. Further investigation on the effect of *in vivo* exposure of BPA and natural steroid hormones on *Carassius auratus* lymphocyte proliferation is needed.

Macrophages play an important role as accessory cells in the immunological system by cooperating with T cells in aiding the response of B cells to antigens. Our results showed that BPA (0.005–50 mg/L), estradiol (0.005–800 mg/L) and hydrocortisone (0.005–500 mg/L) enhanced *Carassius auratus* macrophage proliferation in response to PHA, while higher doses of BPA (500–1000 mg/L) inhibited it. Corticosteroids, such as cortisol, have been long thought to be immunosuppressive in salmonids. However, Pagniello *et al*. (2002) reported that 0.1 and 1.0 mg/L of cortisol prevented rainbow trout macrophage cell line, RTS11, proliferation while the proliferation of RTS11 was enhanced in co-culture with the spleen stroma cell line (RTS34st) or in medium conditioned by RTS34st. These results suggested that proliferation of rainbow trout macrophages was modulated by cortisol, but the effect was regulated by the cellular microenvironment, possibly through the release of cytokines. The other research revealed that the proliferation of human bone marrow progenitor cells for colony forming unit granulocyte-macrophages (CFU-GM) was induced at low and inhibited at high cortisol concentrations (Rinehart *et al*., 1997). Our results that hydrocortisone enhanced macrophage proliferation more at low concentration and less at high concentrations may be explained by the above analysis.

We also observed the effects of *in vitro* exposure of BPA, estradiol and hydrocortisone on *Carassius auratus* macrophage respiratory burst using indicators including appearance time of RLU and maximal values of RLU, as described by Betoulle *et al*. (2000) about the effect of lindane on *in vitro* respiratory burst in rainbow trout phagocytes. However, we thought that the above two indicators may not be enough to compare the effects of various concentrations, and decided to calculate the area that represented the total chemiluminescence units in 15 min by the Newton method (Liu and Yi, 1999). The equation is:

$$S_{\mathrm{T}} = (t/2)\left(y_1 + y_n + 2\sum_{i=2}^{n-1} y_i\right) \quad (1)$$

S_T is the total squares, t is the interval time, and y is the RLU. From 0.05 to 500 mg/L of hydrocortisone, the S_T values are 14.01, 38.02, 28.58, 17.38 and 5.91 respectively. Compared to the control (26.05), macrophage respiratory burst was inhibited at the lowest and two highest doses and induced at two higher doses, the maximal induction occurring at 0.5 mg/L. From 0.05 to 500 mg/L of estradiol, the S_T values were 1.58, 2.88, 6.55, 3.59 and 2.36 respectively. Compared to the control (1.00), macrophage respiratory burst was enhanced, the maximal induction occurring at 5.0 mg/L. From 0.005 to 50 mg/L of BPA, the S_T values were 18.40, 24.78, 34.41, 33.01 and 9.37 respectively. Compared to the control (26.05), macrophage respiratory burst was inhibited at the two lowest and one highest doses and induced at two higher doses, the maximal induction occurring at 0.5 mg/L.

In carp macrophages, respiratory burst was elicited gradually by *in vitro* estradiol from 0.1 to 1000 nmol/L, whereas *in vitro* exposure to cortisol (1.0–1000 nmol/L) significantly suppressed respiratory burst in a dose-dependent manner (Yamaguchi *et al*., 2001). Following *in vitro* exposure to estradiol and cortisol, respiratory burst of goldfish macrophages, as measured by the NBT reduction assay, was induced more significantly at low doses than at high doses (Wang *et al*., 1995), consisting with our results. Moreover, Winston and Di Giulio (1991) reported the mechanisms that may account for the toxic effects of high doses. A large amount of reactive oxygen species (ROS) could be generated by macrophages in response to treatment with these chemicals. The oxidative stress generated by ROS could induce a major inflammatory reaction that causes cellular damage.

Sugita-Konishi *et al*. (2003) reported that BPA is immunotoxicity to mice. It reduces non-specific host defense such as neutrophils phagocytic activity and production of IL-6. In our study, BPA showed immunotoxicity to lymphocyte proliferation, macrophage proliferation and respiratory burst of fish. Compared to two natural steroid hormones, BPA had inhibitory effects at high doses, as did hydrocortisone while having stimulatory effects at low doses, as did estradiol. The stimulatory effect of BPA was, however, much weaker than natural estrogen. It could be that estradiol had higher affinity to the estrogen receptor than BPA (Kloas *et al*., 2001).

There have been several reports that estrogen and EDCs such as diethylstilbestrol, dioxin and 2,4-dichlorophenol did not behave in a dose-dependent manner but in the nonmonotonic dose-effect relationship (vom Saal *et al*., 1997; Markowski *et al*., 2001; Cavieres *et al*., 2002). It was also observed that two natural steroid hormones and BPA have low dose effects that do not occur at high doses in our study. Besides, it should be noted that there is obvious response difference between macrophage and lymphocyte. Welshons *et al*. (2003) also reported wide dose-response range required to observe such dual effects by natural and xenobiotic estrogens for MCF-7 cell, which can be 1000- to 100,000-fold. Such can be seen in the dose-response test of macrophage to estradiol (Fig.3b). It is suggested that mechanisms of nonmonotonic dose response for EDCs should be addressed in the future.

4 Conclusions

The present study showed that BPA and natural steroid hormones (estradiol and hydrocortisone) were immunotoxicity to *Carassius auratus* following *in vitro* exposure, the functional changes of lymphocyte and macrophage in *Carassius auratus* may be different between low and high dosages. The immunotoxicity of BPA may influence the ability of fish to defend against infectious diseases.

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