Supporting Information

The *in vitro* Immune Modulatory Effect of Bisphenol A on Fish Macrophages *via* Estrogen Receptor α and Nuclear Factor-κB Signaling

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The total of 9 pages, 4 figures and 2 tables.

MATERIALS AND METHODS

Primary macrophages from head kidney

Red common carps (*Cyprinus carpio*) were acclimated to laboratory conditions in dechlorinated tap water at room temperature for at least two weeks and then healthy fish were chosen for the experiments. Before the experiments, fish were anesthetized using 0.05% tricaine methane sulfonate and sanitized with 70% alcohol. Blood was removed maximally from the caudal vein using syringes. The method for isolation of primary macrophages from carp head kidney was modified based on Elkamel et al.[1] The head kidney was aseptically extracted from fish and washed two-three times with cell isolation and washing media (CTWM) which was optimized based on Miller and McKinney[2], and contained Hank's Balanced Salt Solution (Gibco BRL), heparin (10 U/mL), penicillin and streptomycin (1%). The tissue was cut into pieces using sterile stainless-steel scissors and forceps, filtered through a 100-pm nylon mesh and homogenized in phenol-red free Leibowitzs-15 medium (Gibco BRL) supplemented with heparin (10 U/mL), penicillin and streptomycin. The tissue suspension was centrifuged three times at 1000rpm for 5 min and resuspended in culture medium. The cells were counted with a hemocytometer and adjusted to a density of 1×10^7 cells/mL and plated in 96-well microplates (Corning, USA) at 100 μL/well, or in 25 cm^2 cell culture flasks (Corning, USA) at 3 mL/flask. After overnight incubation at 26°C, monolayers of adherent cells (about 1% of the seeded cells) in the microplates and culture flasks were washed twice with culture medium to remove unattached cells, and then they were used for the chemical exposure.

Bactericidal activity and phagocytic capability of primary macrophages.

The bactericidal activity of primary macrophages was determined against gram-positive *Staphylococcus aureus* (CGMCC 1.363) and gram-negative *Escherichia coli* (CGMCC 1.2389) and *Vibrio parahaemolyticus* (CGMCC 1.1615) following a modified protocol described by the previous study.[3] Primary macrophages were adjusted to a density of 10^4 cells per well in a 96-welled flat-bottomed plate and exposed to the BPA-supplemented culture medium for 6 h. After exposure, the culture supernatant was removed completely, and the cells were rinsed three times with antibiotic-free culture medium, and then inoculated with freshly prepared bacterial culture at 10^4 CFU / well (1:1), which was collected from an agar slope culture followed by rinsing with antibiotic-free culture medium three times and then dilution with antibiotic-free cell culture medium. A negative control containing only bacteria in the cell culture medium was submitted to the same procedure. After incubation for 24 h at 26°C, the supernatant from each well was transferred to a new well plate to be prepared for measuring absorbance at 600 nm which reflected the bactericidal activity of the macrophages.

The phagocytic capability of the primary macrophages was measured following the modified protocol described by the previous study.[4] The unattached primary macrophages were separated from 34%/51% (v/v) Percoll density gradient (GE, USA) using centrifugation. After exposure to BPA-supplemented culture medium for 6 h, the macrophages were washed three times and resuspended in 0.85 mL of antibiotic-free culture medium. Fifty mL of freshly thawed ice-cold normal serum and 0.1 mL of bacteria were added to each well ensuring the proportion of

macrophages to bacteria was 1:10. After cultivation on a Labquake shaker at 8 rpm for 90 minutes at 26°C, cells were washed four times to remove unbound bacteria. Final suspensions of macrophages were resuspended in 200 μL of PBS and applied to slides with a cytocentrifuge. The slides were then stained using a Diff-Quik Kit (Nanjing Jian Cheng Bioengineering Institute, China) for analysis under 100× oil-immersion microscopy (Axio Observer A1, ZEISS). The phagocytosis of macrophages was evaluated using a phagocytic index following the formula: phagocytic index = (percentage of macrophages containing at least one bacterium) × (mean number of bacteria per positive cell).

REFERENCES

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(2) Miller, N. W., and McKinney, E. C. *In vitro* culture of fish leukocytes. *Biochem. Mol. Biol. Fishes* **1994**, 3, 341–353.

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(4) Campbell, P. A.; Canono, B. P.; Drevets, D. A. Measurement of bacterial ingestion and killing by macrophages. Curr. Protoc. *Immunol.* **2001**, 14.16. 11-14.16. 13.

FIGURES

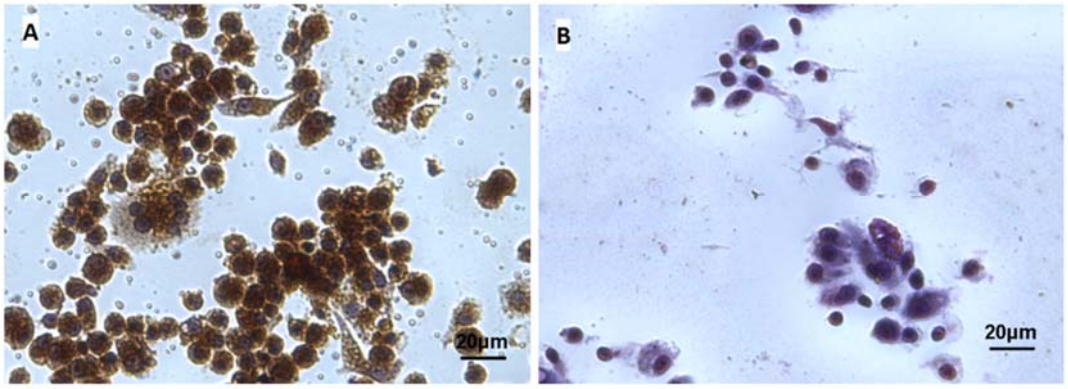

Figure S1. Different staining methods for macrophage identification. A: α-naphthyl butyrate esterase staining; B: Wright - Giemsa staining

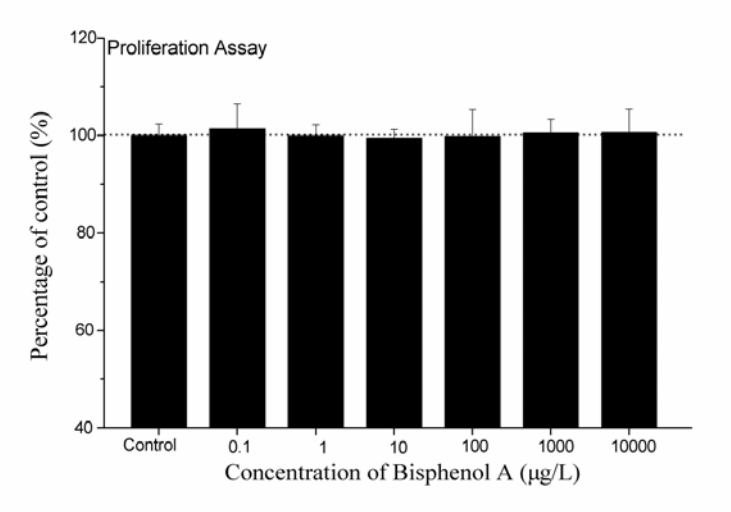

Figure S2. Proliferation assay of primary macrophages upon 6 h-exposure to bisphenol A. Values are means of the standard error of means (n=6) relative to the control. Significant differences versus the control are indicated as $*p < 0.05$ (ANOVA, Dunnett's test).

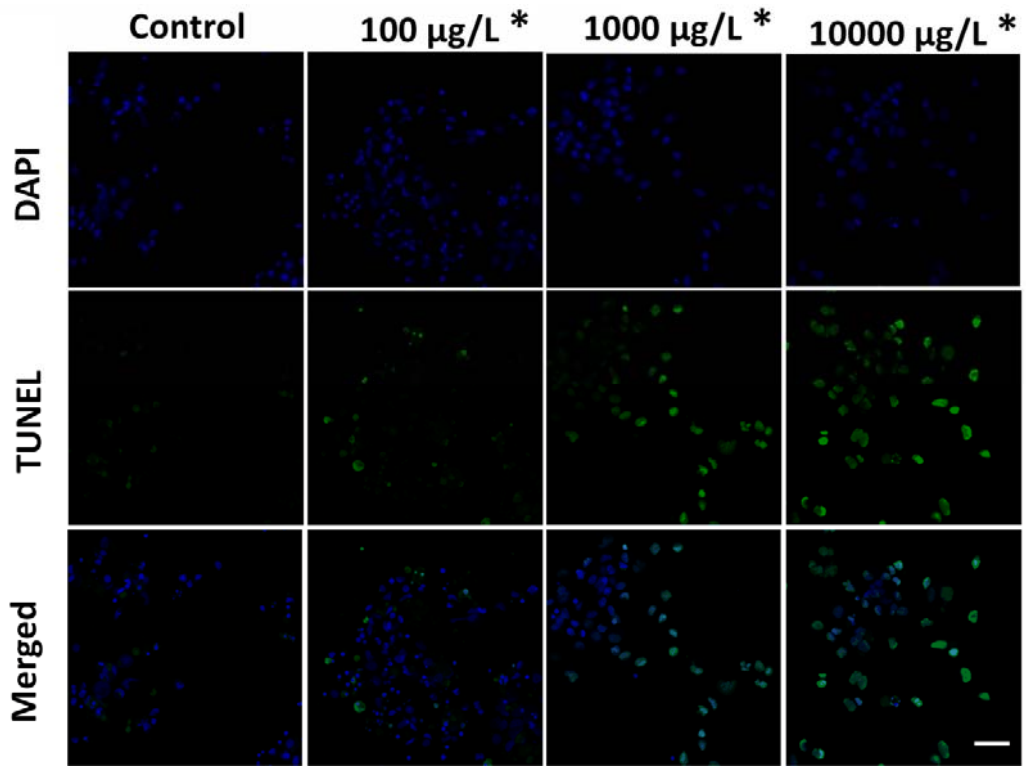

Figure S3. The representative fluorescent images showed a significant increase in TUNEL positive nuclei (green) in the 100, 1,000 and 10,000 µg/L treated samples compared to the control (D, Scale bar=20 µm). Significant differences versus control are indicated as *$p < 0.05$ (ANOVA, LSD's test).

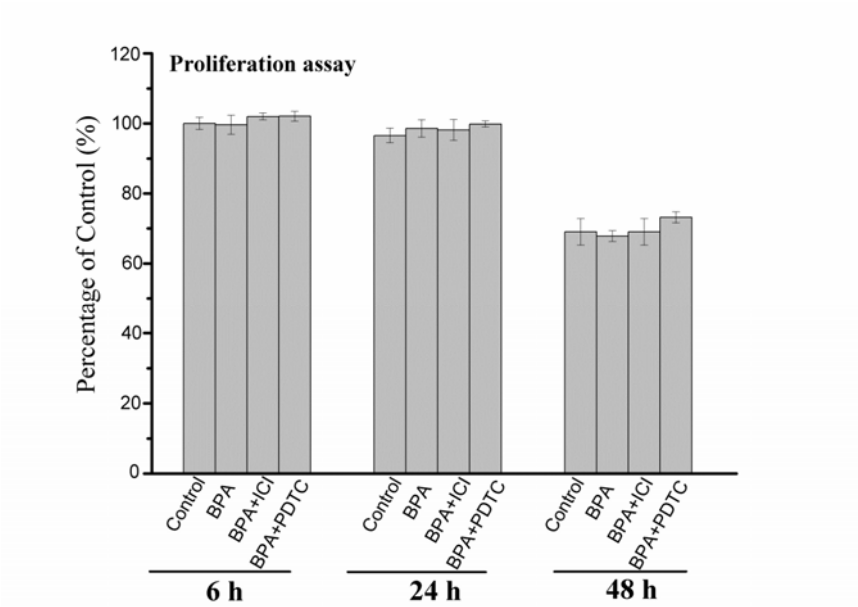

Figure S4. Proliferation assay of primary macrophages upon 48-h exposure to bisphenol A (BPA, 1,000 μg/L), a mixture of BPA (1,000 μg/L) and ICI 182,780 (50 μM), and a mixture of BPA (1,000 μg/L) and pyrrolidine dithiocarbamate (PDTC, 50 μM). Values are means of the standard error of means (n=6) relative to the control. Significant differences versus the control are indicated as $*p < 0.05$ (ANOVA, Dunnett's test).

Tables

Table S1. Primers used for real time PCR.

Gene name	GenBank accession number	Sequence of the primer (5'-3')
40S ribosomal protein S11	AB012087.1	F: GTTCTCGCTGTTGAAGGAAGTGG R: TTGCGGATGTAATGCAAGTAGTC
Interleukin-1β	AJ245635.1	F: AAGGAGGCCAGTGGCTCTGT R: CCTGAAGAAGAGGAGGCTGTCA
Interleukin-10	AB110780.1	F: AGCGGGATATGCGGAAATGTAGG R: TGCCAAATACTGCTCGATGTACTTAA
Interleukin-6 subfamily-like cytokine (*M17*)	AY102632.1	F: AGCTAAATTCAGAATGATCCTCGCTAT R: GCAGAAACTCCTTCAGGTGGGTG
Estrogen receptor α	AB334722.1	F: CACAGCCGCCCATACACCGAGAT R: GGAAGCCTGGTACTTTCTTAGCC
Hepcidin	JX273644.1	F: AGAGTGAAGCACCACAGGAGAAC R: GGAGACAGCCTGCATTTATACCC
Nuclear factor-κB p65	XP_005463218.1	F: TATGAAGCAGACCTACAG A: TCTTGGCATCAGGAATA

Table S2. Correlation coefficients between all tested parameters in the primary macrophages after 6 h-exposure to bisphenol A

	BA (S.)	BA (E.)	BA (V.)	LYSO	ROS	NO·	iNOS	TNOS	*IL-1β*	*IL-10*	*M17*	*Hepcidin*	TUNEL
BA (E.)	0.481*												
BA (V.)	0.238	0.719*											
LYSO	-0.352	-0.513*	-0.535*										
ROS	-0.391	-0.129	-0.276	0.118									
NO·	-0.219	-0.073	0.161	-0.376	0.223								
iNOS	-0.324	0.027	0.098	-0.371	0.493*	0.347							
TNOS	-0.151	0.204	0.319	-0.500*	0.075	0.492*	0.519*						
IL-1β	-0.219	0.193	0.231	-0.516*	0.493*	0.450*	0.591*	0.734*					
IL-10	-0.158	0.297	0.324	-0.581*	0.574*	0.430	0.623*	0.779*	0.796*				
M17	-0.329	0.033	0.121	-0.452*	0.442*	0.625*	0.623*	0.661*	0.837*	0.760*			
Hepcidin	-0.234	0.188	0.207	-0.592*	0.298	0.487*	0.669*	0.683*	0.784*	0.823*	0.684*		
TUNEL	-0.044	0.250	0.258	-0.594*	0.283	0.475*	0.579*	0.750*	0.781*	0.839*	0.764*	0.803*	
ERα	-0.013	0.327	0.318	-0.578*	0.199	0.423	0.566*	0.705*	0.892*	0.829*	0.742*	0.855*	0.733*

*$p < 0.05$ based on Spearman's test.

BA = bactericidal activity measured by number of extracellular bacteria; BA (*S.*) = bactericidal activity for *Staphyloccocus aureus subsp. Aureus*; BA (*E.*) = bactericidal activity for *Escherichia Coli*; BA (*V.*) = bactericidal activity for *Vibrio parahaemloyticus*; LYSO = lysozyme; ROS = reactive oxygen species; NO = nitric oxide; iNOS = induced nitric oxide synthase; TNOS = total nitric oxide synthase; *IL-1β = interleukin-1β*; *IL-10 = interleukin-10*; *M17 = interleukin-6 subfamily-like cytokine M17*; *ERα = estrogen receptor α.*