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Suppressive immunoregulatory effects of three antidepressants *via* inhibition of the nuclear factor-κB activation assessed using primary macrophages of carp (*Cyprinus carpio*)

Wenhui Qiu [a,b,c,1], Minghong Wu [a,1], Shuai Liu [a], Bei Chen [b], Chenyuan Pan [a], Ming Yang [a,*], Ke-Jian Wang [b,*]

[a] School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, China
[b] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, Fujian 361005, China
[c] School of Environmental Science & Engineering, Southern University of Science and Technology, Shenzhen, Guangdong 518055, China

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ABSTRACT

Antidepressants, having been applied for the treatment of major depressive disorder and other conditions for decades, are among the most commonly detected human pharmaceuticals in the aquatic environment. This study evaluated the immunotoxicity of acute exposure to environmentally relevant concentrations of amitriptyline, fluoxetine and mianserin using an *in vitro* primary macrophage model isolated from red common carp (*Cyprinus carpio*), and also explored their potential mechanisms of action. A potential suppressive immunoregulatory effect of antidepressant exposure was suggested based on the observed suppressive effects on oxidative stress parameters, bactericidal activity, NO production, and NO synthase activity, as well as pro-inflammatory cytokine gene expression, and a significant stimulatory effect on anti-inflammatory interleukin-10 and interferon cytokine gene expression and ATPase activities in macrophages after 6 h-exposure to three individual antidepressants and a combination thereof. Notably, we also found these effects were significantly associated with a corresponding decrease in nuclear factor-κB (NF-κB) activity after antidepressants exposure, and the NF-κB antagonist significantly restrained the effects of antidepressants on gene expression of cytokines, indicating that antidepressants could alter the response of various immune-associated components *via* the inhibition of NF-κB. Moreover, time-dependent lethal concentrations of three antidepressants on primary macrophages were firstly determined at mg/L levels, and the synergetic effects of antidepressant mixtures were suggested and in particular, for some parameters including total antioxidant capacity and cytokine genes expression, they could be significantly affected by antidepressants exposure at concentrations as low as 10 ng/L, which together thereby revealed the potential risk of antidepressants to aquatic life.

1. Introduction

The release of human pharmaceuticals into aquatic ecosystems has become a serious environmental problem during the last few years (Togola and Budzinski, 2008). Pharmaceuticals are designed to cure and treat disease, improve health, and increase life span, but the active pharmaceutical ingredients and their metabolites can be released into the environment through patient use or disposal. They ultimately reach the aquatic environment and will likely have an effect on the physiology of aquatic organisms (Cunningham et al., 2006). Antidepressants are among the most commonly detected human pharmaceuticals in the aquatic environment (Fong and Ford, 2014). Several studies have reported that common methods of removal of antidepressants in most water treatment plants are ineffective, which leads to continuous contamination and increasing concentrations of these drugs in water (Lajeunesse et al., 2008). Over the last decade, numerous studies have reported the concentrations of antidepressant drugs ranging from ng to μg/L in various aquatic systems worldwide, including freshwater, estuaries, the open ocean, and drinking water, among others (Calisto and Esteves, 2009; Fong and Ford, 2014). Also, antidepressants are widely detected in the surface river water in Chinese megacities: fluoxetine was detected up 21 ng/L in Beijing (Yuan et al., 2013) and the amitriptyline was found at a maximum of 3.7 ng/L in Shanghai (Wu et al., 2015). The number of occurrence and accumulation of antidepressants promptly rose awareness of health issues of aquatic organism (Silva et al., 2012).

There are many theories regarding the negative effects of antidepressants on the immune system (Eyre and Baune, 2012; Hannestad et al., 2011; Wong et al., 2008). For example, the immune related parameters after amitriptyline treatment have been observed and

* Corresponding authors.
E-mail addresses: mingyang@shu.edu.cn (M. Yang), wkjian@xmu.edu.cn (K.-J. Wang).
[1] These authors contributed equally.

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suggested to be associated with the inhibition of the release of pro-inflammatory cytokines and an decrease of nitric oxide production on the immune cells (Vismari et al., 2012). Consistently, the therapeutic role of the activity of fluoxetine and mianserin on cytokine levels has been reported in humans and murines (Avitsur et al., 2015; Manikowska et al., 2014; Sacre et al., 2010). Most studies concerning the immune modulatory effects of antidepressants thus far have been performed in human and rodent models, either *in vivo* or *in vitro*; however, the adverse effects on aquatic organisms have not been thoroughly studied. The immune system is crucial to the protection of aquatic organisms against pathogens and various stresses in the aquatic environment. Our previous study revealed significant alterations of the nitric oxide (NO) content, nitric oxide synthase (NOS) activity, and oxidative stress upon exposure to amitriptyline on zebrafish embryos, which implied possible adverse effects of antidepressants on the immune system of fish (Yang et al., 2014). Moreover, several measures of decreased immunocompetence, including phagocytosis, levels of reactive oxygen species (ROS), esterase activity, and lysosomal membrane destabilization in primary cultures of abalone hemocytes have been significantly induced upon exposure to four antidepressants at an ecological relevant concentration, implying that waterborne antidepressant contamination may significantly alter the functions of immune cells in aquatic organisms (Minguez et al., 2014).

Macrophages produce a wide range of biologically active molecules, including ROS and various cytokines and chemokines, as well as growth factors involved in antigen presentation, phagocytosis, and immunomodulation of inflammation (Fujiwara and Kobayashi, 2005). They are responsible for both non-specific defense and specific defense, and are one of the most important lines of defense against the outside environment. Therefore, the evaluation of macrophage function is considered as a fast and simple method for investigating the effects of antidepressants on the immune system (Leonard, 2001). In the present study, we employed the primary macrophages from head kidney of red common carp (*Cyprinus carpio*) to evaluate the immune modulatory effects of amitriptyline, fluoxetine, and mianserin, which belong to three different classes of antidepressants, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and tetracyclic antidepressants (TeCAs), respectively. It has been recently reported the alteration of cytokine expression in macrophages was associated with the nuclear factor-κB (NF-κB) signaling pathway (Tak and Firestein, 2001). Similarly, we investigated the potential roles of NF-κB transcription factors in antidepressant effects on fish macrophages. In this study, we revealed for the first time that low-dose antidepressant exposure and mixed antidepressant exposure in a simulated natural environment could significantly disturb the immune response of fish primary macrophages *in vitro*.

2. Materials and methods

2.1. Chemicals

Amitriptyline hydrochloride (CAS number 549-18-8; 98 +%) and mianserin hydrochloride (CAS number 21535-47-7; 98 + %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluoxetine hydrochloride (CAS number 56296-78-7; 98 +%) was purchased from CNW Technologies (Germany). The stock solution was prepared by dissolving the chemicals in Milli-Q to a concentration at 10 g/L. Fresh stock solution was made every week. All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) and Sangon (Shanghai, China).

2.2. Primary macrophages from head kidney

Primary macrophages were isolated from the head kidney of red common carp according to the method reported in our previous study (Qiu et al., 2016). Fish, weighing 300–500 g, were purchased from a fish farm in Shanghai, China and acclimated to laboratory conditions for 2 weeks before the experiments. Briefly, fish were anesthetized using 0.05% tricaine methane sulfonate and sanitized with 70% alcohol. Blood was then removed maximally from the caudal vein using syringes. The head kidney was aseptically extracted from fish and washed two-three times with cell isolation and washing media 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-μm nylon mesh and homogenized in Leibowitzs-15 medium (Gibco BRL) supplemented with heparin and penicillin and streptomycin (1%). The tissue suspension was centrifuged three times at 1000 rpm 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. All procedures were conducted following the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of Shanghai University of China.

2.3. Experimental design

2.3.1. Time-dependent lethal concentrations. Primary macrophages were randomly exposed to amitriptyline, fluoxetine, and mianserin for 6, 12, and 24 h in a series of diluted solutions. The exposure concentrations of each antidepressant were 0, 1, 3, 5, 10, 25, 50, 75, and 100 mg/L, which were based on the reported 24 and 48 h toxicity data for cultured rat pheochromocytoma cells and our previous research on amitriptyline (Kolla et al., 2005; Yang et al., 2014). Cytotoxicity was measured at 6, 12, and 24 h using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA) following the manufacturer's instructions to determine time-dependent lethal concentrations.

2.3.2. Sublethal exposure. We observed the no-observed effect concentration (NOEC) of antidepresants for 6 h-exposure was under 1 mg/L, however, the viability of primary macrophages was not impaired after 6-h exposure to 100 μg/L antidepressants (Fig. S1). Primary macrophages were exposed to amitriptyline, fluoxetine, mianserin at sublethal concentrations of 10 ng/L, 1 μg/L, and 100 μg/L, respectively, for 6 h. To determine whether and how the mixtures of amitriptyline, fluoxetine, and mianserin affected the immune system, the primary macrophages were coexposed at low (10 ng/L amitriptyline, 10 ng/L fluoxetine, and 10 ng/L mianserin), medium (1 μg/L amitriptyline, 1 μg/L fluoxetine, and 1 μg/L mianserin), and high (100 μg/L amitriptyline, 100 μg/L fluoxetine, and 100 μg/L mianserin) concentrations, paralleling the single chemical treatments. After exposure, the primary macrophages were examined in subsequent bioassays.

We also used lipopolysaccharide (LPS) to activate macrophages before antidepressant exposure to confirm the effects of the antidepressants on cytokines. First, primary macrophages were activated by LPS from *P. aeruginosa* 10 (Sigma-Aldrich, USA) for 6 h at the concentration of 50 μg/mL. Next, the concentrations of antidepressants with significant effects on cytokine gene expression (100 μg/L amitriptyline, 100 μg/L fluoxetine, 100 μg/L mianserin, and the 100 μg/L mixture) were selected and macrophages were exposed for 6 h parallel with the LPS positive control groups that were treated with a culture medium for 6 h after activation by LPS. After exposure, the primary macrophages were examined for several typical cytokines.

An NF-κB antagonist experiment was designed separately using coexposure of macrophages for 6 h to 100 μg/L amitriptyline, 100 μg/L fluoxetine, 100 μg/L mianserin, and a 100 μg/L of the mixture with an NF-κB antagonist pyrrolidine dithiocarbamate (PDTC, Sigma) at 1 μM.

If the PDTC blocked the actions of antidepressants, it would suggest which cellular pathways mediated the effects of these antidepressants on gene expression of cytokines.

2.4. Biochemical assays

The protein level of each sample was determined using the Bradford assay (Beyotime Institute of Biotechnology, China). Intracellular ROS content was determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescein diacetate (Beyotime Institute of Biotechnology, China) to fluorescent dichlorofluorescein. Total antioxidant capacity (T-AOC), lysozyme activity, total NO level, activities of total NO synthase (TNOS) and induced NO synthase (iNOS), and the content of Na^+K^+-ATPase, $Ca^{2+}Mg^{2+}$-ATPase, and T-ATPase were measured following the protocols of commercially available kits (Nanjing Jiancheng Bioengineering Institute).

2.5. RNA isolation, reverse-transcriptase, and quantitative polymerase chain reaction

RNA was extracted using an RNAprep pure kit (Tiangen Biotech, China). Reverse-transcriptase reactions were performed on 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit. Real-time quantitative PCR (qPCR) was performed using the FastStart Universal SYBRGreen Master (ROX) kit. The primers specific for target genes and the reference gene endogenous *40s ribosomal protein s11* are listed in Table S1 (SI). A Ct-based relative quantification with efficiency correction normalizing to the reference gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6. Antimicrobial activity and phagocytic capability of primary macrophages

The antimicrobial activity and phagocytic capability of the primary macrophages was determined against gram-negative *Escherichia coli* (CGMCC 1.2389) following the methods of our previous study (Qiu et al., 2016). Primary macrophages were adjusted to a density of 10^4 cells per well in a 96-well flat-bottomed plate and exposed to the antidepressant-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 subjected 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 using the unattached primary macrophages separated from 34%/51% (v/v) Percoll density gradient (GE, USA) using centrifugation. After exposure to antidepressant-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 min 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).

2.7. Statistical analysis

Data are shown as means ± standard error of the means. All data were normalized using the Kolmogorov-Smirnov one-sample test and Levene's test. Statistical analyses were performed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA). The intergroup differences were assessed using a one-way analysis of variance (ANOVA) followed by Dunnett's test or Turkey's test. The level for statistical significance was set at $p < 0.05$ and is indicated with an asterisk.

3. Results

3.1. Time-dependent lethal concentrations

Mortality was determined after 6, 12, and 24 h of primary macrophage exposure to various concentrations of amitriptyline, fluoxetine, and mianserin during the 24 h-exposure period, as shown in Fig. 1. Concentrations at which 50% (LC_{50}), 20% (LC_{20}), and 5% mortality (LC_5) occurred among the tested embryos were determined by Probit analysis (Table 1). These results were confirmed by four independent replicates. The LC_{50} for primary macrophages after 6 h exposure to amitriptyline, fluoxetine, and mianserin were estimated as 76.1, 58.0, 15.6 mg/L, respectively, by Probit analysis. The viability of primary macrophages was not impaired after 6-h exposure to sublethal concentrations of antidepressants based on the cytotoxicity assays (Fig. S1).

3.2. Oxidative stress indices

Generally, cellular ROS content and T-AOC were inhibited in primary macrophages after a 6-h exposure to amitriptyline, fluoxetine, mianserin, and their mixture. ROS content was significantly decreased following exposure to 100 μg/L fluoxetine and all amitriptyline/fluoxetine/mianserin coexposure groups compared to the control (Dunnett's test, $p < 0.05$), as shown in Fig. 2A. In terms of T-AOC level, significant inhibition was observed in the 1 μg/L and 100 μg/L amitriptyline concentrations, all fluoxetine exposure groups, the 100 μg/L mianserin concentration, and all amitriptyline/fluoxetine/mianserin coexposure groups compared with the control (Fig. 2B).

A roughly concentration-dependent decrease in NO levels and iNOS and TNOS activity was observed in macrophages following the 6-h exposure to antidepressants, as shown in Fig. 2C. In comparison with the control, the levels of NO were significantly decreased following exposure to the 1 μg/L and 100 μg/L amitriptyline/fluoxetine/mianserin coexposure groups. INOS activity was significantly reduced for the 100 μg/L mianserin concentration and all amitriptyline/fluoxetine/mianserin coexposure groups. In terms of TNOS activity, significant reduction was observed in the 1 μg/L and 100 μg/L fluoxetine concentrations, the 1 μg/L and 100 μg/L mianserin concentrations, and all amitriptyline/fluoxetine/mianserin coexposure groups.

3.3. Expression of cytokine genes

Gene expression of several cytokines was assessed to evaluate the effects of amitriptyline, fluoxetine, mianserin and their mixture on primary macrophages after 6-h exposure. Overall, the mRNA levels of the tested pro-inflammatory cytokine genes, including *interleukin-1β (il-1β)*, *interleukin-6* (*il-6*), *interleukin-6 subfamily-like cytokine M17* (*m17*), *interleukin-11* (*il-11*), *interleukin-12 p35* (*il-12 p35*), *interleukin-12 p40* (*il-12 p40*), and *tumor necrosis factor-a (tnf-a)* were dramatically inhibited in an approximately concentration-dependent manner following antidepressant exposure (Fig. 3A, Table S2). However, interestingly, the effects of anti-inflammatory and interferon cytokine genes in response to the 6-h exposure to amitriptyline, fluoxetine, mianserin, and their mixture on primary macrophages were different. The expression of *il-10* and interferon cytokine genes was induced in a concentration-dependent manner in primary macrophages after antidepressant

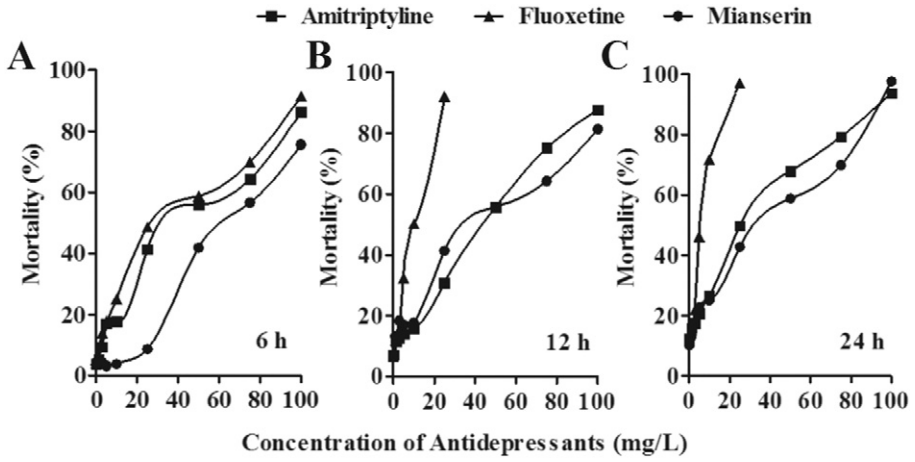

Fig. 1. Dose-response of amitriptyline, fluoxetine and mianserin to primary macrophages of red common carp. Data are shown as means (n = 6) of mortality of the macrophages after exposure to 0, 1, 3, 5, 10, 25, 50, 75, and 100 mg/L of antidepressants at 6 h (A), 12 h (B), 24 h (C).

exposure (Fig. 3A, Table S2). Gene expression of chemokine, including *cxcl-8* and *cxc-chemokine*, were not significantly different following antidepressant exposure (Fig. 3B).

Furthermore, pro-inflammatory cytokine gene expression, including *il-1β*, *il-12 p35*, *il-11*, *il-12 p40*, *il-6*, and *m17*, and chemokine gene expression, including *cxcl-8* and *cxc-chemokine*, were significantly induced after activation by LPS for 6 h compared to the control (Fig. 3C). The above genes appeared to significantly decline compared to the LPS-positive control after antidepressant exposure, and tended to return to the control level. Correspondingly, the gene expression of anti-inflammatory cytokines changed in an inverse pattern as shown in Fig. 3C. The levels of *il-10* and inf-*γ1* appeared to significantly increase after antidepressant exposure compared to the LPS-positive control. These results indicated that antidepressants inhibited pro-inflammatory cytokines, but stimulated anti-inflammatory cytokines and interferon cytokines, which was consistent with the results following exposure to antidepressants alone.

3.4. Bactericidal activity and phagocytic capability

The bactericidal activity of primary macrophages against *Escherichia coli* was inhibited in a concentration-dependent pattern, judging from the increase of bacterial density based on the OD 600 value (Fig. 4A). The bactericidal activity was significantly decreased on exposure to 100 μg/L amitriptyline, 100 μg/L fluoxetine, 1 and 100 μg/L mianserin, and all coexposure groups compared to the control (Dunnett's test, $p < 0.05$). Correspondingly, the phagocytic capability of primary macrophages was changed in an inverse pattern as shown in Fig. 4B after expose to the high concentrations of antidepressants. Consistent with the bactericidal activity, the calculated phagocytic index of primary macrophages was significantly decreased in the highest concentrations of amitriptyline, fluoxetine, mianserin, and their mixture and the pictures for phagocytosis of cells were shown in Fig. 4C.

Table 1
Dose-response for the primary macrophages upon exposure to amitriptyline, fluoxetine and mianserin for 6, 12 and 24 h.

Exposure time	Mianserin (mg/L)			Amitriptyline (mg/L)			Fluoxetine (mg/L)		
	LC_{50}	LC_{20}	LC_5	LC_{50}	LC_{20}	LC_5	LC_{50}	LC_{20}	LC_5
6 h	76.1	19.0	5.1	58.0	16.5	5.0	15.6	4.0	1.1
12 h	34.0	4.8	0.7	33.8	6.6	1.4	9.4	2.4	0.7
24 h	21.7	3.0	0.4	17.4	3.0	0.6	6.2	1.3	0.3

The concentration that would cause 5%, 20% and 50% mortality of tested populations calculated by Probit analysis method.

3.5. Na^+K^+-ATPase, $Ca^{2+}Mg^{2+}$-ATPase and T-ATPase activities

Triphosphatases (ATPases) are a class of enzymes that catalyze the decomposition reaction releases energy for driving other chemical reactions and widely used in all known forms of life, may represent an important link between immune system (Borsellino et al., 2007; Chen et al., 2010; Pasupuleti et al., 2011). We observed increase for ATPase content after 6-h exposure to amitriptyline, fluoxetine, and mianserin and their mixture, as shown in Fig. 5. Compared to the control, Na^+K^+-ATPase was significantly induced in 100 μg/L amitriptyline/fluoxetine/mianserin coexposure groups, $Ca^{2+}Mg^{2+}$-ATPase in 1 μg/L and 100 μg/L amitriptyline/fluoxetine/mianserin groups and T-ATPase in 100 μg/L amitriptyline, 1 μg/L fluoxetine and 100 μg/L amitriptyline/fluoxetine/mianserin groups.

3.6. Role of nuclear factor-κβ signaling pathway in mediating immune modulatory effect of antidepressants

A significant decrease of *nf-κb* (*p65*) expression was observed in primary macrophages upon 6-h exposure to different concentrations of antidepressants (Fig. 6A). The viability of primary macrophages was not significantly impaired during 6 h exposure to antidepressants and coexposure to NF-κB antagonist compared to the control (SI Fig. S2). As shown in Fig. 6B, the pro-inflammatory cytokine gene expressions were significantly inhibited upon exposure to antidepressants. However, upon coexposure with PDTC, the inhibition level of pro-inflammatory cytokines was significantly induced after 6 h by the antagonist effects of NF-κB inhibitor. Corresponding to the results of pro-inflammatory cytokine genes expression, the induction levels of anti-inflammatory cytokine genes were all significantly decreased (Dunnett's test, $p < 0.05$) by coexposure with PDTC (Fig. 6C). In addition, the concentration-dependent inhibition of *nf-κb* (*p65*) expression after antidepressants exposure in macrophages, was observed to correlate with all tested immune response related parameters significantly (Table S3). The results indicated that immune response mediator NF-κB antagonist PDTC could significantly mediated the immune response of the antidepressants.

4. Discussion

In the present study, we evaluated the immune effects of environmentally relevant concentrations of antidepressants on primary macrophages in fish. The main findings of this study are that amitriptyline, fluoxetine, mianserin, and their mixture have a significant suppressive effect on ROS content, T-AOC activity, bactericidal activity, NO level, iNOS and TNOS activities and expression of pro-inflammatory cytokine

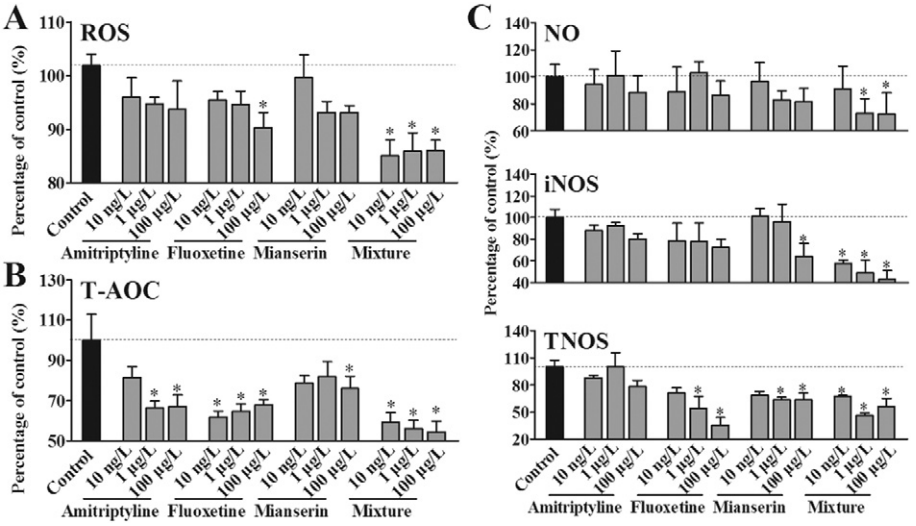

Fig. 2. Changes in the production of reactive oxygen species (ROS) (A), total antioxidative capacity (A-TOC) (B), as well as nitric oxide (NO), induced and the total nitric oxide synthase activities (iNOS and TNOS) (C) in the primary macrophages following 6 h-exposure to amitriptyline, fluoxetine, mianserin, and their mixture. Values are means of percentage ± standard deviation (n = 3) relative to the control. Significant differences versus control are indicated as $^{*}p < 0.05$ (ANOVA, Dunnett's test).

genes, and instead have a significant stimulatory effect on expression of anti-inflammatory *il-10*, interferon cytokine genes, and ATPase activities. The findings of this study suggest that various antidepressive drugs, including tricyclic, SSRIs and tetracyclic antidepressants, may have negative immunoregulatory effects on non-target aquatic animals, according to the similar results reported by Kenis and Maes (2002) in human and animal models. We also found these effects were associated with a corresponding decrease in NF-κB activity, suggesting that antidepressants affect regulation of the immune cells through the inhibition of NF-κB activation, which was consistent with previous observations on NF-κB activity affected by antidepressants using the *in vitro* human hippocampal progenitor cells (Horowitz et al., 2015). In addition,

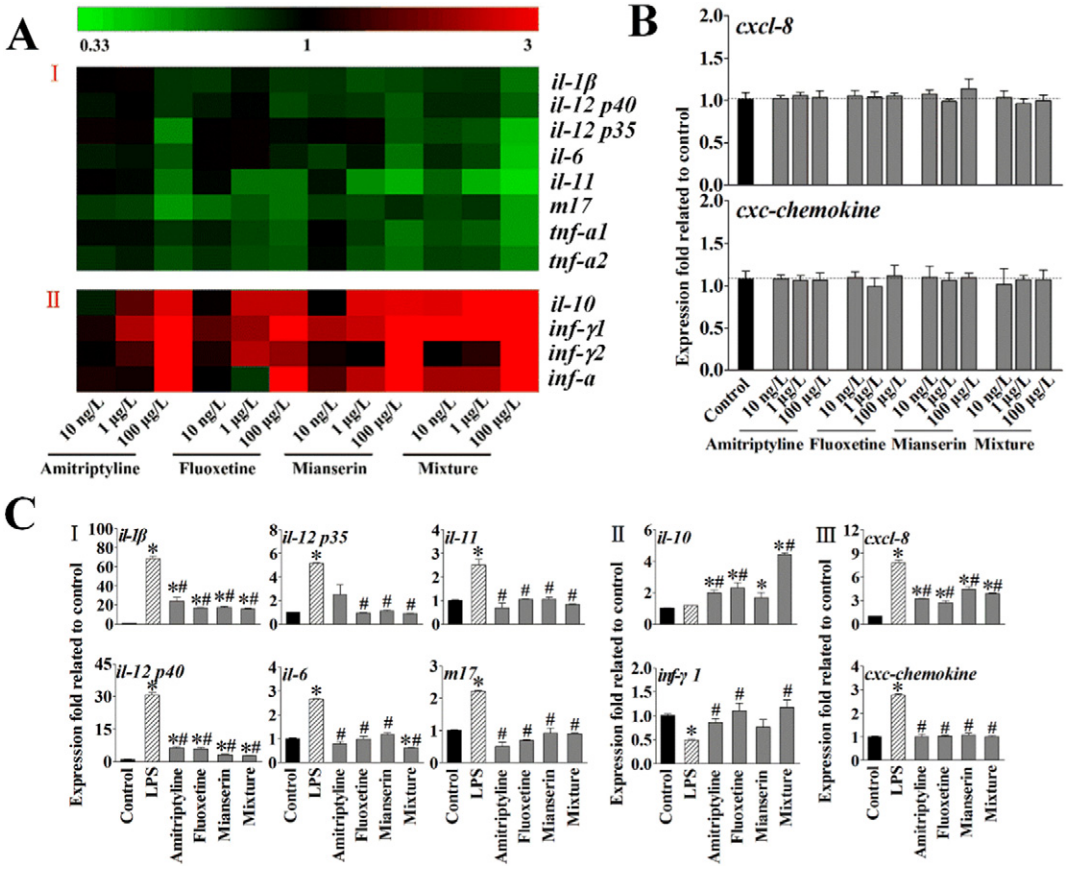

Fig. 3. Changes in the expression of cytokines (A, heat map) and chemokines (B) in the primary macrophages following 6 h-exposure to amitriptyline, fluoxetine, mianserin and their mixture. (C) Primary macrophages were activated by lipopolysaccharide (LPS) at 50 μg/mL for 6 h before antidepressant exposure for 6 h. The data are mRNA expression change (fold) in the treatment groups relative to the control. The heat map (A) uses the color changes to represent the expression fold changes. Values in B and C are means ± standard deviation (n = 3). Significant differences *versus* control are indicated as $^{*}p < 0.05$ (ANOVA, Dunnett's test). Significant differences *versus* LPS are indicated as $\#p < 0.05$ (ANOVA, Dunnett's test).

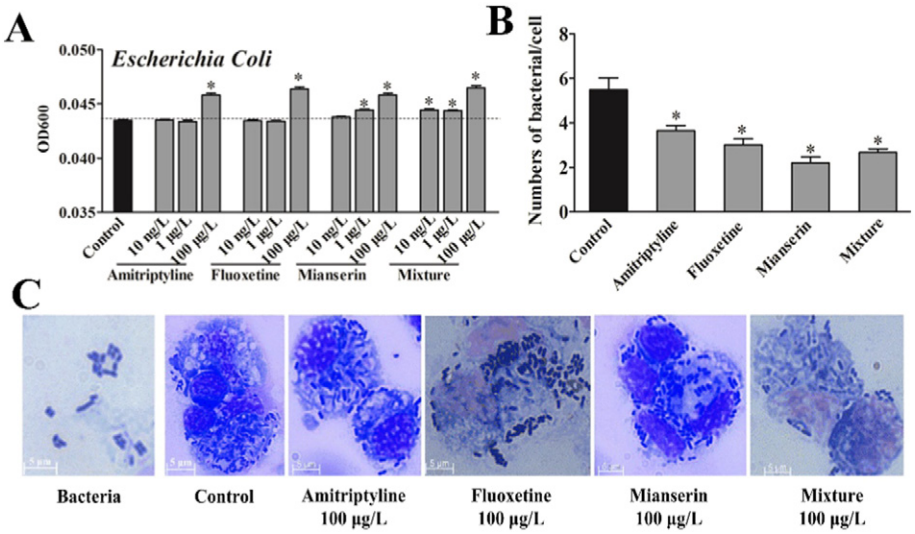

Fig. 4. Changes in antibacterial activities against *Escherichia coli* after 6 h-exposure to amitriptyline, fluoxetine, mianserin and their mixture. (A) Bactericidal activity was assayed by measuring the absorbance of extracellular bacteria at 600 nm. (B) Phagocytosis was measured by counting the number of intracellular bacteria. (C) The representative images comparing the phagocytic activities between the control group and the 100 μg/L of amitriptyline, fluoxetine, mianserin and their mixture group (scale bar = 5 μm). Values are means ± standard deviation (n = 6). Significant differences *versus* control are indicated as $^{*}p < 0.05$ (ANOVA, Dunnett's test).

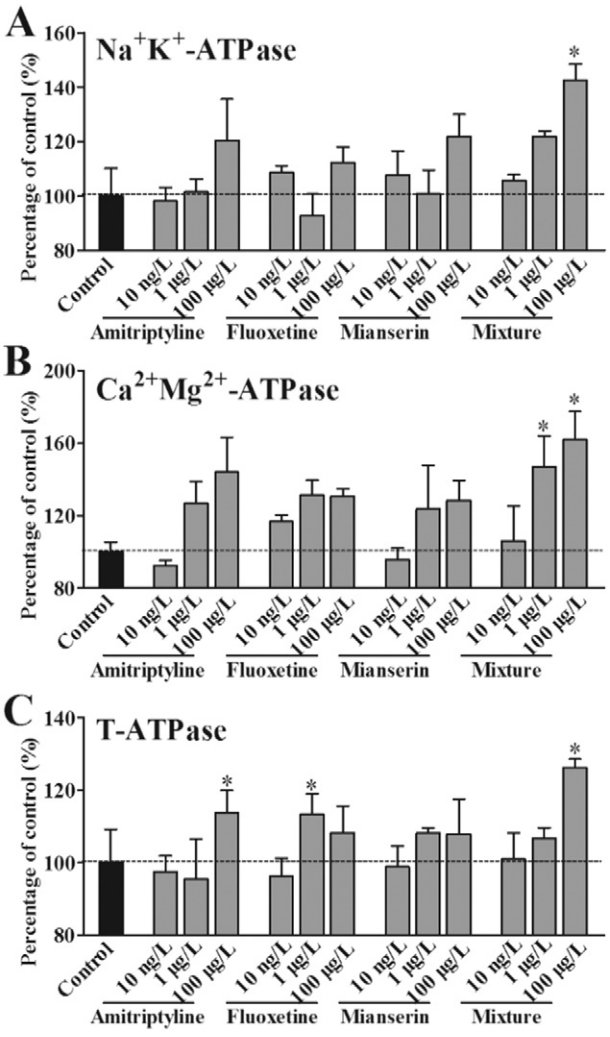

Fig. 5. Changes in the activities of $Na^{+}K^{+}$-ATPase (A), $Ca^{2+}Mg^{2+}$-ATPase (B) and T-ATPase (C) in the primary macrophages upon 6 h-exposure of amitriptyline, fluoxetine, mianserin and mixture. Values are means of the percentage ± standard deviation (n = 3) relative to the control. Significant differences *versus* control are indicated as $^{*}p < 0.05$ (ANOVA, Dunnett's test).

significant correlations between these measured parameters were revealed, which suggested that a dynamic coordinated regulation of physiological responses was evoked while they were adapting to increased stress of amitriptyline exposure.

There are relatively few studies reported on the antidepressants toxicity to the aquatic organism. The LC_{50} of amitriptyline, fluoxetine, and mianserin for 24 h exposure were 17.4, 6.2, and 21.7 mg/L, respectively. Compared to results from previous study that 48 h LC_{50} of primary abalone hemocytes to amitriptyline is 45.24 mg/L (Minguez et al., 2014), the red carp primary macrophages were more sensitive to antidepressants exposure. Furthermore, in one family of antidepressants, three compounds may have different acute toxicities with a structure activity relationship of fluoxetine (SSRIs) > amitriptyline (tricyclic) > mianserin (tetracyclic). According to Derijks et al. (2008) the reason for different acute toxicities of antidepressants may be classified either on different receptor binding profiles or different molecular structure or norepinephrine neurotransmitter systems.

Antidepressant exposure significantly inhibited pro-inflammatory cytokine genes and induced *il-10* and interferon cytokine genes in macrophages, consistent with previous observations in mice after antidepressant treatment including fluoxetine and paroxetine (Kenis and Maes, 2002; Ohgi et al., 2013). There is now evidence that major depression is accompanied by an up-regulation of the inflammatory responses and animal models studies and human cytokine immune therapy suggest that pro-inflammatory cytokines induce depressive symptomatology (Dowlati et al., 2010; Leonard, 2014). Our results suggest that antidepressants alleviate depressive symptoms through changes in cytokine production or action; specifically suppression of pro-inflammatory cytokine and induction of anti-inflammatory cytokine production. However, it has been confirmed that the above changes in cytokine production promoted an overall immunosuppressive cytokine phenotype (Connor et al., 2005; Maes et al., 1999), suggesting that antidepressants suppress immune function while alleviating symptoms of depression. In addition, nitric oxide, acting as an important pro-inflammatory mediator modulating immune response and involved in many physiological and pathological processes (Hou et al., 1999), was significantly reduced after concentrations of antidepressant exposure, implying an immunosuppressive effect of amitriptyline, fluoxetine, mianserin, and their mixture on primary macrophages (Hwang et al., 2008; Vismari et al., 2012).

Both the housekeeping functions and the specialized activities involved in targeting antigens of immune cells such as macrophages

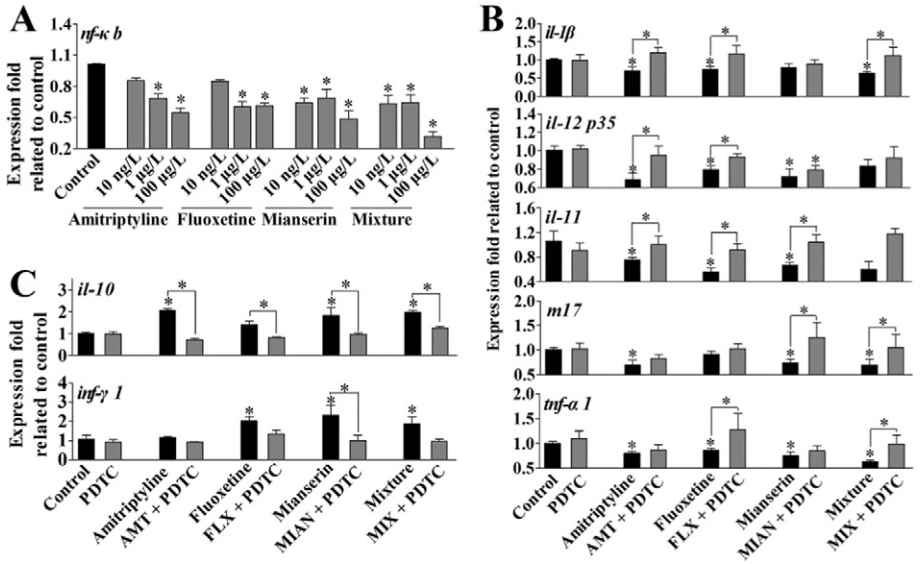

Fig. 6. Role of the nuclear factor-κB (NF-κB) in mediating effects of amitriptyline, fluoxetine, mianserin, and their mixture on cytokines expression in primary macrophages. (A) Effects on *nf-κb* (*p65*) mRNA levels are shown after 6 h-exposure to antidepressants. Effects on the pro-inflammatory cytokine genes (B) and anti-inflammatory cytokine genes (C) expression following 6 h-exposure to amitriptyline (100 μg/L), fluoxetine (100 μg/L), mianserin (100 μg/L), their mixture (100 μg/L), and a mixture of the antidepressants (100 μg/L) and NF-κB antagonist pyrrolidine dithiocarbamate (PDTC, 1 μM). The data are mRNA expression change (folds) in the treatment groups relative to the control. Values are means ± standard deviation (n = 3). Significant differences *versus* control are indicated as $^{*}p < 0.05$ (ANOVA, Dunnett's test). Significant differences between intergroup crossed with a line are indicated as $^{*}p < 0.05$ (ANOVA, Turkey's test).

depend on their energy supply (Buttgereit et al., 2000). The decrease of ATPases may lead proper immune functions fail without adequate energy, but overexpression of ATPases will break the energy balance in the body, increase the cell acute stress, and exert a negative impact on the immune functions (Buttgereit et al., 2000). What is more, the biological activities of overexpression of extracellular ATP are multiple, including mitogenic stimulation and induction of cell death. Different from other previous studies that antidepressants including amitriptyline, fluoxetine and mianserin caused a significant decrease on ATPases (Zanatta et al., 2001). Our results revealed that ATPases were significantly induced after high concentrations of antidepressants treatment, which is consistent with the effect of benzo(a)pyrene exposure on the response of ATPases in juvenile European sea bass (Lemaire-Gony et al., 1995), suggesting that primary macrophages are more sensitive to antidepressants exposure and the responses could mainly be linked to the acute stress caused by high concentration injection, which may also lead to a possible adverse effect on the immune defense (Lemaire-Gony et al., 1995).

Upon exposure to 100 μg/L of amitriptyline, fluoxetine, mianserin, and their mixture, most measured parameters in the primary macrophages were significantly affected. Particularly, some parameters such as on total antioxidant capacity, the activities of NO synthase and expression of cytokine genes could be affected at concentrations as low as 10 ng/L, six orders of magnitude lower than the LC_{50} value of antidepressants. It should be noted that the fish immune system is sensitive to waterborne toxicant exposure, and the alteration of their immune homeostasis would significantly affect their survival and development. Therefore, although the presence of antidepressants in aquatic environments has been reported at low ng/L levels (Calisto and Esteves, 2009; Fong and Ford, 2014), their potential risks for fish species that might have been chronically exposed to these chemicals would be significant (Silva et al., 2012). Moreover, our results demonstrated that the mixture of antidepressants might have synergetic effects on immune regulation in fish primary macrophages. Considering the joint exposure to multiple antidepressants in the actual exposure scenario in some polluted waters (Lajeunesse et al., 2011), the chronic impacts of the antidepressants on aquatic organisms should not be underestimated. In addition, previous studies (Bringolf et al., 2010; Schultz et al., 2010) show that the bioaccumulating activities of some antidepressants are as high as 70–80 times in fish organisms, suggested that the ecotoxicological risk of those antidepressant pollution to fish species and other non-target aquatic organisms would be tremendous.

The exact mechanisms by which antidepressive drugs exert their activities on immune function, including antimicrobial activity and cytokine action, are still unknown. Interestingly, the concentration-dependent inhibition of *nf-κb* expression after antidepressant exposure in macrophages, which was correlated with most immune parameters in our study, was consistent with previous *in vitro* observations on the HT22 cell after amitriptyline and fluoxetine treatment (Post et al., 2000) and on rat primary neuron cell expose to fluoxetine (Zhang et al., 2012). NF-κB is one of the primary transcription factors and a key player regulating the inflammatory response involved in the synthesis and release of pro-inflammatory cytokines (Tak and Firestein, 2001). It has been identified as a key mediator of the effects of stress on depressive behavior (Koo et al., 2010) and found to be upregulated in depressed patients (Horowitz et al., 2015; Pace et al., 2006). In particular, monoaminergic antidepressants have been shown to reduce NF-κB activity in rat glial cultures (Bielecka et al., 2010) and brains (Zhu et al., 2008). Similarly, amitriptyline and fluoxetine have also been shown to reduce NF-κB activity in colon tumor cells (Stopper et al., 2014), as well as in human ovarian carcinoma cell line (Lee et al., 2010). Therefore, the regulation of NF-κB may be a common mechanism of action proposed for the immunomodulatory properties of antidepressant.

5. Conclusions

In short, this study revealed the immunomodulatory effect of antidepressants on fish macrophages in a concentration-dependent manner within a relatively low and environmentally relevant concentration range. A suppressive immunoregulatory effect of antidepressants was observed, and the involvement of NF-κB pathway was suggested in its action mechanism. Our findings firstly provide evidence that antidepressants interfere with the immune regulation of fish macrophages under *in vitro* conditions, further strengthen our understanding of antidepressants' immunotoxicity in fish.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document, associated with this article can be found, in online version.

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