Fish and Shellfish Immunology 86 (2019) 125–134

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Interruption of immune responses in primary macrophages exposed to nonylphenol provides insights into the role of ER and NF-KB in immunotoxicity of Persian sturgeon

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ARTICLE INFO

Keywords:
Nonylphenol
Immunotoxic
Acipencer persicous

ABSTRACT

The severe decline in population of sturgeons due to pollution highlighted poor understanding about the immunotoxicological responses of sturgeons. This study was designed in three experiments to find out how nonylphenol (NP) interrupts some pro-inflammatory immune parameters in macrophages from Persian sturgeon (*Acipencer persicous*) as the oldest vertebrate model conserving intact innate immune system. After determination of IC_{50} values of NP (200 μM), some pro-inflammatory immune parameters and induced apoptosis in macrophages at low dose (10 nM) and high dose (100 nM) of NP and of 17β estradiol (E2) (positive control) were determined after 6, 24 and 48 h of the exposure (as the first experiment). The two doses of NP induced pro-inflammatory reaction and apoptosis with strong correlations, whereas this result was observed more obviously in high dose of E2. In the second experiments, the macrophages were exposed to the two doses of NP along with estrogen receptor alpha (ERα) antagonist, which consequently decreased the induction of pro-inflammatory reactions. Similarly, in the third experiment, NF-KB and ERα antagonists were used and pro-inflammatory reactions decreased compared to the control group ($P < 0.05$). Decreasing correlation between immune parameters following the second and third experiments verified interaction between ERα and NF-KB pathways. Thus, NP could be immune disrupter and apoptosis inducer in sturgeon macrophages in vitro, even in low dose. For the first time, this study revealed that NP can induce pro-inflammatory reactions in macrophages derived from sturgeons.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are man-made compounds that can disturb biochemical processes related to natural hormones and result in homeostatic, reproductive, developmental and behavioral changes in vertebrates, especially in fish [1,2]. One of the most EDCs recognized as potentially hazardous factors for fish physiology and its immune function [3] is estrogen-like endocrine disrupting chemicals (EEDCs) [4,5]. Nonylphenol (NP), as a subset of alkylphenols and EEDCs [6] is produced environmentally through degradative reaction of nonylphenol ethoxylates (NPEs), which are important industrial compounds in making cleaners, detergents and plastics [7]. The NP compared to its parent compounds is more resistant and lipophilic, tending to be accumulated in aquatic environment [8] and consequently posing a serious threat to fish.

Some previous studies illustrated that EEDCs such as NP may affect the immune system of fish [9,10]. Since the chemical structure of NP has similarities to that of endogenous steroid hormones especially 17β-estradiol (E2), NP may interact with the ligand-binding domain of estrogen receptors (ERs) subtypes that are expressed in piscine immune cells [5,11]. In fish, innate immunity serves as the first line of defense against invading pathogens [12] and probably have more important role than that in mammals with more developed acquired immune system [13,14]. In the both animals, however, estrogens have immune-modulating role via ERs expressed in immune cells [11].

Head kidney macrophages (HKMs) as the most important components of the fish innate immune system [15,16] invade pathogens by engulfing and eliminating them through producing reactive oxygen species [17]. Previous studies showed that ERs are expressed on fish macrophages and regulate their activity by estrogen [18,19]. Some

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https://doi.org/10.1016/j.fsi.2018.11.038
Received 3 August 2018; Received in revised form 13 November 2018; Accepted 15 November 2018
Available online 16 November 2018

other studies also reported that bisphenol A as another EED affects macrophages activity, and showed that this compound has potential to interfere with macrophage function and modulate immune response under in vitro condition [20–22]. Nevertheless, only few studies elaborated how nonylphenol affects macrophage activity and interacts with the innate immune system of fish.

Persian Sturgeon (*Acipenser persicus*) as a living fossil, has maintained some evolutionary characteristics between chondrichthyes and osteichthyes [23]. Sturgeon fish with some primitive characteristics are assumed to be phylogenetically related to extinct ancestors of teleost fish [24]. These fish possess some unique features of immune systems [25], have long life and presumably high potential to fight against pathogens in their lifespan to remain alive [26]. Therefore, sturgeons have developed efficient defense mechanisms extensively related to leucocyte-producing tissues [25–27]. Having this features and intact innate immune system [28] introduces the sturgeons as an ideal model in evolutionary study of immunity system, if similar studies be carried out in higher vertebrates. In addition, over the past decades, natural population of Persian sturgeon has suffered a severe decline, due to multiple anthropogenic factors such as the contaminants [29]; and urban sewage containing EDCs is the major contaminant in the Caspian sea area of Iran [30].

Based on the reasons mentioned above, studying the regulation of the immune response and understanding the effect of nonylphenol on the reaction of immunity cells from Persian sturgeon seems to be required. In the present study, effect of high and low doses of nonylphenol (as treatment) and 17β estradiol (as positive control) on the activity (respiratory burst and phagocytosis potential) and the apoptosis level of the macrophage from Persian sturgeon (*Acipencer persicous*) were assessed in vitro; and ERα and NF-KB antagonists were used to understand how nonylphenol affects the macrophage activity through these routes.

2. Materials and methods

2.1. Animals

Healthy Persian sturgeon (40–50 g) were obtained from Eslami farm (Sari, Mazandaran Province, Iran) and then acclimated to dechlorinated tap water at room temperature under a natural light cycle for 2 weeks. The fish were fed with commercial fish food twice a day and fasted for 24 h before macrophage sampling.

2.2. Macrophage isolation and in vitro culture conditions

The fish were killed by decapitation, and head kidneys aseptically were removed (All experiments were performed according to Babol University Animal Scientific Procedure Act.) and transferred into microtubes containing cold L-15 medium (Sigma-Aldrich, UK) supplemented with 10 U/mL heparin (SigmaAldrich), 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamicin (Gibco™). After initially cutting tissue into small pieces using sterile scissor, it was passed through a nylon cell strainer (100 μm). The cell suspension was layered on 54% Percoll® (Sigma-Aldrich.UK) according to the method described by Secombes et al. (1990) [31]. Then the cells at the medium-Percoll® interface were washed twice by centrifugation at 200*g* for 10 min at 4 °C and resuspended in L-15 complete medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin (Gibco™) and 1% fetal bovine serum (FBS). After incubation overnight, non-adherent cells were removed by washing three times with PBS. Macrophages were then cultured in L-15 complete medium containing 10% of FBS at 23 °C. Trypan blue (Sigma-Analytical) exclusion test was used for assaying viability of cells. For identifying primary macrophages, Wright- Giemsa staining method was used.

2.3. Cytotoxicity

The cytotoxic effect of nonylphenol and 17β estradiol was examined using the MTT assay [32,33]. Briefly, viable macrophages (1×10^5) were seeded into each well and treated with different concentrations (10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 100, 200 and 300 μM) of NP and E_2 dissolved in 0.005% DMSO (v/v) for 24 h. After the exposure, the plate was washed with PBS and incubated in complete medium (100 μL) containing MTT (0.5 mg/ml) dissolved in PBS for 4 h at 23 °C. The supernatants were then discarded and DMSO (150 μL) added and mixed thoroughly to dissolve dark blue formazan crystals. The optical density was recorded at 570 nm (Awareness, Stat fax-2100, USA).

2.4. Experimental design

Experimental design for the macrophage exposure to NP and E_2 included 3 experiments. In the first experiment, the macrophages (approximately 1×10^6 cells/mL for each test) were divided into 4 groups, exposed to high (100 nM) and low doses (10 nM) of NP and E2 (according to IC50) and the macrophage activities (burst respiration and phagocytosis) and the apoptosis were measured at 6, 24 and 48 h (NP concentration employed in this experiment was chosen based on the least and the highest dose without significant cytotoxicity). In the second experiment, the macrophages were co-exposed to 100 nM NP and 50 μM ER antagonist ICI 182,780 (Sigma-Aldrech,UK) for 24h [34], and the macrophage activity was assayed. This process was also performed for E2. The third experiment was exactly like the second one, but 50 μM NF-κB antagonist pyrrolidine dithiocarbamate (PDTC, Sigma UK) was used along with ICI (50 μM). In parallel with each experiment, blank and vehicle control groups (0.005% DMSO in culture medium) were used. The exposure solution was replaced completely every 24 h.

2.5. Detection of superoxide anion (ROS)

Nitroblue tetrazolium (NBT, Sigma-Aldrich, UK) test was used for measuring ROS production in the medium culture [35]. Briefly, 1 mg/mL NBT (Sigma-Aldrich, UK) was added to the culture plate of the experiment, and after 3 h incubation, washed once with 70% methanol to remove extracellular formazan. Then, 2 M KOH (120 μL) and DMSO (140 μL) were added. The optical density was measured by ELISA reader at 630 nm (Awareness, Stat fax-2100, USA).

2.6. Nitric oxide production

NO synthesis in the culture supernatants were determined by using Griess method. After treatment with NP and E2, macrophages were washed, and then stimulated by lipopolysaccharides (LPS) (LPS, Sigma-Aldrich, USA) at concentration of 20 μg/mL for 72 h. Then, cell-free supernatant was collected and assayed for nitrite according to previous studies [36,37]. Briefly, equal volumes of Griess reagent (0.1% NEDD, 1% Sulphanilamide and 2.5% H_3PO_4; Merck, Germany) and collected medium were mixed in a 96-well culture plate. After 10 min incubation at room temperature, absorbance was measured at 540 nm by ELISA plate reader. The concentration of nitrite in the cell-free supernatant media was quantified using the standard curve of sodium nitrite.

2.7. Phagocytosis assay

After treatment in each experiment, *E. coli* bacteria (CGMCC 1.2389) were cultured for 24 h and the bacterial suspension was prepared according to Seeley et al. [38]. Briefly, bacteria were fixed in 10% formalin, washed 3 times with PBS, and resuspended (10^5 cell/mL) in PBS. The macrophages were exposed to the bacteria for 1 h and stained with Wright-Giemsa (Talaye Teb Azma, Iran). For evaluating the phagocytosis of macrophages, phagocytic index was used following the formula:

Phagocytic index = (percentage of macrophages containing at least one bacterium) × (mean number of bacteria per positive cell) [39].

2.8. Macrophage apoptosis

The apoptosis level of primary macrophages after exposure to various concentrations of nonylphenol was quantified using an AnnexinV-FITC/PI apoptosis kit (BioLegend, San Diego, CA) following the supplier's protocol. After the treatment, cells were collected and rinsed with cell staining buffer (BioLegend) and then resuspended in 100 µL Annexin-binding buffer. The suspension was incubated with 2.5 µL Annexin V-FITC for 15 min and then 2.5 µL propidium iodide (PI) for 10 min in dark at room temperature. FITC or PI positive cells were determined by flow cytometer (BD FACSCalibur™, USA) using appropriate filters.

2.9. Statistical analysis

Quantitative data were presented as mean ± standard deviation and were tested for normality via Kolmogorov-Smirnov Test. One-way and two-way ANOVA tests were used to compare different experimental groups ($P < 0.05$), followed by Tukey's test. IC_{50} calculations were carried out using standard probit analysis techniques. All analyses were performed using OriginPro 2016, Version b9.3.226.

3. Results

3.1. Determination of the half maximum inhibitory concentration (IC_{50} values)

After exposure of the macrophages to different concentrations of NP and E2 ranging from 1 nM to 300 µM for 24 h, IC_{50} values of NP was determined as 200 µM. According to Fig. 1, the viability significantly decreased with increasing concentrations of NP and E2 from 1 to 300 µM and 10–300 µM ($P < 0.05$), respectively. The cell survival decreased from 97% to 23% after 24 h exposure to different concentrations of NP ranging from 1nM to 300µM.

3.2. First experiment

In the first experiment, the production of ROS and nitrite oxide significantly ($P < 0.05$) increased compared to control and blank groups after exposure to the both does of E2 (Fig. 2 A (ROS production) and B (Nitric Oxide production)) in the macrophages. Apart from 48 h exposure, the previous results was also observed for NP exposure (Fig. 3 A and B). While high dose of E2 and NP significantly decreased phagocytosis index compared to the control, low dose of E2 and NP had no and enhancing effect on this index in the macrophages, respectively (Figs. 2 and 3C (Phagocytic index)). Apoptotic responses of the macrophages were observed ($P < 0.05$) in the both doses of NP and only high dose of E2 (Figs. 2 and 3 D (Percentage of apoptotic cells)).

After exposure to NP, some nuclei of macrophage cells were condensed and broke into smaller pieces, and fragmentation of plasma membrane and finally apoptosis occurred (Fig. 4).

3.3. Second experiment

After co-exposure of macrophages to estrogen receptor antagonist (ICI) and two doses of NP, no significant change ($P > 0.05$) was observed in ROS production (Fig. 5 A (ROS production) and B (nitric oxide production)). Phagocytic activity in the macrophages significantly reduced after 6 h co-exposure to ICC and NP (Fig. 5C (phagocytic index)).

3.4. Thrid expriment

After coexposure of the macrophages to PDTC, ICI, and low and high doses of NP, production of ROS significantly reduced. This result was also observed ($P < 0.05$) in nitrite oxide experiment after coexposure to only high dose of NP (Fig. 6 A and B). In addition, phagocytic activity of the macrophages significantly decreased after the coexposur in both doses (Fig. 6C).

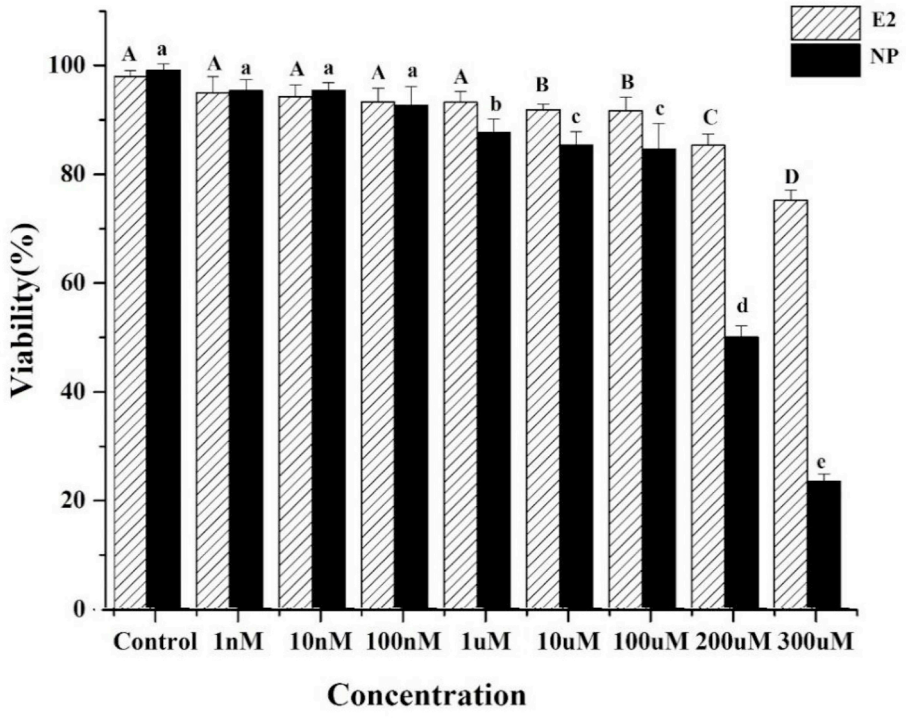

Fig. 1. Nonylphenol (NP) and 17β-etstradiol (E2) cytotoxicity assay of the primary macrophages after 12 h of the exposure. Significant differences versus the control are indicated as $p < 0.05$ (mean ± SD). Different letters show significant difference for each compound.

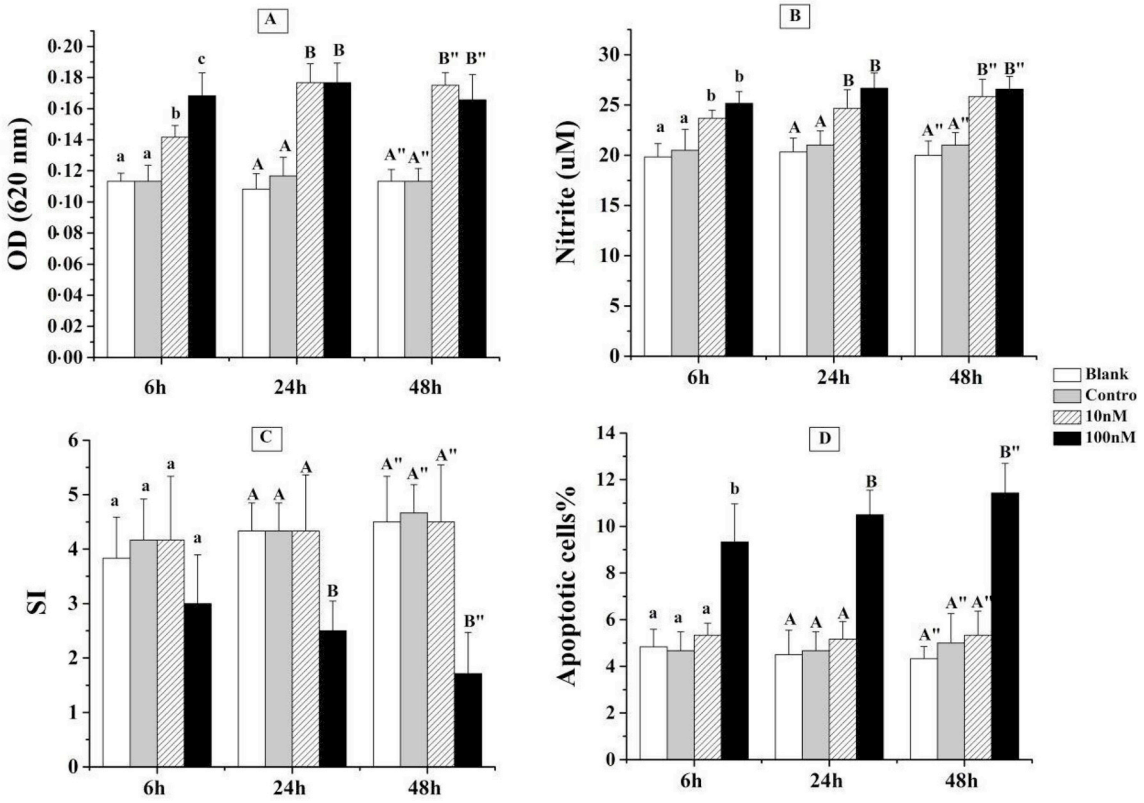

Fig. 2. The macrophages activity and the apoptosis after exposure to high and low doses of 17β estradiol (E2): A) ROS production. B) Nitric Oxide production. C) Phagocytic index. D) Percentage of apoptotic cells. Significant differences versus control and blank groups are indicated $P < 0.05$ (mean ± SD).

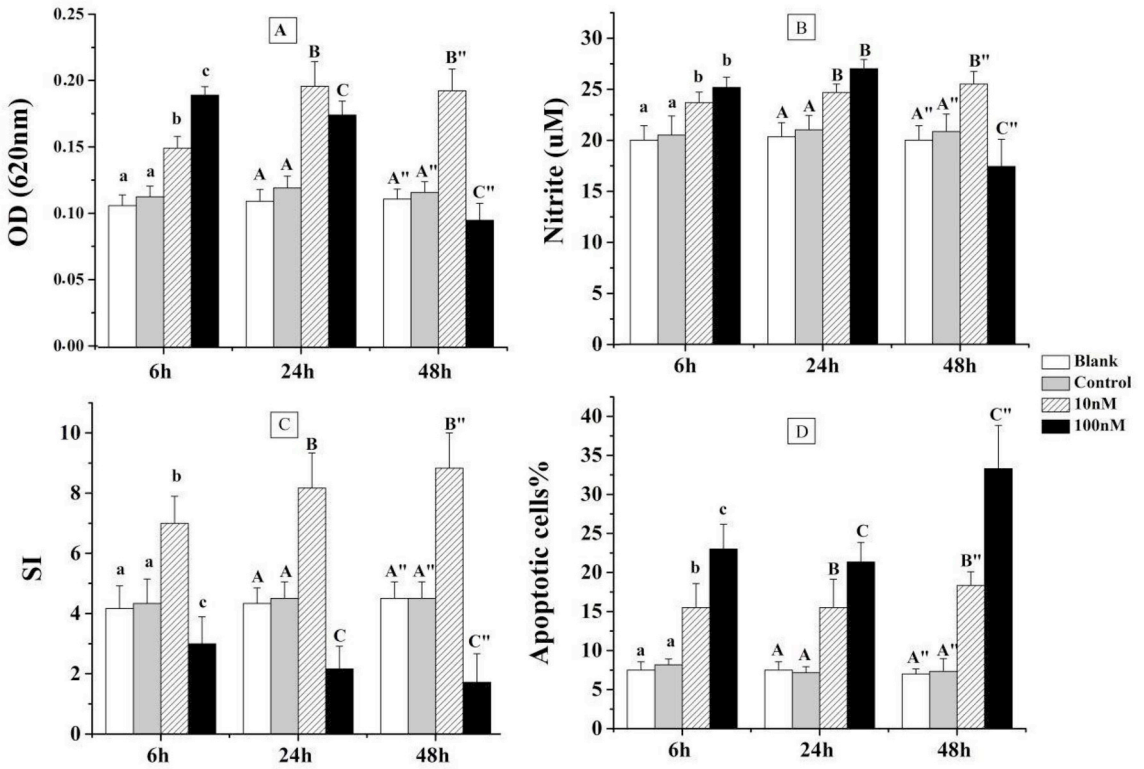

Fig. 3. The macrophage activity and the apoptosis after exposure to high and low doses of nonylphenol (NP): A) ROS production. B) Nitric Oxide production. C) Phagocytic index. D) Percentage of apoptotic cells. Significant differences versus control and blank groups are indicated $P < 0.05$ (mean ± SD).

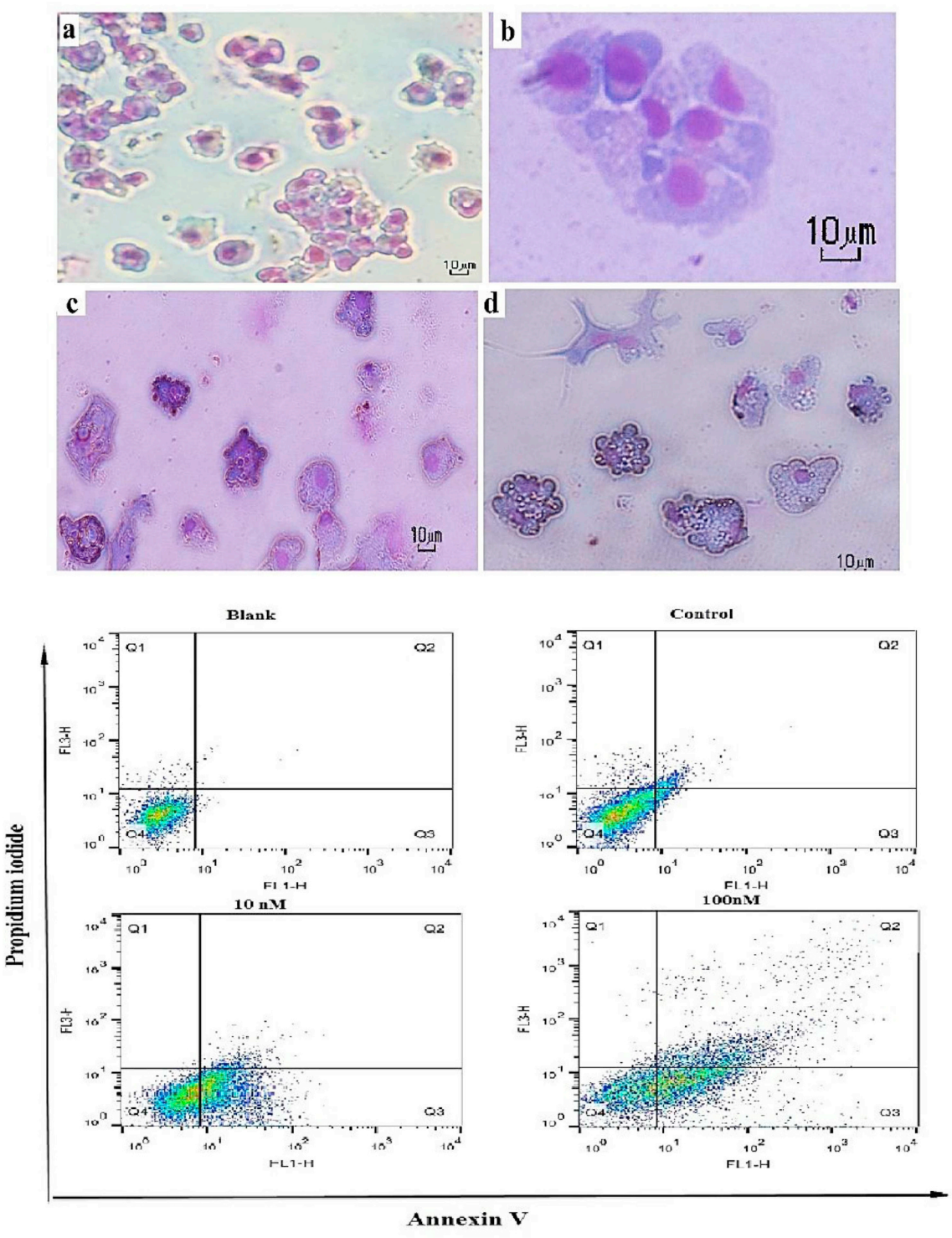

Fig. 4. Up: The morphology of head kidney macrophages from the Persian sturgeon after 24 h exposure. a) Macrophages of blank group. b) Macrophages of Control group. c) Macrophages exposed to 10 nM nonylphenol (NP). d) Macrophages exposed to 100 nM nonylphenol. Down: Histogram plot of apoptotic macrophages after 24 h exposure to nonylphenol (NP) was quantified by flowcytometry and cells were stained with propidium iodide (PI) and annexin V-FITC.

4. Discussion

The current study was going to use the primary macrophages from Persian sturgeon as a biological model to find out how low and high concentrations of NP can affect immune parameters of macrophage in vitro. This case is interesting because in vitro studies about immunotoxic potential of xenoestrogens in ancient fish was poorly studied. Our study demonstrated that the cell viability after exposure to the same dose of NP and E2 was different. Macrophage viability began to decline ($P < 0.05$) after exposure to 1 µM NP and 10 µM E_2 and reached less than 50% and 80% in dose of 300 µM NP and E2, respectively. It may be resulted from different cytotoxic mechanisms of each compound.

In the first experiment, the results showed that the both doses of E2

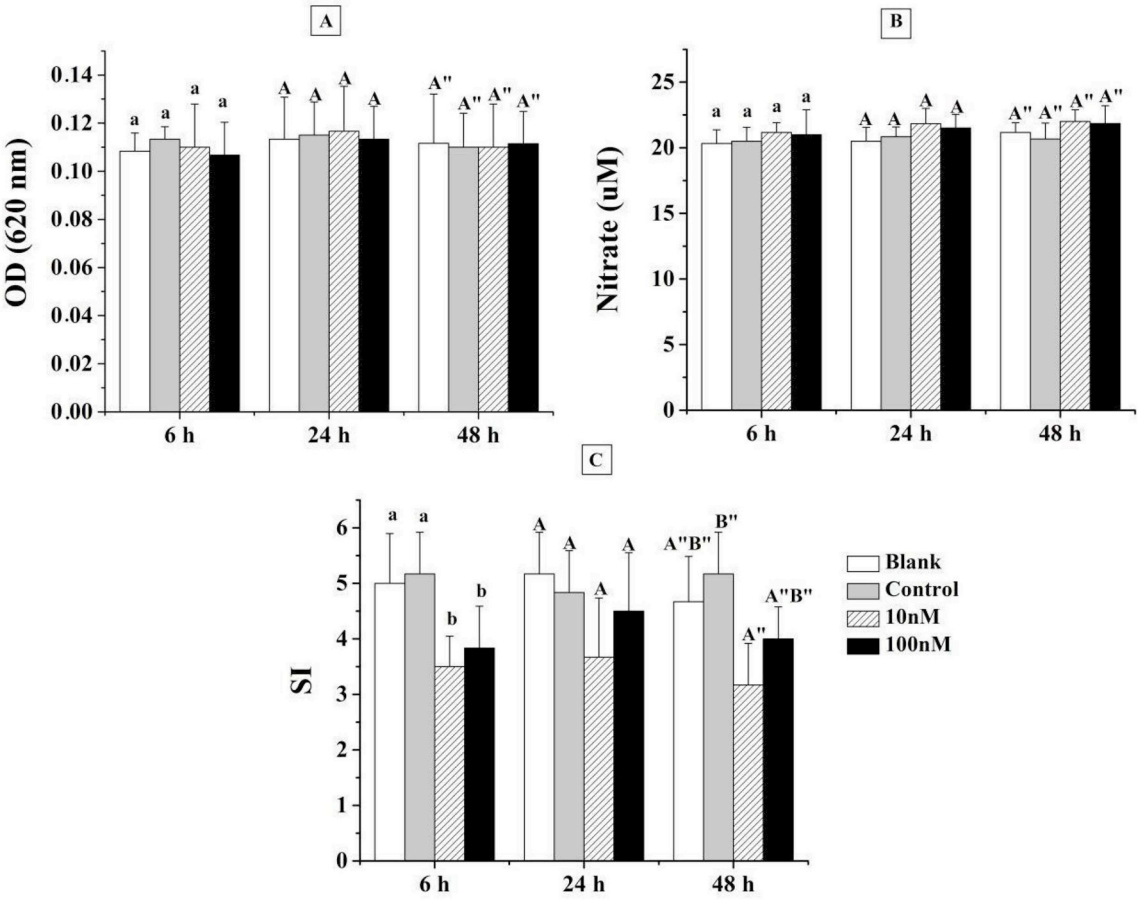

Fig. 5. Production of superoxide anions (A and B) and phagocytic activity (C) in Persian sturgeon macrophages co-treated with nonylphenol (NP) and estrogen antagonist ICI. Significant differences versus control are indicated as $P < 0.05$ (mean ± SD).

significantly increased production of ROS and NOS in the macrophages. Increasing in the respiratory burst activity in macrophages after treatment with estrogen indicates immunomodulatory role of estrogen, as reported in previous studies [40–42]. The investigation showed that estrogen can increase transcription of inducible nitric oxide synthase (iNOS) in rat macrophages [40] therefore estrogen is potential enhancer of NOS activity in macrophages. From a mechanistic point of view, E2 can increase mitochondrial Ca^{2+} in macrophages and stimulate mtNOS (mitochondrial nitric oxide synthase) and subsequently increase production of ROS via NO-dependent inhibition of cytochrome c oxidase [43]. Therefore, high dose of E2 caused high generation of NO and ROS and subsequently peroxynitrite formation. This production can induce apoptotic process [44]. According to the result, high dose of E2 can result in harmful oxidative stress and the cell apoptosis, in contrast to low dose of E2 exposure with no significant effect on phagocytosis and apoptosis of the macrophages. It has been shown that exposure to E2 can suppress transcription of bcl-2 and cyclin A, as important factors in anti-apoptotic process and cell cycle progression, respectively, in mammals [45]. Moreover, Wang and Belosevic (1995) reported that exposure of carp kidney leucocytes to high dose of estradiol significantly suppressed the phagocytic activities [46]. At the present study, negative correlation ($r = -0.77$) between apoptosis and phagocytosis in the macrophages exposed to high dose of E2 confirmed this report (Fig. 7).

Normal concentration of E_2 in immature Persian sturgeon body is 0.78 ± 0.1 ng/ml (2.86 nM) [47] and body macrophages are continuously exposed to this low level of E_2; in contrast to in vitro system with one point-in-time cell stimulation. This limitation does not allow stimulation of macrophages over extended periods and thus, higher concentration of E_2 than the physiological level is used in in vitro studies [46]. Probably, no coordination in correlation between immune parameters after in vitro exposure of sturgeon macrophage to E2 results from this concept. Immunomodulatory of E2 in macrophages is not as simple as presence or absence of this hormone. Surprisingly, immune effect of E2 can be varied by nuance in assay design, species, age, sex and stage of reproductive cycle. Thus, E2 may appear immune inhibitory or immune stimulatory based on the exposure concentration [3].

The macrophages exposed to NP in first experiment had a different story. Most immune parameters had significant and strong correlations (Fig. 7), indicating harmonic immune response in macrophages after exposure to NP. Low dose of NP caused significantly increase in superoxide anion production, phagocytosis potential and even apoptosis in the macrophages. Pervious study suggested that overproduction of NO and ROS in the cells exposed to NP promote inflammatory responses [48]. Fish, similar to mammals, have two phenotype of macrophages (M1 and M2) [49], and activated M1 macrophages produce pro-inflammatory reaction, secret superoxide onions and facilitate

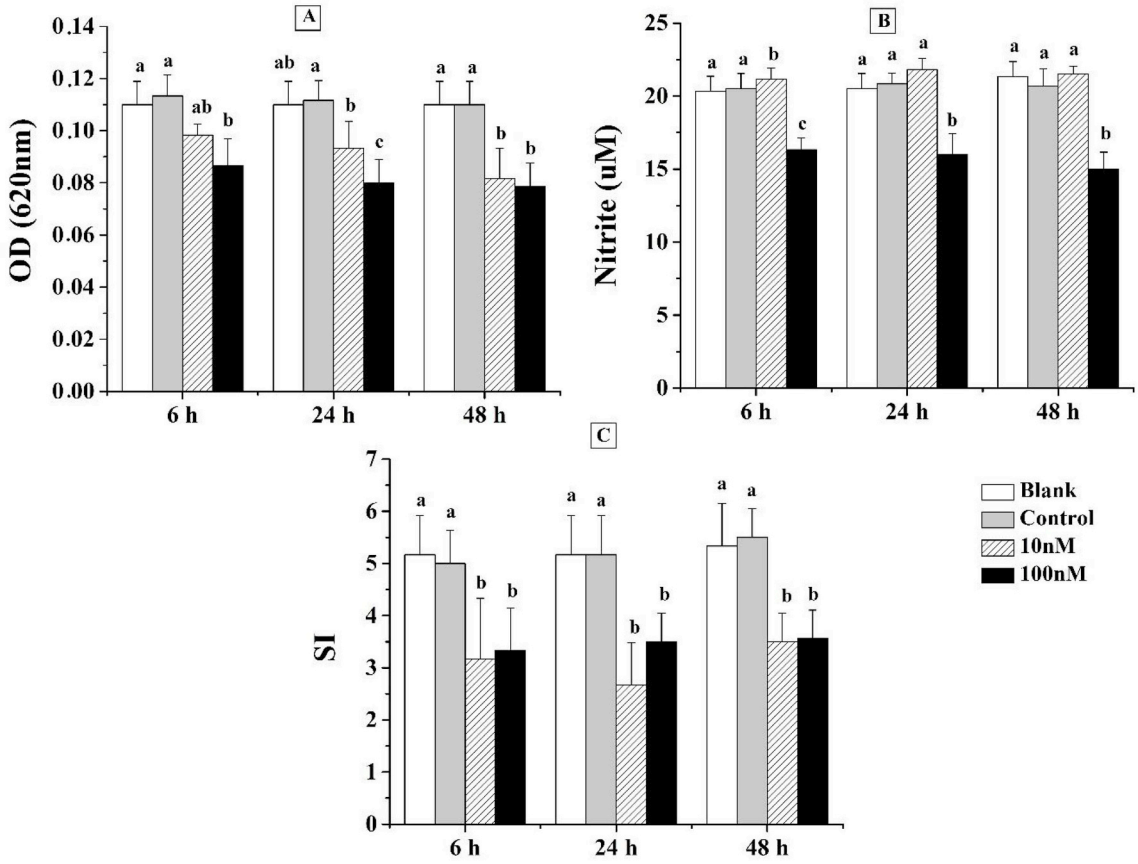

Fig. 6. Production of superoxide anions (A and B) and phagocytic activity (C) in the Persian sturgeon macrophages co-exposure to nonylphanol (NP), PDTC and ICI. Significant differences versus control are indicated as $P < 0.05$ (mean ± SD).

antimicrobial activity [50]. After exposing macrophages to environmental harmful compounds, they respond with switching from one functional phenotype to another one [51]. NP have potential to affect polarization of macrophages [52] and low dose of NP may stimulate M1 macrophage response. Thus, the results indicate that Persian sturgeon macrophages were activated and their released pro-inflammatory products increased ROS and phagocytic potential [53] after exposure to low dose of NP. Those observations are consistent with the findings of Yang et al. (2015) reporting that low dose of Bisphenol A significantly increased superoxide anion and phagocytosis activity [20]. The mechanism explaining how low dose of NP affect polarization and macrophage activity cannot be determined without further studies.

Most of the EEDCs, including NP can interfere with the normal estrogen signaling pathway by interacting with two forms of estrogen receptors (ERα and ERβ) [54]. It was suggested that some xenoestrogens shows greater affinity for ERα compared to ERβ, but this case is inverse for natural estrogen [55]. On the other hand, ERα is the main primary estrogen nuclear receptor expressed and function in macrophage biology [56] and may be targeted by harmful compounds. Some reports showed that NP and E2 interact with the same part of a ligand-binding domain of ER but they are not always the same reaction at the molecular level [57,58], because conformational changes in the ER binded to E2 is completely different with that binded to NP. This phenomenon causes different responses of ERα at gene expression level [59] and subsequently different immune reactions.

In the second experiment, after coexposure of macrophages to NP and ICI, production of superoxide anions was not observed in the absence of ER and strong correlation between immune parameters was disappeared (Fig. 8).

In the second experiment, inhibition of NP binding to ERα by ICI can interference ER-dependent signaling cascade and consequently NF-κB mediated gene transcription activation or NF-κB DNA binding [21]. Since the NF-κB pathway in macrophages has main role in redox regulation and ROS generation [60], in the second experiment no change in superoxide anions was observed due to no crosstalk between ER and NF-KB.

In third experiment, the role of NF-KB was completely removed by PDTC antagonist and ultimately led to decreasing transcription of immune parameter genes in macrophages. This supposition was confirmed by significant correlation between ROS and NO in third experiment (Fig. 8). However, further works might be required to clear accurate molecular mechanisms underlying potential crosstalk between ER and NF-KB in macrophages of primitive fish exposed to EEDCs, and to find more number of pieces for solving this complex puzzle. Previous studies demonstrated that NP can affect estrogen-dependent signaling pathway including nuclear factor-κB, as a key regulator of proinflammatory gene expression [61,62]. Also, Katsu et al. (2008) reported that ER gene expression in ancient fish exposed to EDCs was different compared with that in other fish due to different phylogeny [63]. Therefore, prediction of EDC effects on all fishes through simple examination of binding and

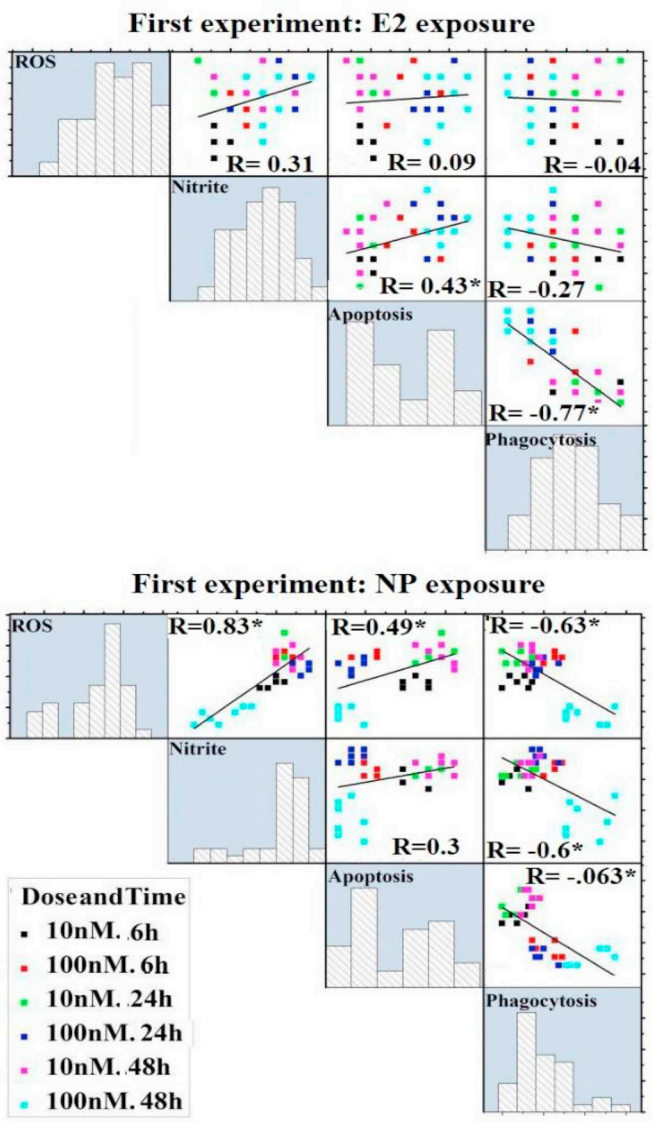

Fig. 7. Correlation coefficients between all tested immune parameters and apoptosis in the primary macrophages after exposure to two doses of 17β estradiol (E2) nonylphenol (NP). The sign (*) shows P < 0.05, based on Pearson's test.

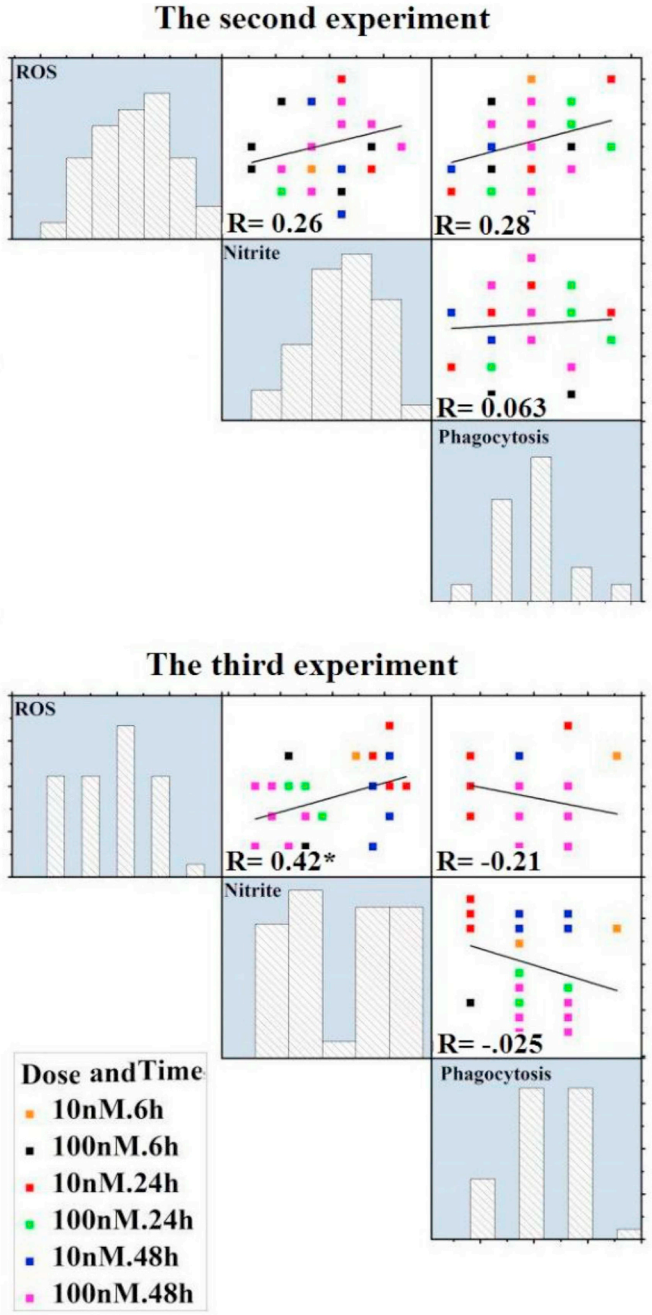

Fig. 8. Correlation coefficients between all tested immune parameters in sturgeon primary macrophages after coexposure to two doses of nonylphenol and ICI (the second experiment) and co exposure to two doses of nonylphenol, ICI and PDTC. The sign (*) shows P < 0.05 based on Pearson's test.

activating the receptor in a few fishes is not possible [63]. Understanding how EEDCs influence immune system in sturgeons as ancient and endanger fish is more important.

In conclusion, the present study showed that NP and E2 can induce pro-inflammatory responses and apoptosis on primary macrophages from Persian sturgeon in dose and time dependent manner, along with more cytotoxic effect of NP than that of E2. This study for the first time focused on dynamic pro-inflammatory actions of NP on sturgeon macrophages as ancient fish through interference in crosstalk between ER and NF-KB; and can provide basic molecular immunotoxicity data inside evolutionary reference points for effect of NP on macrophages in other vertebrate such as human.

Acknowledgments

The authors would like to thank Professor Christopher j Secombes from Scottish Fish Immunology Research Centre, University of Aberdeen for valuable advice on this research. We also thank Dr. Rashid Alijani Ardeshir from Department of Marine Biology, Khorramshahr

University of Marine Science and Technology in Iran for scientific and structural edition on the manuscript. This study was supported financially by Khorramshahr University of Marine Science and Technology in Iran with grant number 16546.

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