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Effects of heat shock protein inducer on Hsp70 gene expression and immune parameters during *Streptococcus iniae* infection in a Persian sturgeon fry

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Abstract

Heat shock proteins (HSPs) as stress-related factors play a fundamental role in innate and adaptive immune responses in fish, which can be considered as strong candidates for the development of new methods for fish disease prevention. It has been proven that Pro-Tex® as a heat shock protein inducer (HSPi) reduces harmful effects of cellular stress by increasing the Hsp70 protein production. We evaluated the effects of Pro-Tex® as an HSPi in a Persian sturgeon, (*Acipenser persicus*) exposed to a pathogenic bacterium. Therefore, *A. persicus* fries were pre-treated with 25.00, 50.00 and 100 mg L^{-1} of Pro-Tex® and then, injected with *Streptococcus iniae ATCC29178.* The Hsp70 gene expressions were determined in various organs including liver, gill and intestine and lysozyme (LYZ) activities along with supplemental levels of complement component 3 (C3) and immunoglobulin M (IgM) were also determined in sturgeon blood in days 3 and 7 after infection. Expression of Hsp70 gene was increased during the first three days of infection and then, it was found to be down-regulated during the infection process. Also, levels of LYZ activity, C3 and IgM increased in a concentration-dependent manner; but these parameters decreased after 7 days. Our data suggest that induction of Hsp70 is a promising approach in modulation of immune response in *A. persicus* and it might be employed to confer protection in fish against bacterial infections.

Introduction

Sturgeon is listed as a critically endangered species by the International Union for Nature Conservation. The impact of sturgeon aquaculture has limited due to various factors including illegal catching, spawning area destruction and water pollution.[1,2] Bacterial infections are regarded as major limiting factors in the development of fish aquaculture. *Streptococcus iniae* is a Gram-positive bacterium and a leading cause of dangerous diseases in fresh and marine water fish. Streptococcal infections cause mortality, decreased growth and unmarketable appearance; resulting in high economic losses in the aquaculture industry.[3,4]

The impacts of traditional methods such as anti-microbial treatments are not significant in the inhibition and cure of aquatic diseases and their over-use has led to an increased rate of bacterial resistance to drugs in the environment. The considerable disadvantages of using antibiotics confirm the requirement for developing alternative treatment strategies. Some alternatives include novel vaccination methods and probing new immune-stimulating treatments. Many reports have already mentioned the efficacy of heat shock proteins (HSPs) to regulate innate and adaptive immune responses, suggesting that they may be strong candidates for development of a new disease inhibition approach. The HSPs are a family of highly conserved intra-cellular proteins having a vital role in response to abiotic stresses such as high temperature, oxygen poverty and heavy metal ions as well as biotic stresses like pathogens.[5] The HSPs are also called molecular chaperones due to their role in the successful intra-cellular localization, folding secretion, assembly regulation and decay of other proteins.[6] The HSP families include Hsp90, Hsp70, Hsp60 and smaller Hsps.

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Among them, Hsp70 family is a highly conserved and the best-studied group of HSPs in aquatic animals. It has also been proven that Hsp70 displays potent immuno-modulatory effects on innate and acquired immunities, apoptosis and many functions of the inflammatory process.[7] Thus, non-lethal heat shock proteins and preparations of exogenous HSP inducers (HSPi) are used to enhance resistance to pathogens in fish.[8]

It has been recently demonstrated that one of the methods for non-stressful production of HSP is using chemical compounds such as Pro-Tex®, the active molecule of Tex-OE®, which is a patented plant-based compound from the skin of tropical cactus (*Opuntia ficus indica*). The Tex-OE® compounds have been shown to contribute to the decrease in trauma and physical stresses associated with husbandry events such as transportation and vaccination. Thus, HSPi might be beneficial for the prevention and treatment of bacterial fish diseases including golf ball disease, a disease caused by *Streptococcus* spp.[8-12]

Several reports are available regarding the modulation of innate and adaptive immune systems by Hsp70 in fish.[12-16] Lysozyme (LYZ), complement component 3 (C3) and immunoglobulin M (IgM) are important factors of innate and adaptive immune systems and are of interest for their importance in the fish immune system. However, no study has investigated the effect of this gene modulation on LYZ activity and C3 and IgM levels in fish tissues.

Over 3 million fingerlings of *Acipenser persicus* are released into the Caspian Sea every year to protect the natural resources of this valuable species. Released fish are exposed to various stresses weakening them leading to high mortality.[17] Bacterial and viral infections are among the factors causing high morbidity and mortality at the time of release in the sea. Thus, we investigated the effect of Tex-OE® (as an HSPi) application on the expression of Hsp70 gene in the liver, gill and intestine and its contribution to the response of immune parameters including LYZ activity, C3 and IgM levels during *S. iniae* infection in a Persian sturgeon.

Materials and Methods

Fish. Healthy individuals of *A. persicus* with the mean weight of 15.00 – 20.00 g were obtained from Shahid Beheshti Sturgeon Breeding and Rearing Center, Guilan, Rasht, Iran, in July 2016. The use of animals was conducted following the ethical and legal codes supervised by University of Guilan, Rasht, Iran (Ethical code; 106528). Fish were maintained at the center for aquaculture at University of Guilan (Rasht, Iran) to acclimate to laboratory conditions (T~ 20.00 ˚C; pH~ 7.44; DO~ 7.89 mg L^{-1}) for two weeks before the experiment. During maintenance, fish were fed with live food including *Artemia* biomass and *Chironomus* larvae, twice a day, at approximately 2.00% of body weight. No feed was provided in 24 hr prior to the experimental challenge. In each group, 15 fish were randomly divided into 50.00 L tanks and three replicates of the tanks were designed for each treatment. During the exposure time, the water was continuously monitored for temperature, dissolved oxygen, pH and conductivity. In order to evaluate previous bacterial diseases among the fish, some samples were randomly obtained from different fish organs, streaked on blood agar medium and incubated at 28.00 ˚C for 48 hr.

The HSP-inducing product. Nopal endurance capsules containing Tex-OE® as an extract of *O. ficus* (Source Naturals Inc., Santa Cruz, USA) were dissolved in sterile distilled water at 2.00g L^{-1} to prepare a fresh stock for each experiment.

The HSP-inducing compound and experimental challenge. Two separate analyses for the performance of HSPi were followed. In experiment 1, fish were treated with HSPi (25.00, 50.00 and 100 mg L^{-1}) for a period of 2 hr at 20.00 ˚C to assay the effect of HSPi on expression of Hsp70 gene.[1] Fish were washed with sterile water and allowed to recover for 2 hr at 2.00 ˚C. Non-treated fish that did not receive HSPi were considered as controls. In the second experiment, *S. iniae ATCC29178,* a bacterial pathogen, was used for experimental infection of fish. Each fish was intraperitoneally injected by 100 µL of a bacterial suspension containing *S. iniae* with concentrations of 5.00 × 10^6 CFU mL^{-1}. Bacterial concentration was selected based on previous studies.[18,19] The fish that did not receive HSPi but were infected with bacterial cells were regarded as non-pre-treated control. The fish that neither received HSPi nor infected with bacteria were regarded as control. In brief, fish were anesthetized with clove powder (0.50 g L^{-1}), the blood samples were collected from the caudal vein of the fish on the 3rd and 7th day after infection of each treatment and then, the plasma was separated. Also, the liver, gills and small intestine were sampled and stored at – 80.00 ˚C for subsequent use.

The RNA extraction and cDNA synthesis. Total RNA was extracted from the liver, gill and intestine by Trizol reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. To eliminate DNA contamination, the total RNA was treated with RNase-free DNase. The quality of RNA samples was determined by electrophoresis on 1.00% agarose gel and their quantity was determined using the spectrophotometric method (NanoDropOne-C. Ramsey, USA). Then, cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany). The expression of Hsp70 gene in each tissue was evaluated using quantitative real time polymerase chain reaction (qRT-PCR) by LightCycler® 96 System (Roche Diagnostics, Mannheim, Germany) using a pair of specific primers as presented in Table 1.

Table 1. Specific primers used for real-time quantitative polymerase chain reaction analysis of *Acipenser persicus* heat shock protein 70 (Hsp70) and GAPDH.

Gene	Sequences of forward and reverse primers (5′→3′)	Time (sec)	Amplicon (bp)	Reference
Hsp70	F: CGCTGGCCTTAATGTTCTCC R: GCGCTTGAACTCTGCAATGA	56	249	Safari *et al.*[2]
GAPDH	F: ACACCCGCTCATCAATCTTT R: AGGTCCACGACTCTGTTGCT	54	114	Akbarzadeh *et al.*[20]

The qRT-PCR amplifications were performed in a total volume of 25.00 µL, containing 12.50 µL of Maxima SYBR Green qPCR Master Mix (Fermentas, Cambridgeshire, UK), 5.50 µL of nuclease-free water, 5.00 µL of cDNA template and 1.00 µL of each primer. The PCR program was subjected to the following thermal profile: Initial denaturation (94.00 ˚C during 5 min), 40 cycles of amplification and quantification (10 sec at 94.00 ˚C followed by 10 sec at 56.00 ˚C and 10 sec at 72.00 ˚C) and cooling (4.00 ˚C). Each reaction was amplified in triplicates. The quantification cycle (Cq) values were determined using LightCycler® 96 Application Software (version 1.1; Roche Diagnostics). N-fold differential expression was calculated using the comparative Cq method,[21] by calculating each Cq average for the triplicate samples. The total Cq amount of the gene for each cDNA was subtracted from the Cq amount of GAPDH (housekeeping gene) to obtain the ΔCq amount. An average of the ΔCq values was determined from the triplicate samples. Subsequently, the ΔΔCq was calculated by subtracting the ΔCq of the samples from the ΔCq of the calibrator. Fold difference was calculated as $2^{-\Delta\Delta Cq}$.[21]

Lysozyme activity. Lysozyme activity of plasma was assessed using a turbidometric method based on the ability of LYZ to lyse the bacterium (*Micrococcus luteus*)[22] with some modifications. Lysozyme activity was measured with 25.00 µL per well of plasma and 175 µL of *M. luteus* (Sigma-Aldrich, St. Louis, USA) at a concentration of 0.20 mg mL $^{-1}$ in 0.50 M phosphate buffered saline (PBS), pH: 6.20, in a 96-well plate in triplicates. The PBS was regarded as a negative control. Optical density (OD) at 530 nm after 1 and 5 min at 22 .00 ˚C was recoded. One unit of LYZ activity was described as a decline in absorbance of 0.001 absorbance units per min.

Complement component C3 assay. Concentration of C3 was evaluated using a sandwich enzyme-linked immunosorbent assay (ELISA) with a Fish ELISA Kit (Hangzhou Eastbiopharm Co., Ltd. Hangzou, China). Plasma was combined with a monoclonal antibody enzyme well which was pre-coated with a fish C3 monoclonal antibody. The plate was incubated at 37 .00 ˚C, after which the C3 antibody, labeled with biotin, was added and mixed with Streptavidin-Horseradish Peroxidase to form an immune complex. After incubation, the plate was washed to remove uncombined enzyme. Then, chromogenic solutions A and B were added, the color of the liquid turned blue and after addition of sulfuric acid (M 2), the color finally changed to yellow. The OD value was measured by an ELISA reader at 450 nm and C3 concentrations were expressed as mg mL^{-1}.[23]

The IgM quantification. Similar to the C3, the IgM levels were assessed using an ELISA kit (Hangzhou Eastbiopharm Co., Ltd.). Also, IgM level was evaluated using a monoclonal antibody (Product No.: F13 that reacts with *Acipencer* spp. IgM) according to the manufacturer's instructions.

Statistical analysis. The normality test (Kolmogorov–Smirnov) was done initially on the raw data of Hsp70 gene expression, the LYZ activity and IgM and C3 levels. Then, data was analyzed by one-way analysis of variance using SPSS (version 20.0; IBM Corp., Armonk, USA). The Duncan *post hoc* test was applied with $p \leq 0.05$ as significantly different. All the values were expressed as a mean ± standard deviation.

Results

Effect of HSPi pre-treatment on Hsp70 gene expression of the Persian sturgeon challenged with *S. iniae*. The expression level of Hsp70 gene in all tissues of the Persian sturgeon treated with HSPi demonstrated gradual up-regulation for all concentrations of HSPi after 3 and 7 days compared to the control group. The Hsp70 gene was over-expressed significantly at the dose of 100 mg L^{-1}; while, it was up-regulated at 25.00 and 50.00 mg L^{-1} through first three days. Expression of Hsp70 gene showed a slight decrease in all tissues after 3 days. The Hsp70 gene in the liver of fish treated with 100 mg L^{-1} of HSPi showed the highest expression after 3 days (25 folds), followed by the gill and intestine with 15 and 11 folds higher than the control, respectively. Further, although the Hsp70 gene was up-regulated in all tissues after seven days, the expression of this gene was lower than that on the day 3 ($p \leq 0.05$; Fig. 1). Expression of the Hsp70 gene in the liver of fish infected with *S. iniae* showed significant up-regulation during first three days after inoculation compared to the control group; however, the degree of up-regulation was decreased during the next four days ($p \leq 0.05$; Fig. 2A). The expression level of Hsp70 gene in the gill of Persian sturgeon was higher than the control group on the 3rd day following the *S. iniae* injection ($p \leq 0.05$); while, the highest level of Hsp70 gene expression after 7 days was recorded only at a concentration of 100 mg L^{-1} (Fig. 2B). The Hsp70 gene in the intestine of fish was up-regulated only at concentrations of 50.00 and 100 mg L $^{-1}$ on the 3rd day; then, it reduced significantly from

day 3 to 7 after infection ($p \leq 0.05$; Fig. 2C). Comparison of the expression of Hsp70 gene in all tissues showed the highest gene expression for Hsp70 on the 3rd day in the liver of fish pre-treated with 100 mg L^{-1}, which was 63.02 folds higher than control. Also, Hsp70 gene was up-regulated during the first three days after infection by 18.90 and 15.40 folds in gill and intestine, respectively. In addition, although Hsp70 gene was up-regulated in all tissues during the first three days, its expression was decreased dramatically during next four days and reached to 0.80, 8.20 and 6.10 folds in the liver, gill and intestine respectively compared to the control (Fig. 2D).

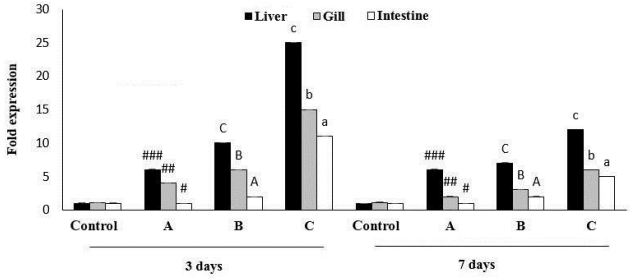

Fig. 1. Expression analysis of Hsp70 gene in liver, gill, and intestine of *Acipenser persicus* treated with various concentrations of HSPi (Groups A= 25.00, B= 50.00 and C= 100 mg L^{-1}) on the days 3 and 7. Fold expression was calculated as $2^{-\Delta\Delta Cq}$. Control group was taken as the calibrator. Results, which are the mean of three replicates, are presented relative to *A. persicus* GAPDH gene expression. Significant differences between the mean values at corresponding concentration points are indicated by different symbols and letters ($p \leq 0.05$).

Lysozyme activity. The level of LYZ activity in the plasma of *A. persicus* pre-treated with HSPi and infected with *S. iniae* is exhibited in Figure 3. The LYZ activity showed a significant steady increase from the control group to dose of 100 mg L^{-1} of HSPi in the Persian sturgeon in response to *S. iniae* ($p \leq 0.05$). Most of LYZ activity was observed at 100 mg L^{-1} during the first three days after infection. Although the activity of LYZ was significantly stronger for all concentrations than the control groups, it decreased sharply from the day 3 to day 7 after infection (Fig. 3).

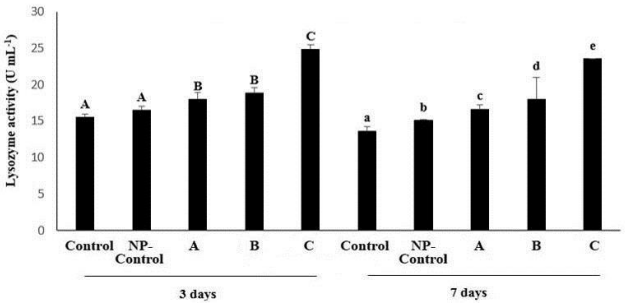

Fig. 3. The level of lysozyme activity in plasma of *Acipenser persicus* pre-treated with HSPi at various concentrations (Groups A= 25.00, B= 50.00 and C= 100 mg L^{-1}) at 3 and 7 days post-infection with *Streptococcus iniae*. The fish that did not received HSPi but were infected with bacterial cells were regarded as non-pretreated control (NP-Control). The fish that neither received HSPi nor infected with bacteria were regarded as control. Data represent the mean ± standard error. Different uppercase and lowercase letters indicate significant difference at each time point, respectively ($p \leq 0.05$).

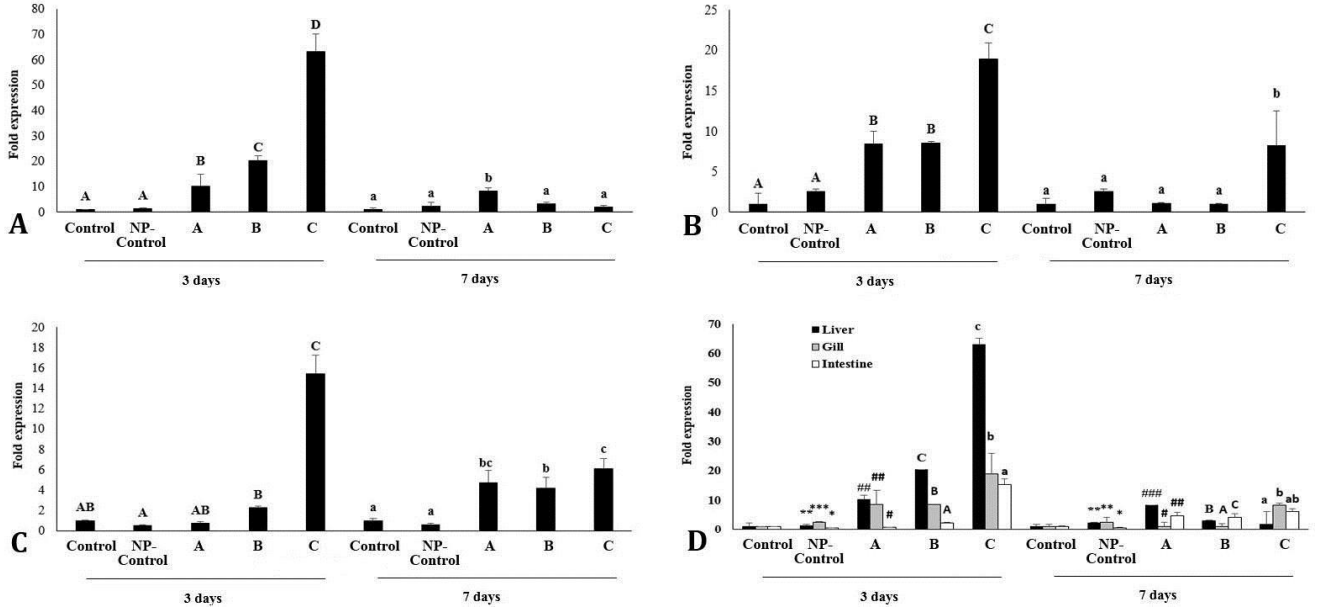

Fig. 2. Expression analysis of Hsp70 gene in the liver (A), gill (B), intestine (C) and all tissues (D) of *Acipenser persicus* pretreated with HSPi at various concentrations (Groups A = 25.00, B= 50.00 and C= 100 mg L^{-1} HSPi) at 3 and 7days post-infection with *Streptococcus iniae*. The fish that did not receive HSPi but were infected with bacterial cells were regarded as non-pretreated control (NP-Control). The fish that neither received HSPi nor infected with bacteria were regarded as control. Fold expression was calculated as $2^{-\Delta\Delta Cq}$. Control group was taken as the calibrator. Data are presented relative to *A. persicus* GAPDH gene expression. Significant differences between the mean values at corresponding concentration points are indicated by different letters and symbol for each group ($p \leq 0.05$).

Complement component 3. The C3 levels in the Persian sturgeon pre-treated with Pro-Tex® and infected with *S. iniae* increased steeply in comparison with the control group; however, it showed significant higher levels in response to 100 mg L^{-1} of HSPi than 25.00 and 50.00 mg L^{-1} at days 3 and 7 after inoculation ($p \leq 0.05$). The highest level of C3 was observed for 100 mg L^{-1} of HSPi during the first three days. Level of C3 infected with *S. iniae* decreased markedly from the day 3 to day 7 (Fig. 4A).

Immunoglobulin M. A significant difference in the IgM level was observed among the treated fish compared to the control group. There was a sharp increase in IgM level in response to different concentrations of HSPi (25.00 mg L^{-1} to 100 mg L^{-1}) in Persian sturgeons after 3 and 7 days ($p \leq 0.05$). The IgM contents reduced markedly from day 3 to day 7 after infection. The highest level of IgM was observed in response to the concentration of 100 mg L^{-1} on day three after injection, which was 0.52 mg mL^{-1} and then in a similar dose on the 7th day (Fig. 4B).

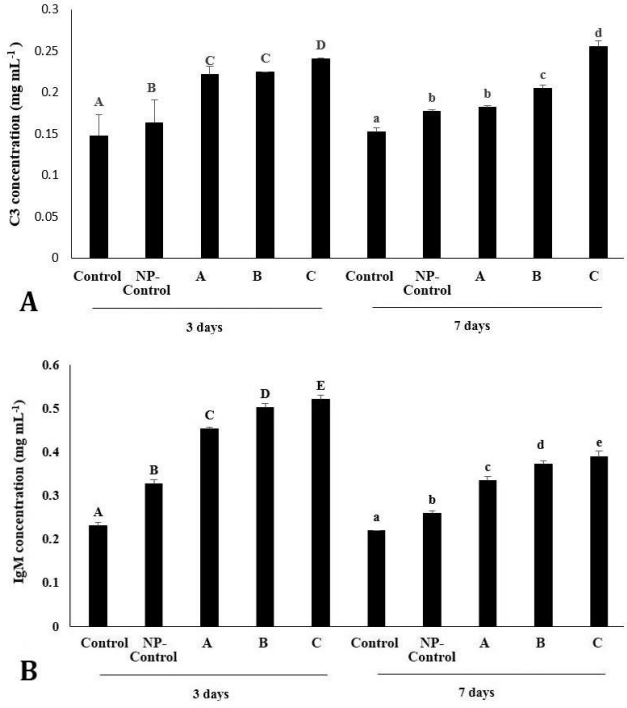

Fig. 4. A) Component C3 level and **B)** Immunoglobulin M (IgM) level in the plasma of *Acipenser persicus* pre-treated with Hspi at various concentrations (Groups A= 25.00, B= 50.00 and C= 100 mg L^{-1}) at 3 and 7 days post-infection of *Streptococcus iniae*. The fish that did not received HSPi but were infected with bacterial cells were regarded as (NP-Control). The fish that neither received HSPi nor infected with bacteria were regarded as control. Data represent the mean ± standard error. Different uppercase and lowercase letters indicate significant difference among the groups at each time point, respectively ($p \leq 0.05$).

Discussion

The study of several animal models has revealed the association between Hsp70 induction and protective immune response against various pathogenic biological stressors, indicating that they may be potent candidates for the development of novel disease inhibitory protocols. Heat shock protein genes play significant roles in the physiology of fish in many ways such as growth and aging, stress physiology and endocrinology, immunology, environmental physiology, stress tolerance and acclimatization.[24]

In the first experiment, an increase in Hsp70 expression was observed in fish treated with HSPi, suggesting that this compound could induce protein expression of the mentioned gene. It was also found that Tex-OE® (HSP-stimulating factor) affects Hsp70 expression patterns in response to *S. iniae* in the liver, gill and intestine of *A. persicus*. Results showed that the Hsp70 gene had various expression profiles in response to different concentrations of HSPi and in different fish tissues. The HSPi concentration and Hsp70 gene expression indicated direct association during the first three days after infection in all tissues; while, there was a sharp decrease in the expression levels of Hsp70 gene in all tissues from day 3 to day 7. The maximum level of Hsp70 expression was observed in the liver at the day 3 after infection with *S. inia*. The increased expression of Hsp70 after bacterial contamination showed its vital role in immunity during exposure to bacteria in fish. The up- or down-regulation of Hsp70 gene might be associated with the levels of bacterial durability in the host and the amount of tissue damage. Different expression patterns of Hsp70 related to tissues, class of HSP families, stressors and time have been reported in previous studies. In one study, Das *et al.*, have reported the expression of seven HSP genes at different times and tissues. Up-regulation of Hsp70 gene in the liver of rohu (*Labeo rohita*) infected with *Aeromonas hydrophila* was similar to our results, with higher Hsp70 expression during the first three days and lower from day 7 and day 15 after infection. Previous studies have shown an increase in superoxide production in *Puntius sarana* during *A. hydrophila* infection.[25] Phagocytes are the major innate immune cells taking part in respiratory or oxidative burst activity by releasing reactive oxygen species (ROS).[26] However, excessive generation of ROS may cause oxidative stress in fish. Over-expression of Hsp70 may result in preservation of cells from oxidative stress-induced damage. High expression during the first three days of infection significantly protects host cells from the effects of oxidative stress associated with diseases.[25,27] Also, HSPi mediate Hsp70 by incipient generations of ROS such as superoxide anion and H_2O_2.[9] Subsequently, the Hsp70 protein exhibits strong effects on innate and adaptive immunities.[28] In agreement with our study, although the

Hsp70 gene was up-regulated in all tissues during the first three days, during the next four days with excessive production of ROS, expression of Hsp70 gene was decreased dramatically.[29,30] Similarly, cyto-protection from *Vibrio alginolyticus* in the silver sea bream (*Sparus sarba Forsskal*) was shown by the decrease in the expression of the Hsp70 family in renal and hepatic tissues at 36 hr after infection.[31]

The Hsp70 as a molecular chaperone folds nascent and partially denatured proteins which raise innate and adaptive immune responses.[8] The HSPi increased LYZ activities of innate immunity, IgM content of adaptive immunity and levels of C3 as a link between innate and adaptive immune responses through Hsp70 induction in *A. persicus* challenged with *S. iniae*. These parameters increased in a concentration-dependent manner and decreased in a time-dependent manner in the Persian sturgeon. The over-expression of immune parameters during the initial 3 days after infection may be related to the feasible participation in processing of bacterial antigens. Induction of stress proteins by HSPi significantly activates the prophenoloxidase system and trans-glutaminase immune gene of the innate system to protect animals against infection.[9] Similar to our study, increased levels of Hsp70 protected coho salmon and shrimps against *Renibacterium salmoninarum* and *Staphylococcus aureus* infections.[32,33] So, according to these studies, Tex-OE® is a potential inducer of Hsp70 in fish that could increase levels of LYZ, complement system proteins and adaptive immunity agents such as IgM during bacterial infections.

In conclusion, our data suggest that HSPi could be responsible for higher expression of Hsp70 and act as a potentially important modulator of immune response in *A. persicus* against *S. iniae*. However, characterization of their impact on the health status and protection of fish against bacterial infections still needs further investigations.

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Conflict of interest

The authors declare that they have no conflict of interest.

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