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The key role of Tumor Necrosis Factor alpha (TNF-α) in vaccinated rainbow trout via irradiated *Ichthyophthirius multifiliis* trophont

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ABSTRACT

In this study, in order to characterize the immune response against *Ichthyophthirius multifiliis* (*I. multifiliis*) in the skin, liver, gills and head kidney of immunized rainbow trout, with two types of killed vaccines (γ-irradiation and formalin inactivation of trophont), the gene expression levels of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α_1 and TNF-α_2) were evaluated. The vaccinated fish showed significant protection against *I. multifiliis* 30 days after the second vaccination. We showed that the pro-inflammatory cytokine, TNF-α_1, was expressed in rainbow trout after vaccination, not only in the skin but also in the head kidney and liver, whereas TNF-α_2 expression was seen in the liver. Also, parasite-related changes in TNF-α_1 expression could be detected only in the gills of fish that were exposed to live *I. multifiliis* trophonts during this experiment. Finally, according to previous reports and the current study, TNF-α_1 could be involved in an immune mechanism that can control *I. multifiliis* infection in vaccinated rainbow trout.

Key words: tumor necrosis factor alpha (TNF-α), *Ichthyophthirius multifiliis,* irradiated vaccine, rainbow trout

Introduction

The protozoan *Ichthyophthirius multifiliis* (*I. multifiliis*) has been diagnosed as one of the most important freshwater ciliate pathogens of a sudden death of fish in one tank on a commercial farm, and generally causes significant economic losses to the aquaculture industry worldwide (AIHUA and BUCHMANN, 2001; MAKI and DICKERSON, 2003). Current strategies for its control depend on the use of chemical agents such as formalin

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to kill the waterborne stages of this parasite. However it is difficult for chemotherapy to control this parasite after penetration into fish's skin and gills. Moreover, the high cost involved in therapy, and the public concern for food and environmental safety are other disadvantages of chemotherapy. Rainbow trout infected with sub-lethal doses of *I. multifiliis* are able to respond immunologically (SIGH et al., 2004a; SIGH et al., 2004b) and generate protection to subsequent infection (WAHLI and MEIER, 1985; SIGH and BUCHMANN, 2001). Thus, vaccination against *I. multifiliis* and immunotherapies can be considered as an alternative to chemical treatments, to prevent mortality in fish (MAKI and DICKERSON, 2003). Protection against protozoan parasites usually involves Th_1 cell-responses with IFN-γ and TNF-α as proinflammatory cytokines being particularly important (OVINGTON et al., 1995). There are a number of protozoan infections in which the antiparasitic role of TNF-α has been well established (OVINGTON and SMITH, 1992). Therefore, the aim of the current investigation was to confirm the role of the TNF-α as an important proinflammatory cytokine in the liver, kidney, gills and skin of rainbow trout vaccinated against *I. multifiliis*.

Materials and methods

Fish. Rainbow trout (*Oncorhynchus mykiss*) weighing 30 - 40 g (parasite-free) obtained from a fish farm in Karaj, Iran, were kept in running water (flow rate 0.5 lit/s) in nine polyethylene tanks (300 L). They were continuously supplied with aerated water, temperature 15 ± 1 ºC, dissolved oxygen 5.2 ppm, under the natural photoperiod (10L:14D). Adaptation to these tanks was performed 14 days, using a commercial pelleted diet (Behparvar, Iran).

Preparation of gamma irradiation vaccine (radio vaccine). Gamma irradiated vaccine (radio vaccine) was prepared as described previously (HEIDARIEH et al., 2015). In brief, fifty fish were infected with *I. multifiliis* via a high dose of collected live trophont (immersion). Exposure was performed in the dark for 8 hours, and they were then transferred to a glass aquarium. Fish were kept for 5 days at 20 °C and then trophont were collected with a 200-mesh sieve (skin). Trophont should be used immediately (HEIDARIEH et al., 2014a).

After collection of trophonts, the gamma cell instrument, Nordian, model 220 with a dose rate of 0.22 Gy/sec and 20469 Ci activity, was used for parasite irradiation. The dose of gamma ray (170 Gray) was used for irradiation of parasite samples. The irradiation process was performed on the parasite samples held on dry ice (HEIDARIEH et al., 2014a).

Preparation of formalin-fixed trophonts (formalinvaccine). Live trophonts were suspended in 3% formalin and incubated at room temperature for 2 hours; treated trophonts were centrifuged at 3000× g for 2 min and the supernatant was removed. Trophonts pellet was washed 3 times with 1 mL of 0.15 M sterile phosphate buffered saline (PBS) (pH

7.4). After the wash, the formalin-treated trophonts were harvested by centrifugation at 3500× g for 3 minutes (HEIDARIEH et al., 2015).

Preparation of gamma irradiated Ergosan extract (alginic acid nanoparticles). Commercial Ergosan (Schering Plough Aquaculture, UK) was suspended in sterile 0.15 M (pH 7.2). The sample was sonicated on ice for 30 min and centrifuged at 5000× g for 15 min. After precipitation in 2.5 volumes of 96% ethanol, and heating at 40˚C, the dried precipitate was then milled to the mesh size of 53-125 μm. The remaining powder was irradiated by cobalt-60 gamma irradiator (PX-30- IssIedovapel, Russia) at a dose rate of 0.22 Gy/sec. The applied dose level was 30 kGy (HEIDARIEH et al., 2012; HEIDARIEH et al., 2014b). Dosimetry was performed using the Fricke reference standard dosimetry system, and after the irradiation process; the irradiated-Ergosan was stored at 4 °C for further tests.

Immunization procedures. 90 parasite-free fish were randomly allocated into 6 groups in triplicate, at a density of 15 fish per each group. The dose rate of vaccine was 100 gamma-irradiation trophont per 150 gram fish body weight (via bath method). The 1st group was immunized with 100 gamma-irradiation (170 Gray) inactive trophont with alginic acid nanoprticle, the 2nd group with 100 gamma-irradiation (170 Gray) inactive trophont, the 3rd group with 100 formalin (3%) inactive trophont with alginic acid nanoparticles, the 4th group with 100 formalin (3%) inactive trophont, the 5th group with 100 live trophont (as the positive control group), and the last group was the negative control (uninfected rainbow trout). Apart from the negative control group, all the other (six) groups received boosts of the same immunization on the 10th day after the first dose of vaccine. The fish in groups 1 to 5 (without the negative and positive control groups) were challenged with 100 live trophonts at 10 days after the second vaccination (as a booster dose) using the bath method.

The aquaria were equipped with biological filtration; water was monitored daily for quality and temperature. Diets were fed to the fish three times per day at a level of 1.5% average fish weight per meal.

Sampling procedures. All samples for this study were taken using the same method as described by SIGH et al. (2004a). Five fish from the vaccinated and control groups were sampled at 30 days following the first vaccination. Fish from each group were gently transferred to a small plastic aquarium containing a mild anesthetic (MS 222, 20 mg/L). In the laboratory, fish were killed quickly with an overdose of MS222 (200 mg/L), whereupon the tissues were aseptically dissected and subsequently snap-frozen in liquid nitrogen. These samples were pre-stored at 4 °C for 24 h, and then stored at -80 °C until RNA purification.

Real-time PCR. PCR primer sets specific for $TNF\alpha_1$ and $TNF\alpha_2$ were designed using the primer 3 program, based on sequences deposited in the Gene Bank (primer sequences and amplicon length listed in Table 1).

Table 1. The sequences of the forward and reverse primer and the amplicon length

Gene	Sequence [5-3]	Amplicon length [bp]
TNFα-1.F	TTCGGGCAAATATTCAGTCG	433
TNFα-1.R	GCCGTCATCCTTTCTCCACT	
TNFα2.F	GGCCTTGAAAATAGCCTTGT	345
TNFα2.R	GCCGTCATCCTTTCTCCACT	
β-Actin.F	TCACCCACACTGTGCCCATCTACGA	295
β-Actin.R	CAGCGGAACCGCTCATTGCCAATGG	

The primer sets were designed in conserved regions of the molecules to enable recognition of all the described isoforms of each gene. β-actin was selected as a reference gene, and subsequently for sample normalization (SIGH et al., 2004). To assess PCR efficiency, serial dilutions of the standard cDNA preparation were used to generate the standard curve for each primer set. The primer efficiency was calculated according to the equation: E ¼ 10 (1/slope). The sequences of the forward and reverse primers, as well as the amplicon length, are listed in Table 2. The constitutive expression of the genes included in the study was tested on a separate pool of cDNA, generated from four non-infected fish (from the negative control group). For cDNA generation, 1 μL of random hexamer primer (2 μg/L) was added to 2 μL extracted RNA. The mixture was incubated in a thermal cycler at 65 °C for 5 min, and then immediately placed on ice for at least 1 min. Then, 10 μL of 2× first standard reaction (10 mM $MgCl_2$, 1 mM Dntp) and 2 μL reverse transcriptase were added, with incubation at 25 °C for 10 min, followed by 50 min at 50 °C, and finally, 85˚ C for 5 min. The threshold value [Ct], defined as the threshold cycle number of PCR at which the sample fluorescent signal passes a fixed threshold above the baseline, was determined manually for each run. Quantitative PCR assays were performed in a Step One™ Real-Time PCR System (Applied Biosystems, USA). Reactions contained 10 μL SYBR® Premix Ex Taq™ (Tli RNase H Plus), ROX plus (TaKaRa, Japan), 1 μL of cDNA, 0.5 μL of forward and reverse primer (100 nM), filled up with ultra-pure water to a final volume of 20 μL. The following cycling conditions were used: One incubation step of 10 min at 95˚C, followed by 45 amplification cycles, which included; 30 s at 94 °C, 60 s at 60 °C. In order to detect the presence of non-specific amplification, control reactions without template were included for each primer set. At the end of each cycle, DNA melting curve analysis was performed in order to confirm the specificity of the PCR products. Melting curves were acquired on the SYBR channel using a ramping rate of one

cycle of 0.5 °C per 30 s for 55 °C - 99 °C. Gene expression of the samples compared to the control was calculated according to the following equation, using REST2009 QPCR software (Qiagen, USA), and the Pfaffl method.

$$Ratio = (\in_{target})\Delta c_{T\ target\ (control\text{-}sample)} / (\in_{RPS11})\Delta c_{T\ target\ (control\text{-}sample)}$$

Statistical analysis. All the measurements were made in triplicate. The results were subjected to analysis of variance (ANOVA) followed by the least significant differences (Tukey) test. Correlation coefficients were significant with $P<0.05$.

Results

The vaccinated fish showed significant protection (*t*-test, $P<0.05$) against *I. multifiliis* 30 days after the first vaccination. Expression data were obtained mainly from the skin, but the liver, gills and head kidney were also investigated with regard to the β-actin gene. The examined genes showed various levels of constitutive expression, but some genes became significantly up- or down-regulated following vaccination.

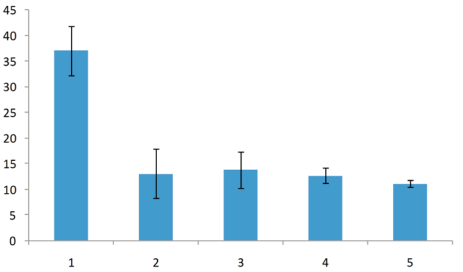

Fig. 1. Expression of TNFα$_1$ in the skin of immunized rainbow trout relative to the β-actin gene

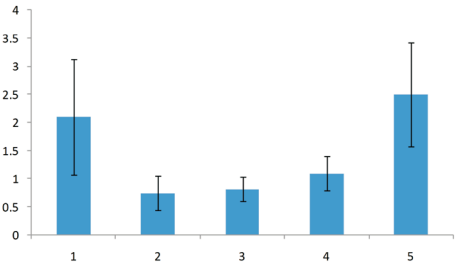

Fig. 2. Expression of TNFα$_2$ in the skin of immunized rainbow trout relative to the β-actin gene

* A graph showing mean and ± SEM bar of three replicates treatments. T1: rainbow trout vaccinated via irradiated vaccine plus alginate nanoparticles; T2: rainbow trout vaccinated via irradiated vaccine; T3: rainbow trout vaccinated via formalin vaccine plus alginate nanoparticles; T4: rainbow trout vaccinated via irradiated vaccine; T5: rainbow trout immunized via live trophont of *Ichthyophthirius multifiliis*.

The current experiment overall showed a significantly increased level of expression of the TNF-α$_1$ gene in the skin, liver and head kidney of immunized rainbow trout relative to the β-actin gene 30 days after the first vaccination (Figs. 1, 3 and 5). Especially after 30 days of vaccination, as shown in Figs. 1 and 5, the expression of the TNF-α$_1$ gene was unexpectedly elevated in the skin and liver of the fish vaccinated with radiovaccine plus alginate nanoparticle, compared to the other groups ($P<0.05$). A significant up-regulation

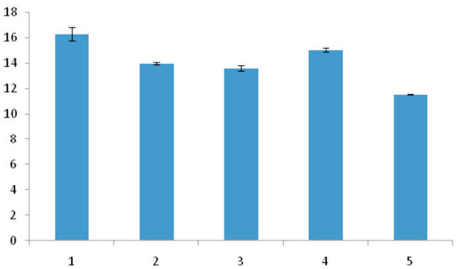

Fig. 3. Expression of TNFα$_1$ in the kidneys of immunized rainbow trout relative to the β-actin gene

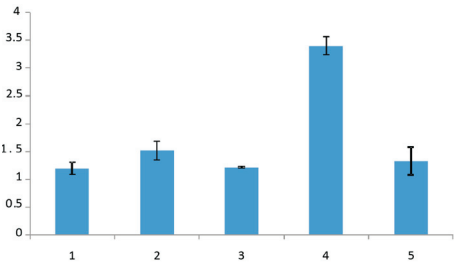

Fig. 4. Expression of TNFα$_2$ in the kidneys of immunized rainbow trout relative to the β-actin gene

* See the explanation below Fig. 1 and Fig. 2

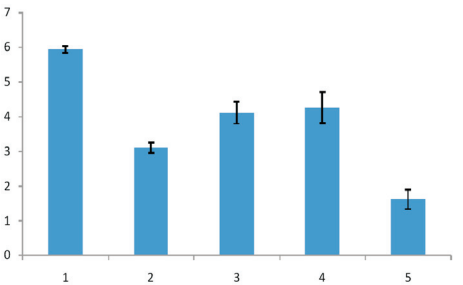

Fig. 5. Expression of hepatic TNFα$_1$ in immunized rainbow trout relative to the β-actin gene

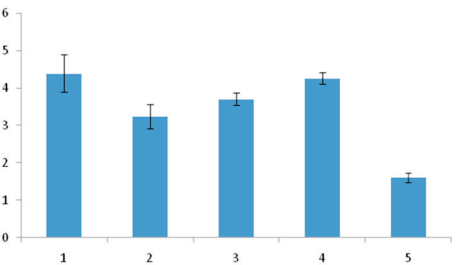

Fig. 6. Expression of hepatic TNFα$_2$ in immunized rainbow trout relative to the β-actin gene

* See the explanation below Fig. 1 and Fig. 2

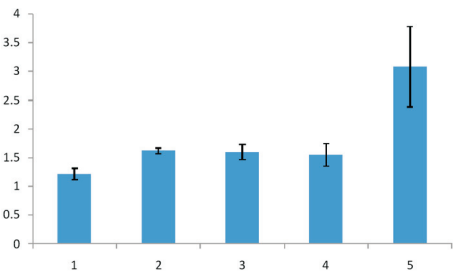

Fig. 7. Expression of TNFα$_1$ in the gills of immunized rainbow trout relative to the β-actin gene

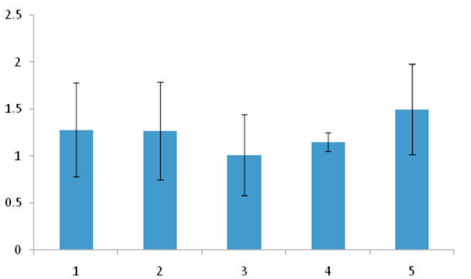

Fig. 8. Expression of TNFα$_2$ in the gills of immunized rainbow trout relative to the β-actin gene

* See the explanation below Fig. 1 and Fig. 2

of transcription of the cytokine TNFα_2 was detected in head kidney of the group that received formalin vaccine ($P<0.05$) (Fig. 4). We also found a significant increase in the TNF-α_1 gene expressed in the gills of rainbow trout immunized using live trophonts of *I. multifiliis* ($P<0.05$) (Fig. 7). In the skin and head kidney of the immunized rainbow trout, the TNF-α_1 gene was expressed much more strongly than the TNF-α_2 gene (Figs. 1-4). Furthermore, there was no significant difference in the expression of the TNF-α_2 gene in any of the gill tissues in the groups under all forms of treatment ($P>0.05$) (Fig. 8).

Discussion

The elicitation of acquired protective immunity following natural infection by *I. multifiliis* suggests that the development of a vaccine is feasible (HEIDARIEH et al., 2015; BUSCHKIEL, 1910; HINES and SPIRA, 1974).

In this study, to characterize the immune response against *I. multifiliis* in the skin, liver, gills and head kidney of immunized rainbow trout, with two types of killed vaccines (γ-irradiated and formalin inactivated trophonts), the gene expression levels of the pro-inflammatory cytokines TNF-α_1 and TNF-α_2 were evaluated. The vaccinated fish showed significant protection against *I. multifiliis* 30 days after the first vaccination.

SIGH et al. (2004a) showed that TNF-α was expressed during an infection with *I. multifiliis* at 26 days following infection. Also, the expression profile of tumor necrosis factor receptor-associated factor 6 in grass carp (*Ctenopharyngodon idella*) in the skin, gills, head kidney and spleen, showed that these molecules were significantly up-regulated after infection with *I. multifiliis* in all tissues tested (ZHAO et al., 2013). Therefore, the expression of TNF-α throughout the period of infection suggests that these molecules may play a role in the recruitment and maintenance of inflammatory cells in the skin (DICKERSON, 2012; SIGH et al., 2004a; SIGH et al., 2004b).

In the current study, we showed that the pro-inflammatory cytokine, TNF-α_1, is expressed in rainbow trout during vaccination with an irradiated vaccine plus alginic acid nanoparticles, not only in the skin but also in the head kidney and liver, whereas TNF-α_2 expression was seen only in the liver. Also, parasite-related changes in TNF-α_1 expression could be detected in the gills of fish exposed to live *I. multifiliis* trophonts during this experiment.

HUTSON (1993) and SUESCUN et al. (2003) reported that macrophages could be one of the cell types expressing this TNF-α. It seems that the expression of this gene could be crucial for recruitment of the relevant immune cells necessary for initiation of the immune reactions needed to clear the infection. Moreover, protection against protozoan parasites usually involves Th_1 cell-responses, with IFN-γ and TNF-α as proinflammatory cytokines being particularly important (OVINGTON et al., 1995). Also, there is a number of protozoan infections in which the antiparasitic role of TNF-α has been well established (OVINGTON and SMITH, 1992).

In conclusion, this study clearly showed that TNF-α_1 could be involved in immune mechanisms that can control *I. multifiliis* infection in rainbow trout vaccinated via an irradiated vaccine plus alginic acid nanoparticles.

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AKBARI, M. K., V. TAGHIZADEH, M. HEIDARIEH, A. HAJIMORADLOO: Ključna uloga faktora tumorske nekroze alfa (TNF-α) u kalifornijskih pastrva cijepljenih ozračenim trofontom parazita *Ichthyophthirius multifiliis*. Vet. arhiv 87, 229-237, 2017.

SAŽETAK

Istražen je imunosni odgovor u koži, jetri, škrgama i bubregu kalifornijskih pastrva cijepljenih dvjema vrstama inaktiviranih cjepiva pripravljenih od parazita *Ichthyophthirius multifiliis*. Jedna je bilo pripravljena ozračivanjem trofonta γ-zrakama, a druga njegovim ubijanjem formalinom. Istražena je razina genske ekspresije proupalnih citokina odnosno faktora tumorske nekroze α (TNF-α_1 i TNF-α_2). Cijepljene ribe pokazivale su značajnu zaštitu protiv *I. multifiliis* 30 dana nakon drugog cijepljenja. Pokazalo se da je proupalni citokin TNF-α_1 bio izražen u pastrva nakon cijepljenja ne samo u koži već i u bubregu i jetri, dok je ekspresija TNF-α_2 bila dokazana samo u jetri. U ovom je pokusu također ustanovljeno da se promjene u ekspresiji TNF-α_1 mogu dokazati samo u škrgama riba izloženima živim trofontima *I. multifiliis*. Na osnovi prijašnjih izvješća i ovog istraživanja može se zaključiti da bi TNF-α_1 mogao biti upleten u imunosne mehanizme za kontrolu invazije vrstom *I. multifiliis* u cijepljenih kalifornijskih pastrva.

Ključne riječi: faktor tumorske nekroze alfa, TNF-α, *Ichthyophthirius multifiliis,* cjepivo, ozračivanje, kalifornijska pastrva