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Variations of kidney, liver and spleen total sialic acid levels in immunized rainbow trout (*Oncorhynchus mykiss*) against *Ichthyophthirius multifiliis*

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ABSTRACT

The aim of this study is to evaluate the effects of irradiated trophonts of *Ichthyophthirius multifiliis* and alginate/calcium phosphate nanoparticles on total sialic acid levels in immunized rainbow trout kidney, liver and spleen tissues. The 15 fish tissues of the treated and control groups were sampled at 30 days following the first immunization. Tissue samples were homogenized and centrifuged. The total sialic acid levels of the tissue samples were determined using a commercially available Quantitation Kit. Significant increases in kidney, spleen and liver TSA levels were determined in the treated groups in comparison to the controls. The immunosupportive effects of sialic acid were illustrated by the elevation in the amount of TSA in the kidneys, liver and spleen of the immunized fish. Also, the significant increase in the TSA levels in the rainbow trout treated with calcium phosphate nanoparticles could be attributed to the Ca-binding capacity of the glycerol side chain of the sialic acid. Finally, the results of this study showed that sialic acid may be a mediator in the development of long-circulating nanocarriers to advance the field of vaccine delivery in fish.

Key words: alginate nanoparticles; calcium phosphate nanoparticles; total sialic acid; rainbow trout; gamma- irradiated *Ichthyophthirius multifiliis*

Introduction

Sialic acid is the biologically most important monosaccharide unit of glycoconjugates. Sialic acids are present in different types of vertebrate tissues of all classes (CABEZAS,

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1973). Sialic acids were found to be components of glycoproteins in the epithelial mucus of several fish species (ASAKAWA, 1974). FLETCHER et al. (1976) demonstrated histochemically that sialoglyco proteins may have functional significance in the epithelia of freshwater teleosts. Also, quantitative and qualitative differences in sialic acid levels may be an important health/disease indicator (BASSAGANAS et al., 2014). In many inflammatory and infectious diseases of domestic animals, SA values have been analyzed (KARAGENC et al., 2005), but, there are no data on SA levels in immunized fish against *Ichthyophthirius multifiliis*.

The protozoan *Ichthyophthirius multifiliis* (*I. multifiliis*) was diagnosed as one of the most important freshwater ciliate pathogens in the sudden death of fish in a tank on a commercial farm, and in general it causes significant economic losses to the aquaculture industry worldwide (AIHUA and BUCHMANN, 2001; MAKI and DICKERSON, 2003). Chemotherapy is not sufficient to control this parasite after penetration of the theront stage of the *Ichthyophthirius multifiliis* into the fish skin and gill epithelium. Thus, immunization against *I. multifiliis* and immunotherapy are considered as an alternative to chemical treatments to prevent mortality in fish (MAKI and DICKERSON, 2003).

The present study is concerned with evaluation of sialic acid levels from the kidney, spleen and liver tissues of rainbow trout immunized 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, at a temperature of 15 ± 1 ºC, with dissolved oxygen 5.2 ppm under a natural photoperiod (10L:14D). Adaptation to these tanks was performed for 14 days, with a commercial pelleted diet (Behparvar Co., Tehran, Iran).

Preparation of gamma-irradiated trophonts. Gamma irradiated trophonts were prepared as described previously (HEIDARIEH et al., 2015). In brief, fifty fish were infected with *I. multifiliis* via a high dose of live collected trophonts. The live trophonts of the parasite were obtained from heavily infected rainbow trout caught from a local Karaj fish farm. Exposure was performed in the dark for 8 hrs and they were then transferred to a glass aquarium. The fish were kept for 5 days at 20 °C and the trophonts were then collected with a 200-mesh sieve (skin). Trophonts should be used immediately (HEIDARIEH et al., 2014a).

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

Preparation of gamma irradiated alginate nanoparticles. In order to prepare alginate nanoparticles, the remaining powder, following sonication and heating (at 40 °C) of commercial Ergosan (Schering Plough Aquaculture, UK), was irradiated at a dose rate of 0.22 Gy/sec and the dose level applied was 30 kGy (HEIDARIEH et al., 2012; 2014a,b).

Preparation of calcium phosphate nanoparticles. Calcium phosphate nanoparticles can be synthesized using the simple method described by Sheihzadeh et al., 2017. In brief, the components of a formulation of 12.5 mM calcium chloride, 12.5 mM dibasic sodium phosphate, and 15.6 mM sodium citrate were mixed together and stirred for 48 h, with 30-min sonication (SHEIKHZADEH et al., 2017).

Immunization procedures. Two hundred and twenty-five (225) parasite free fish were randomly allocated into 5 groups in triplicate at a density of 15 fish per aquarium, equipped with biological filtration. The vaccine dose rate was 100 gamma-irradiation trophonts per 150 gram fish body weight (via the bath method) (SHEIKHZADEH et al., 2016). The1st group was immunized with 100 gamma-irradiation (170 Gray) inactive trophonts, the 2nd group immunized with alginate nanoparticles, the 3rd group immunized with10,000 live trophonts (as the positive control group), and the 4th group was the negative control (uninfected rainbow trout). Apart from the negative control group, all the other groups were boosted with the same immunization on the 10th day after the first dose of vaccine. The fish (apart from the negative and positive control groups) were challenged with 100 live trophonts by a second vaccination 10-days later (as a booster dose) via the bath method for 12 hrs.

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

Sampling procedures. All samples for this study were taken using the same method described by SIGH et al. (2004). The samples of 15 fish from the immunized and control groups were taken 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, the fish were killed quickly with an overdose of MS222 (200 mg/L) whereupon the tissues were aseptically dissected, immediately frozen in liquid nitrogen (-196 °C) and stored at -80 °C for biochemical evaluation of TSA levels.

Homogenization of tissues. The frozen tissues stored in nitrogen were placed in an Eppendorf tube containing 300 mL of PBS and manually homogenized using an appropriate manual pestle. The tubes were then centrifuged (3000 g for 10 minutes), and the supernatant obtained after centrifugation was transferred into another Eppendorf tube and frozen at -20 °C.

Analysis of the tissues samples. The tissues' total sialic acid levels were determined using a commercially available Sigma Sialic Acid Quantitation Kit (Sigma-Aldrich

Chime GmbH) and by an Olympus AU 600 auto-analyzer (Olympus Optical Corp, Shizuoka-ken, Japan). Each TSA level determination was performed three times for each single 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. The level for statistical significance was $P < 0.05$.

Result

A significant increase in kidney, spleen and liver TSA levels was determined in the immunized groups in comparison to the controls ($P<0.05$) (Figs. 1-3). Furthermore, the values of kidney, liver and spleen TSA levels were significantly higher in the group treated with calcium phosphate nanoparticles as compared to the irradiated trophonts and alginate nanoparticles groups ($P<0.05$) (Figs. 1-3).

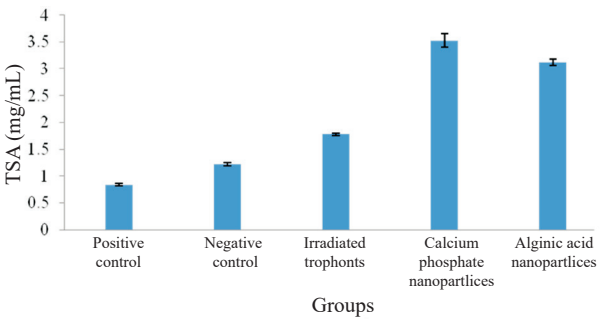

Fig 1. The results of rainbow trout kidney tissue total sialic acid analysis are shown for different treatment groups

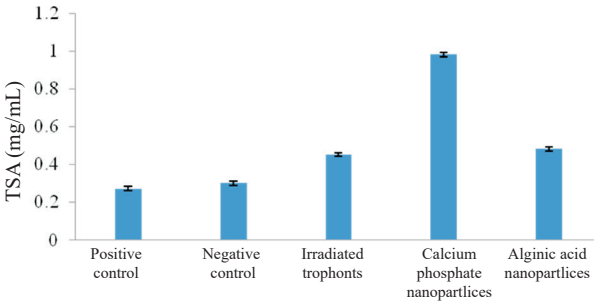

Fig 2. The results of rainbow trout liver tissue total sialic acid analysis are shown for different treatment groups

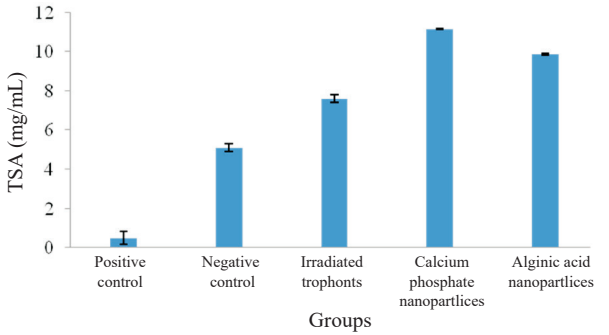

Fig 3. The results of rainbow trout spleen tissue total sialic acid analysis are shown for different treatment groups

Discussion

To our knowledge, this is the first study in which TSA levels have been investigated in the tissues of immunized rainbow trout against *I. multifiliis* using irradiated parasite trophonts, alginate and calcium phosphate nanoparticles. A previous study revealed that the concentration of TSA in the serum increases in infected fish via the *I. multifiliis* parasite compared with healthy ones (AZMIZADEH, 2016).

Sialic acid (SA) is known to be the one of derivatives of neuraminic acid as an acetylated derivative that is widely distributed throughout all vertebrate tissues, body fluids and in higher invertebrate species (GUZEL et al., 2008). It perches on the terminal location of monosaccharides attached to cell surface glycoconjugates (NAZIFI et al., 2010), which play a crucial role in various biological processes, and aberrant sialylation is closely associated with many diseases, particularly cancers. SAs can contribute to various forms of cell-cell and cell-pathogen interaction, such as cell adhesion, signal transduction, and immune responses (SCHAUER, 2009). Furthermore, SA values have been analyzed in many inflammatory and infectious diseases of domestic animals (KARAGENC et al., 2005), but no assessment has been observed regarding TSA levels in immunized fish against a parasitic disease.

In the present study, a considerable increase in kidney, spleen and liver TSA levels was determined in immunized fish, particularly the nanoparticles groups in comparison to the controls. One possibility may be the capability of the important biological functions of this monosaccharide, TSA levels, in regulating an alternative pathway of complement activation leading to enhancement of adaptive and innate immune responses (FEAROM, 1978; KAJANDER et al., 2011; MERI and PANGBURN, 1990; RAM et al., 1998; FERREIRA et al., 2010).

On the other hand, the development of advanced nanomaterials in creating controllable drugs, which makes them highly suitable for delivery, has presented considerable challenges over the past two decades. However, recognition, synthesis, transmission and distribution of nanomaterials within diverse types of host is also critical. It is important to note the rapid blood clearance limits of nanomaterial accumulation at target delivery sites; nanoparticle accumulation in macrophages within clearance organs initiates inflammatory responses, inducing toxicity (ALBANESE et al., 2010; CARTER and DRISCOLL, 2001; CHO et al., 2007; DRISCOLL, 2000; GAZI and MARTINEZ-POMARES, 2009; NISHANTHA et al., 2011; ORR et al., 2011; PAJARINEN et al., 2013; PARK and PARK, 2009; SHARMA et al., 2010; VELARD et al., 2013). However, sialic acid-covered nanoparticles are expected to facilitate inhibition of immune cell activation, which allows RES escape by rendering the nanoparticles prolonged circulation in the blood stream, and reduced uptake by the immune system. The major serum protein complement factor H also recognizes sialic acid as a "self" marker, which helps to reduce immune recognition and phagocytic recognition (FEARON, 1978; KAJANDER et al., 2011; MERI and PANGBURN, 1990; RAM et al., 1998; FERREIRA et al., 2010). In this study, the immunosupportive effects of sialic acid may be illustrated by the elevation in the amount of TSA in the kidney and spleen of immunized fish via nanoparticles. On the other hand, the slight increase in TSA levels in the rainbow trout immunized with calcium phosphate nanoparticles compared to the fish immunized with alginate nanoparticles could be attributed to the Ca-binding capacity of the glycerol side chain of the sialic acid (BEHR and LEHN, 1972). Given these results, the increased levels of TSA in immunized rainbow trout tissue, especially in the groups treated with nanoparticles, it appears sialic acid may be used as a mediator in the development of long-circulating nanocarriers to advance the field of vaccine delivery in fish.

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Conflicts of interest

We declare that we have no conflict of interest.

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HEIDARIEH, M., S. MOODI, O. SAFARI: Varijacije ukupne razine sijalične kiseline u bubrezima, jetri i slezeni imunizirane kalifornijske pastrve (*Oncorhynchus mykiss*). Vet. arhiv 89, 223-232, 2019

SAŽETAK

Cilj ovoga istraživanja bio je procijeniti učinak ozračenog trofonta *Ichthyophthirius multifiliis* i nanočestica alginata / kalcijeva fosfata na ukupnu razinu sijalične kiseline u bubrezima, jetri i slezeni imunizirane kalifonijske pastrve. Uzeti su uzorci 15 riba iz pokusne i kontrolne skupine 30 dana nakon imunizacije. Uzorci tkiva su homogenizirani i centrifugirani. Ukupna razina sijalične kiseline u uzorcima tkiva utvrđena je komercijalnim kitom za kvantifikaciju. U pokusnim skupinama u usporedbi s kontrolnom utvrđen je statistički znakovit porast ukupne razine sijalične kiseline u bubregu, jetri i slezeni. Imunopotporni učinci sijalične kiseline prikazani su porastom ukupne razine sijalične kiseline u bubrezima, jetri i slezeni imunizirane ribe. Također, statistički znakovit porast ukupne razine sijalične kiseline u kalifonijske pastrve kojoj su davane nanočestice kalcijeva fosfata, mogao bi se povezati sa sposobnošću postranog lanca glicerola sijalične kiseline za vezanje kalcija. Rezultati ovoga istraživanja pokazali su da sijalična kiselina može posredovati u razvoju dugocirkulirajućih nanonositelja kako bi se pospješila imunizaciju riba.

Ključne riječi: nanočestice alginata; nanočestice kalcijeva fosfata; ukupna razina sijalične kiseline; kalifornijska pastrva; gama-ozračen *Ichthyophthirius multifiliis*