

Original Research Article

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Elevation of Nitric Oxide Level in Rohu (*Labeo rohita*) in Response to Immunization with Whole Antigens of Fish Ectoparasite, *Argulus siamensis*

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ABSTRACT

Keywords

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Argulosis, caused predominantly by *Argulus siamensis* is a threatening ectoparasitic disease of Indian carp aquaculture. Vaccination against this parasite is a safe alternative to the harmful chemicals used for its control. Nitric oxide (NO), a signaling molecule plays an important role in immune mediated functions in different parasitic diseases. NO mediates immune response through cytokine production and gives protection against parasitic diseases by vaccination or immunization. In the present study, the level of NO production in response to *Argulus siamensis* whole antigen immunization in rohu (*Labeo rohita*) was assessed by Griess method in serum and two tissue samples (kidney and liver). There was significant increase in NO in serum (39.27 vs 15.57 nmol/ml), kidney (0.66 vs 0.17 nmol/mg tissue) and liver (0.61 vs 0.16 nmol/mg tissue) in immunized fish compared to the control fish. Further the immunized fish were confirmed for the presence of antibody against the *Argulus* parasite by dot blot method. The results possibly confirm the increased level NO possessing protective or immune-related function against this parasitic disease.

Introduction

Nitric oxide (NO) is a small molecule that regulates multiple physiological functions in animals (Nahrevanian and Amini, 2009), including immunological functions in both innate and adaptive responses (Bogdan *et al.*, 2000). NO is produced from amino acid L-arginine by an enzyme called nitric oxide synthase (NOS) that exists in three different isoforms. Only one is an inducible form of NOS (iNOS) found in numerous cell types

including phagocytic cells and is rapidly expressed in response to stimuli such as proinflammatory cytokines (Burgner *et al.*, 1999). In mammals, phagocytic cells are known to produce NO in response to stimulation by pathogens or their components, and this is suggested to be an important antimicrobial effector against bacteria, viruses and parasites (Bogdan, 2001). Many common human parasites have been shown to elicit host iNOS induction and the subsequent initiation of immune mechanisms, resulting in

the expulsion of the parasite (Wink *et al.*, 2011). Inducible NO responses have also been demonstrated in fish phagocytes similar to mammalian phagocytes (Whyte, 2007). Enhanced NO responses have been reported in several microbial infections (Campos-Perez *et al.*, 2000; Acosta *et al.*, 2005) including parasites (Saeij *et al.*, 2002) in fish. The production of more amount of nitric oxide during parasitic infestation may possess protective response in the host body against the parasite.

Parasitic diseases are the major factors hindering the high productivity in carp farming in India. The different parasitic infestations along with other secondary infections affect mass population of fish resulting in mortality and loss to the fish farmers. Among different ectoparasites, *Argulus siamensis*, a branchiuran parasite is a major threat to the Indian carp farming (Sahoo *et al.*, 2013). Normally the parasite is controlled by application of various chemicals in the fish ponds, which also possess detrimental effects on fish health as well as human beings. Hence, alternate safe and effective method of control e.g. vaccination has to be devised. Among different effector mechanisms of parasitic infestation, nitric oxide (NO) has been shown to play a major role in parasitic diseases in fish. Thus the present study was carried out to know whether there will be any effect of immunization of whole parasitic antigens on nitric oxide levels in the immunized fish.

Materials and Methods

Maintenance of rohu (*Labeo rohita*)

Experimental fish (*L. rohita*) of 50-100 g size were obtained from ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India farm and were kept in 500 l tank in the wet laboratory. The fish were left for

acclimatization for 7 days prior to experimentation. Those were given ad libitum feeding with a commercial pellet feed. Before experimentation, the fish were checked properly to be devoid of any infection.

Immunization of rohu with *Argulus* antigens and collection of serum

The whole homogenate of *Argulus* parasites was prepared for immunization of rohu. Ten numbers of fish were immunized with *Argulus* antigens following our previously standardized method (Das *et al.*, 2018a). In brief, each fish was injected three times at 14 days intervals with 50 µg protein emulsified with Freund's adjuvants. After 14 days of last booster dose, the fishes were bled before sacrificing (to collect tissues as detailed later) and serum separated by centrifugation at 8000 rpm for 20 min and preserved at -20 °C. Control fish were similarly injected with TBS (20 mM TrisHCl buffer, pH-7.4 with 0.15 M NaCl) alone; serum prepared and preserved. MS-222 was used as anaesthetic during handling of fish.

Detection of anti-*Argulus* antibody in immunized rohu serum by dot blot

Dot blot was carried out on the nitrocellulose membrane to detect the anti *Argulus* antibody in immunized rohu serum. *Argulus* homogenate sample was placed in two nitrocellulose membranes each having concentration of protein at 4 µg/2 µl. Two µl of TBS was also placed in both the nitrocellulose papers as negative control. The membranes were blocked with 5% skim milk (prepared in TBS) for 2 h. Subsequently, the membranes were incubated sequentially with rohu serum (*Argulus* immunized serum or control serum in 1:2000 dilution), guinea pig anti-rohu IgM serum (1:2000) and goat anti-rabbit ALP conjugate (1:5000) (Genei, India) for 1 h each as per the protocol of Das *et al.*,

(2018b). Washing of the blot with TBST (TBS with 0.1% tween 20) was carried out 3 times at 5 min intervals after incubation with each reagent. Finally, the membranes were developed with substrate, BCIP/NBT (MP Biomedicals, OH, USA) for development of colour.

Preparation of sample from liver and kidney

After 14 days of last booster dose, the fishes of both the groups were dissected after euthanizing the fish with heavy dose of anaesthesia. The organs viz., liver and kidney were collected and weighed. The tissues were processed by making it 10% with TBS. Then the tissue were homogenized by Super FastPrep-1 homogenizer (MP Biomedicals, OH, USA) using lysing matrix B at a speed of 25 (4000 cycles per min) for 10 s with addition of protease inhibitor cocktail (Promega, WI, USA). The homogenate was centrifuged at 10,000rpm for 30 min and the supernatant was collected for NO estimation.

Estimation of nitric oxide

Nitric oxide concentration in serum and tissue homogenates was estimated by using Griess reagent following Halonen *et al.*, (1998). The Griess method is an indirect measurement of NO production that involves spectrophotometric determination of nitrite levels. In brief, the Griess reagent was prepared by adding 1:1 proportion of 1% sulphanic acid in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine in distilled water. For estimation of nitric oxide, 150 µl of appropriately diluted sample was mixed with 50 µl of Griess reagent and diluted with 1.3 ml of distilled water. The tubes were incubated at room temperature for 30 min and the absorbance was measured at 548 nm in spectrophotometer (BioSpectrometer basic, Eppendorf, Germany). The molar

concentration of nitrite in the samples was determined from a standard curve generated using known concentrations of sodium nitrite (1-100 µM).

Statistical analysis

Mean and standard error for two groups of fish were calculated using Microsoft Excel.

The difference between both control and treated groups was calculated at 95% confidence interval and significance at $p < 0.05$ with help of unpaired t-test using online GraphPad software.

Results and Discussion

The experimental fish remained apparently healthy all throughout the experimental period. Initially, the production of antibody against *Argulus* antigens was verified in dot blot experiment, where a clear dot could be observed with the serum from immunized group compared to the control group (Fig. 1). This indicated that the immunized *Argulus* antigens were successful in eliciting antibodies in rohu, *L. rohita*.

The NO estimation in serum and tissue samples was carried out by Griess reaction, a well-accepted colorimetric method for measuring NO levels (Miranda *et al.*, 2001). Rohu serum and two tissues viz., kidney and liver were selected for estimation of NO level in the present experiment. Serum has been used by various researchers to study the NO level in various fish species (Acosta *et al.*, 2005; Yeh and Klesius, 2013). Kidney tissue was selected for NO activity as it is the principal immune organ in fish responsible for phagocytosis, antigen trapping and processing activity, and formation of IgM and immune memory through melanomacrophagic centres (Kum and Sekkin, 2011). Liver, besides its metabolic functions has also been reported to

be actively involved in immune defence in teleosts (Secombes and Wang, 2012) and hence, we also selected liver tissue for estimation of NO in response to *Argulus* antigen injection. Barroso *et al.*, (2000) conclusively reported the presence of inducible nitric oxide synthase (iNOS) activity in kidney and liver of rainbow trout (*Oncorhynchus mykiss*) tissue implying the capability of these cells in generating nitric oxide and playing a potential role in fish defense mechanisms.

In the experiment the NO level was found to be significantly increased in serum, kidney

and liver samples of the immunized group of fish compared to the control group. In serum sample, the immunized group of fish showed the average NO value of 39.27nmol/ml compared with the value of 15.57nmol/ml in the control group (Fig. 2). Nitric oxide level detected in kidney also showed significant increase in immunized fish (avg. 0.66nmol/mg tissue) compared to control fish (avg. 0.17nmol/mg tissue) (Fig. 3). Similarly, the NO level in liver tissue was significantly more in immunized group of fish (avg. 0.61nmol/mg tissue) as compared to control group fish (avg. 0.16nmol/mg tissue) (Fig. 4).

Fig.1 Dot blots showing development of antibody in *Argulus*-immunized rohu; 1. *Argulus* antigen and 2. TBS; developed with A. control serum and B. immunized serum

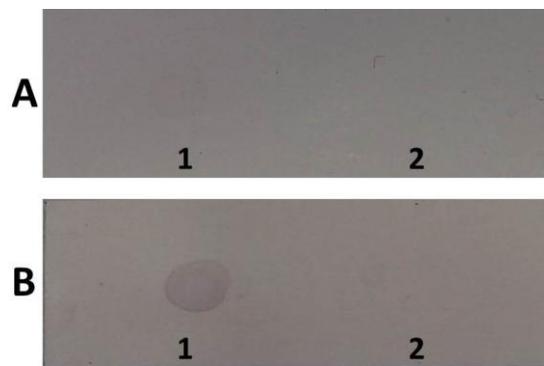


Fig.2 Estimation of nitricoxide (NO) in serum samples of immunized rohu. *indicates statistically significant from control

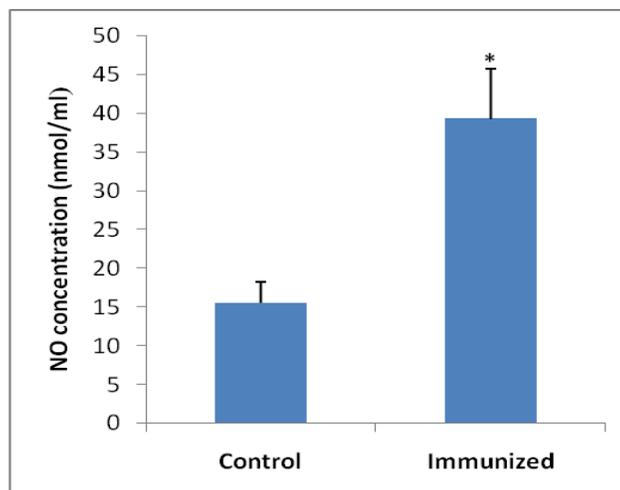


Fig.3 Estimation of nitric oxide (NO) in kidney samples of immunized rohu. *indicates statistically significant from control

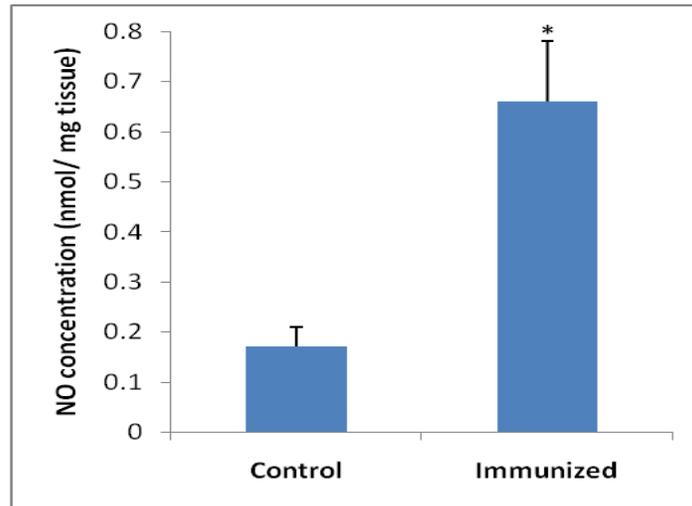
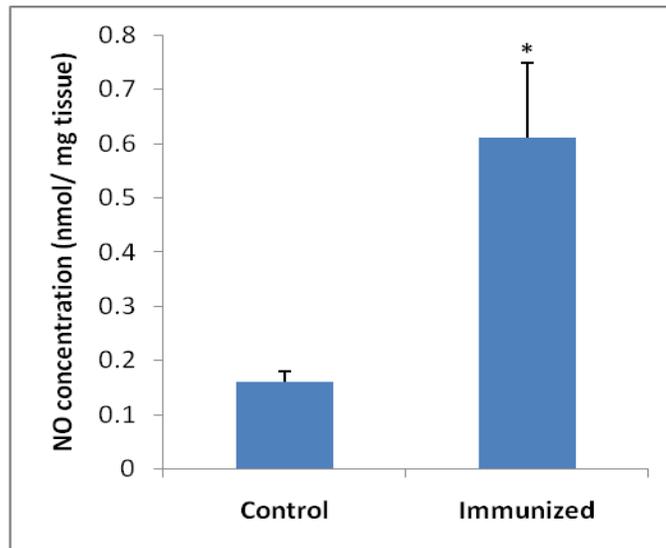


Fig.4 Estimation of nitricoxide (NO) in liver samples of immunized rohu. *indicates statistically significant from control



In the present experiment, the nitric oxide (NO) levels in control and immunized rohu serum and tissue samples were evaluated as a measure of innate immune response to the injected *Argulus* antigens. The plasma NO levels as an indicator of innate immunity has also been measured in other non-mammalian vertebrate such as eider ducks (Bourgeon *et al.*, 2007). In fish also NO is produced at high levels particularly by macrophages through its

activation, which is integral to its antimicrobial immunity to a range of pathogens (Grayfer *et al.*, 2018). The present study showed that more amount of NO was produced in the immunized compared to the control group of fish, possibly by the continuous activation of macrophages by adjuvanted antigens. The effect is further corroborated by the development of antibodies as detected in dot blot. Hosein *et*

al., (2015) similarly reported a significant increase in serum NO levels in cows vaccinated with *Brucella abortus* compared to unvaccinated control. A similar observation was also made by Campos-Perez *et al.*, (2000) while studying the serum NO levels in fish, rainbow trout (*Oncorhynchus mykiss*). Immunization with a killed *Renibacterium salmoninarum* preparation in FIA significantly increased NO levels after challenge with the pathogen in comparison to the control. Acosta *et al.*, (2005) also observed an increased NO response in gilthead seabream juveniles vaccinated and challenged with *Photobacterium damsela* subsp. *Piscicida* (*Pdp*) and concluded that vaccination resulted in an enhanced NO response to infection with *Pdp*. Furthermore, the level of protection of fish to experimental challenge with virulent *Pdp* also correlated with the level of the NO responses. Canthaboo *et al.*, (2002) have conclusively proved that NO plays an important role in effecting protection against *Bordetella pertussis* challenge. Similar responses have also been observed with parasitic infestations. Moreira *et al.*, (2016) reported an increase in intracellular NO in monocytes from dogs vaccinated against visceral leishmaniasis until six months post-vaccination, after interaction with *L. chagasi* promastigotes. In addition, the increased level of nitric oxide production has also been accounted in most of the internal parasitic infestations that had protective immunity against the disease (Wink *et al.*, 2011). It may be due to the production of pro-inflammatory cytokines that predisposes to the increased synthesis of NO, which mediates host protection through either direct parasite killing or by limiting parasite growth (Brunet, 2001). In our earlier study, a similar injection of whole antigens of *Argulus* showed some degree of protection against the parasite challenge (Das *et al.*, 2018a). Thus, the fish immunized with the *Argulus* antigens in the present study

produced higher amount of nitric oxide indicating the possible role of NO in providing protection against the *Argulus* parasite.

The present study showed a statistically significant elevation in nitric oxide levels in serum, kidney and liver tissues of rohu (*L. rohita*) in response to whole antigens of *Argulus* parasites which possibly aids in protective response against this parasite.

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