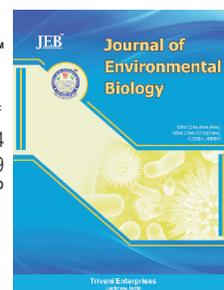


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Evaluation of cellular induction, soluble components of proteins and expression of pro-inflammatory genes in *Labeo rohita* fingerlings



Abstract

Aim : To investigate the acute inflammatory response in *Labeo rohita* induced by carrageenan (red seaweeds)

Methodology : The *Labeo rohita* fingerlings were injected with 50 μ l of carrageenan and the inflammatory response was examined at 3, 6, 12, 24, 48 and 96 hr post injection. The cellular markers that includes thrombocyte, macrophage, monocyte, granulocyte (neutrophils) and lymphocyte counts, serum myeloperoxidase activity, nitroblue tetrazolium (NBT) assay, total protein, albumin and globulin, C-reactive protein (CRP) and cortisol were examined. Additionally, the mRNA expression of proinflammatory cytokine genes IL-1 β and TNF- α and complement (C3) were analyzed by real time PCR.

Results : Analysis of fish fingerlings showed significantly ($p < 0.05$) higher thrombocyte and macrophage count in the inflammatory exudates of carrageenan injected group. The neutrophil and monocyte number were correlated with the inflammatory response and higher counts were observed during 12 hrs and beyond, whereas lymphocyte counts were lower in the treated group as compared to control. The NBT assay and myeloperoxidase activity that indicates the neutrophils activation were significantly ($p < 0.05$) upregulated in the treatment group. The C- reactive protein (acute phase protein), globulin, proinflammatory genes, i.e., IL-1 β , TNF- α and complement C3 were recorded significantly ($p < 0.05$) higher in the fishes injected with carrageenan as compared to control.

Interpretation : The carrageenan induces acute inflammatory response in *Labeo rohita* and the results provide a basic experimental model to further study the activity of anti-inflammatory drugs and mediators of inflammation.

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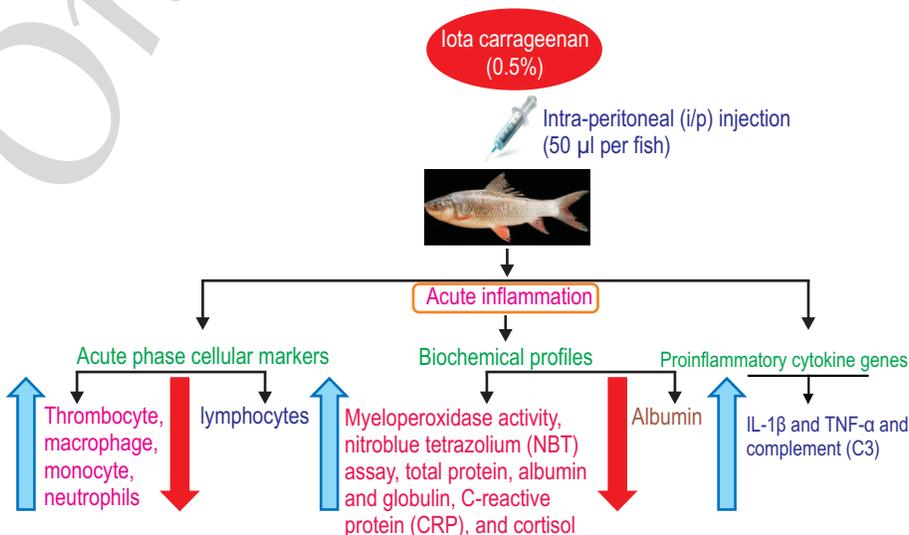
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Introduction

Inflammation is a general term used to indicate body response towards any perceived cellular injury. Injury to cells may arise due to physical and chemical agents, immunological reactions, external agents, infectious microorganism and genetic defects (Zweifach *et al.*, 2004). The first observation study on inflammation in fishes was conducted by Metchnikoff (1905), who studied phagocytosis by injection of guinea pig erythrocytes into the visceral cavity of *Carassius auratus*. Later, Weinreb (1958) reported increased phagocytic activity, following injection of *Bacillus anthracis* and kerosene in rainbow trout, *Salmo gairdneri irideus*.

The inflammatory process is mediated by various chemical mediators derived from plasma proteins and cells. In fact, various processes like increase in the number of circulating white blood cells (leucocytosis), presence of various acute phase proteins i.e. C-reactive protein, serum amyloid A and fibrinogen, etc. appears and play important role during inflammation (Gurenlian, 2009). The proinflammatory cytokines i.e., interleukin (IL-1 β) and tumor necrosis factor- α (TNF- α) are predominantly produced by activated immune cells such as macrophages and are involved in the amplification of inflammatory reaction. The complement cascade system of enzymatic proteins are activated during acute inflammatory response and C3 particularly (chemotactic for neutrophils) help in increase of vascular permeability and release of histamine from mast cells (Chauhan *et al.*, 2006). There are several reports demonstrated the sequential acute inflammatory response in higher vertebrates, however very limited information is available in fishes including observations from Matushima *et al.* (2006) and Martins *et al.* (2006) that shows injection of carrageenan in the swim bladder of *O. niloticus* and *Piaractus mesopotamicus* (pacu), leads to accumulation of thrombocytes, macrophages as well as a small number of granulocytes. Bozzo *et al.* (2007) observed accumulation of lymphocytes and thrombocytes during inflammatory response induced by thioglycolate, *Escherichia coli* lipopolysaccharide (LPS) and heat-inactivated *Aeromonas hydrophila*. Kumar *et al.* (2014) reported increase in hematological, biochemical and innate immune parameters in carrageenan incorporated feed.

Carrageenan a polysaccharide prepared by alkaline extraction and modification from red seaweeds (Rhodophyceae) and generally used for thickening, suspending, gelling and as a binder in aquaculture feeds (Kaushik *et al.*, 1991). Carrageenan is also reported to cause inflammation in different animal models (Margret *et al.*, 2009; Kumar *et al.*, 2014); hence, it was chosen as inflammatory inducing agent in the present study. Teleost fish exhibit inflammatory reactions which closely resemble those of mammals, but the mechanism of inflammation in fish are less understood as compared to mammals. Therefore, in this study, using *Labeo rohita* fingerlings the acute inflammatory response

was evaluated and cellular induction and soluble components of acute phase proteins and expression of proinflammatory genes was analysed.

Materials and Methods

Experimental design : The *Labeo rohita* fingerlings of average length 15 ± 0.79 cm per fish and weighing 19 ± 0.95 g per fish was purchased from Khopoli (Government fish farm), Maharashtra, India. The fingerlings were acclimatized in 1000 l fiber reinforced plastic (FRP) circular tanks filled with adequately aerated freshwater at ambient temperature (26-28°C) for 15 day with continuous aeration. For experiment trails, the fishes were divided (equally and randomly) into two groups (control, treatment) and each group was maintained in triplicates containing 60 fish total, following a completely randomized design (CRD). The fishes were anesthetized with clove oil (Merck, Germany) with 4-5 mg l⁻¹ prior to injection and injected intra-peritoneal with 50 μ l per fish l-carrageenan (0.5%) (lota carrageenan, type-II, Sigma-Aldrich) dissolved in 100 ml of sterile saline solution (0.85%) in the treatment group and 50 μ l per fish sterile saline solution in the control group. The study was done for 96 hrs and sampling was carried out at 3, 6, 12, 24, 48 and 96 hr for hematological, serum biochemical and molecular parameters. At each time point nine fishes were sacrificed and blood samples and kidney tissue were collected. Tissue of three animals were pooled and such three pools were used to study the gene expression.

Hematological studies : Blood smears were prepared immediately from whole blood. Methanol fixed blood smears were stained with Field stain A and B (Himedia, India). Thrombocyte count was done by the method of periodic acid-schiff (PAS) (McManus, 1946) with slight modification. The blood smear was fixed in Gendre solution and after cooling it for 5 min dipped in 1 % periodic acid for 20 min, Schiff for 55 min, washed with sulphur water three times for 3 min and then quickly washed with distilled water. Finally, stained with haematoxylin for 5 min and washed with water (Martins *et al.*, 2009). The percentage of macrophages, lymphocytes, neutrophils and thrombocytes were determined by counting the cells from smear. The total number of cells was determined by a binocular microscope (Olympus, Japan) with a haemocytometer.

Biochemical profiles : The collected blood was centrifuged at 3000 x g for 10 min at 28°C. The separated serum was collected in sterilized eppendorf tubes and analyzed for different parameters. Serum total protein was estimated by biuret method (Reinhold, 1953) using a kit (Qualigen, India), albumin was estimated by bromocresol green binding method (Doumas *et al.*, 1971); and globulin content was calculated by subtracting albumin values from total serum protein. The activity of cortisol was determined by cortisol enzyme immunoassay kit (Catalog No. 500360) procured from Cayman Chemical Company, USA

and the activity of serum CRP was determined using Fish high sensitivity C - reactive protein (hs-CRP) ELISA kit (Catalog No. CSB-E06854f) procured from CUSABIO BIOTECH CO., LTD., China. The myeloperoxidase activity present in serum was measured according to the methods of Quade and Roth (1997) and Nitroblue tetrazolium (NBT) assay was determined by the method of Secombes (1990) as modified by Stasiack and Baumann (1996).

Immune-related gene expression study

RNA isolation and cDNA synthesis : Approximately, fifty milligram of kidney tissue was taken into a screw-capped tube containing glass beads which was subsequently utilized for extraction of total RNA using TRI reagent Trizol™ (Invitrogen, USA). One microgram of total RNA was used for cDNA synthesis using first strand cDNA synthesis kit (Fermentas, India). The reverse transcription reaction was carried out in 20 µl by adding 4 µl of 5x reaction buffer (250 mM Tris-HCL; pH 8.3, 250 mM KCl, 20 mM MgCl₂, 50 mM DTT), 2 µl (10 mM each dNTP) mix of dNTP, 1 µl (100 mM) of random hexamer primer, 1 µl (20 units per µl) of RiboLock RNase inhibitor, 2 µl (20 units per µl) of M-MULV reverse transcriptase. The solution was mixed thoroughly, spun and incubated at 25 °C for 5 min and 37 °C for 1 hr. The reaction was terminated by heating at 70 °C for 5 min. The resulting cDNA was stored at -20 °C for further use.

Real time PCR (qPCR) : PCR reaction (20 µl) was performed with the gene specific primers and 10 µl of 2x master mix (Fermentas, India) containing 0.05 units per µl Taq polymerase, 4 mM MgCl₂ and 0.04 mM of each dNTP. To this 8 µl of nuclease free water, 0.5 µl forward and reverse primers each (25 picomoles per µl) and 1.0 µl of template were added. The threshold cycle (C_T) value (observed from real time PCR) was determined using automatic setting on the ABI 7500 real time PCR system (Applied biosystem, USA). The difference in the C_T value between IL-1β, TNF-α and C3 gene and the corresponding internal control β-actin gene (Table 1), ΔC_T (C_T C3, C_T IL-1β, C_T TNF-α, C_T β-actin) was calculated and relative expression level of target gene to β-actin was described using the equation 2^{Δ-ΔCT}, where 2 is equal to PCR efficiency (Su et al., 2008). For determining the fold

change, the 2^{Δ-ΔCT} formula proposed by Pfaffl (2001) was used.

Statistical analysis : The data were statistically analyzed by statistical package SPSS version 16 and the mean values were compared by one way ANOVA followed by Duncan's multiple range test (DMRT) to determine the significant differences between the means. Comparisons were made at 5% (*p* < 0.05) probability level.

Results and Discussion

The first clinical signs in carrageenan injected fish appeared at 12 hr and on second day, fishes were lethargic and disinclination to move as well as loss of equilibrium. Examination of moribund fishes (in carrageenan injected group) at necropsy, revealed abdominal distension and hyperemia at the ventral side. The liver in the treatment group fishes was pale, mottled and slightly swollen. After 48 hr, there was accumulation of straw to pinkish coloured ascitic fluid at the injection site and petechial hemorrhage in the peritoneal cavity.

Thrombocytes are the major cell group reported in the carrageenan induced inflammatory response; they have immune function in body and play a role in phagocytic activity in fishes (Hill and Rowley, 1996). Monocytes are motile and phagocytic in nature that can engulf bacteria and cellular debris. Monocytes move quickly to sites of inflammation in the tissues and differentiate into macrophages to elicit an immune response (Ingersoll et al., 2011). Thrombocyte count increased significantly (*p* < 0.05) in experiment after 3 hr and beyond, and high value was observed during 24 hr. However, presence of macrophage in the exudates observed after 12 hr and cells resembling lymphocyte were rarely observed in the inflammatory exudates of carrageenan injected group (Table 2). The percentage of neutrophils along with monocyte increased significantly (*p* < 0.05) in the experiment 6 hrs onwards and continued to increase till the end of the experiment periods, whereas the lymphocyte percentage decreased significantly (*p* < 0.05) throughout the experimental periods when compared to the control (Table 3). The result is supported by work from Martins et al. (2009), which

Table 1 : Primers used in the study and their optimum annealing temperatures and sizes of PCR amplicons

| Target gene | Primer name | Primer sequence (5' to 3') | Optimum annealing temperature (°C) | Size of PCR amplicon (bp) | Accession no. of target gene |
|-------------------------|-------------|-------------------------------------|------------------------------------|---------------------------|------------------------------|
| Complement component C3 | C3CCF | CCC TGGACA GCATTATCACTC | 60.0 | 155 | AM773825 |
| | C3CCR | GAT GGT CGC CTG TGT GGT | | | |
| IL-1β | IL-1βrohuF | ATC TTG GAGAAT GTGATC GAA GAG | 57.5 | 561 | AM932525 |
| | IL-1βrohuR | GATACG TTT TTG ATC CTCAAG TGT GAA G | | | |
| TNF-α | TNF α F | CCAGGC TTT CAC TTCAGG | 51.65 | 181 | FN543477 |
| | TNF α R | GCC ATAGGAATC GGAGTAG | | | |
| β-actin | CCBA1F | GAC TTC GAG CAG GAGATG G | 55.3 | 181 | AY531753 |
| | CCBA1R | CAAGAA GGA TGG CTG GAA CA | | | |

Table 2: Total thrombocyte and macrophage count in the inflammatory exudates during various sampling period of *Labeo rohita*

| Time period (hr) | Cell types | | |
|------------------|------------------|-------------------|-------|
| | Macrophage count | Thrombocyte count | Other |
| 3 | 10 | 69 | 0 |
| 6 | 15 | 74 | 0 |
| 12 | 13 | 81 | 5 |
| 24 | 6 | 92 | 2 |
| 48 | 12 | 87 | 1 |
| 96 | 15 | 85 | 0 |

showed that carrageenan injected fish have increased number of immune cells including thrombocytes, macrophage, neutrophils and eosinophilic granular cell in inflammatory site (Martins et al., 2009).

The change in concentration of total protein level in serum represents the dynamics of protein synthesis (albumins and globulins) that actively participate in the inflammation process. In the study, total protein and globulin levels in serum (Table 4) increased significantly ($p < 0.05$) upto 48 hr, however its level decreased later 96 hr in the experiment. While, the albumin level (Table 4) in the serum decreased significantly ($p > 0.05$) in the treatment group upto 48 hrs afterwards an increased was observed during the experiment. The A:G ratio (Table 4) in the treatment groups showed significantly ($p < 0.05$) lower value as compared to the control group. The present study is supported by Moghazy et al. (2010) who observed that during inflammation albumin level decreased in the circulation, until the inflammatory process resolves.

The level of cortisol is an important biomarker for stress and is reported to influence the inflammatory response (Aronson, 2009). It has also been observed that cortisol plays an important role in reducing the inflammatory process in the body (Aronson, 2009). The cortisol level in the treatment groups increased significantly ($p < 0.05$) 3 hr and peak value was observed at 6 hr, but later decreased in the experiment (Fig. 1A). C-reactive protein is

widely studied acute phase protein found in the body fluids of vertebrates and invertebrates, associated with the inflammation acute phase response (Wu et al., 2003). It plays an active role in the immune system (Roy et al., 2017) and activation of classical complement pathway (De Haas et al., 2000). The CRP level in the serum showed a gradual rise in I-carrageenan injected fish during the experimental periods and a significant ($p < 0.05$) increase was observed after 3 hr and high value was observed at 48 hrs in the treatment groups and there was about 8 fold increase in the CRP level in the treatment group as compared to the control group (Fig. 1B). Results were supported by Magnadottir et al. (2011) who observed acute phase response (APR) in Atlantic cod (*G. morhua*) following intra-muscular injection of turpentine oil.

NBT and myeloperoxidase activity are the key indicator of neutrophils activation. The myeloperoxidase utilizes hydrogen peroxidase during respiratory burst activity to produce hypochlorous acid (Dalmo et al., 1997) and serve as important enzyme having anti-microbial activity. Whereas, NBT reduction product perform excellent indicator of health status or the immunization effectiveness in fish (Anderson et al., 1992), which are obtained after reaction with superoxide. The NBT and myeloperoxidase activity in the treatment group showed significantly ($p < 0.05$) higher values as compared to control. The NBT activity in the treatment group increased significantly ($p < 0.05$) 3 hr onwards and reached the maximum level at 24 hr and thereafter decreased during the experiment (Fig. 1D). Moreover, the myeloperoxidase activity showed similar trend and increased significantly ($p < 0.05$) 3 hr and reached peak level at 24 hr, while afterwards decreasing in the experiment (Fig. 1C). In fact, the role of macrophages, monocytes and neutrophils to produce reactive oxygen radicals by respiratory burst activity is well characterized (Dalmo et al., 1997), the myeloperoxidase and NBT assay supports the result of differential count demonstrating that there was an increase in neutrophils and monocyte count post injection and the highest value was recorded 24 hr.

In the present study, up regulation in the expression of studied proinflammatory genes was observed (Fig. 2). Expression of genes IL-1 β , TNF- α and C3 from inflammatory

Table 3: Total differential leukocyte count during various sampling period of *Labeo rohita*

| Time period (hr) | Monocyte count (%) | | Neutrophil count (%) | | Lymphocyte count (%) | |
|------------------|--------------------|-------------------|----------------------|-------------------|----------------------|-------------------|
| | Control | Intraperitoneal | Control | Intraperitoneal | Control | Intraperitoneal |
| 3 | 9.03 \pm 0.05 | 9.42 \pm 0.06 | 11.64 \pm 0.02 | 11.12 \pm 0.14 | 79.33 \pm 0.14 | 79.46 \pm 0.21 |
| 6 | 8.69 \pm 0.06 | 9.48 \pm 0.13* | 11.67 \pm 0.04 | 12.42 \pm 0.11* | 79.64 \pm 0.11 | 78.1 \pm 0.15* |
| 12 | 9.06 \pm 0.14 | 12.32 \pm 0.12* | 11.78 \pm 0.04 | 13.64 \pm 0.25* | 79.16 \pm 0.21 | 74.04 \pm 0.02* |
| 24 | 9.54 \pm 0.03 | 14.06 \pm 0.02* | 12.02 \pm 0.22 | 14.54 \pm 0.31* | 78.44 \pm 0.13 | 71.4 \pm 0.12* |
| 48 | 9.54 \pm 0.05 | 10.52 \pm 0.01* | 11.25 \pm 0.24 | 13.86 \pm 0.22* | 79.21 \pm 0.11 | 75.62 \pm 0.06* |
| 96 | 8.68 \pm 0.25 | 8.25 \pm 0.16 | 11.91 \pm 0.02 | 11.22 \pm 0.04 | 79.41 \pm 0.06 | 80.53 \pm 0.05* |

Mean \pm SE values in the columns with superscript (*) are significantly different ($p < 0.05$) with control

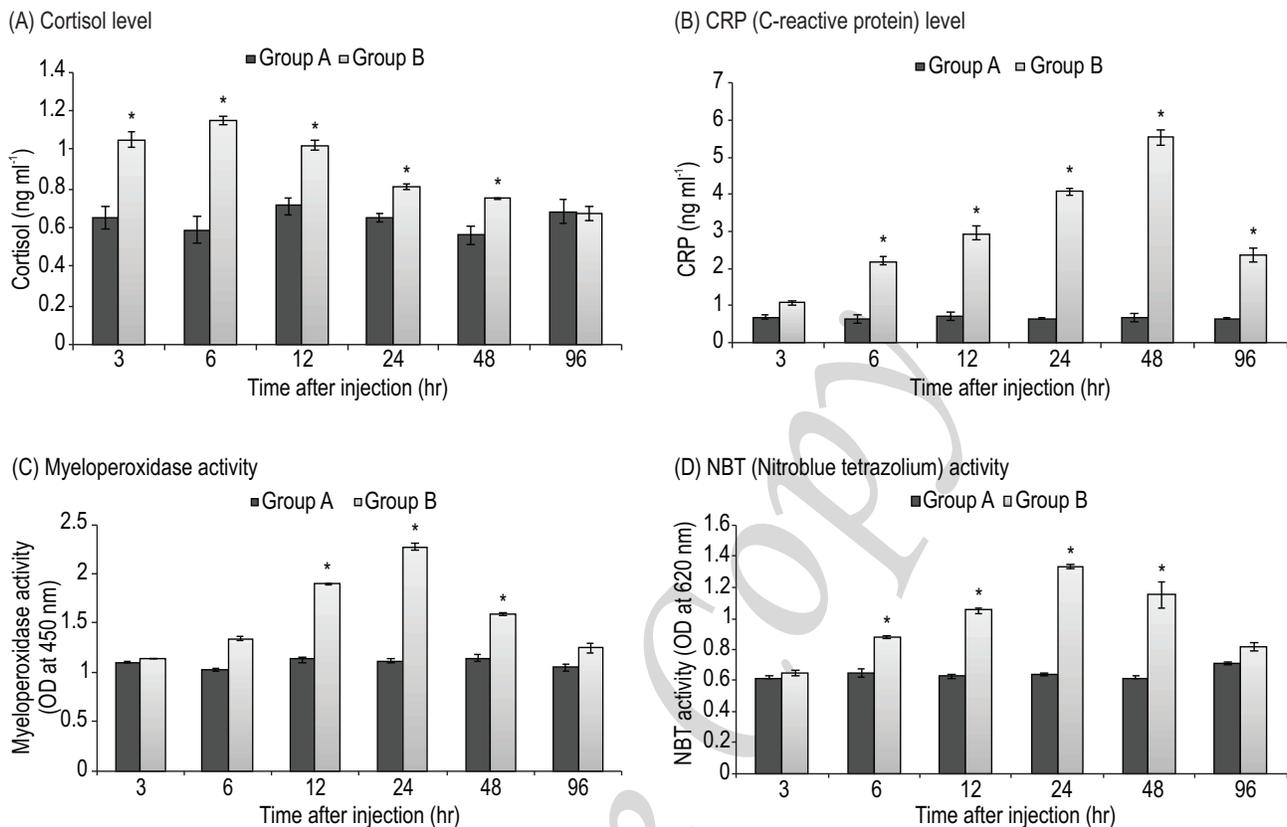


Fig. 1 : Serum biochemical profile of *Labeo rohita* during various sampling (values with superscript *) are significantly different ($p < 0.05$) and express as mean \pm SE)

Table 4 : Total protein, albumin, globulin levels and A:G (Albumin:Globulin) ratio during various sampling period of *Labeo rohita*

| Time period (hr) | Total protein (gdl ⁻¹) | | Albumin (gdl ⁻¹) | | Globulin (gdl ⁻¹) | | A:G ratio | |
|------------------|------------------------------------|--------------------|------------------------------|---------------------|-------------------------------|--------------------|-------------------|--------------------|
| | Control | Intraperitoneal | Control | Intraperitoneal | Control | Intraperitoneal | Control | Intraperitoneal |
| 3 | 2.402 \pm 0.016 | 2.561 \pm 0.057 | 1.307 \pm 0.03 | 1.397 \pm 0.007 | 1.095 \pm 0.02 | 1.164 \pm 0.05 | 1.194 \pm 0.029 | 1.200 \pm 0.696 |
| 6 | 2.410 \pm 0.01 | 2.510 \pm 0.074* | 1.440 \pm 0.015 | 1.260 \pm 0.0177* | 0.970 \pm 0.033 | 1.250 \pm 0.061* | 1.485 \pm 0.015 | 1.008 \pm 0.97* |
| 12 | 2.420 \pm 0.047 | 3.160 \pm 0.14* | 1.560 \pm 0.01 | 1.110 \pm 0.04* | 0.860 \pm 0.052 | 2.050 \pm 0.10* | 1.814 \pm 0.01 | 0.541 \pm 0.28* |
| 24 | 2.260 \pm 0.098 | 3.570 \pm 0.029* | 1.36 \pm 0.007 | 1.02 \pm 0.061* | 0.9 \pm 0.094 | 2.55 \pm 0.032* | 1.511 \pm 0.008 | 0.400 \pm 0.014* |
| 48 | 2.500 \pm 0.067 | 3.890 \pm 0.058* | 1.460 \pm 0.011 | 0.910 \pm 0.078* | 1.040 \pm 0.059 | 2.980 \pm 0.028* | 1.404 \pm 0.004 | 0.305 \pm 0.076* |
| 96 | 2.270 \pm 0.055 | 3.480 \pm 0.065* | 1.340 \pm 0.014 | 1.190 \pm 0.021* | 0.930 \pm 0.069 | 2.290 \pm 0.080* | 1.441 \pm 0.014 | 0.520 \pm 0.013* |

Mean \pm SE values in the columns with superscript (*) are significantly different ($p < 0.05$) with control

induced kidney exhibited a pattern of rapid increase in expression and quantification of these genes relative to β -actin transcript in kidney showed significantly ($p < 0.05$) higher level of expression level in the I-carrageenan injected *L. rohita* as compared to control fish. The expression level of IL-1 β (Fig. 2A) and TNF- α (Fig. 2B) were observed significantly up-regulated by 6.4 and 4.3 folds and highest value were recorded at 6 hr, afterwards expression decreased to normal levels at 96 hr. A significant

increase in expression level of C3 by 4.5 folds was marked at 12 hr as compared to control fish value. The highest level of C3 expression was observed at 12 hr (Fig. 2C). The results are supported by Covello *et al.* (2009) who observed that proinflammatory cytokines such as TNF- α , IL-1 β and IL-8 are important markers of an inflammatory response. Mohanty and Sahoo (2010) reported up-regulation of IL-1 β , TNF- α and C3 genes from 6 to 12 hr post-challenge during *Edwardsiella tarda*

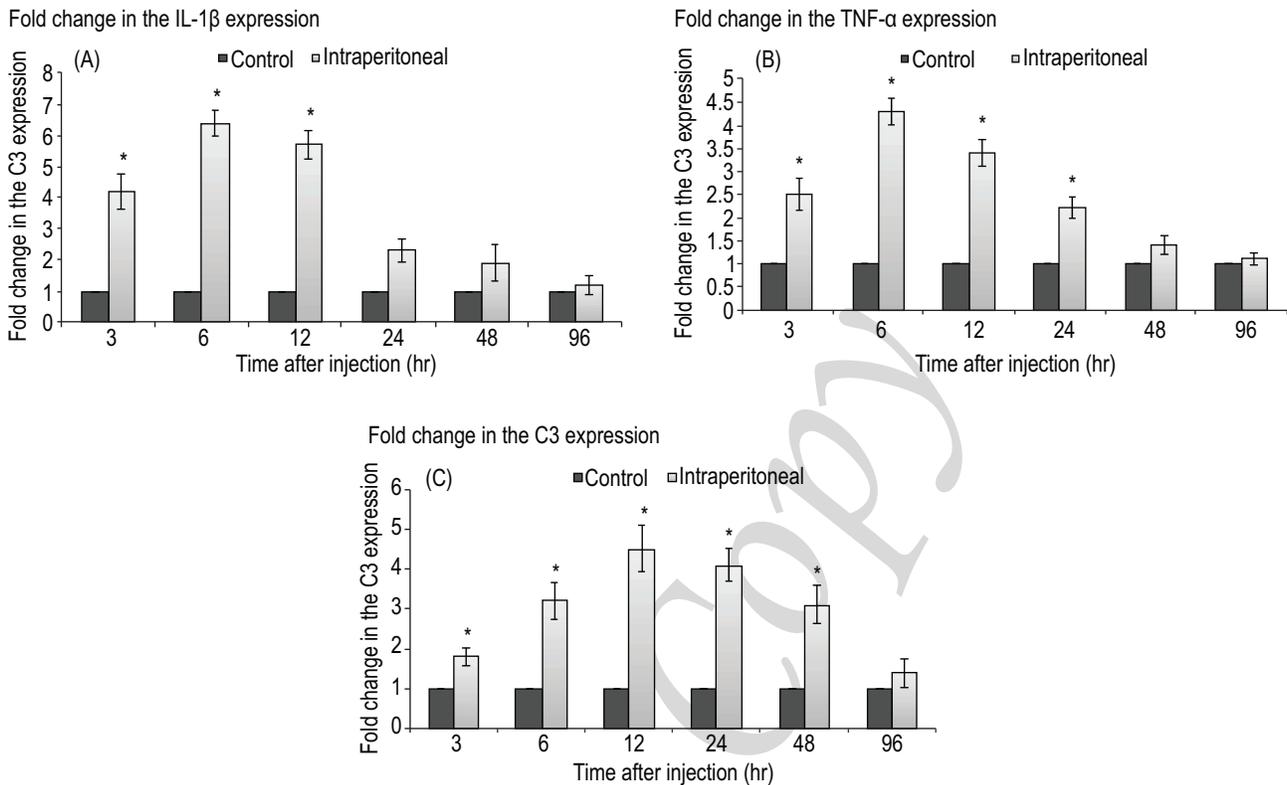


Fig. 2 : Expression of proinflammatory genes of *Labeo rohita* during various sampling (values with superscript (*)) are significantly different ($p < 0.05$) and express as mean \pm SE)

infection. Audunsdottir *et al.* (2012) reported that expression of complement component C3, interleukin-1 β (IL-1 β) and transferrin amplified by the turpentine injection in cod (*G. morhua*). Complement molecule C3 was observed to increase in the plasma of rainbow trout within 10 min of initiation of acute stress (Jorgensen *et al.*, 1993).

Increase in number of thrombocyte, macrophage, neutrophils, monocyte, cortisol and CRP levels, up-regulation in expression of IL-1 β , TNF- α , C3 genes and conversely a decrease in albumin value and lymphocyte count studied in the present study might play an important role in the inflammatory process and show a very close resemblance to the mammalian inflammatory response. These results build to the understanding of acute inflammatory process in *L. rohita* and provide a basic experimental model to study the activity of anti-inflammatory drugs and mediators of inflammation.

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References

- Anderson, D.P., T. Moritomo and R. de Grooth: Neutrophil, glass-adherent, nitro blue tetrazolium assay gives early indication of immunization effectiveness in rainbow trout. *Veterinary Immunopathology*, **30**, 419-429 (1992).
- Aronson, D.: Cortisol — Its role in stress, inflammation and indications for diet therapy. *Today's dietitian*, **11**, 38 (2009).
- Audunsdottir, S.S., B. Magnadottir, B. Gisladdottir, Z.O. Jonsson and B. T. Bragason: The acute phase response of Atlantic cod (*Gadus morhua*): Expression of immune response genes. *Fish Shellfish Immunol.*, **32**, 360-567 (2012).
- Bozzo, F.R., J.R.E. Moraes, F.R.D. Moraes and G.T. Pereira: Kinetics of cellular component in inflammatory response induced by different stimuli in the Swim Bladder of Pacu, *Piaractus mesopotamicus* Holmberg 1887 (Characidae). *J. World Aquacul. Soci.*, **38**, 302-308 (2007).
- Chauhan, L.S., C.S. Chauhan and C.P. Jain: Inflammation Mediators: A Review. *Pharmaceutical Reviews*, **4**, (2006).
- Covello, J.M., S. Bird, R.N. Morrison, S.C. Battaglene, C.J. Secombes and B.F. Nowark: Cloning and expression analysis of three striped trumpeter (*Latris lineata*) pro-inflammatory cytokines, TNF-alpha, IL-1beta and IL-8, in response to infection by the ectoparasitic, *Chondracanthus goldsmidi*. *Fish and Shellfish Immunol.*, **26**, 773-786 (2009).
- Dalmo, R.A.K., Ingebrigtsen and J. Bogwald: Non-specific defence

- mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *J. Fish Dis.*, **20**, 241-273 (1997).
- De Haas, C.J., E.M.M. van Leeuwen, T. van Bommel, J. Verhoef, K.P.M. van Kessel and J.A.G. van Strijp: Serum amyloid P component bound to Gram-negative bacteria prevents lipopolysaccharide-mediated classical pathway complement activation. *Infection and Immunity*, **68**, 1753-1759 (2000).
- Doumas, B.T., W.A. Watson and H.C. Biggs: Albumin standards and the measurement of serum albumin with bromocresol green. *Clin. Chem. Acta.*, **31**, 87-96 (1971).
- Hill, D.J. and A.F. Rowley: The thromboxane mimetic, U-46619, induces the aggregation of fish thrombocytes. *Brazilian J. Haematol. Oxford*, **92**, 200-211 (1996).
- Ingersoll, M.A., A.M. Platt, S. Potteaux and G.J. Randolph: Monocyte trafficking in acute and chronic inflammation. *Trends Immunology*, **32**, 470-477 (2011).
- Gurenlian, J.R.: Inflammation: The relationship between oral health and systemic disease. Dental Assistant (Chicago, Ill.: 1994), **78**, pp. 8-10 (2009).
- Jorgensen, J.B., G. Sharp, C.J. Secombes and B. Robertsen: Effect of a yeast cell wall glucan on the bactericidal activity of rainbow trout macrophages. *Fish and Shellfish Immunology*, **3**, 267-277 (1993).
- Kaushik, S.J., J. Breque and D. Blanc: Requirements for protein and essential amino acids and their utilization by Siberian sturgeon (*Acipenser baeri*). In: *Acipenser* (Ed.: P. Williot); CEMAGREF, Bordeaux, France, pp. 25-39 (1991).
- Kumar, V., K. Kumar, R.P. Raman, K.P. Prasad, S. Roy, S. Kumar and N. Kumar: Haematological and histopathological changes during carrageenan induced acute inflammatory response in *Labeo rohita* (Hamilton, 1822) fingerlings. *Int. J. Curr. Microbiol. App. Sci.*, **3**, 794-802 (2014).
- Kumar, V., S. Kumar, P.K. Pandey, R.P. Raman, K.P. Prasad, S. Roy, A. Kumar and K. Kumar: Growth and hemato-immunological response to dietary i-carrageenan in *Labeo rohita* (Hamilton, 1822) juveniles. *The Israeli J. Aquaculture-Bamidgeh.*, **66**, 971-981 (2014).
- Magnadottir, B., S.S. Audunsdottir, B.T. Bragason, B. Gisladdottir, Z.O. Jonsson and S. Gudmundsdottir: The acute phase response of Atlantic cod (*Gadus morhua*): Humoral and cellular response. *Fish Shellfish Immunol.*, **30**, 1124-1130 (2011).
- Margret, R., Jothibai, S. Kumaresan and S. Ravikumar: A preliminary study on the anti-inflammatory activity of methanol extract of *Ulva lactuca* in rat. *J. Environ. Biol.*, **30**, 899-902, (2009).
- Martins, M.L., D.M.Yb. Myiazaki, Mc. Tavares-Dias, Jd. Fenerick Jr, EME. Onaka, F.Rf. Bozzo, R.Yg. Fujimoto and F.R. Moraes: Characterization of the acute inflammatory response in the hybrid tambacu (*Piaractus mesopotamicus* male × *Colossoma macropomum* female) (Osteichthyes). *Braz. J. Biol.*, **69**, 957-962 (2009).
- Martins, M.L., F.R. Moraes, R.Y. Fujimoto, E.M. Onaka, F.R. Bozzo and J.R.E. Moraes: Carrageenan induced inflammation in *Piaractus mesopotamicus* (Osteichthyes: Characidae) cultured. *Brazil. B. Inst. Pesca.*, **32**, 31-39 (2006).
- Matushima, E.R., A.L. Filho, C.T. Kanamura and I.L. Sinhorini: Experimental chronic granulomatous inflammatory process in fish: A morphological, ultrastructural and immunocytochemical study. *Braz. J. Vet. Res. Anim. Sci.*, **43**, 152-158 (2006).
- McManus, Jfa.: Histological demonstration of mucin after periodic acid. *Nature*, **158**, 202-202 (1946).
- Metchnikoff, E.: Immunity in infective diseases. University Press (1905).
- Mohanty, B.R. and P.K. Sahoo: Immune responses and expression profiles of some immune-related genes in Indian major carp, *Labeo rohita* to *Edwardsiella tarda* infection. *Fish and Shellfish Immunol.*, **28**, 613-621 (2010).
- Pfaffl, M.W.: A new mathematical model for relative quantification in real time RT-PCR. *Nucl. Acids Res.*, **29**, 2001-2007 (2001).
- Moghazy, A.M., O.A. Adly, A.H. Abbas, T.A. Moati, O.S. Ali and B.A. Mohamed: Assessment of the relation between prealbumin serum level and healing of skin-grafted burn wounds. *Burns*, **36**, 495-500 (2010).
- Quade, M.J. and J.A. Roth: A rapid direct assay to measure degranulation of bovine neutrophils primary granules. *Vet Immunol. Immunopathol.*, **58**, 239-248 (1997).
- Ramos, F. and A.C. Smith: The C-reactive protein (CRP) test for the detection of early disease in Fishes. *Aquaculture*, **14**, 261-266 (1978).
- Reinhold, J.G.: Manual determination of serum total protein albumin and globulin fractions by Biuret method. In: Standard Method of Clinical Chemistry (Ed.: M. Reiner). Academic Press, New York, p. 88 (1953).
- Roy, S., V. Kumar, V. Kumar and B.K. Behera: Acute phase proteins and their potential role as an indicator for fish health and in diagnosis of fish diseases. *Prot. Pept. Lett.*, **24**, 78-89 (2017).
- Secombes, C.J.: Isolation of salmonid macrophage and analysis of their killing activity, In: Techniques in Fish Immunology (Eds: J.S.T.C., Stolen, D.P. Fletcher, B.S. Anderson and W.B. Van M Winkel). SoS Publication., New Jersey, pp. 137-152 (1990).
- Stasiack, A.S. and C.P. Bauman: Neutrophil activity as a potent indicator of concomitant analysis. *Fish Shellfish Immunol.*, **37**, 539-542 (1996).
- Su, J. D. Oanh, R.E. Lyons, L. Lecton, M.C.W. Van Hulton, S. Tan, L. Song, K.V. Rajendran and P.J. Walker: A key gene of the RNA interference pathway in the black tiger shrimp, *Penaeus monodon*: identification and functional characterization of Dicer-1. *Fish and Shellfish Immunol.*, **24**, 223-233 (2008).
- Weinreb, E.L.: Studies on the histology and histopathology of the rainbow trout, *Salmo gairdneri irideus*. I. Hematology: Under normal and experimental conditions of inflammation. *Zoologica*, **43**, 145-154 (1958).
- Wu, Y., S. Wang and X. Peng: Serum acute phase response (APR)-related proteome of loach to trauma. *Fish and Shellfish Immunol.*, **16**, 381-389 (2003).
- Zweifach, B.W., A.L. Nagler and L. Thcaas: The inflammatory process. *J. Exp. Med.*, **104**, 881-896 (2004).