

## Original Research Article

### Haematological and histopathological changes during carrageenan induced acute inflammatory response in *Labeorohita* (Hamilton, 1822) fingerlings

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#### ABSTRACT

Inflammation is a non-specific protective immune response and the study was designed to evaluate the acute inflammatory response induced by I-carrageenan in Rohu, *Labeorohita*. The carrageenan (0.5%) was intraperitoneally injected 50 µl/fish and the inflammatory response was studied at 3, 6, 12, 24, 48 and 96 hours post injection by observation of hematological parameters i.e total erythrocyte count (TEC), total leukocyte count (TLC), haemoglobin concentration (Hb) and tissue samples for histopathological changes. Carrageenan injection revealed typical inflammatory response at the site of inflammation and continued to increase till 96 hours post injection. There was significant decrease in the TEC and Hbconcentration, whereas the TLC were increased up to 48 hours and thereafter decreased. Carrageenan injected fish showed typical inflammatory sign at the site of injection with ultra-structural changes in the liver, kidney and muscle tissue. Alteration of hematological and histopathological changes during carrageenan injected fishes will help in better understanding of inflammatory response during infection process in the native fish.

#### Keywords

Hematological,  
Carrageenan,  
Acute  
inflammation,  
Histopathological

## Introduction

Rohu, *Labeorohita* (Hamilton, 1822), a major candidate species for aquaculture in India as well as in other south-east Asian countries (FAO, 2001) contributed more than 0.95 million tones production during 2006 (FAO, 2008). At present, information on immune status of the Indian major carps is rare (Sahoo *et al.*, 2005); this lack of knowledge is a major obstacle for establishment of effective

preventive measures against broad spectrum of infectious agents.

Inflammation is a general term used for body response towards any cellular injuries. Cell injury may occur due to trauma, genetic defects, physical and chemical agents, tissue necrosis, foreign bodies, immune reactions and infections (Zweifach *et al.*, 1965). The first

observations on inflammation in fishes were made by Metchinikoff (1905), who studied phagocytosis by injection of guinea pig erythrocytes into the visceral cavity of *Carassius auratus*. Prior to this, Mesnill (1895) recorded *Bacillus anthracis* phagocytised by mononuclear leucocytes of fish. In the haematological count, Ravikant (2003) described the anaemic condition in *Catla catla* due to the haemorrhage caused by mechanical injury on muscle tissues. The finding of Thomson and Fowler (1981) suggest that carrageenan has some haemolytic property in rats which results in decrease of TEC (total erythrocyte count) and haemoglobin content and also proliferation of WBC in rat response to carrageenan. Chadzinska *et al.*, (2000) observed increased number of total leukocytes after injection with thioglycollate in *Carassius auratus*. Ravikant (2003) also reported increase in TLC of the injured group of fishes as compared to control group. In the histopathological changes, Fontaine and Lightner (1974) observed extensive tissue destruction in the heart and abdominal muscle with inflammatory agent. Weinreb (1958) also described histological changes induced by turpentine in rainbow trout.

Carrageenan is a polysaccharides prepared by alkaline extraction and modification from red seaweeds (*Rhodophyceae*), mostly of the genus *Chondrus*, *Eucheuma*, *Gigartina*, *Iridaea* and mainly used for thickening, suspending and gelling in food products. Vinegar *et al.* (1987) has described sequential pathway for the inflammatory response in the rat by giving injection of Carrageenan. Injection of carrageenan in Nile tilapia, *Oreochromis niloticus* and pacu, *P. mesopotamicus*, induces the accumulation of thrombocytes followed by macrophages and granulocytes (Matushima and Mariano, 1996; Martins *et al.*, 2000; Martins *et al.*, 2006) and increased in total number of cells in the inflammatory exudates (Martins *et al.*, 2009).

Teleost fish exhibits inflammatory reactions which closely resemble those of mammals, but the mechanism of inflammation in fish are less understood than in mammals, while the leukocytes associated with inflammatory responses of fish has close morphological similarity to those of mammals.

However, it is not yet clear how comparable they are in function like there are clear analyses of acute inflammatory response in mammals by testing White blood cells, red blood cells and by observation of histological changes. Keeping this in consideration, the present experiment was designed to evaluate the inflammatory response in *Labeo rohita* fingerling and hypothesis was made that during acute inflammatory response there is change in hematological parameters and histology of different tissues.

## Materials and Methods

### Experimental animal

*L. rohita* (Hamilton, 1822), fingerlings of average length  $15 \pm 0.79$  cm and weighing  $19 \pm 0.95$  g, were procured from a Government fish farm at Khopoli, Maharashtra, India and were stocked in FRP (fiber reinforced plastic) circular tanks (1000 L) filled with adequately aerated freshwater. The fishes were acclimatized at ambient temperature ( $26-28^{\circ}\text{C}$ ) for 15 days with continuous aeration. They were fed twice daily with a diet (rice bran and mustard oil cake in the ratio of 1:1) @ 4% body weight at 9.00 hrs and 16.00 hrs respectively. The optimum physicochemical parameter of water: dissolved oxygen ( $6.88 \pm 0.56$  mg  $\text{l}^{-1}$ ), total dissolved solids ( $0.98 \pm 0.10$  g  $\text{l}^{-1}$ ), BOD ( $40.00 \pm 1.60$  mg  $\text{l}^{-1}$ ), COD ( $120.00 \pm 9.00$  mg  $\text{l}^{-1}$ ), ammonia ( $0.068 \pm 0.008$  mg  $\text{l}^{-1}$ ) and pH ( $7.14 \pm 0.77$ ) were maintained throughout the experiment period.

## **Experimental design**

The experiment was performed in 500L FRP tanks in the CIFE department wet laboratory. The fishes were equally and randomly divided into two groups (control, treatment) and each group was maintained in triplicate set containing 50 nos. of fishes, following a completely randomized design (CRD). The fish were anesthetized with clove oil (Merck, Germany) @ 4-5 ppm prior to their injection and the treatment group were injected intra-peritoneal (i/p) @ 50µl/fish with 0.5% of I-Carrageenan (Iota Carrageenan, type-II, SIGMA-ALDRICH, Life science, USA, P.Code-1001068408) dissolved in 100 ml of sterile saline solution (0.85%) and the control group were kept in tanks without injection. The experiment was conducted for 96 hours and the sampling for various haematological and histopathological parameters was carried out at 3, 6, 12, 24, 48 and 96 hours post injection. For each sampling 8 fishes were selected randomly from each tank and analyzed for various parameters.

## **Collection of blood for haematological Studies**

Blood from the fishes were drawn with the help of a sterilized 2 ml hypodermal syringe and 24 gauge needles directly from the caudal vein containing 2.7 % EDTA (Qualigens, India) as an anticoagulant. Before drawing blood, fishes were anaesthetized with clove oil. The haematological studies were conducted for total erythrocyte count (TEC), total leukocyte count (TLC) and haemoglobin concentration (Hb). The TEC and TLC were determined using Neuber's haemocytometer (Feinoptik, Germany) with RBC diluting fluid (Himedia, India) (RBC diluting fluid contains sodium citrate, formalin and distilled water) for TEC and WBC diluting fluid (Himedia, India) (WBC diluting fluid contains 0.025 gm

Gentian violet, 2 ml Glacial Acetic Acid & 100 ml Distilled water) for TLC. The Hb concentration was analyzed following the cyanomethemoglobin method (Snell and Marini 1988) using Drabkins fluid (Qualigens, India).

## **Collection of tissue for pathomorphological studies**

At necropsy, the site of injection and other organs were examined for haemorrhage, discoloration and other gross abnormalities. The collected tissue sample (liver, Kidney and muscle) were fixed in 10% neutral buffered formalin (NBF) for histopathology and processed by routine paraffin embedding technique. Five microns thick section was cut using microtome (Leica RM 2125 RT, Germany) and stained with haematoxylin and eosin. Pathological changes manifested in the tissue sections were observed and recorded using a light binocular microscope (Olympus CX-31, Japan).

## **Statistical analysis**

The data were statistically analysed by statistical package SPSS version 16 in which data were subjected to one-way ANOVA and Duncan's multiple range test (DMRT) was used to determine the significant differences between the means. Comparisons were made at 5% probability level.

## **Results and Discussion**

The present study revealed the alteration in haematological parameters with histopathological changes in the tissue of *L. rohita* injected by carrageenan. Clinically, the primary aim of the experiment was to delineate vascular as well as cellular change during inflammatory response. So, more emphasis is being given here to look into alterations in haematology and

histopathology with respect to external chemical agent. The findings of haematology and histopathology during acute inflammatory response have been reported first time for Indian major carps.

### Haematological parameters

There was significant decrease ( $P > 0.05$ ) in haemoglobin concentration (Hb) (fig 1) and total erythrocyte count (TEC) (fig 2) in carrageenan injected group in comparison to control group. The TEC and Hb content started decreasing after 3 hours and continued till 96 hours post injection. Total erythrocytes count (TEC) varies with species to species, usually ranges between  $1.05 \times 10^6/\text{mm}^3$  to  $3.0 \times 10^6/\text{mm}^3$  and generally affected by stress and environmental temperature. The total erythrocyte count and haemoglobin content showed significant decrease on 48 and 96 h of sampling in treatment group as compared to control. The result is in agreement with the finding suggests that carrageenan has property of inducing inflammation, which was reported in rat by Thomson and Fowler (1981). It also suggests that carrageenan possibly has some haemolytic property which results in decrease of TEC and haemoglobin. Ravikant (2003) in *Catlacatla* described that fish may undergo anaemic condition due to the haemorrhage caused by mechanical injury on muscle tissues.

The total leukocyte count (TLC) (fig 3) in treatment group significantly increased ( $p < 0.05$ ) after 3 hours and reached maximum level at 48 h post injection and remained high till the end of the study (96 h post injection). White blood cells (WBC) also known as immune cells, important in providing defence against immune-complexes (Albertset *al.*,2006). Change in TLC (Total leukocyte count) along with other immunological parameters can be

considered as an indicator of the health status of fish. The TLC increased from 3 hours post injection up to 48 hours and highest count were observed at 48 hours, afterwards decreased in the treatment group as compared to the control group. The similar observation was recorded by Chadzinskaet *al.* (2000) found that increased number of total leukocytes after injection with thioglycollate in the peritoneal cavity of *Carassius auratus* in relation to non-injected fish. Ravikant (2003) also reported that there is increase in TLC in the injected group of fishes as compared to control group.

**Table.1** The total blood count in *Labeorohita* post injection.

| Time period(hr.) | Treatment |                          |                          |
|------------------|-----------|--------------------------|--------------------------|
|                  |           | Control                  | Intra-peritoneal         |
| 3                | RBC       | 2.28 <sup>b</sup> ±0.015 | 2.15 <sup>a</sup> ±0.051 |
|                  | WBC       | 32.75 <sup>a</sup> ±0.19 | 32.83 <sup>b</sup> ±0.29 |
|                  | Hb        | 8.74 <sup>b</sup> ±0.069 | 8.48 <sup>a</sup> ±0.086 |
| 6                | RBC       | 2.22 <sup>b</sup> ±0.02  | 2.03 <sup>a</sup> ±0.015 |
|                  | WBC       | 31.61 <sup>a</sup> ±0.26 | 33.03 <sup>b</sup> ±0.35 |
|                  | Hb        | 8.82 <sup>b</sup> ±0.13  | 7.68 <sup>a</sup> ±0.11  |
| 12               | RBC       | 2.17 <sup>b</sup> ±0.08  | 1.94 <sup>a</sup> ±0.02  |
|                  | WBC       | 32.18 <sup>a</sup> ±0.44 | 33.47 <sup>b</sup> ±0.22 |
|                  | Hb        | 8.82 <sup>b</sup> ±0.09  | 7.17 <sup>a</sup> ±0.13  |
| 24               | RBC       | 2.24 <sup>b</sup> ±0.02  | 1.83 <sup>a</sup> ±0.02  |
|                  | WBC       | 32.70 <sup>a</sup> ±0.42 | 34.27 <sup>b</sup> ±0.43 |
|                  | Hb        | 8.80 <sup>b</sup> ±0.12  | 6.92 <sup>a</sup> ±0.05  |
| 48               | RBC       | 2.30 <sup>b</sup> ±0.01  | 1.77 <sup>a</sup> ±0.01  |
|                  | WBC       | 32.18 <sup>a</sup> ±0.25 | 39.46 <sup>b</sup> ±0.21 |
|                  | Hb        | 8.82 <sup>b</sup> ±0.12  | 4.76 <sup>a</sup> ±0.06  |
| 96               | RBC       | 2.26 <sup>b</sup> ±0.02  | 1.73 <sup>a</sup> ±0.01  |
|                  | WBC       | 32.29 <sup>a</sup> ±0.50 | 39.05 <sup>b</sup> ±0.20 |
|                  | Hb        | 8.80 <sup>b</sup> ±0.22  | 4.56 <sup>a</sup> ±0.13  |

\* Significant difference between control and treatment group at respective day ( $P < 0.05$ )  
 Total Erythrocyte Count (TEC)( $\times 10^6$  cells/ $\text{mm}^3$ ),  
 Haemoglobin (Hb) (mg/dl) and Total Leucocyte count (TLC) ( $\times 10^3$  cells/ $\text{mm}^3$ )

The similar observation was recorded by Chadzinskaet *al.* (2000) found that increased number of total leukocytes after injection

with thioglycollate in the peritoneal cavity of *Carassius auratus* in relation to non-injected fish. Ravikant (2003) also reported that there is increase in TLC in the injected group of fishes as compared to control group. Thomson and Fowler (1981) observed that carrageenan acts as a natural chemical substance capable of inducing production or proliferation of TLC in rat. Vinegar *et al.* (1987) has given a sequential 43 step pathway scheme for the inflammatory response in the rat by giving interdermal injection of Carrageenan. Injection of carrageenan into the swim bladder of Nile tilapia and pacu, *P. mesopotamicus*, induced the accumulation predominantly thrombocytes, macrophages and a small number of granulocytes (Matushima and Mariano, 1996; Martins *et al.*, 2000; Martins *et al.*, 2006). Carrageenan injected fish, showed increase in the total number of cells in the inflammatory exudates and with highest migration of

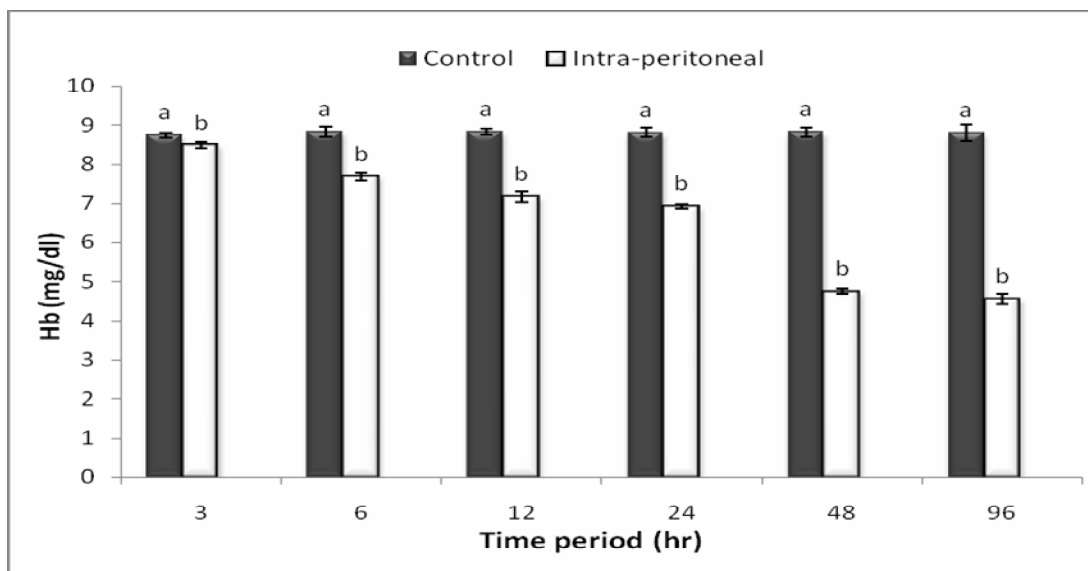
macrophage to the inflammatory site (Martins *et al.*, 2009).

### Pathomorphological studies

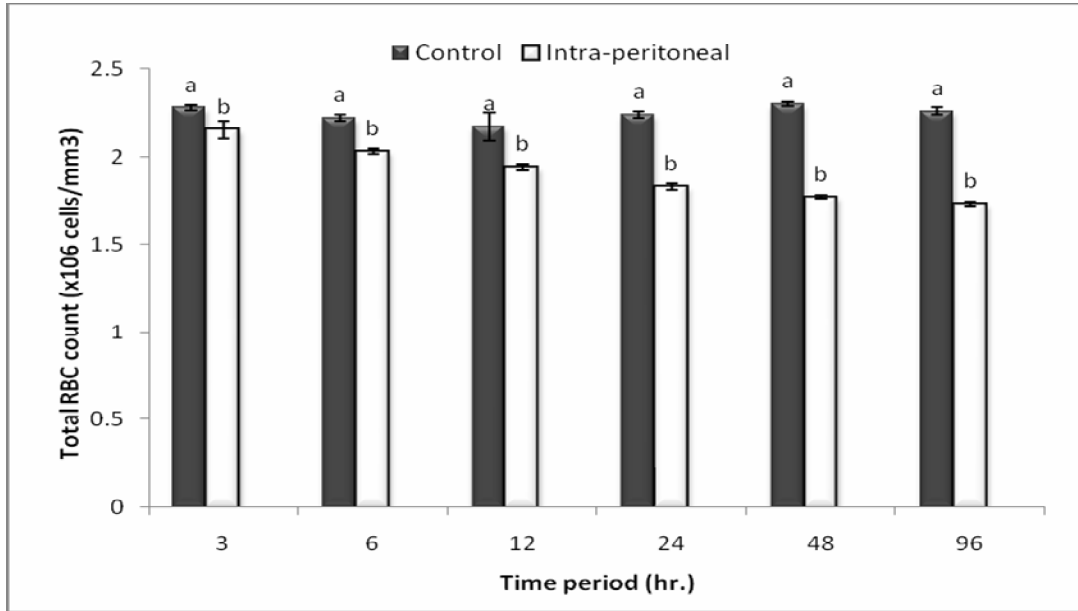
#### Gross pathology

Initial clinical sign in carrageenan injected fish appeared at 12 hr post injection. External sign in both the treatment group of fish on second day typically indicated lethargy and disinclination to move and loss of equilibrium. At necropsy, external examination of moribund fish revealed hyperemia at the ventral side and abdominal distension in carrageenan injected fishes. All sampled fish after 48 hours in carrageenan injected group showed accumulation of ascitic fluid (straw to pinkish colored) at the injection site, and petechial haemorrhage in the peritoneal cavity. Kidney and liver were pale, mottled and slightly swollen.

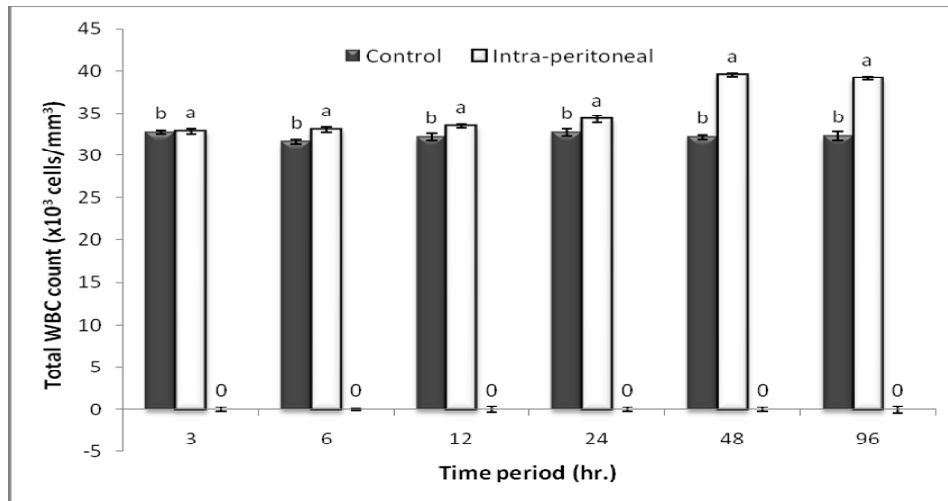
**Fig.1** Total Hb concentration with control and treatment groups in *L. rohita* during different sampling hours (values are mean ± SE). Mean values with different superscript within a column for a parameter is significantly different, ( $p < 0.05$ )



**Fig.2** Total TECwith control and treatment groups in *L. rohita* during different sampling hours (values are mean±SE). Mean values with different superscript with in a column for a parameter is significantly different, ( $p < 0.05$ )

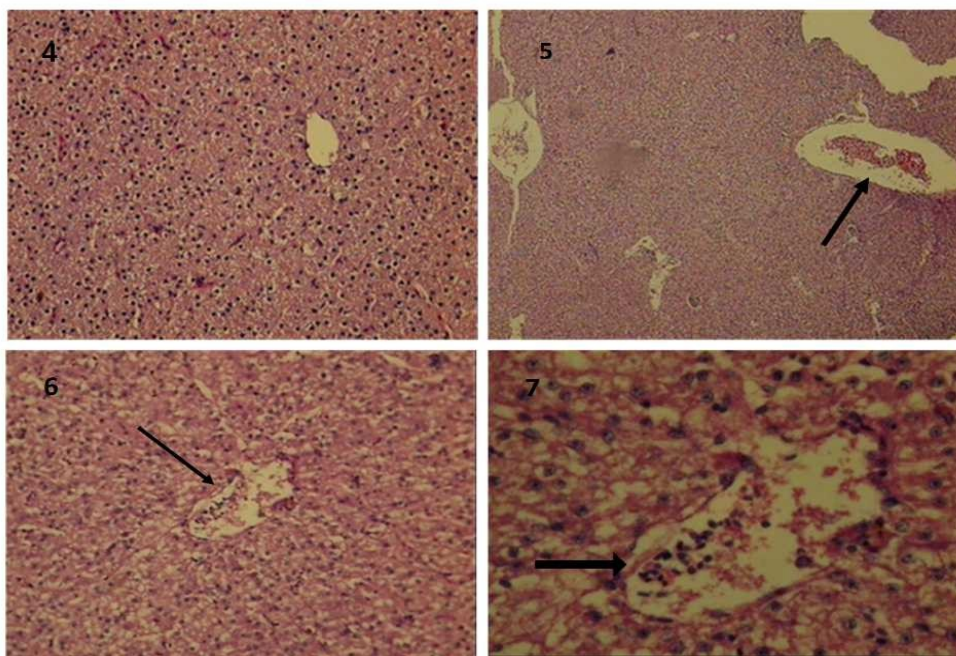


**Fig.3** Total TLCwith control and treatment groups in *L. rohita* during different sampling hours (values are mean±SE). Mean values with different superscript with in a column for a parameter is significantly different, ( $p < 0.05$ ).

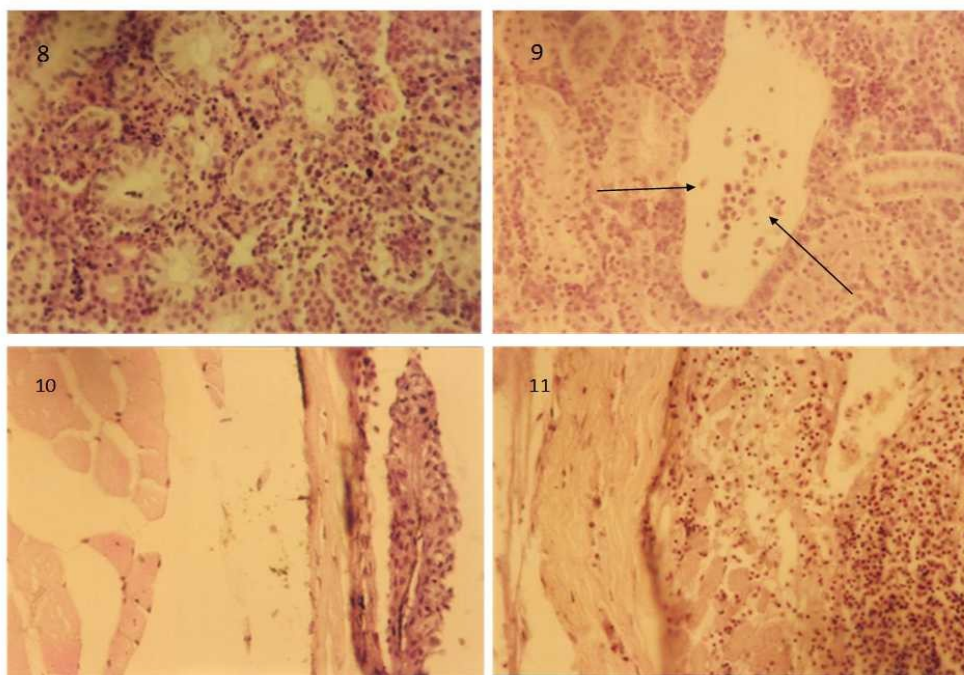




**Fig.4** Liver tissue (control group) - showing intact hepatocytes and appearing normal, H&E (40x), **Fig 5:** Liver tissue (48 hours post injection) showing dilated blood vessels engorged with blood cells, H&E (10x), **Fig 6:** Liver tissue (48 hours post injection) showing loss of nucleus in the hepatocyte and moderate necrosis near the margin of central vein, H&E (40x), **Fig 7:** liver tissue (48 hours post injection) showing marked necrosis near the margin of central vein in higher magnification, H&E (100x).



**Fig.8** Posterior kidney (24 hours post injection) showing accumulation of mononuclear cells in the interstitium, **Fig.9** Kidney (24 hours post injection) showing dilated blood vessels with aggregation of few polymorphs and macrophages (arrow), H&E (160X). **Fig10:** Muscle tissue (24 hours post injection) showing separation of epidermis from the muscle tissue with thickening of the epidermis, H&E (160X), **Fig11:** Muscle tissue (24 hours post injection) showing marked necrosis with leucocytic infiltration at the site of the infection, H&E, (160X).



## Histological changes

The control group of fishes showed normal histological detail of the liver, kidney and muscle throughout the experiment. In carrageenan injected group, the liver tissue (fig 4, 5, 6, 7) showed dilation of the arteries and vein in the portal triad with thickening of the bile duct epithelium second day post injection. The hepatocytes were moderately swollen with prominent margins and karyorrhexis.

On the fourth day after the injury, liver tissue showed micro-hemorrhagic spots. Larger area of hemorrhage was also observed in the parenchyma and pancreatic tissue. In kidney (fig 8, 9) carrageenan injected fish, second day post injection, brownish pigment resembling haemosiderosis was evident at the cortical region. Accumulation of mononuclear cell in the interstitium was also observed. On the fourth day after the injection, widespread haemorrhage could be seen in kidney tissue with accumulation of mononuclear cell in the interstitium. In the muscle tissue (fig 10, 11) carrageenan injected fish, second day after the injection, the myofibrils were separated from the epidermis by a wide gap. The histopathological study showed marked dilation of arteries and thickening of the bile duct which revealed that carrageenan may act as a vasodilator. This effect of vasodilation could easily be observed under histopathological examination characterized by focal to diffuse haemorrhages in the capillaries of liver tissues at some place and widespread haemorrhage in kidney. The liver tissue study revealed edematous hepatocytes with marked vacuolation at places and also moderate degenerative changes. The kidney tissue at the interstitium exhibited dilated blood vessels which contained

polymorphs and macrophages. This may be due to vasodilation in response to carrageenan. Similarly, the muscle tissue at the site of injection exhibited leucocytic infiltration followed by necrotic myositis clearly indicating the property of carrageenan as an indicator of inflammation. Similar results were observed by Fontaine and Lightner (1974) in *Penaeus setiferus*, who observed that extensive tissue destruction is distinguishable in the heart and the abdominal muscle even at 120 days post injection with inflammatory agent, i.e., turpentine. Weinreb (1958) also described histological changes induced by intraperitoneal injection with turpentine in rainbow trout, *Salmo gairdneri irideus*. Aleksandrovet al. (1986), in hamster where they observed considerably increased diameter of arterioles and venules in carrageenan treated animals and the dilation occurred on 3<sup>rd</sup> day and subsided by 5<sup>th</sup> day of treatment.

Interestingly up-regulation in TLC and down regulation of TEC and haemoglobin concentration along with ultra-structure changes in the liver, kidney and muscle during acute inflammatory response induced by carrageenan has been reported here for the first time in fish species. Thus, up-regulation of the TEC and down-regulation of TEC along with alteration in histology of liver, kidney and muscle studied here might be playing important role in the inflammatory process and shows a very close resemblance with the mammalian inflammatory response. Our results add to the understanding of the Indian major carp inflammatory process and provide a basic experimental model to study the activity of anti-inflammatory drugs and mediators of inflammation. The result of this study suggested that carrageenan act as a potent inflammation inducing agent in fishes. This study will



also help to find out anti-inflammatory drug that can be used in fishes in inflammatory condition.

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