Effect of parenteral endotoxin administration on the immuno-haematological responses of catfish, *Heteropneustes fossilis*

S.K. Nayak *, N. Jahan, S. Pattnaik

Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University, (Erstwhile North Orissa University), Mayurbhanj, Baripada, Odisha 757003, India

**ARTICLE INFO**

**Keywords:**
Endotoxin
Lipopolysaccharide
*Heteropneustes fossilis*
Immunity
Haematology

**ABSTRACT**

Endotoxin, the outer cell wall membrane lipopolysaccharide component of the Gram-negative bacteria is a factor responsible for a number of complications/disorders and plays important role in the associated with pathophysiological complications and pathogenesis of many diseases in animals. Unlike higher animals which are extremely sensitive to endotoxin, fish are found to be resistant to endotoxic shock and earlier studies though limited have demonstrated the patho-physiological, immuno-endocrinological and immuno-neurological effects of LPS/endotoxin in aquatic animals including fish. Herein in the present investigation, the effect of pure endotoxin on immuno-haematological parameters of stinging catfish, *Heteropneustes fossilis* ranging from 50-60 g was studied by intraperitoneally injecting 0.1, 0.05 and 0.01 mg endotoxin per fish. *H. fossilis* yearlings were found to resist the endotoxin concentration up to 0.1 mg without any mortality. While, no change in immune parameters was recorded in stinging catfish injected with low dose of endotoxin (0.01 mg), most of the immune parameters were found to be significantly elevated in catfish injected with 0.05 mg endotoxin. Different serum and immune parameters like protein, globulin, lysozyme, respiratory burst activity, myeloperoxidase activity, natural agglutination titre were found to be significantly high (*p* < 0.01) at a dose of 0.05 mg endotoxin per fish. On the contrary, most of these parameters were decreased at high dose *i.e.*, 0.1 mg endotoxin per fish, thereby indicating the immuno-suppressive effect of the endotoxin. The findings of the modulation of innate immunity also corroborated with the results of *Aeromonas hydrophila* pathogen challenge study with highest percent of mortality in group injected with 0.1 mg endotoxin per fish and least percentage in group injected with 0.05 mg endotoxin per fish.

1. Introduction

Endotoxin is the highly conserved outer cell wall membrane lipopolysaccharide (LPS) component of Gram-negative bacteria which is involved in pathogenesis of many diseases. Endotoxin (synonym ‘LPS’) being an amphiphilic cell surface antigen and common to all Gram-negative bacteria comprised of components that confer both immunogenic and endotoxic properties to the molecule [1–3]. While, higher vertebrates often experience endotoxic shock during Gram negative bacterial infections, various patho-physiological complications including cytotoxic, hypotension, thrombocytopenia, hypoferremia, sepsis, liver disease, vascular disease, etc are often induced by endotoxin in sensitive hosts [4–12]. Among various bacterial cell surface structures (fimbriae, flagella, capsule or LPS/endotoxin), endotoxin/LPS is believed to one of the most potent stimulators of innate immunity [13]. It can execute immuno-stimulatory or suppressive effect on host [14] and its profound effect on immune components like T cells, B cells, macrophages etc and production of antibody, cytokines etc are well demonstrated [15–16].

Higher animals are extremely sensitive to endotoxin even at a very low concentration but lower vertebrates like frog and fish are found to be resistant to endotoxic shock [17]. Although fish are often reported to be resistant to endotoxin, the constant exposure to Gram-negative bacteria and their endotoxin can affect the health status of fish. It is already demonstrated that the LPS is responsible for the pathogenicity of several Gram-ve bacterial pathogens in several aquatic animals including fish [18]. Further, endotoxin/LPS in pure or crude form could induce stress leading to a wide variety of metabolic effects, patho-physiological, immunological, immuno-endocrinological and neuro-immunological effects in aquatic vertebrates including fish [7–9,19,20]. However, literatures in this regard are very limited in different fish species and many times experimental data are conflicting and divergent [6,9,19–22].

---

* Corresponding author.
E-mail address: sukanantanayak@rediffmail.com (S.K. Nayak).

https://doi.org/10.1016/j.fisirep.2021.100022
Received 9 July 2021; Received in revised form 10 August 2021; Accepted 27 August 2021
Available online 29 August 2021
2667-0119/© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license
Therefore, the present study investigates the effects of endotoxin at varying concentration on the hematology, innate immunity and protection of stinging catfish, *Heteropneustes fossilis* a catfish species of considerable economical importance.

### 2. Materials and methods

#### 2.1 Fish

Stinging catfish, (*Heteropneustes fossilis*) of weight ranging from 50-60 g were used in the present investigation.

#### 2.2 Endotoxin

Pure *E. coli* endotoxin (*E. coli* 055:B5 endotoxin) procured from Cambrex, (Cambrex Bioscience Walkersville Inc., Walksville, USA), was used in the present investigation.

#### 2.3 Experimental design

*H. fossilis* yearlings were acclimatized under laboratory conditions 15 days prior to the start of the experiment. Ten yearlings were maintained per tank (1000 L) and fed with artificial carp diet with daily two – third water exchange. Ten catfish per group in duplicate set were intra-peritoneally injected with 0.1ml of endotoxin in such a manner that individual fish in a particular group received 0.1 mg endotoxin, 0.05 mg endotoxin and 0.01 mg endotoxin while another group injected with same amount of phosphate buffered saline (PBS, pH 7.2) was kept as control group. Blood was collected from *H. fossilis* from all groups at 7th and 15th days post injection (dpi) to assess various immunohaematological parameters. During the entire study period the alkalinity (75–80 mg mL⁻¹), pH (7.4 - 7.8) and temperature (27°C to 28°C) of the rearing tanks were found to be within the permissible limit.

#### 2.4 Haematological parameters

##### 2.4.1 Collection of blood

Blood from *H. fossilis* in different groups was collected aseptically by caudal fin after 7th and 15th dpi. One part of the blood was collected and mixed with anticoagulant (heparin solution) to evaluate different haematological parameters like total erythrocytes count, total leucocytes count and haemoglobin content were done as per the method of Blaxhall and Daisley [23]. Similarly another part was allowed to clot at room temperature for 45 min. After that it was centrifuged at 2000 × g for 5 min. The supernatant was collected for evaluating different serum parameters.

##### 2.4.2 Differential leucocytes count (DLC)

The slides were stained with combination of Wright’s and Giemsa stains. At first, modified Wright’s stain was applied into the smears and kept for 10 min. It was followed by dilution (1:10 with distilled water, pH 7.4–7.2) and kept for another 20 min. the smear was then stained with diluted Giemsa (1:10 in distilled water) for 90 min. finally, the slides were washed, air dried and observed under the microscope (×1000). Different types of leucocytes were counted on randomly selected fields from the smear.

##### 2.4.3 Total erythrocytes count (TEC)

Blood was drawn up to 0.5 marks in RBC diluting pipette of haemocytometer (Hi media, India). The pipette was immediately filled up to the 101 mark with RBC diluting fluid. The pipette was shaken for 30 s and from it few drops of diluted blood were expelled. Then tip of the pipette was touched to Neubauer’s slide and cover slip junction. Diluted blood was drawn inside automatically by capillary action and counting was done under 10 × objective lens of microscope. The Neubauer’s slide was divided into ruled areas of 1 sq. mm with the center 1 sq. mm divided into 25 groups of 16 small squares each. The cells within the boundaries of five of these small squares (80 smallest squares) were counted. The total erythrocyte count was done using the following formula

\[
\text{TEC} = \text{total number of cells in five small squares} \times 10,000 / \text{cu. mm of blood}
\]

##### 2.4.4 Total leucocytes count (TLC)

The blood was sucked up to 0.5 mark and diluted up to 11 mark with diluting fluid (0.1 N HCl containing 1% Giemsa) in the pipette meant for WBC counting. The counting was done in four big squares at the four corners of the Neubauer’s slide each of which contain 16 small squares.

##### 2.4.5 Hemoglobin content

Total haemoglobin percentage was calculated as per the method of Blaxhall and Daisley [23]. blood was drawn up to 20 mm mark of pipette of haemoglobinometer and then put inside the calibrated test tube prefilled up to 10 mm mark with 0.1N HCL. the samples were gently mixed with a glass rod for 3–5 min for lysis. To it, 0.1 N HCL was gradually added and diluted till the colour matched with the side tubes and the results as obtained from the graduated tube directly were expressed in gram percentage of blood.

#### 2.5 Serum parameters study

##### 2.5.1 Total serum protein, albumin and globulin content

The serum protein content of various groups was estimated as per the standard method of Bradford [24]. Briefly, 0.2 ml of serum from individual fish of various experimental groups was separately added to 2.0 ml commissa blue-g-reactent (1% blue g. 5% ethanal and 10% phosphoric acid) and the absorbance was read at 550 nm. The protein content was calculated by regression analysis with bovine serum albumin as standard. The albumin content was estimated spectrophotometrically using standard kits (Glaxo, India). The globulin content was estimated as per the following method. 50 µl saturated ammonium sulphate solution was added drop wise to 50 µl serum followed by vortexing. Centrifugation was done at 10,000 × g for 5 min. Then, 20 µl of this sample was dissolved with 80 µl carbonate-bicarbonate buffer (pH 9.3) and the globulin content was determined by estimating the protein content as done above. Finally the albumin:globulin (A:G) ratio was calculated.

##### 2.5.2 Serum enzymatic activities

Different serum enzymatic activities such as Alkaline phosphatase, Serum glutamate pyruvate transaminase (SGPT) and Serum glutamate oxaloacetate transaminase (SGOT) were estimated as per standard enzyme estimation method by using commercial kit [25].

##### 2.5.3 Myeloperoxidase activity

The myeloperoxidase activity was studies as per the method of Quade and Roth [26]. Briefly, 15 µl of serum was diluted in 135 µl of Hank’s balanced salt solution (Ca²⁺ and Mg²⁺ free) and to it 50 µl of 20 mM 3, 3'-, 5'-, 5'- tetra methyl benzidine and 5 mM H₂O₂ were added. The reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid and the optical density (OD) was read at 450 nm in a spectrophotometer.

##### 2.5.4 Lysozyme activity

A turbidimetric assay utilizing lyophilized *Micrococcus lysodeikticus* (Sigma, USA) was done to determine lysozyme activity in serum as described by Shankaran and Shanto [27] and Studnicka et al. [28] with slight modifications. *M. lysodeikticus* at a concentration of 0.2 mg ml⁻¹ (in 0.02 M sodium citrate buffer) was added to serum samples at 1:10 ratio. Immediately after adding *M. lysodeikticus*, initial OD was taken at 450 nm. After incubating for 1 h at 240°C, OD was taken. Lysozyme activity was expressed as units/ml where one unit is defined as the decrease in absorbance of 0.001 min⁻¹.
2.5.5. Respiratory burst assay

The respiratory burst activity was measured by the reduction of NBT by intracellular super oxide radicals [29]. Briefly, 100 µl of haemoglobin blood from fish of each group was mixed in 100 µl of 0.2% NBT (Sigma, USA) solution for 30 min at 250C. After incubation, 50 µl from the above mixture was added with 1ml of N.N., Diethymethyl formamid (Qualigens, India) and then centrifuged at 3 000 × g for 5 min. The optical density of the supernatant was measured at 540 nm in a spectrophotometer.

2.5.6. Bacterial agglutination activity

The natural bacterial agglutinating activity of the sera samples of all the groups was studied in ‘U’ shaped microtitre plates. Two-fold serial dilution of 50 µl serum of fish was made with equal volume of PBS (pH 7.2) in each well, to plates were incubated overnight at room temperature. The titre was calculated as the reciprocal of the highest dilution of serum complete agglutination of the bacterial cells.

2.5.7. Hemagglutination activity

The haemagglutination activity of serum samples from various endotoxin injected and control groups was carried out using a standardized method [30]. This assay was done in ‘U’ shaped microtitre plates by serial two-fold dilution of 50 µl serum (inactivated at 45° C for 30 min) with PBC (pH 7.2). Then 50 µl of freshly prepared 1 % New Zealand white rabbit RBC suspension was added to each well. The plates were kept at room temperature (28–300C) for 2 h or over night at 4° C, in case agglutination was not visible within 2 h. The titre was calculated as the reciprocal of the highest dilution of serum showing complete agglutination of RBC.

2.6. Challenge study

After one week of final sampling, fishes from all the experimental groups were challenged with virulent Aeromonas hydrophila pathogen to find out the mortality pattern. Approximately, 0.1 ml of overnight grown A. hydrophila @ 10^6 CFU ml^-1 (LD50 dose) was intra-peritoneally injected to all the endotoxin injected and control fishes. After challenge the mortality was recorded up to 10 days post challenge to find out the percentage of mortality.

2.7. Statistical analysis

The statistical analysis system (SAS) software (version 6.12) was used to analyse all the data. One-way analysis of variance (ANOVA) followed by DMRT was done to compare the variations in various serum and immune parameters at significance level of difference at 0.01 % level in different endotoxin injected groups [31].

3. Results

No mortality or abnormality could be recorded in any of the endotoxin injected groups before the pathogen challenge study. H. fossilis yearlings were found to resist the endotoxin use at 0.1 mg endotoxin per fish. Fish were found to intake feed actively during feeding and no sign and symptoms or abnormalities of disease/injury during the experiment.

3.1. Haematological parameters

The RBC content (× 10^6 cell ml^-1) was lowest in group injected with 0.1 mg endotoxin at 7th dpi. However, the RBC counts didn’t vary significantly in any of the groups at both the sampling period. On the other hand, the WBC content (× 10^3 cell ml^-1) was significantly lowered (p < 0.01) in group injected with 0.1 mg endotoxin at both 7th and 15th dpi while the count was highest in group injected with 0.05 mg endotoxin. No variation in the count could be recorded in 0.01 mg endotoxin injected and control groups. Similarly, no significant variation (p > 0.01) in the haemoglobin content could be recorded in any of the experimental groups and the content varied from 9.3 to 10.0 gm percentage in all groups (Table 1).

Similarly, the differential leucocytes count also varied among the groups. The lymphocytes percentage was significantly lowest (p < 0.01) in group injected with 0.1 mg endotoxin irrespective of sampling period. Conversely, the percentage of lymphocytes, monocytes and neutrophils were significantly higher (p < 0.01) in 0.05 mg endotoxin injected group as compared to other groups (Table 2). The percentage of monocytes and neutrophils did not vary significantly (p > 0.01) among the 0.01 mg endotoxin injected and control groups. Similarly, the percentage of neutrophils was least (p < 0.01) in group injected with 0.1 mg endotoxin irrespective of sampling periods from the other groups.

3.2. Serum and Immunological parameters

3.2.1. Protein, Albumin and Globulin

The effect of endotoxin on different blood serum and immune parameters of H. fossilis injected with endotoxin is presented in Table 3. Among different serum parameters, serum protein content was least recorded in control group at both the sampling period. No significant difference (p > 0.01) among the serum protein content could be recorded in control, 0.1 mg and 0.01 mg endotoxin injected groups at 7th dpi. On the other hand, serum protein content was significantly high (p < 0.01) in group injected with 0.05 mg endotoxin as compared to that of other groups. The mean (± SD) serum protein content of fishes of this group was 3.46 (± 0.2) mg ml^-1 and 4.29 (±0.19) mg ml^-1 at 7th and 15th dpi, respectively.

The albumin content was least (1.32 ± 0.18 mg ml^-1) in control group while it was highest (1.72 ± 0.18 mg ml^-1) in 0.05 mg endotoxin injected group at 7th dpi. However, the albumin content did not vary significantly (p > 0.01) in all endotoxin injected groups irrespective of its concentration at both 7th and 15th dpi.

The globulin content was significantly low (1.27 ± 0.03 mg ml^-1) in 0.1 mg endotoxin injected group at 7th dpi but found to be increased (2.07 ± 0.08 mg ml^-1) at 15th dpi. Significantly high (p < 0.01) globulin level was recorded in 0.05 mg endotoxin injected group at both sampling periods. The mean (±SD) globulin content of this group was 1.75 (± 0.19) mg ml^-1 and 2.47 (± 0.19) mg ml^-1 at 7th and 15th dpi, respectively. No significant difference (p > 0.01) among the globulin level in 0.01 mg endotoxin injected and control groups could be recorded. Similarly, no significant difference (p > 0.01) among the A:G ratio could be recorded among all groups except 0.05 mg endotoxin injected group (Table 3).

3.2.2. ALP level

The ALP level was found to increase in all the endotoxin injected groups but significant increased (p < 0.01) in 0.1 mg endotoxin injected group at 7th dpi and maintained at a high level up to 15th dpi. The ALP content of fishes in this group was 0.49 (± 0.04) U ml^-1 and 0.38 (± 0.06) U ml^-1 serum at 7th and 15th dpi, respectively. However, the ALP level did not vary significantly (p > 0.01) in other groups (Fig. 1a).

3.2.3. SGOT and SGPT level

The endotoxin didn’t affect the SGOT level in any of the injected groups and no significant difference (p > 0.01) in the SGOT level was found to in any of the groups irrespective of sampling periods. Similarly, no difference in the SGPT level irrespective of sampling periods was found among the experimental groups except in 0.1 mg endotoxin injected group (Fig. 1b). The SGPT level was significantly high (p < 0.01) with 0.18 (± 0.006) U ml^-1 and 0.19 (± 0.005) U ml^-1 serum in this group at 7th and 15th dpi, respectively (Fig. 1c).

3.2.4. Myeloperoxidase activity

The myeloperoxidase activity was significantly high (p < 0.01) in
Fish and Shellfish Immunology Reports 2 (2021) 100022

Table 1
The mean (± SD) of total erythrocyte count (TEC), total leucocytes count (TLC) and hemoglobin content in *H. fossilis* injected with various concentrations of endotoxin at 7th and 15th days post injection (dpi).

| Parameters            | 0.01 mg  | 0.01 mg  | 0.05 mg  | 0.05 mg  | 0.1 mg  | 0.1 mg  | Control |
|-----------------------|-----------|-----------|-----------|-----------|---------|---------|---------|
|                       | 7thdpi    | 15thdpi   | 7thdpi    | 15thdpi   | 7thdpi  | 15thdpi | 7thdpi  |
| TEC (× 10⁸ cells/ml)  | ±         | ±         | ±         | ±         | ±       | ±       | ±       |
| TLC (× 10⁹ cells/ml)  | ±         | ±         | ±         | ±         | ±       | ±       | ±       |
| Haemoglobin (gm %)    | ±         | ±         | 0.24 b    | ±         | 0.31 a  | ±         | ±       |

*Superscripts indicate significance difference among different endotoxin injected groups with respect to specific sampling period.

Table 2
The mean (± SD) of differential leucocyte counts of *H. fossilis* injected with different concentrations of endotoxin at 7th and 15th days post injection (dpi).

| Types of cells | 0.1 mg  | 0.05 mg  | 0.01 mg  | Control |
|----------------|-----------|-----------|-----------|---------|
|               | 7thdpi    | 7thdpi    | 7thdpi    | 7thdpi  |
| Lymphocytes (%) | ±         | ±         | ±         | ±       |
| Monocyte (%)   | ±         | ±         | ±         | ±       |
| Neutrophil (%) | ±         | ±         | ±         | ±       |

*Superscripts indicate significance difference among different endotoxin injected groups with respect to specific sampling period.

Table 3
Effect of different endotoxin concentrations on various blood serum parameters of *H. fossilis* at 7th and 15th days post injection (dpi).

| Groups   | Protein (mg/ml) | Albumin (mg/ml) | Globulin (mg/ml) | A/G ratio |
|----------|-----------------|-----------------|------------------|-----------|
|   0.1 mg | 3.07 ± 0.41 b   | 1.62 ± 0.15     | 1.78 ± 0.24      | 0.46 b    |
|   0.05 mg| 3.46 ± 0.39 a   | 1.28 ± 0.18     | 1.82 ± 0.15      | 0.16 ± 0.1 |
|   0.01 mg| 3.22 ± 0.27 b   | 1.64 ± 0.19     | 1.94 ± 0.16      | 0.11 b    |
| Control  | 2.93 ± 0.29 b   | 1.32 ± 0.18     | 1.58 ± 0.43      | 0.15 b    |

*Superscripts indicate significance difference in the mean (± SD) values of individual serum parameters among different endotoxin injected groups with respect to specific sampling period.

0.05 mg endotoxin injected group at both sampling period. The mean (±SD) OD value of 1.32 (± 0.3) and 1.75 (± 0.19) was recorded at 7th and 15th dpi, respectively. On the contrary, no significant difference in the mean (±SD) OD value could be recorded in other groups at 15th dpi (Fig. 2a).

3.2.5. Lysozyme level
No significant difference (p > 0.01) in the serum lysozyme level in control, 0.1 mg and 0.01 mg endotoxin injected groups could be recorded at 7th and 15th dpi except in 0.1 mg endotoxin, the content was significantly low at 15th dpi. However in group injected with 0.05 mg endotoxin, the serum lysozyme level with mean (±SD) value of 160 (± 6.0) and 176 (± 5.0) units per ml serum was found to be significantly high (p < 0.01) from that of other groups at both 7th and 15th dpi, respectively (Fig. 2b).

3.2.6. Respiratory burst activity
The respiratory burst activity as measured by NBT reduction assay was significantly high (p < 0.01) in group injected with 0.05 mg endotoxin with mean (±SD) OD values of 0.362 (± 0.03) and 0.382 (± 0.026) at 7th and 15th dpi, respectively. On the other hand, the mean (±SD) OD value was least (0.195 ± 0.02) in group injected with 0.1 mg endotoxin at 7th dpi but the level was increased at 15th dpi. No significant difference (p > 0.01) in the bacterial agglutination titre could be recorded in 0.05 mg endotoxin injected and control groups irrespective of sampling periods (Fig. 2c).

3.2.7. Bacterial agglutination titre
No significant difference (p > 0.01) in the bacterial agglutination titre could be recorded among groups injected with 0.1 mg, 0.01 mg endotoxin as well as control group at 7th and 15th dpi. On the contrary, the agglutination titre of the 0.05 mg endotoxin injected group was significantly high (p < 0.01) at both the sampling periods (Table 4).

3.2.8. Haemaglutination titre
The haemaglutination titre was higher in all endotoxin injected groups but not at significant level (p > 0.01) except in group injected with 0.05 mg endotoxin. Significantly high haemagglutinating activity with a titre value (Log₂) of 4.3 (± 0.57) was recorded in group injected with 0.05 mg endotoxin at 15th dpi (Table 4).
3.3. Challenge study

On challenging with virulent *A. hydrophila* after one week of 2nd sampling, highest percentage of mortality (70%) was recorded in 0.1 mg endotoxin injected group followed by 60% mortality in both 0.01 mg endotoxin injected and control groups. However, significantly least percentage of mortality (40%) was recorded in 0.05 mg endotoxin injected group (Fig. 3).

4. Discussion

Endotoxin, the major virulence factor of many Gram-ve bacteria, is responsible for the clinical manifestations of diseases in host. It can induce innumerable biological effects ranging from simple response to intense reactions which may even lead to death in several animals including humans. Higher vertebrates are highly sensitive to endotoxin even at very low concentration. Unlike higher vertebrates, lower vertebrates like fish and frogs are found to resist endotoxic shock [17]. Herein the effect of varying doses of endotoxin on certain blood and serum biochemical and immunological responses of stinging catfish *H. fossilis* was studied.

Herein in this study, *H. fossilis* yearlings were found to resist the toxicity of endotoxin up to the dose of 0.1 mg endotoxin per fish. Earlier, researchers have demonstrated the resistance pattern of different fish species towards LPS/endotoxin [7,21]. While, Wedemeyer et al. [7] demonstrated the resistivity of coho salmon, *Oncorhynchus kisutch* and rainbow trout, *Oncorhynchus mykiss* towards *Escherichia coli* and *Aeromonas salmonicida* endotoxin up to 80 mg per ml, Nayak et al. [21] found Indian major carp, rohu, *Labeo rohita* to be resistant to bacterial endotoxin up to 20 EU per fish. The resistance of *H. fossilis* to endotoxin could be attributed to its preference for living conditions in very dirty and muddy areas leading to resistance either due to routine exposure to aquatic environment where they are intimately associated with Gram-ve bacteria or serum factors that conferred the sensitivity to toxin in mammals [32].

The patho-physiological effects in a host in response to endotoxins/LPS are predominantly dose-dependent. It is believed that with increased dose, the peak effect becomes more pronounced, and the duration of the effects prolonged [33]. In this present investigation, the lower dose of endotoxin (i.e., 0.01 mg) failed to modulate serum biochemical and immunological parameters of *H. fossilis*. The level of various immune parameters were not changed significantly and were either at par with control group or after an initial enhancement at 7th dpi, decreased to normal level as that of control at 15th dpi. However, at 0.05 mg of endotoxin concentration, the responses enhanced significantly but at higher dose (0.1 mg endotoxin) detrimental effects were

![Fig. 1. (a–c). Effect of different endotoxin concentrations on various serum enzyme parameters of *Heteropneustes fossilis* at 7th and 15th days post injection (dpi) a) Alkaline phosphatase activity; b) SGOT level; c) SGPT level *: superscripts indicate significant difference in the mean (± SD) values of individual enzyme content at P < 0.01 among different endotoxin injected and control groups at specific sampling period.](image-url)
recorded in catfish.

Literatures on the effects of LPS/endotoxin on fish serum biochemical parameters are limited and often contradictory. While, Selvaraj et al. [34] recorded decreased protein and globulin level by injecting crude A. hydrophila LPS to common carp, Cyprinus carpio, Nayak et al. [21] demonstrated enhanced protein and globulin level by injecting pure E. coli endotoxin to L. rohita. On the contrary, no significant difference among serum parameters like total protein, albumin and globulin content in any of the endotoxin injected groups could be recorded. Such differences could be attributed to the fish species used as well as dose and source of LPS since different bacteria produce different morphologically heterogeneous LPS molecules.

The effect of toxicant on serum enzymatic activity is one of the most important biochemical parameters, which is affected under stress [35]. Like higher vertebrates, the blood chemistry parameters varied in the response to stress with anemia, polycythemia, leucopenia or leucocytosis depending upon the stress nature [36]. In this study endotoxin especially at high dose act as a potential stress or with significant reduction in TLC, TEC, DLC (lymphocytes, neutrophils) levels especially at 7th dpi. Similarly, it was demonstrated that various serum enzymes increase in the fish in response to stress [37–42]. Herein in this study, various serum enzymatic parameters like transamines and ALP values enhanced significantly in response to high dose of endotoxin (0.1 mg endotoxin per fish) in H. fossilis. But these parameters didn’t vary significantly at lower dose (0.01 mg endotoxin per fish). However, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) which are involved in transamination reactions in living system, didn’t vary significantly in endotoxin injected catfish, H. fossilis.

Table 4
Agglutinating and haemagglutinating activity of serum of H. fossilis injected with different concentrations of endotoxin at 7th and 15th days post injection (dpi).

| Groups     | Agglutination titre (Log_{2}) | Haemagglutination titre (Log_{2}) |
|------------|-------------------------------|-----------------------------------|
|            | 7th dpi | 15th dpi | 7th dpi | 15th dpi |
| 0.1 mg     | 2.8±1.1 | 2.3±1.0  | 3.3±0.75 | 2.0±0.0  |
| 0.05 mg    | 3.6±0.75* | 4.0±0.5* | 3.3±0.75 | 4.3±0.57*|
| 0.01 mg    | 2.6±0.57  | 2.6±0.57  | 2.0±0.0  | 2.0±0.0  |
| Control    | 2.6±0.57  | 2.0±0.0  | 1.6±0.5  | 1.6±0.5  |

*Superscripts indicate significance difference in the mean (± SD) titre values (Log_{2}) among the different endotoxin injected groups with respect to specific sampling period.

![Fig. 2. (a–c). Effect of different endotoxin concentrations on various non-specific immune parameters of Heteropneustes fossilis at 7th and 15th days post injection (dpi); (a) Respiratory burst activity; (b) Myeloperoxidase activity; (c) Lysozyme content.](image-url)
Fig. 3. The mortality percentage of *Heteropneustes fossilis* injected with various doses of endotoxin challenged after 15th days post injection with virulent *Aeromonas hydrophila* @ $10^6$ CFU ml$^{-1}$

*: Superscript indicates significance difference (p $\leq 0.01$) in the mortality percentage from other groups.

rainbow trout, *Oncorhynchus mykiss* [46,47], Indian major carp, rohu, *L. rohita* [21].

Similarly, myeloperoxidase, a hemoprotein stored in primary azurophilic granules of neutrophils and secreted during activation of neutrophils, plays an important role in the defense of the organism. In this study, the significant rise in myeloperoxidase activity at 0.05 mg endotoxin per fish injected group showed the activation of neutrophils by endotoxin. Earlier Nayak et al. [21] also demonstrated that the respiratory burst activity and myeloperoxidase activity in *L. rohita* injected endotoxin to increase significantly at dose dependent manner.

Among other non-specific immune parameters, endotoxin/LPS are also reported to induce lysozyme expression leading to increase its concentration in the serum and mucus of animals [48]. Lysozyme which plays an important role in piscine protective mechanism [49], was found to be unchanged at lower dose followed by increase and then decreased at higher dose. Significantly high lysozyme level was recorded in fish injected with 0.05 mg endotoxin. Earlier findings also indicated the stimulation of plasma lysozyme activity by LPS in fish like Atlantic salmon (*Salmo salar*), Indian major carp, *L. rohita* [21,48].

Likewise, the agglutination titre in irrespective of endotoxin injected groups indicated a possible enhancement of natural agglutinin level in serum of the treated fish due to leucocyte proliferation and differentiation. Furthermore, hemagglutination activity is believed to be an important biological function of LPS [50] and this is also apparent from the findings of the present study i.e., irrespective of doses agglutinating activity of *H. fossilis* serum increased in responses to endotoxin.

Endotoxin/LPS can activate the immune system in different eukaryotic species [51] and also responsible for production of cytokines, Pro-inflammatory cytokines and inflammatory effector substances such as nitric oxide [9,52,53]. In fish, it often acts a potent stimulator of the cellular immunity and protective antigen to several fish pathogens [54]. LPS have shown a tendency towards high distribution in haematopoetic organs like head-kidney and spleen [55] and dietary supplementation or bath immersion of LPS/endotoxin can effectively stimulate piscine immunity [10,21,43].

Nonetheless, the disease protective capacity of endotoxin/LPS alone and/or in combination with other agents was also earlier reported in several fish species [21,34,56–62]. In this study, the variations /changes of non-specific immune parameters among various endotoxin injected groups were also reciprocated in the challenge study. On challenge with virulent *A. hydrophila*, significantly lowest mortality (40%) was recorded in the group injected with 0.05 mg endotoxin per fish but it was 70% in 0.1 mg endotoxin injected group. The survivability of fish appears to be dependent upon the concentration of endotoxin/LPS. The low concentration of endotoxin/LPS is effective in inducing higher protection [46,54,62,63]. Earlier, Nya and Austin [46] demonstrated high relative percent survivability of rainbow trout fed with low dose of LPS and less survivability in high LPS fed fish against *A. hydrophila*. Similar type of trend was also observed in *Labeo bata* fed with 50,100 and 150 mg *E. coli* LPS kg$^{-1}$ feed against *Edwardsiella tarda* [63].

5. Conclusions

The research on bacterial endotoxin mostly focused to understand in detail the cellular and molecular mechanism(s) by which this unique highly conserved microbial component contributes to the changes in the immune response to Gram-negative bacterial infections. Herein we have demonstrated the dose dependent variation among several immune parameters. While immunomodulating effect of endotoxin at 0.05 mg per fish was recorded, higher dose i.e., 0.1 mg per fish was found to suppress the immune status of the host.

Compliance with ethical standards

All the animal experiments conducted in this present study were in accordance with the ethical guidelines and approval of the university.

Declaration of Competing Interest

The authors declare that they have no known competing interests

Acknowledgement

The authors duly acknowledge the Head of the Department, for his support.

References

[1] A.N. Nakahla, A.J. Szalai, J.H. Banoub, K.M.W. Keough, Serum anti-LPS antibody production by rainbow trout (*Oncorhynchus mykiss*) in response to the administration of free and liposomally incorporated LPS from *Aeromonas salmonicida*, Fish Shellfish Immunol. 7 (1997) 367–401.
[2] C.R. Raetz, C. Whitfield, Lipopolysaccharide endotoxins, Annu. Rev. Biochem. 71 (2002) 635–700.
[3] R.E. Bishop, Fundamentals of endotoxin structure and function, Contrib. Microbiol. 12 (2005) 1–27.
