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Chronic amoxicillin exposure affects *Labeo rohita*: assessment of hematological, ionic compounds, biochemical, and enzymological activities

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Abstract

Labeo rohita were exposed to amoxicillin at a concentration of 1 mg/L (Treatment -I) and 0.5 mg/L (Treatment-II) for a period of 35 days. Numerous alterations were found in amoxicillin treatment groups when compared to the control group. Hemoglobin (Hb), hematocrit (Hct), and erythrocytes (RBCs) levels were significantly ($P < 0.05$) decreased. Leukocytes (WBC), mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) levels were significantly ($P < 0.05$) increased. In the plasma and gill tissues, ionic compounds (sodium, potassium, and chloride) levels were significantly declined throughout the treatment period. The plasma biochemical profiles were significantly altered: glucose level remained low (except at the end of 7th day in Treatment -I) till 35 days of the treatment period. Biphasic trend occurred in the protein level, significant increase was observed on 7th and 28th day (Treatment -I and -II), and 35th day (Treatment -I), and in remaining days its level was found to be decreased. Glutamate oxaloacetate

transaminase (GOT) activity in the plasma was inhibited significantly, whereas in the gill, liver, and kidney tissues the enzyme activity was elevated. Plasma glutamate pyruvate transaminase (GPT) activity was inhibited throughout the study period. GPT activity in the gill was found to be elevated during the treatment period. Liver GPT activity was elevated in all the treatments except 28th (Treatment-I) and 35th day (Treatment-I, and II). GPT activity in the kidney was elevated (except 14th day in Treatment-II). Lactate dehydrogenase (LDH) activity was inhibited in plasma (except 14th day in Treatment-II), gill, liver (except 7th day in Treatment-I), and kidney tissues significantly ($P < 0.05$). The present study emphasizes that amoxicillin at 1 and 0.5 mg/L concentrations affects the hematological/biochemical/electrolytes/enzymological parameters of fish and these biomarkers serve as an effective test system for environmental risk assessment of pharmaceuticals in the aquatic environment.

Keyword: Toxicology

1. Introduction

Diseases and pharmaceuticals lay parallel to each other. Pharmaceuticals have a broad spectrum of applications in human and veterinary therapeutics. Every year nearly 2,00,000 tons of antibiotics produced and utilized throughout the world (Ok et al., 2011). The extensive usage of pharmaceuticals has contributed to the ubiquitous nature of these emerging contaminants (Kurunthachalam, 2012). Pharmaceuticals and their metabolites can be released into the wastewater system after consumption (Zhang et al., 2018). Moreover, the techniques employed in wastewater treatment plants may not be sufficient to remove antibiotics thus leading to their introduction into the aquatic ecosystem. The presence of pharmaceuticals in surface waters, groundwater, and even marine systems have been confirmed, and they range at ng/L or µg/L levels (Tran et al., 2017). The pervasive nature of pharmaceutical residues in the water bodies is a serious environmental burden, i.e., they affect the biota even at µg/l levels (Corcoran et al., 2010). The toxicological implications of the persistent pharmaceuticals in the aquatic environment remain unknown for the most part.

Among pharmaceuticals, antibiotics are extensively prescribed medications. Due to their extensive usage and occurrence in the aquatic environment these compounds are now recognized as emerging pollutants. Even a low level of antibiotics in the water bodies could enhance microbial resistance, which results in adverse health problems to humans (Liu et al., 2018; Ramesh et al., 2018a). Amoxicillin, an aminopenicillin derivative, is a prophylactic drug belonging to β-lactams drug groups that are habitually prescribed medication against microbial infections. It is used as a single drug or in combined forms with clavulanic acid. Its log Kow is

0.87, pKa: 3.23 (acidic) and 7.43 (basic), and is not lipophilic, thus easily absorbed in the intestine. FAO/WHO categorized amoxicillin as “highly important antimicrobial agent” based on their usage both in human and veterinary medicine. Amoxicillin was considered as the drug of choice in 71 countries from 2000 to 2010 (Van Boeckel et al., 2014). Amoxicillin (30 mg/kg) is also used as a standard regimen during cardiopulmonary bypass surgery in humans (Vargas et al., 2004). The drug amoxicillin is very quickly absorbed and is excreted in urine in unchanged form. The mode of action of amoxicillin is similar to other members of penicillin, which inhibits the enzymes (penicillin binding proteins) involved in peptidoglycan biosynthesis in the bacteria.

Furthermore, amoxicillin is employed as one of the chief bactericides (against gram positive and negative bacteria) in the aquaculture practices. It is highly effective in contradiction of the growth of streptococcosis, furunculosis, and pasteurellosis in fishes (Langin et al., 2009). Due to their high usage, these antibiotics are a big threat to the aquatic environment in England and Korea (Jones et al., 2002; Lee et al., 2008). Watkinson et al. (2009) quantified the concentration of amoxicillin in the Australian river waters (200 ng/L), hospital effluents (900 ng/L), and in urban wastewater effluents (50 ng/L). High concentration (64 ng/L to 1670 ng/L) of amoxicillin has been detected in urban wastewater effluents of Hong Kong (Minh et al., 2009). Similarly, amoxicillin was detected up to 283 ng/L in Spain (Rodríguez-Navas et al., 2013) and 1.88–20 ng/L in sewage effluents of Italy (Andreozzi et al., 2004). However, there is a paucity of data on long term toxicity studies of these compounds on aquatic organisms (Park and Choi, 2008).

Biomarkers are widely used to monitor the environmental quality and the health status of the aquatic organisms. Hematological, inorganic ions, biochemical, and enzymological responses were measured as first line biomarkers (Ramesh et al., 2014). Blood parameters such as Hb, Hct, RBC, WBC, MCV, MCH, and MCHC were employed as health indicators in toxicology. Biochemical parameters such as glucose and protein levels and inorganic ions (Na^+ , K^+ , and Cl^-) are the indicators of energy levels of the fish. Enzymological parameters such as LDH, GOT and GPT serves as feasible biomarkers to study the integrity of the immune system and tissue damage in fish (Liu et al., 2015; Poopal et al., 2017).

In fish, blood act as a carrier for biological materials in the body. Gills are directly contact with waterborne contaminants. The liver is an important organ, a site for most of the metabolism process (Yang et al., 2017). Metabolized product and protein re-absorption occur at the kidney. These entire media are highly susceptible to environmental stressors. Assessment of these parameters in the blood and in tissues could provide valuable/comprehensive information on the health status and response of an organism to environmental stress.

Kotwani and Holloway (2013) studied the trends in the use of antibiotics in residents of New Delhi, India and reported that among the highly prescribed antibiotics, amoxicillin and its combinations were predominantly used in primary, secondary, and in tertiary care level hospitals. Countable numbers of reports have been documented on the toxic effects of amoxicillin on fishes (Laville et al., 2004; Rao et al., 2013; Oliveira et al., 2013). However, studies on the toxicity of amoxicillin in freshwater fishes particularly on Indian cultivable fishes are scanty. Hence, the present study is intended to study the potential toxic effects of amoxicillin at environmentally relevant concentrations on certain health biomarkers of edible fish, *Labeo rohita*. The fish, *Labeo rohita* is one of the important fish used for carp polyculture system in India. To generate the information on the ecological risks of amoxicillin at chronic exposure, in this study we exposed (35 days) *L. rohita* fingerlings to environmental relevant/low concentrations of amoxicillin (1 mg/L and 0.5 mg/L) and its effect on hematological/biochemical/electrolytes/enzymological parameters were assessed.

2. Materials and methods

The experimental setup and handling of individuals were performed as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA) and Organization of Economic Co-operation and Development (OECD). The Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore 46, Tamil Nadu, India, has been registered with the Committee for Control and Supervision of Experiments on Animals (CPCSEA) (Ref.: 14134/2006, dated 21 November 2006), Government of India.

2.1. Experimental animal and water quality parameters

Healthy fingerlings, *L. rohita* (average weight of 6.0 ± 0.5 g and length of 7.5 ± 0.5 cm) were collected in well aerated polyethene bags from TamilNadu Fisheries Development Corporation, Aliyar, TamilNadu, India, and immediately transported to the lab. Fish were maintained in a cement tank (1000 L capacity) and fed with rice bran, corn flour, wheat flour and groundnut oil cake (ratio 1:1:1:1) in dough form once in a day. After two weeks of acclimatization, fish were maintained in glass aquaria as stock. During the acclimation period, the hydrological features (APHA (American Public Health Association), 2005) such as; temperature 26.2 ± 1.5 °C, pH 7.1 ± 0.05 , salinity 0.27 ± 0.7 ppt, dissolved oxygen 6.6 ± 0.04 mg/L, and total hardness 17.1 ± 0.8 mg/L were recorded throughout the study period. No mortality was found during the acclimation period. The water in the aquarium was renewed daily by removing three fourth of the water along with excess feed and fecal matters. Feeding was ceased 24 h before the initiation of the treatments.

2.2. Toxicant and chronic toxicity study

All the chemicals used in the experiment were analytical grade, Amoxicillin (CAS: 61336-70-7) (purity ≥ 97.0) was purchased from Sigma Aldrich, and other chemicals and reagents were purchased from Loba Chemie Pvt. Ltd. Mumbai, India. Amoxicillin stock solution was prepared by dissolving 1g in 1000 ml of tap-water. From this stock solution, appropriate quantity was taken and dissolved to get the desired concentration of toxicity solution.

Healthy fingerlings (150 Nos) from the stock were equally distributed (50 Nos) in three glass tanks (100 L capacity). Then 1 mg/L and 0.5 mg/L of amoxicillin was added to the glass tank and identified as Treatment I and Treatment II, respectively. The remaining tank was maintained as control. Three replicates were maintained for each concentration and control group. The glass tanks were aerated and the concentration of amoxicillin (1 mg/L, and 0.5 mg/L) was renewed daily after performing the cleaning process. The study was conducted for 35 days. At the end of every 7, 14, 21, 28, and 35th day, fingerlings from each group (Treatment I, and II, and control) were sacrificed for further analysis. During the experiment period (35 days) no mortality was observed.

2.3. Collection and preparation of samples

At the end of every stipulated period, fingerlings from control and amoxicillin treated (Treatment I, and II) groups ($n = 10$), were segregated and the blood was collected by heart puncture using prechilled heparinised 26 gauge needled-syringe (medical grade disposable). The collected blood samples were immediately transferred to heparinized Eppendorf vials (at ice cold condition). A portion of the whole blood was used for hematological analysis. The remaining blood samples were centrifuged in a cooling centrifuge at 10,000 rpm for 20 min, and the plasma was separated and stored at 4 °C. After blood was drawn, the organs such as gills, liver, and kidney were excised and stored at ice cold condition. 100 mg of each tissue was homogenized with 0.25 M sucrose solution. The homogenates were centrifuged for 20 min at 6,000 rpm at 4 °C and a clear supernatant was obtained. The collected plasma and supernatant were used for the estimation of inorganic ions (Na^+ , K^+ , and Cl^-), biochemical (plasma glucose and protein), and enzymological (AST, ALT, and LDH) parameters.

2.4. Hematological analysis

Hb concentration (g/dl) was estimated by following the cyanmethemoglobin method of [Drabkin \(1946\)](#). Hct was determined by following the microhematocrit method and expressed in %, as described in [Nelson and Morris \(1989\)](#). RBC and WBC counts were determined using Neubauer's hemocytometer, as described in [Rusia](#)

and Sood (1992). Erythrocyte indices of fish viz., MCV, MCH, and MCHC were calculated based on standard formulas, as follows.

$$\text{MCV (fL)} = \text{Hct (\%)} / \text{RBC count in millions/mm}^3 \times 10$$

$$\text{MCH (picograms)} = \text{Hb (g/dl)} / \text{RBC count in millions/mm}^3 \times 10$$

$$\text{MCHC (g/dl)} = \text{Hb (g/dl)} / \text{Hct (\%)} \times 100$$

2.5. Assay on inorganic ions

Na^+ and K^+ were estimated by the method of Maruna (1958) and Cl^- was estimated by the method of Young et al. (1975) and Tietz (1990) using standard kit procedures.

2.6. Biochemical analysis

To estimate the level of plasma glucose, 5 mL of O-Toluidine reagent was mixed with 0.1 mL of sample. Simultaneously a blank (5 mL of O-Toluidine reagent and 0.1 mL of deionized water) and standard (5 mL of O-Toluidine reagent and 0.1 mL of glucose standard) were also prepared. Samples were kept in a water bath (hot) for 10 min, cooled under running tap water and the colour intensity was observed against blank using UV-spectrophotometer (630 nm). The results were expressed in mg/100 mL (Cooper and Mc Daniel, 1970).

The protein level of the sample was determined following the method of Lowry et al. (1951) using bovine serum albumin as standard. To 0.10 mL of sample, a volume of 0.90 mL of deionized water was added. Simultaneously, 1 mL of deionized water in a glass tube was used as the blank. The contents were mixed with 5 mL of solution C (mixture of 50 mL of solution A (2 g of sodium carbonate dissolved in 100 ml of 0.1 N NaOH) and 1 mL of solution B (500 mg of copper sulfate dissolved in 100 mL of 1% sodium potassium tartarate solution)), and kept undisturbed at room temperature for 10 min. Later, 0.5 mL of Folin-phenol reagent was mixed with the content and kept for 15 min at room temperature. The optical density of the contents was determined using UV-spectrophotometer (720 nm) and expressed in $\mu\text{g/mL}$.

2.7. Enzyme assay

GOT and GPT activities were estimated by the method of Reitmen and Franckel (1957). To 50 mL of supernatant and plasma, 0.25 mL of buffering agent (aspartate for GOT and alanine for GPT) was added and incubated (37°C) for 1 min. 0.25 mL of 2,4-DNPH was mixed with the contents and kept undisturbed at room temperature for 20 min. Then, 2.5 mL of NaOH (0.4 N) was added and mixed well. After 10 min, the optical density of the contents was measured using UV-spectrophotometer at 505 nm. LDH activity was determined by following the method of Tietz (1976). To 10 μL of sample, 1000 μL of buffer pyruvate was mixed. The colour intensity of the

content was measured for 2 min at 340 nm. Simultaneously, the standard curve was also performed, and the values were interpreted. The activities were expressed in IU/L.

2.8. Statistical analysis

The results of the present study were expressed as means of five individuals of each parameter and the standard error was also measured. The significant differences between the control and treatments were compared using ANOVA followed by Duncan's Multiple Range Test.

3. Results

When fingerlings were exposed to different concentrations of amoxicillin minor behavioral changes such as fast swimming, movement towards the corners of the tank, and settlement at the bottom of the tank were noticed.

3.1. Effects of amoxicillin on hematological profile

The hematological profiles of *L. rohita* exposed to chronic concentration (Treatment I: 1 mg/L, and Treatment II: 0.5 mg/L) of amoxicillin and the control group were noted and the values were tabulated in Table 1. Significant ($P < 0.05$) decrease in the levels of Hb, Hct, and erythrocytes were observed in the blood of amoxicillin exposed fingerlings than the control group, for a period of 35 days. Whereas, the values of leukocytes, and erythrocyte indices such as MCV, MCH, and MCHC were significantly increased in amoxicillin treated groups throughout the exposure period when compared to the control group.

3.2. Effects of amoxicillin on inorganic ions

Throughout the study period, there was a significant ($P < 0.05$) decrease in the level of ionic compounds (Na^+ (Fig. 1), K^+ (Fig. 2), and Cl^- (Fig. 3)) in the plasma, and gill tissues of amoxicillin exposed groups when compared to control groups.

3.3. Effects of amoxicillin on biochemical parameters

Table 2 depicts the levels of glucose and protein in plasma of *L. rohita* exposed to different concentrations of amoxicillin and control groups. Hypoglycemic condition was noticed in the plasma of fingerlings exposed to the chronic concentrations of amoxicillin in both the treatments (except 7th day of Treatment I), the values were found significant ($P < 0.05$) with the control group. Plasma protein levels of the fingerlings of amoxicillin exposure indicate biphasic responses throughout the study

Table 1. Hematological parameters of *Labeo rohita* exposed to amoxicillin.

Parameter	Exposure period in days	Control	Treatment –I (1 mg/L)	Treatment –II (0.5 mg/L)
RBC (million/cu.mm)	7	6.63 ± 0.007 ^a	5.96 ± 0.007 ^c (-10.10)	6.06 ± 0.007 ^b (-08.59)
	14	6.65 ± 0.007 ^a	5.51 ± 0.007 ^c (-17.14)	5.92 ± 0.007 ^b (-10.97)
	21	6.65 ± 0.007 ^a	5.10 ± 0.007 ^c (-23.30)	5.80 ± 0.007 ^b (-12.39)
	28	6.72 ± 0.007 ^a	5.08 ± 0.023 ^c (-24.40)	5.82 ± 0.007 ^b (-13.39)
	35	6.72 ± 0.007 ^a	4.40 ± 0.142 ^c (-34.52)	5.21 ± 0.007 ^b (-22.47)
WBC (1000/cu. mm)	7	33.34 ± 0.002 ^b	38.72 ± 0.003 ^a (16.13)	38.89 ± 0.002 ^a (16.64)
	14	33.27 ± 0.003 ^c	38.09 ± 0.003 ^a (14.48)	37.76 ± 1.001 ^b (13.49)
	21	33.31 ± 0.004 ^c	39.80 ± 0.005 ^a (19.48)	38.91 ± 0.003 ^b (16.81)
	28	33.22 ± 0.003 ^c	41.11 ± 0.098 ^a (23.75)	39.51 ± 0.003 ^b (18.93)
	35	33.45 ± 0.003 ^c	37.83 ± 0.088 ^b (13.09)	40.11 ± 0.003 ^a (19.91)
Hemoglobin (g/dl)	7	13.86 ± 0.023 ^a	13.36 ± 0.024 ^a (-3.60)	13.20 ± 0.046 ^a (-04.76)
	14	13.81 ± 0.038 ^a	11.43 ± 0.038 ^c (-7.23)	13.13 ± 0.159 ^b (-04.90)
	21	13.83 ± 0.041 ^a	12.75 ± 0.041 ^b (-7.80)	12.17 ± 0.052 ^b (-12.00)
	28	13.87 ± 0.049 ^a	11.65 ± 0.049 ^b (-16.0)	13.39 ± 0.051 ^a (-03.43)
	35	13.88 ± 0.040 ^a	13.14 ± 0.040 ^a (-5.33)	13.20 ± 0.110 ^a (-04.89)
Hematocrit (%)	7	41.12 ± 0.195 ^a	39.32 ± 0.195 ^b (-4.37)	38.64 ± 0.442 ^c (-06.03)
	14	40.82 ± 0.222 ^a	38.12 ± 0.222 ^b (-6.61)	38.12 ± 0.665 ^b (-06.61)
	21	40.94 ± 0.290 ^a	37.30 ± 0.290 ^c (-8.89)	38.52 ± 0.066 ^b (-05.91)
	28	41.14 ± 0.290 ^a	34.02 ± 0.290 ^c (-7.30)	39.0 ± 0.060 ^b (-05.20)
	35	41.28 ± 0.312 ^a	33.38 ± 0.312 ^c (-9.13)	35.18 ± 0.033 ^b (-14.77)
MCV (fL)	7	62.02 ± 0.303 ^c	65.97 ± 0.147 ^a (06.36)	63.76 ± 0.793 ^b (02.80)
	14	62.38 ± 0.305 ^c	69.18 ± 0.172 ^a (10.90)	64.39 ± 1.184 ^b (03.22)
	21	63.40 ± 0.448 ^c	73.13 ± 0.140 ^a (15.34)	66.40 ± 0.183 ^b (04.73)
	28	61.21 ± 0.398 ^c	67.97 ± 2.471 ^a (11.04)	67.01 ± 0.172 ^b (09.47)
	35	61.70 ± 1.117 ^c	76.15 ± 2.299 ^a (23.41)	67.52 ± 0.726 ^b (09.43)
MCH (picograms)	7	20.91 ± 0.048 ^c	22.40 ± 0.065 ^a (07.17)	21.78 ± 0.415 ^b (04.16)
	14	20.77 ± 0.044 ^c	23.80 ± 0.074 ^a (14.58)	22.18 ± 0.193 ^b (06.78)
	21	21.09 ± 0.068 ^c	25.0 ± 0.070 ^a (18.53)	23.91 ± 0.158 ^b (13.37)
	28	20.64 ± 0.066 ^b	23.28 ± 0.855 ^a (12.79)	23.01 ± 0.243 ^a (11.48)
	35	20.74 ± 0.065 ^c	26.08 ± 0.810 ^a (25.74)	23.37 ± 0.095 ^b (12.68)
MCHC (g/dl)	7	33.72 ± 0.121 ^a	33.97 ± 0.022 ^a (0.741)	34.16 ± 0.20 ^b (1.304)
	14	33.85 ± 0.102 ^b	34.40 ± 0.044 ^a (1.624)	34.45 ± 0.218 ^a (1.772)
	21	33.79 ± 0.150 ^b	34.18 ± 0.051 ^a (1.154)	34.26 ± 0.079 ^a (1.390)
	28	33.72 ± 0.132 ^b	34.25 ± 0.229 ^a (1.571)	34.34 ± 0.091 ^a (1.838)
	35	33.62 ± 0.171 ^b	34.25 ± 0.273 ^a (1.873)	34.60 ± 0.241 ^a (2.914)

Values are means ± S.E of five individual observations, (+) denote percent increase over control, (-) Denotes percent decrease over control, Different labels above bars indicate significant differences at $P < 0.05$ between groups.

period. The values were statistically significant ($P < 0.05$) when compared to the control group.

3.4. Effects of amoxicillin on enzymological parameters

Activities of plasma and tissue (gills, liver, and kidney) enzymes of control and amoxicillin exposed groups were illustrated in Fig.4, 5, and 6. The plasma GOT activity was found to be inhibited significantly ($P < 0.05$) in the amoxicillin exposed groups than the control group. Whereas in gill, liver, and kidney tissues, the activity

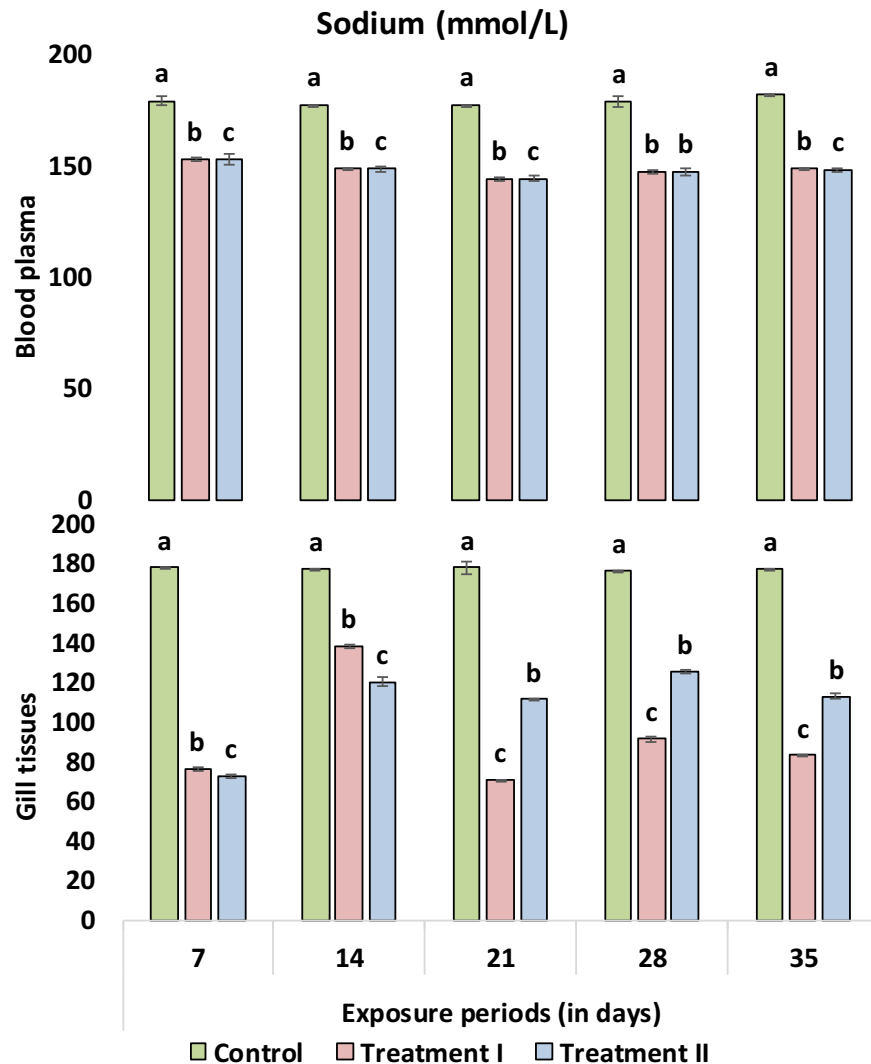


Fig. 1. Sodium level of *L. rohita*, different labels above bars indicates significant differences at $P < 0.05$ between groups. The vertical line in the bars indicates standard error.

of GOT was higher in amoxicillin treated groups. The elevation of GOT activities was statistically significant ($P < 0.05$) when the values were compared to control groups (Fig. 4).

GPT activity in the plasma of amoxicillin exposed fingerlings was inhibited throughout the study period (35 days), the values were statistically significant ($P < 0.05$) when compared to control groups. GPT activity in the gill tissues was activated in Treatment I and II, than control groups. Liver tissues of amoxicillin exposed fingerlings showed an elevated activity of GPT, except 28th day of Treatment I, and 35th day of Treatment I, and II (Fig. 5). Whereas in kidney tissues GPT activity was increased in Treatment I and II (except 14th day). GPT activities in the different tissues were significant ($P < 0.05$) when related to control groups.

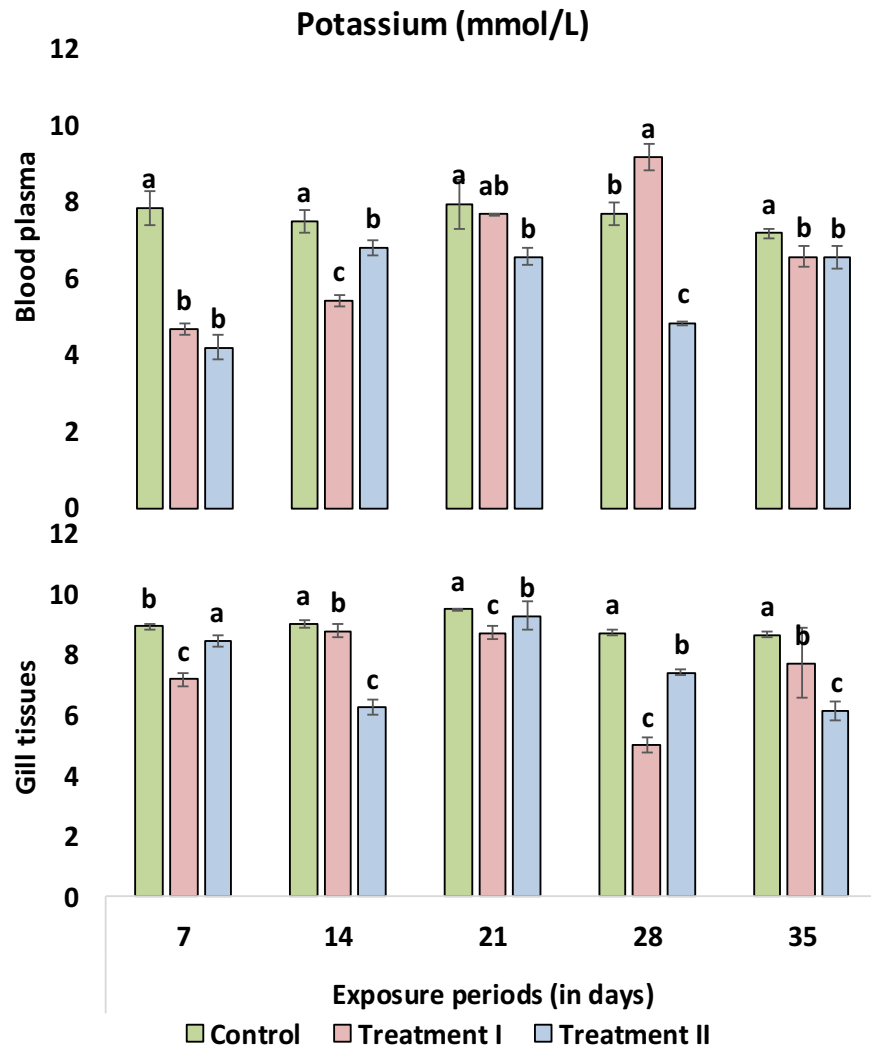


Fig. 2. Potassium level of *L. rohita*, different labels above bars indicates significant differences at $P < 0.05$ between groups. The vertical line in the bars indicates standard error.

Unlike transaminase activity, a significant ($P < 0.05$) decline in the activity of LDH was observed in the plasma, gill, liver, and kidney tissues of amoxicillin treated fingerlings throughout the study period (except Treatment I at 7th day) (Fig. 6).

Alterations in the hematological, inorganic ionic compounds, biochemical, and enzymological activities in the plasma and tissues (gill, liver, and kidney) of fingerlings during chronic exposure of amoxicillin was maximum in Treatment I (1 mg/L). It is an indication of concentration-based responses of fingerlings to amoxicillin toxicity.

4. Discussion

The contamination of pharmaceuticals and their metabolites in the aquatic environment is a serious threat to aquatic biota. Recently many scientific communities have

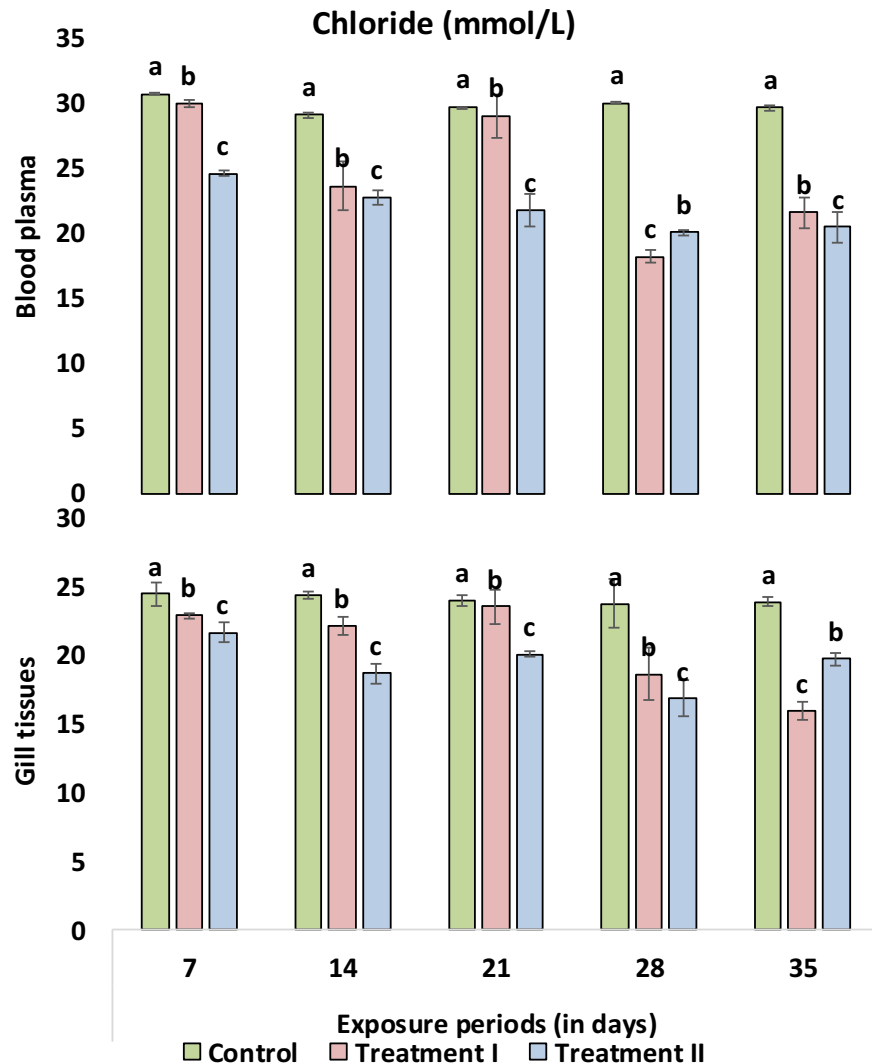


Fig. 3. Chloride level of *L. rohita*, different labels above bars indicates significant differences at $P < 0.05$ between groups. The vertical line in the bars indicates standard error.

deviated towards the toxicity research on pharmaceuticals (Fabbri, 2015). We have already reported the impacts of few pharmaceuticals on fresh water edible fishes (Ambili et al., 2013; Saravanan et al., 2014; Ramesh et al., 2018a,b; Renuka et al., 2018). Fish farmers use unapproved chemicals to maintain the health status of fishes by preventing and treating disease outbreaks (Nyambok and Kastner, 2012). The diverse use of antibiotics in fish farming and their potential impacts on aquatic ecosystems have raised an environmental concern (Rico et al., 2012). This class of antibiotics comprise more than 50 products (Mompelat et al., 2009), and amoxicillin is just one of the antibiotics. Amoxicillin is also used as a growth regulator, and in commercial feeds to prevent microbial contamination (as mentioned in Carballreira et al., 2012). The occurrence of amoxicillin in the surface water may be due to its higher excretion rate (80–90 %) as an unchanged compound (Aydin and

Table 2. Biochemical profile of *Labeo rohita* exposed to amoxicillin.

Parameter	Exposure period in days	Control	Treatment -I (1 mg/L)	Treatment -II (0.5 mg/L)
Glucose (mg/100ml)	7	81.99 ± 0.604 ^b	82.19 ± 0.439 ^a (0.243)	78.43 ± 0.313 ^c (-04.34)
	14	81.94 ± 0.866 ^a	72.65 ± 0.24 ^b (-11.33)	72.17 ± 0.399 ^b (-11.92)
	21	82.80 ± 0.206 ^a	68.49 ± 1.02 ^c (-17.28)	77.87 ± 0.846 ^b (-05.95)
	28	82.14 ± 0.598 ^a	67.43 ± 1.435 ^c (-17.90)	77.17 ± 0.268 ^b (-06.05)
	35	82.50 ± 0.254 ^a	54.98 ± 1.159 ^c (-33.35)	79.51 ± 0.150 ^b (-03.62)
Protein (g/ml)	7	1.11 ± 0.001 ^b	1.62 ± 0.024 ^a (45.94)	1.68 ± 0.139 ^a (51.35)
	14	1.86 ± 0.003 ^a	1.57 ± 0.025 ^b (-15.59)	1.77 ± 0.020 ^a (-04.83)
	21	1.89 ± 0.004 ^a	1.55 ± 0.021 ^b (-17.98)	1.55 ± 0.047 ^b (-17.98)
	28	1.14 ± 0.003 ^b	1.73 ± 0.005 ^a (51.75)	1.31 ± 0.057 ^b (14.91)
	35	1.83 ± 0.023 ^b	2.24 ± 0.033 ^a (22.40)	1.76 ± 0.005 ^b (-03.82)

Values are means ± S.E of five individual observations (+) denote percent increase over control (-) Denotes percent decrease over control, Different labels above bars indicate significant differences at $P < 0.05$ between groups.

Talinli, 2013). Matozzo et al. (2016) reported that the level of amoxicillin in the aquatic ecosystem is expected to be detectable (at µg/L), which can create an environmental risk in the water.

The use of multi-biomarker approaches is greatly valuable both in laboratory and field studies (Guimaraes et al., 2012). Changes in hematological, ionic compounds, biochemical, and enzymological parameters play a vital role in ecotoxicological evaluation of the chemicals in the environment (Lyons et al., 2010). Hb, Hct, RBCs, and WBCs as well as hematological indices such as MCV, MCH, and MCHC, are routinely used biomarkers with a wide potential for application in biomonitoring and aquatic toxicity studies. In the present study, decline in the Hb content of the drug exposed group may be due to insufficient oxygen supply, and an indication of hypochromic microcytic anemia. Haem content has a significant role in energy metabolism, thus their changes could alter the mechanism of energy production. Decline in the value of Hb content could also be due to the inhibitory effect of the drug on the enzyme system responsible for hemoglobin synthesis. Swelling of RBCs or the reduction in the rate of formation of RBCs could decline the Hct level in the organisms. The observed decrease in the RBC count indicates anemia, impaired osmoregulation and gill damage (Poopal et al., 2017), caused by the drug amoxicillin. Fish exposed to amoxicillin showed leucocytosis, which is an indication of activation of immune system during abnormal conditions in organisms. This could result to protect them from amoxicillin toxicity. The leucocytes from the spleen could also increase their count in the blood.

The levels of erythrocyte indices reveal the normal or anemic condition of the fingerlings. MCV of the fingerlings were elevated, this could be due to the hypoxic condition created by the drug. The RBC swelling could contribute higher MCV in the circulation. MCH value could increase as a result of alterations in Hb, normally towards anemic condition. Increased MCHC value indicates a protective response of

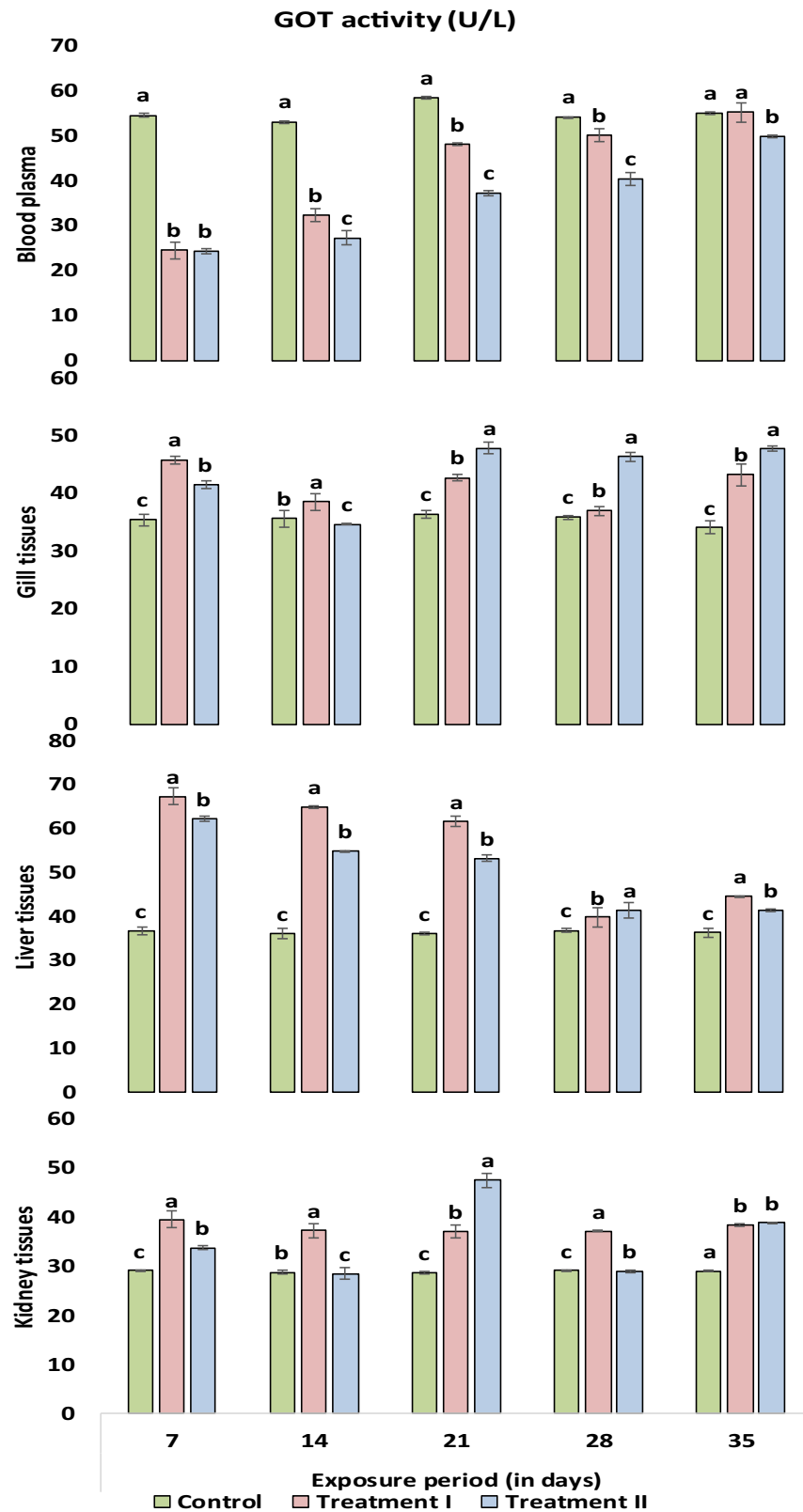


Fig. 4. GOT activity of *L. rohita*, different labels above bars indicate significant differences at $P < 0.05$ between groups. The vertical line in the bars indicates standard error.

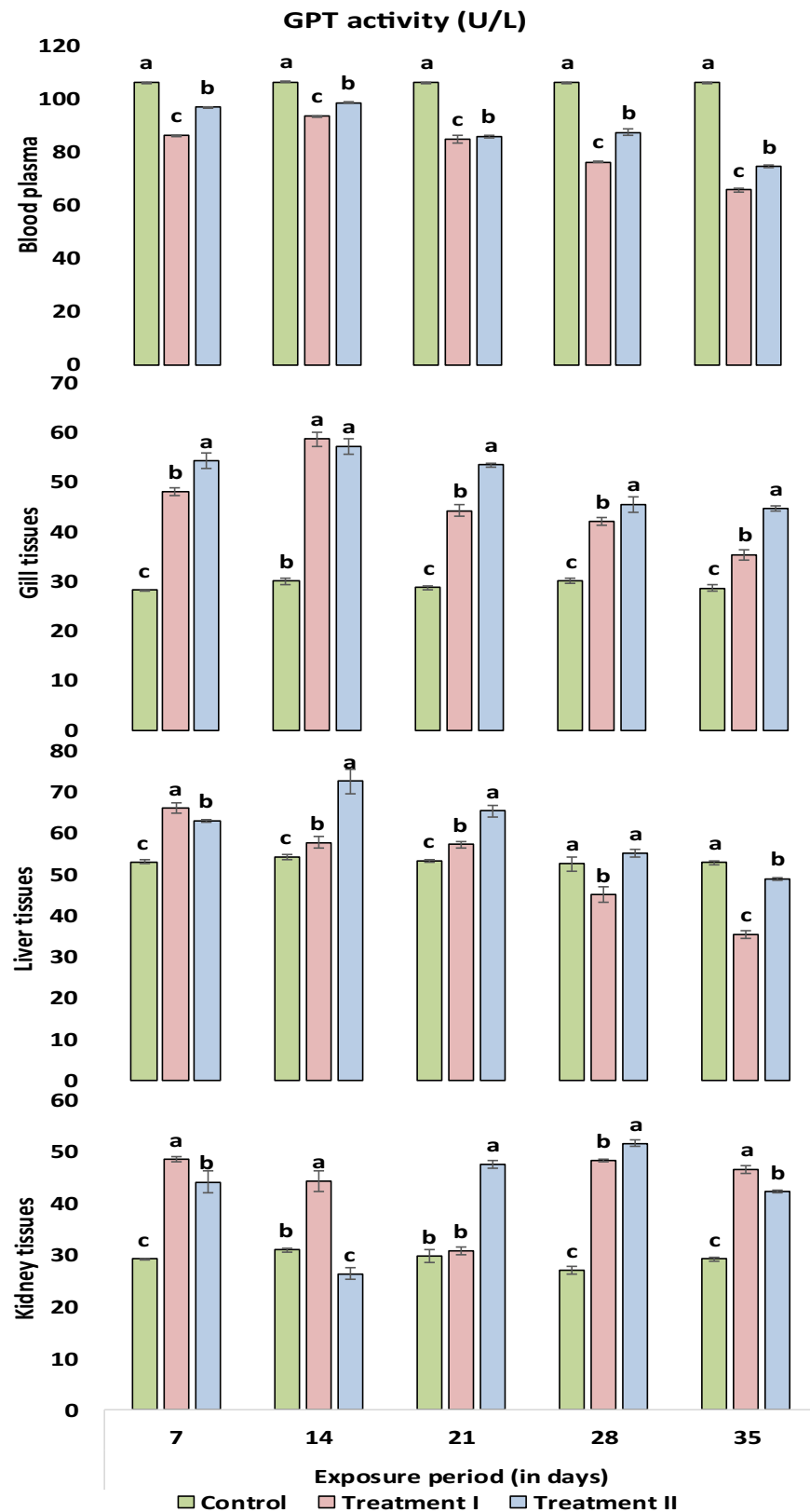


Fig. 5. GPT activity of *L. rohita*, different labels above bars indicate significant differences at $P < 0.05$ between groups. The vertical line in the bars indicates standard error.

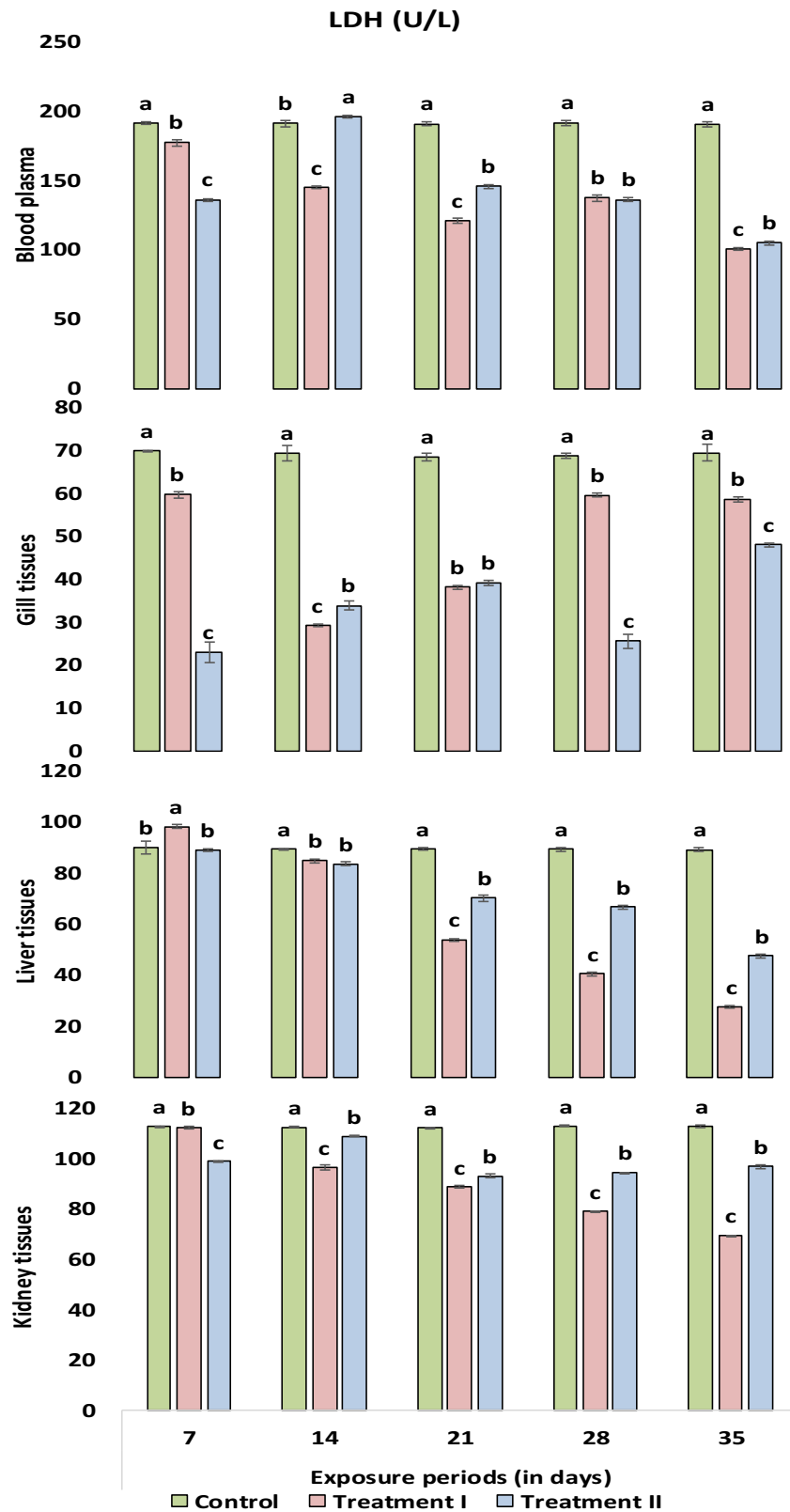


Fig. 6. LDH activity of *L. rohita*, different labels above bars indicate significant differences at $P < 0.05$ between groups. The vertical line in the bars indicates standard error.

the fish against the drug toxicity. This situation occurs due to congenital spherocytosis (hemolytic anemia), where the Hb concentration outside the cell is higher (Li et al., 2011). Rossi et al. (2010) reported that the glucose phosphate isomerase (GPI) deficiency could lead to failure of the system that removes free radicals generated by amoxicillin, thereby resulting in oxidation of hemoglobin and destabilization of red cell membranes, with acute hemolysis and severe hemoglobinuria.

Ionic compounds balance is essential because freshwater fish are hyperosmotic to their environment. Freshwater fishes maintain their regular physiological process and body fluid homeostasis with the help of ion regulation process (Hwang and Lee, 2007). Gills of fish maintain pH of body fluid, the osmotic pressure of the body, nitrogenous wastes, and regulation of water influx and ion efflux. In the present study the significant drop in plasma and gill Na^+ , K^+ , and Cl^- ions indicate the impairment of uptake mechanisms of inorganic-ionic compounds at the gill (Sathya et al., 2012). Moreover, the drug may alter the membrane permeability attributing to a lesser intake of ionic compounds into the body. This could create intra- and extra-cellular fluid imbalance that resulted in hyponatremia, hypochloremia, and hypocalcemia in the fingerlings (Wood et al., 1996; Remen et al., 2008). Waterborne chemicals could act on the gill membrane and alters the permeability, which results in ion loss and decrease the plasma ion concentration (Uddin et al., 2016).

Improper carbohydrate metabolism could alter the glucose level in the blood. Glucocorticoids could increase the blood glucose level due to stimulation by the toxicants. Endocrine system releases cortisol and catecholamine (stress hormones) in the circulatory system as a preliminary stress response. This could shoot up the glucose level in plasma as a subsequent response. Endocrine disruption effects of several pharmaceuticals have been reported by many authors. Generally antibiotic residues in water may cause a decrease in reproductive activity of the aquatic organisms (Kang et al., 2006). Production of vitellogenin (Vtg) has been reported in Japanese medaka (*Oryzias latipes*) exposed to amoxicillin (Kim and Kim, 2007). Respiratory dysfunction during stress in fish could also raise the blood glucose level (Pakhira et al., 2015). The decrease in the glucose level (hypoglycemia) might be resulted from the utilization of glucose during hypoxic situation or decrease in glycogenolysis in the hepatic or reduced glucose absorption rate in the intestine (Remyla et al., 2008). In the present study, hyperglycemic condition in the blood of amoxicillin exposed fingerlings indicates supply of energy for the process of elimination of the drug and activation of other metabolic activities in the body themselves. Substrate depletion and depletion of stored energy in fish exposed to chronic stress result in lowering of glucose level in the blood (West et al., 1993).

Fluctuations in the plasma protein level emphasize impaired protein synthesis mechanism in the liver (Kavitha et al., 2010). Proteins serve as a vital source of energy during abnormal conditions. This could alter their levels in the blood.

Moreover, antibiotics have high affinity for protein. β -lactams are haptens that covalently bind with proteins to perform an immune action against infection (Ariza et al., 2014). Amoxicillin forms haptenation with the protein molecules towards activation of immunological process that could be a potential reason for the decline in the protein content in the blood. During physical/chemical stress condition heat shock protein levels involves in cope up the stress which could alter the protein (Simide et al., 2016). Catabolism of protein and osmotic imbalance under stress condition could alter the protein in the blood, and the transfer of body fluids in the intracellular compartment may increase the protein content (Pakhira et al., 2015). Elizalde-Velázquez et al. (2017) reported that the amoxicillin induced oxidative stress in brain, gill, liver and kidney of *C. carpio* during acute exposure.

Enzymological parameters are the primary indicator of the stress in the fish. Hence enzymological activities were considered as an “alarm” in the assessment of health status of the aquatic environment. Enzymes are pollution biomarkers because they play a catalytic role in metabolism and detoxification of chemicals in the body. Pharmaceuticals may alter the transaminase and other enzymes in the tissues, which are responsible for cellular processes. Hence, enzyme assays are widely used in most of the pharmaceutical toxicity assessment (Woo et al., 2018). Moreover, the health status of the vital organs, such as gills, liver, and kidney could be monitored using the enzyme activities. GOT or AST, GPT or ALT and LDH are the enzymes found in heart, liver, kidney, skeletal muscles and erythrocytes. GOT and GPT participate in transamination reactions. Likewise, LDH is an oxidative enzyme which is important for glycolytic activity. The alterations in the enzymes are used as organ health indicators of chemical exposure.

Decrease of AST and ALT activities indicate the deficiency of amino acids and α -ketoglutarate in fish under stress condition (Ramesh et al., 2018b). Structural changes in the cell organelles could decrease the level of transaminases. Improper protein and carbohydrate metabolism could alter the level of transaminase activities. Kim et al. (2011) reported that amoxicillin affects the liver and could activate the enzyme. Elevated transaminases are an indication of hepatomegaly, which is caused due to amoxicillin toxicity. Moreover, high level of transaminases could improve the synthesis/disturbance in cell integrity, by which the enzyme concentrations in the cells do not allow for distribution, this could increase the internal enzyme concentration (Yang et al., 2013). Increase in GPT and GOT activity in the organs of fish exposed to toxicants indicate that the fish developed alleviating mechanism to compensate the impaired metabolism (Woo et al., 2018). An elevation in GPT activity in various organs (gill, liver and muscle) was also noted in *Cyprinus carpio* exposed to pharmaceutical drug carbamazepine (Malarvizhi et al., 2012). Rao et al. (2013) found that rohu fingerlings subjected to 0.25 $\mu\text{g/L}$ to 80 $\mu\text{g/L}$ of amoxicillin for 5 days resulted in increased enzyme activities (AST, ALT, LDH, ACP and

ALP) in the liver and muscle tissues. Any variation in these intracellular enzymes may hinder the synthesis of aspartate aminotransferase protein due to the accumulation of the drug (Santos et al., 2010).

LDH is a tetrameric enzyme which is mainly situated in the cytoplasm. It is essential for stress mediated energy metabolism under hypoxic condition. LDH can be employed as a biomarker for monitoring the chemical exposure and stress in fish (Tugiyono and Gagnon, 2002). It acts as a good indicator for assessing the toxicity of a chemical. Various processes could alter LDH activity, which includes leakage of blood cells, improper energy metabolism, and inclusion of isozymes due to cell damage. In the present study, there is a reduction in the LDH activity in plasma, gill, liver, and kidney which is in line with the study of Yadav et al. (2007). Probably, the inhibition of LDH in the present study during chronic treatment may be due to impaired carbohydrate metabolism. LDH is generally associated with cellular metabolism, and so, its inhibition might have resulted from ion imbalance or due to the formation of enzyme inhibitor complex (Sayed et al., 2011). Furthermore, a decrease in LDH activity may be due to lower metabolic rate resulting from the toxicity buildup of the toxicants (Renuka et al., 2018).

5. Conclusion

The results of the present study indicate that amoxicillin could cause significant changes in hematological, ionic levels, biochemical, and enzymological parameters of *Labeo rohita*, upon chronic exposure. The alterations of these parameters could be effectively used as potential biomarkers for the risk assessments of pharmaceuticals in the aquatic environments. The present study could provide a baseline information on the potential effects of antibiotics on non-target organisms, especially on fish under chronic exposure. Further exploration of molecular toxicity studies would provide the better understanding of the amoxicillin action of antibiotics on organisms.

Declarations

Author contribution statement

Sathisaran Umamaheswari: Conceived and designed the experiments; Performed the experiments, Analyzed and interpreted the data.

Siva Shankar Renuka: Analyzed and interpreted the data.

Mathan Ramesh: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Rama Krishnan Poopal: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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