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Research Article

Effect of some Immunostimulants on Clinicopathological Findings of African Catfish *Clarias gariepinus* Infected with Motile Aeromonas Septicemia

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Abstract

This work was carried out to study the impact of *Aeromonas hydrophila* (*A. hydrophila*) on African catfish (*Clarias gariepinus*) with immunostimulants (Biogen and Ropadiar) being used for control trials. One hundred and twenty catfish (*Clarias gariepinus*) were randomly allocated to 6 equal treatment groups as follows: G_1 and G_4 received the control diet without any additives; G_2 and G_5 received 2 gm Biogen supplement/kg diet; G_3 and G_6 received 3 ml Ropadiar/kg diet. At 45th day of experiment, G_4, G_5 and G_6 were challenged using virulent strain of *A. hydrophila.* Clinicopathological investigation showed significant decrease in red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV)% and a significant increase in the number of white blood cells (WBCs) and lymphocytes in all infected groups. Serum total protein and albumin showed a significant decline in infected groups with non-significant increase in globulin concentration. Immunological investigation revealed a significant rise in interleukin-1β (IL1β) and interleukin-10 (IL10) in non-treated infected group. While, infected treated groups showed a significant decline in IL1β and IL10 as compared with the non-treated infected group. Regarding antioxidant parameters, serum malondialdehyde (MDA) and nitric oxide (NO) levels increased significantly, while, total antioxidant capacity (TAC) decreased significantly in infected group. Whereas, infected treated groups showed a significant decrease in MDA and NO with a significant increase TAC levels as compared with the non-treated infected group. Histopathological results were fully reported in different experimental groups.

Conclusion: Dietary supplementation of Biogen and Ropadiar could improve the humoral defence mechanism and anti-oxidant response in fish, which in turn can aid in the control and prevention of A. hydrophila infection.

Keywords: *A. hydrophila; Biogen; Catfish; Hematological; Immunomodulatory; Ropadiar; Biochemical*

Introduction

Aeromonas hydrophila, is responsible for the disease known as Motile Aeromonad Septicemia (MAS), which characterized by various pathological symptoms such as dermal ulceration, fin hemorrhages and rots, hemorrhages and necrosis of the visceral organs [1]. In humans, *A. hydrophila* infection has been correlated with gastroenteritis and localized wound infection [2] and therefore this bacterium is of the main public health concern.

Antimicrobial drugs are currently the most commonly used technique for controlling *A. hydrophila* infection in cultured fish**.** These antimicrobials promote the growth of drug-resistant microorganisms and leave antibiotic residues in the fish and adverse environmental impacts [3]. Commercial vaccines, could be the most efficient technique to control fish diseases by activating the specific immune

response of the fish [4]. However, they are costly and may not be accessible to all species and against emerging diseases. An alternative approach, besides vaccine development, is dietary modulation of immune responses and disease resistance of aquaculture species [5].

Probiotics are described as cultures of live microorganisms that benefit the host (humans and animals) by improving the properties of the native microflora [6]. Among these products, Biogen® product, which is an immunostimulant, improved the health conditions, enhanced the growth and immunity as well as enabled the fish to overcome artificial infection- induced stress [7]. Another form of immunostimulants have also been tested in fish feeds is Plant based feed additives [8]. Phytogenic extracts containing phenolic and flavonoid chemical compounds, which improve the structure of gut microflora, enhance the immune function and resistance of human and animals to pathogens. Among these additives, carvacrol and thymol, essential oils from oregano (*Origanum vulgare*), which not only act as growth promoters [9] but also as antioxidants and antimicrobials [10]. This work was conducted to study the impact of *A. hydrophila* on African catfish (*Clarias gariepinus*) and its related hematological, biochemical, immunological, pathological and antioxidant changes with trials for control using immunostimulants (Biogen and Ropadiar) via experimental studies.

Materials and Methods

A total of 120 apparently healthy African catfish (*Clarias gariepinus*); with average body weight (165 ± 15); were divided into 6 equal groups of 20 fish each. Fish were acclimatized with dechlorinated water for 14 days in a full glass aquarium (100 × 50 ×50 each). A 12:12 h light: dark period maintained and air stones for supplemental aeration. Water temperature was maintained at 25 ± 1. A basal diet of 30% crude protein was fed twice daily, at a rate of 3% of body weight for 60 days. G_1 and G_4 obtained a control diet with no additives; G_2 and G_5 received the basal diet with 2 gm Biogen supplement; G_3 and G_6 received the basal diet with 3 ml Ropadiar supplement.

Feed additives: Biogen (*Bacillus subtilis natto* not less than 1 x 10^{11}) was acquired from Samu Median Co. Ltd., China. Ropadiar solution 20% (Oreganum natural oil 20%, contributing 120.4 gm carvacrol and 8.0 gm thymol as active components) was received from Ropapharm International BV., India. Both additives have been thoroughly mixed with the basal fish diet.

Bacterial strain: A well-identified virulent *A. hydrophila* strain was supplied from Dept. of Microbiology, Animal Health Research Institute, El Doki, Giza, Egypt. Bacterial cultures were grown in nutrient broth (Oxoid, UK) at 37°C for 24h. Then the isolate was sub cultured on Tryptone Soya Agar, Rimler-shot media (Oxoid, UK) incubated at 37°C for 24 to 48h. The isolate has been verified to be *A. hydrophila* through biochemical identification of the reisolated organism, like those reported [11]. The bacterial suspension was diluted in sterile phosphate buffer saline (PBS) at a concentration of 1.5 x 10^8 CFU according to the McFarland scales [12].

Experimental infection: Fish in groups 4, 5 and 6 were inoculated with 0.5 ml saline suspension containing 1.5 × 10^8 CFU of *A. hydrophila* by intra-peritoneal injection at 45 days of experimental period; infected fish were kept under observation for 15 days.

Clinical examination: The experimented fish was examined according to Schaperclaus., *et al* [13].

Blood samples: Random five fish were assigned for sampling at 2 and 10 days post-infection. Blood was collected from the caudal vein of fish and serum was separated and preserved at -20 until biochemical, immunological and antioxidant parameters were determined.

Hemogram: Total RBCs and WBCs [14], blood hemoglobin [15], packed cell volume [16], the percentage and absolute values for different WBC cells [17], were estimated.

Biochemical parameters: serum alanine aminotransferase (ALT) [18], serum alkaline phosphatase (ALP) [19], serum total protein [20], serum albumin [21], serum globulin [22] and creatinine [23].

Immune parameters: Fish IL-1β and IL-10 was determined using ElISA kits Cat. No: MBS700230, MBS044038 obtained from MyBioSource, Inc., San Diego, California, USA.

Analysis of antioxidant parameters: Serum NO [24], serum MDA [25], serum TAC [26] were determined using commercial kits obtained from Biodiagnostic kits, Egypt.

Histopathological examination: Specimens from liver, kidney and spleen were collected from fish of different groups and histopathological technique was done according to Drury and Wallington [27].

Statistical analysis

Analysis was carried out to all collected samples using the SPSS program 16 (SPSS, Richmond, VA, USA) as described by Dytham [28]. One way analysis of variance (ANOVA) and Duncan's Multiple Range test [29] was used to determine differences between treatments (mean at significance level $P \leq 0.05$).

Results

The clinical signs of infected catfish (*Clarias gariepinus*) after experimental challenge with *A. hydrophila* revealed sluggish movement and swimming close to the water surface at the terminal stage, greyish white lesion and hemorrhagic patches a long with fin erosions and skin ulceration. Fish of Biogen infected group showed sluggish movement, grayish white and/or hyperemic lesions. While Ropadiar infected group showed no clinical signs compared to the infected non-treated group.

In our study, infection with *A. hydrophila* induced a significant reduction in RBCs, Hb and PCV%, significant increase in WBCs, lympho-

Groups	Time of sampling	RBCs (10^6/µl)	Hb (g/dl)	PCV %
Control	2 days	2.83 ± 0.04 ^{b}	9.85 ± 0.03 ^{b}	29.15 ± 0.60 ^{b}
(G_1)	10 days	2.79 ± 0.04 ^{b}	9.97 ± 0.14 ^{b}	29.45 ± 0.35 ^{b}
Biogen	2 days	3.03 ± 0.005 ^{a}	10.49 ± 0.11 ^{a}	31.55 ± 0.29 ^{a}
(G_2)	10 days	3.13 ± 0.05 ^{a}	11.12 ± 0.20 ^{a}	33.70 ± 0.60 ^{a}
Ropadiar	2 days	2.80 ± 0.02 ^{b}	9.80 ± 0.14 ^{b}	29.00 ± 0.23 ^{b}
(G_3)	10 days	2.81 ± 0.04 ^{b}	10.11 ± 0.08 ^{b}	30.02 ± 0.61 ^{b}
Infected	2 days	2.30 ± 0.02 ^{d}	7.65 ± 0.05 ^{d}	22.80 ± 0.69 ^{d}
(G_4)	10 days	1.73 ± 0.03 ^{d}	5.42 ± 0.17 ^{d}	20.80 ± 0.43 ^{d}
Biogen infected	2 days	2.45 ± 0.03 ^{c}	8.17 ± 0.11 ^{c}	24.72 ± 0.64 ^{c}
(G_5)	10 days	2.04 ± 0.05 ^{c}	6.50 ± 0.21 ^{c}	23.85 ± 0.06 ^{c}
Ropadiar infected	2 days	2.34 ± 0.04 ^{d}	7.80 ± 0.15 ^{d}	23.40 ± 0.58 ^{cd}
(G_6)	10 days	1.84 ± 0.05 ^{d}	5.85 ± 0.25 ^{d}	21.53 ± 0.59 ^{d}

Table 1: *Erythrogram profile in different experimental groups at 2 and 10 days post-infection.*

cytes, monocytes and eosinophils at 10 days post-infection. While, Biogen treated group (G_2) revealed a significant increase in RBCs, Hb and PCV, as compared with the control. In addition to, a significant increase in lymphocytes in Biogen and Ropadiar supplied groups (G_2, G_3) compared to the control (Table 1 and 2).

Groups	Time of sampling	WBCs (10^3/µl)	Heterophils (10^3/µl)	Lymphocytes (10^3/µl)	Monocytes (10^3/µl)	Eosinophils (10^3/µl)
Control	2 days	23.67 ± 0.44 ^{b}	8.44 ± 0.47 ^{b}	13.72 ± 0.62 ^{a}	1.03 ± 0.16 ^{b}	0.47 ± 0.01 ^{a}
(G_1)	10 days	24.17 ± 0.45 ^{b}	8.70 ± 0.36 ^{a}	13.92 ± 0.36 ^{c}	1.13 ± 0.10 ^{b}	0.40 ± 0.08 ^{b}
Biogen	2 days	25.17 ± 1.92 ^{b}	8.42 ± 0.91 ^{b}	15.28 ± 1.28 ^{a}	1.06 ± 0.10 ^{b}	0.41 ± 0.06 ^{a}
(G_2)	10 days	26.67 ± 1.20 ^{b}	8.77 ± 0.12 ^{a}	16.30 ± 1.15 ^{b}	1.15 ± 0.09 ^{b}	0.44 ± 0.07 ^{b}
Ropadiar	2 days	25.24 ± 0.17 ^{b}	8.65 ± 0.71 ^{b}	15.16 ± 0.67 ^{a}	1.01 ± 0.15 ^{b}	0.42 ± 0.08 ^{a}
(G_3)	10 days	26.83 ± 0.60 ^{b}	8.59 ± 0.58 ^{a}	16.63 ± 0.40 ^{bc}	1.17 ± 0.19 ^{b}	0.45 ± 0.10 ^{b}
Infected	2 days	37.58 ± 2.05 ^{a}	21.17 ± 2.42 ^{a}	13.78 ± 0.35 ^{a}	2.01 ± 0.20 ^{a}	0.49 ± 0.09 ^{a}
(G_4)	10 days	35.67 ± 0.88 ^{a}	8.79 ± 0.07 ^{a}	23.91 ± 0.90 ^{a}	2.26 ± 0.24 ^{a}	0.71 ± 0.02 ^{a}
Biogen infected	2 days	34.33 ± 1.76 ^{a}	18.35 ± 1.28 ^{a}	13.71 ± 0.55 ^{a}	1.82 ± 0.03 ^{a}	0.46 ± 0.12 ^{a}
(G_5)	10 days	36.50 ± 0.76 ^{a}	8.89 ± 0.49 ^{a}	24.81 ± 0.35 ^{a}	2.07 ± 0.16 ^{a}	0.73 ± 0.01 ^{a}
Ropadiar infected	2 days	34.50 ± 1.89 ^{a}	18.27 ± 1.01 ^{a}	13.70 ± 0.96 ^{a}	2.06 ± 0.19 ^{a}	0.47 ± 0.14 ^{a}
(G_6)	10 days	37.67 ± 1.33 ^{a}	8.27 ± 0.26 ^{a}	26.15 ± 1.35 ^{a}	2.01 ± 0.17 ^{a}	0.75 ± 0.03 ^{a}

Table 2: *Leukoogram profile in different experimental groups at 2 and 10 days post-infection.*

Groups	Time of sampling	ALT (U/L)	ALP (U/L)	T.P. (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Creatinine (mg/dl)
Control	2 days	17.25 ± 0.85 ^{c}	15.75 ± 0.25 ^{c}	4.15 ± 0.12 ^{a}	2.20 ± 0.13 ^{a}	1.95 ± 0.21 ^{a}	0.32 ± 0.04 ^{b}
(G1)	10 days	17.25 ± 0.85 ^{c}	14.50 ± 1.94 ^{bc}	4.36 ± 0.07 ^{b}	2.12 ± 0.20 ^{a}	2.24 ± 0.16 ^{b}	0.29 ± 0.03 ^{c}
Biogen	2 days	15.50 ± 1.44 ^{c}	15.00 ± 0.91 ^{c}	4.47 ± 0.23 ^{a}	2.41 ± 0.20 ^{a}	2.06 ± 0.21 ^{a}	0.23 ± 0.03 ^{b}
(G_2)	10 days	16.35 ± 1.80 ^{d}	13.12 ± 0.72 ^{c}	5.56 ± 0.26 ^{a}	2.52 ± 0.11 ^{a}	3.09 ± 0.34 ^{a}	0.23 ± 0.03 ^{c}
Ropadiar	2 days	16.00 ± 1.41 ^{c}	13.25 ± 1.65 ^{c}	4.30 ± 0.23 ^{a}	2.32 ± 0.19 ^{a}	1.97 ± 0.13 ^{a}	0.27 ± 0.09 ^{b}
(G_3)	10 days	15.51 ± 0.50 ^{d}	12.50 ± 1.19 ^{c}	5.19 ± 0.23 ^{a}	2.32 ± 0.39 ^{a}	2.62 ± 0.08 ^{ab}	0.20 ± 0.06 ^{c}
Infected	2 days	29.88 ± 1.20 ^{a}	43.75 ± 1.89 ^{a}	3.07 ± 0.43 ^{b}	0.90 ± 0.15 ^{b}	2.17 ± 0.31 ^{a}	0.60 ± 0.05 ^{a}
(G_4)	10 days	36.00 ± 1.08 ^{a}	24.48 ± 2.08 ^{a}	3.53 ± 0.24 ^{c}	1.02 ± 0.10 ^{b}	2.50 ± 0.14 ^{ab}	1.12 ± 0.05 ^{a}
Biogen infected	2 days	23.25 ± 0.95 ^{b}	27.50 ± 2.02 ^{b}	3.38 ± 0.08 ^{b}	1.07 ± 0.07 ^{b}	2.30 ± 0.06 ^{a}	0.50 ± 0.06 ^{a}
(G_5)	10 days	25.23 ± 1.93 ^{c}	17.20 ± 0.52 ^{b}	3.49 ± 0.17 ^{c}	1.12 ± 0.13 ^{b}	2.61 ± 0.21 ^{ab}	0.97 ± 0.04 ^{b}
Ropadiar infected	2 days	24.75 ± 2.17 ^{b}	26.00 ± 2.12 ^{b}	3.27 ± 0.10 ^{b}	1.02 ± 0.06 ^{b}	2.25 ± 0.06 ^{a}	0.57 ± 0.03 ^{a}
(G_6)	10 days	31.75 ± 2.72 ^{b}	17.75 ± 0.63 ^{b}	3.22 ± 0.24 ^{c}	0.92 ± 0.10 ^{b}	2.54 ± 0.20 ^{ab}	0.87 ± 0.03 ^{b}

Table 3: *Serum biochemical parameters in different experimental groups at 2 and 10 days post-infection.*

Serum biochemical findings in fish infected with *A. hydrophila*, revealed a significant increase in ALT, ALP, and creatinine levels. While, G_5 and G_6 showed a significant decrease in their level in comparison with the infected non-treated. A significant decrease in the total protein with non-significant increase in globulin concentration was noticed in *Aeromonas* infected groups (G_4, G_5 and G_6). Treated non-infected fish showed significant increase in total protein concentration compared to the control (Table 3).

Groups	Time of Sampling	IL1β (pg/ml)	IL10 (pg/ml)	NO (µmol/L)	MDA (nmol/L)	TAC (mM/L)
Control (G1)	2 days	10.02 ± 0.06 ^{d}	23.16 ± 0.94 ^{d}	25.00 ± 0.72 ^{d}	14.83 ± 0.20 ^{d}	1.70 ± 0.06 ^{a}
	10 days	9.53 ± 0.08 ^{d}	23.05 ± 0.66 ^{d}	25.40 ± 1.17 ^{d}	14.16 ± 0.55 ^{d}	1.77 ± 0.09 ^{a}
Biogen (G_2)	2 days	10.05 ± 0.10 ^{d}	24.10 ± 0.36 ^{d}	23.46 ± 1.90 ^{d}	14.98 ± 0.30 ^{d}	1.67 ± 0.05 ^{a}
	10 days	9.95 ± 0.09 ^{d}	24.05 ± 0.30 ^{d}	22.50 ± 0.90 ^{d}	15.23 ± 0.40 ^{d}	1.69 ± 0.04 ^{a}
Ropadiar (G_3)	2 days	10.06 ± 0.02 ^{d}	23.17 ± 0.41 ^{d}	22.24 ± 0.70 ^{d}	14.88 ± 0.75 ^{d}	1.70 ± 0.03 ^{a}
	10 days	9.77 ± 0.02 ^{d}	23.10 ± 0.22 ^{d}	22.51 ± 0.77 ^{d}	15.23 ± 0.60 ^{d}	1.77 ± 0.01 ^{a}
Infected (G_4)	2 days	26.78 ± 0.39 ^{a}	51.08 ± 0.84 ^{a}	78.50 ± 2.29 ^{a}	43.64 ± 1.23 ^{a}	0.70 ± 0.007 ^{d}
	10 days	21.24 ± 0.33 ^{a}	38.16 ± 1.03 ^{a}	80.50 ± 1.24 ^{a}	36.75 ± 1.26 ^{a}	0.90 ± 0.09 ^{d}
Biogen infected (G_5)	2 days	18.76 ± 1.18 ^{b}	38.18 ± 0.96 ^{b}	66.00 ± 1.16 ^{b}	35.14 ± 1.13 ^{b}	0.87 ± 0.01 ^{c}
	10 days	16.23 ± 0.87 ^{b}	31.48 ± 0.41 ^{b}	71.00 ± 1.14 ^{b}	31.38 ± 2.27 ^{b}	1.023 ± 0.02 ^{c}
Ropadiar infected (G_6)	2 days	15.72 ± 1.54 ^{c}	29.93 ± 0.72 ^{c}	54.50 ± 1.14 ^{c}	26.58 ± 0.42 ^{c}	1.12 ± 0.04 ^{b}
	10 days	13.23 ± 0.84 ^{c}	26.22 ± 0.44 ^{c}	63.49 ± 1.23 ^{c}	20.30 ± 0.43 ^{c}	1.39 ± 0.02 ^{b}

Table 4: *Serum immunological and antioxidant parameters in different experimental groups at 2 and 10 days post-infection.*

A. hydrophila infection elevated the serum level of both IL1β and IL10 in G_4 when compared to the G_1. Infected treated fish showed a significant decline in comparison to G_4. Regarding anti-oxidant parameters, *Aeromonas* infected fish (G_4) showed a significant increase in NO, MDA and a significant decrease in TAC in comparison with the control group (G_1). Infected treated groups (G_5 and G_6) showed a significant decrease in NO, MDA and significant increase in TAC compared with the infected group (G_4) (Table 4).

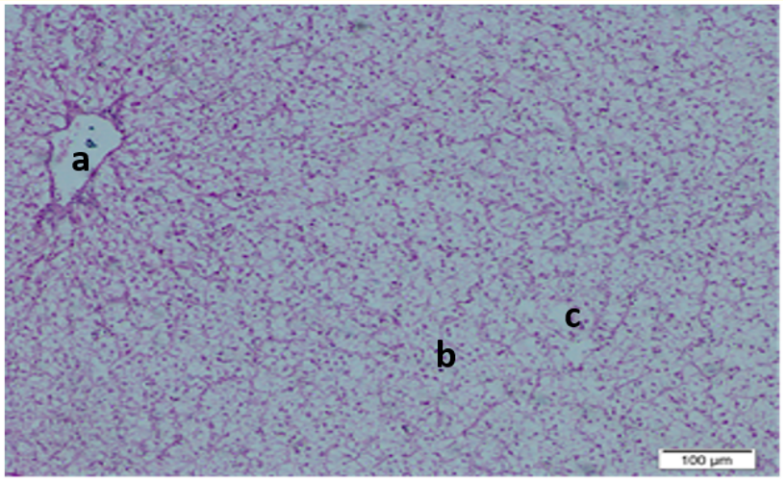

Figure 1: *Liver of catfish (C. gariepinus) infected group, showing a) Hemolysis of erythrocytes, b) Vacuolar degeneration, c) Coagulative necrosis of hepatocytes. H&E stain, X 250.*

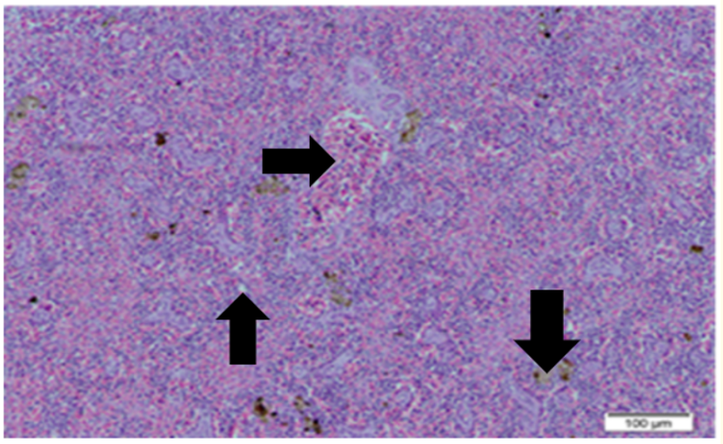

Figure 2: *Spleen of catfish (C. gariepinus), infected group, showing congestion, hemolysis of erythrocytes, degeneration as well as necrosis of melanomacrophage cells. H&E stain, X 250.*

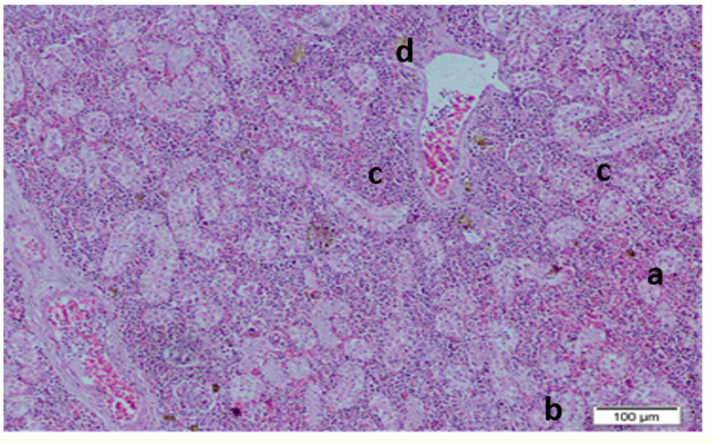

Figure 3: *Kidney of catfish (C. gariepinus) infected group showing a) Tubular nephrosis, b) Coagulative necrosis of renal tubule, c) Focal alternative hyperplasia or depletion, d) Necrosis of some melanomacrophage cells. H&E stain, X 250.*

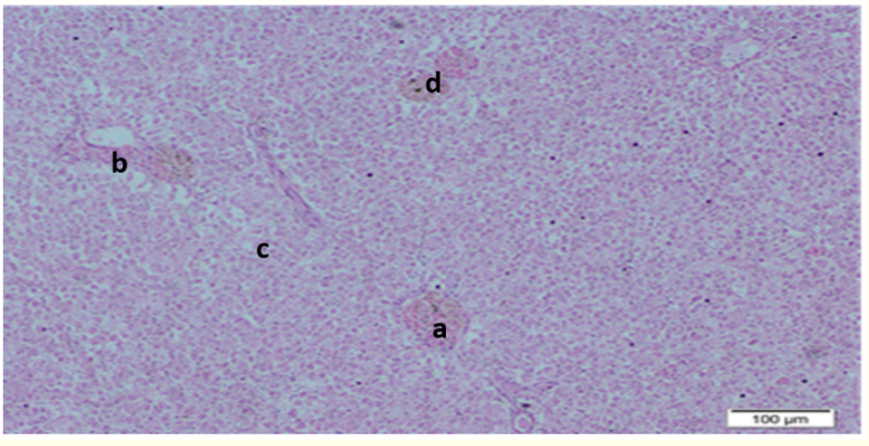

Figure 4: *Liver of catfish (C. gariepinus), Biogen infected group, showing a) Congestion in the hepatic vessels, b) Perivascular mononuclear cell infiltration, c) Vacuolar degeneration of hepatocytes, d) Early activation of melanomacrophage center. H&E stain, X 250.*

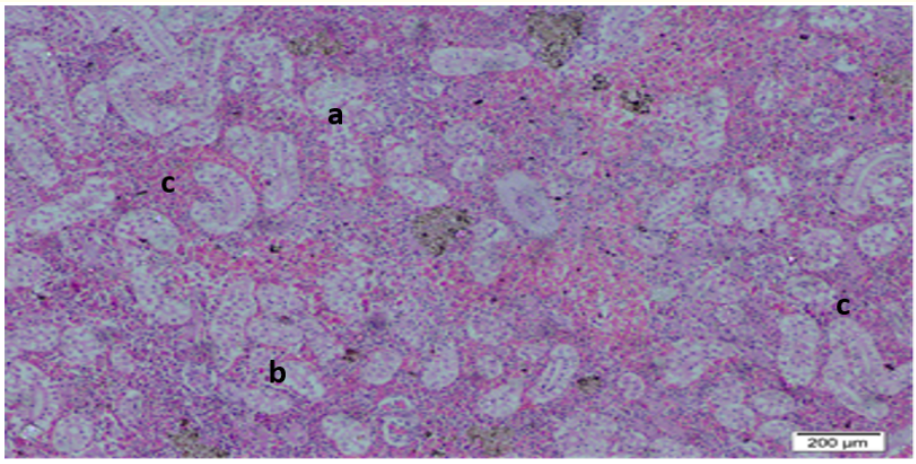

Figure 5: *Kidney of catfish (Clarias gariepinus), Ropadiar infected group showing a) Tubular nephrosis, b) Coagulative necrosis of renal tubule, c) Focal alternative hyperplasia or depletion. H&E stain, X 250.*

The histopathological studies, in *Aeromonas* infected group showed congestion, hemolysis of erythrocytes, vacuolar degeneration as well as coagulative necrosis of hematopoietic tissues (Figure 1-3). While, those treated with Biogen and Ropadiar showed hyperplasia of hematopoietic tissue, tubular nephrosis and activation of melanomacrophage center. The liver showed congestion with mononuclear cell infiltration. Vacuolar degeneration of most hepatocytes has been observed in Biogen treated group (Figure 4 and 5). The spleen showed proliferation of lymphoid follicles and activation of melanomacrophage center.

Discussion

In the present study, *A. hydrophila* infected group demonstrated sluggish motion and swimming close to the water surface at the terminal stage, greyish white lesion, hemorrhagic patches a long with fin erosions and skin ulceration. Cahill (1990) [30] attributed fish hemorrhagic septicaemia and mortality to the toxins, proteases, haemolysins, cholinesterases, enterotoxins and endotoxins generated by *A. hydrophila.*

The hematological investigation revealed a decrease in the number of RBCs count, concentration of Hb and percentage of PCV in the *Aeromonas* infected groups than the normal range. This can be attributed to the severe hemolytic activity of aerolysin and β-hemolysin produced by *A. hydrophila,* which have lytic activities on RBCs leading to anemia [31]. The reduced hemoglobin concentration may be as a result of swelling of RBCs as well as poor mobilization of Hb from the spleen to other hematopoietic organs [32]. Similar results have been recorded in *Channa striatus* [33], and in *Labeo rohita*, post challenge with *A. hydrophila* [34]. Our results were proved by the histopathological results in which the spleen showed congestion, hemolysis of RBCs, degeneration and necrosis of melanomacrophage cells.

Biogen treated group (G_2) revealed a marked rise in RBCs, Hb and PCV relative to the control. Our findings agreed with Agouz and Anwer [35], which linked the higher values to the presence of *B. subtilis* that could enhance immune response and increase the vitality of cells by supplying oxygen to the entire body. Our result came in accordance with the histopathological results, where the spleen of treated groups exhibited dilatation of splenic vessels, a proliferation of lymphoid follicles and activation of melanomacrophage center.

Our results showed a significant increase in WBCs, lymphocytes, monocytes and eosinophils in *Aeromonas* infected groups, at 10 days post-infection. The enhanced WBCs would result from induction of the non-specific defense system [36] and/or enhanced phagocytosis

and cytotoxic activity [37]. Contrarily, Rafiq., *et al*. [38] did not notice any alteration in the differential counts of white blood cells in Tilapia challenged with *A. hydrophila*. The lymphocytosis can be attributed to the antigenic stimulation with enhanced T-lymphocytes by bacterial infection [39]. In comparison with the control, there was significant lymphocytosis in Biogen and Ropadiar treated groups (G_2, G_3). Nearly, same findings were achieved in catfish provided with Biogen [40] and in fish after feeding on thymol and carvacrol diets [41].

Regarding the liver enzyme activities, *Aeromonas* infected group (G_4) showed significantly higher ALT and ALP activities than the control group (G_1). Our results came in harmony with the liver pathology, which displayed vacuolar degeneration as well as coagulative necrosis of hepatocytes. This can be ascribed to toxins and extracellular products; hemolysin, protease, and elastase, produced by *A. hydrophila,* which cause severe necrosis in the liver [42], leads to an extensive release of these enzymes to the blood circulation [43]. Increased serum ALT activity was also recorded in Nile catfish experimentally infected with *A. hydrophila* [44]. The lower values of serum ALT and ALP have been observed in G_5 and G_6. Similar protective effect of *B. subtilis* and carvacrol and thymol on hepatocytes against severe damage, have been reported in fish infected with *A. hydrophila* [45,46]. Our histopathological results showed vacuolar degeneration, perivascular mononuclear cell infiltration and early activation of melanomacrophage center.

Furthermore, the supplementation of Biogen and Ropadiar (G_2 and G_3), significantly decrease the serum ALT and insignificantly decrease the serum ALP activity compared to the control group (G_1), at 10 days post-infection. Significantly lower ALT activity was observed in tilapia fed Biogen [47] and tilapia treated with *Origanium vulgare* extract [48]. The lower value of serum ALT and ALP in Biogen and Ropadiar treated groups, before and after infection, stated the positive protective impact of feed additives, Biogen and Ropadiar on the maintenance of hepatocytes integrity.

Data of serum protein gram showed a significant decrease in total protein, albumin concentration and Albumin/Globulin (A/G) ratio in *Aeromonas* infected groups (G_4, G_5 and G_6) with a non-significant increase in globulin concentration, at 10 days post-infection. The decreased serum total protein may be due to vascular leakage as a result of increased permeability following the release of histamine [49,50] or liver damage or non-specific proteolysis [51] or albumin losses from skin lesions [52]. However, the application of *Bacillus* sp. could significantly increase total protein, albumin and globulin concentration during challenge with *A. hydrophila* [53]. The Biogen and Ropadiar (G_2 and G_3) treated groups showed a significant rise in the serum level of total protein at 10 days post-infection. This may be as a result of enhancing of the non-specific immune response of fish [54]. Higher serum protein and globulin content were recorded in fish provided with *B. subtilis* [55], *Origanium vulgare* extract [48].

Concerning the results of renal function test, there were a significant increase of creatinine in the infected group compared to the control group that indicated alteration induced by bacterial toxins in normal kidney physiology [39]. This came in accordance with tubular nephrosis, coagulative necrosis of renal tubule and focal alternative hyperplasia or depletion of renal tissue. In contrast, a significant reduction in creatinine level was noted in Atlantic salmon suffering from cold water vibriosis [56]. A significant decline in creatinine concentration was noticed in Biogen and Ropadiar infected groups (G_5, G_6) compared to the non-treated infected group (G_4), 10 days post-challenge. These results were proved by histopathological results of kidney tissues of both groups displayed marked proliferation of hematopiotic tissue and melanomacrophage cells.

Examining the expression of cytokines, the *Aeromonas* infected group revealed significantly elevated serum levels of IL-1β and IL10, at 10 days post-infection. This may be attributed to *Aeromonas* cytotoxic enterotoxin or lipopolysaccharide (LPS), a component of Gram-negative bacteria that stimulate the expression of both Tumor necrosis factor- α (TNF-α) and IL-1β in fish [57,58]. These cytokines are the main molecules in the initiation of inflammation, immune response and induction of other cytokines (e.g. IL-6, IL-8 and IL-10) [59]. IL10 is expressed in rainbow trout and carp, soon after LPS stimulation [60].

The Biogen and Ropadiar infected groups showed a significant decrease in IL-1β and IL-10 as compared to the infected group. This may be due to the inhibitory impact of the carvacrol in essential oils and *B. subtilis*, on the expression of proinflammatory cytokines, in LPS -stimulated murine macrophage RAW 264.7 cells [61,62]. In consistent with our study, Lima., *et al.* [63] found that treatment of mice with carvacrol attenuated the paw edema and decreased the IL-1β mRNA expression. However, oral intubation of *B. subtilis* could reduce *A. hydrophila*-induced intestinal mucosal barrier damage and inflammation by restricting up-regulation of IL-1β mRNA levels [64].

The *Aeromonas* infected group showed a significant increase in serum level NO, MDA and significant decrease in TAC when compared to the control group. Also, higher serum NO activity was recorded in rainbow trout infected with *Renibacterium salmoninarum* [65]. This may be due to bacterial products such as lipopolysaccharide are powerful inducers of inducible nitric oxide synthase (iNOS), which enhance NO production [66]. The increased MDA levels reflecting enhanced lipid peroxidation and free radical content by pathogen infection [67], resulting in significant declines in antioxidant defenses [68].

The infected treated groups showed significantly lower level of NO, MDA and higher TAC compared to the infected non-treated group. This may be due to, the antioxidant action of phenolic compounds (carvacrol and thymol) of oregano essential oils, against free radicals that inhibit lipid peroxidation [69]. Moreover, the enhanced total antioxidant capacity could be due to the effective protection of *B. subtilis* against oxidative stress damage caused by *A. hydrophila* infection in fish [68].

Conclusion

Dietary supplementation of Biogen and Ropadiar could improve the humoral defence mechanism and anti-oxidant response in fish, which in turn can aid in the control and prevention of *A. hydrophila* infection.

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