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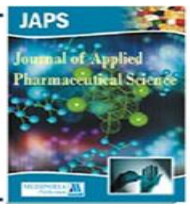

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Haematology of *Cirrhinus mrigala* fed with Vitamin C supplemented diet and post challenged by *Aphanomyces invadens*

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ABSTRACT

Disease outbreak in fish culture system interferes with productivity. Oral Immunostimulant containing vitamin C can result in activating the immune system in a non-specific way thus providing resistance against pathogens. In the present study the fish *Cirrhinus mrigala* was fed with a feed supplemented with vitamin C (100mg/100g) for 40 days and post challenged with two different dilutions (10^2 and 10^5) of *Aphanomyces invadans*. The haematological parameters like TEC, TLC and differential leukocyte counts were analyzed 24hours, 72 hours and 7^{th} day after infection. TEC counts and lymphocyte counts of infected fishes previously fed with control diet decreased significantly (43%, 36%) whereas it was minimal in fishes (21%, 8%) fed with Vitamin C supplemented diet. Post challenged fishes exhibited an increase in TLC in Vitamin supplemented diet (20%, 32%) over control diet fed fishes. In differential leucocyte counts the lymphocytes decreased in both the experimental groups (9%, 11%) whereas neutrophils increased in both, however the increase was higher in control diet fed group (34%). The basophils and eosinophils increased significantly in Vitamin C supplemented diet only (10%, 60%). Thus supplementation of feed with vitamin C enhanced fish's tolerance to infection.

Keywords: Immunostimulant, Haematology, Vitamin C, *Cirrhinus mrigala*, *Aphanomyces invadens*.

INTRODUCTION

Fishes are considered as one of the important food sources for human beings because their flesh contains a high percentage of proteins, calcium and phosphorus. So, there is an increased attention given to fish farms and their diseases. Pathogens such as virus, bacteria & fungus are all causes for concern to aquaculturists. In fish farms many of these pathogens cause wide spread mortality. The use of antibiotics to treat bacterial infections has resulted in a global increase of resistance. For this reason, studies are conducted on Immunostimulants which represent a modern and promising tool in aquaculture as they enhance the resistance of cultured fish to disease and stress. It comprises a group of biological or synthetic compounds that enhance the hormonal and cellular response both in specific and non-specific way (Tewary and Patra, 2004). Immunostimulants also stimulate the natural killer cells, complement and lysozyme antibody responses of fish (Sakai, 1999; Tewary and Patra, 2007). Investigation regarding the health status of a fish can be assessed by haematological parameters (Hickey, 1976). By analyzing blood cells, characteristic clues for diagnosis and prognosis of the diseases may be found (Anderson, 2003). Ascorbic acid is an indispensable and multifunctional micronutrient. It plays important roles in improving immune function (Hardie et al 1991), improving growth (Boonyaratpalin et al 2001), providing good health, feed conversion, survival (Khajarern et al,

1997), resisting stress (Henrique et al., 1998) and oxidation (Shiau et al., 2002). Most fish species are not capable of vitamin C biosynthesis (Chatterjee, 1975) due to the absence of the enzyme L-gulonolactone oxidase necessary for ascorbic acid synthesis (Wilson, 1973). Vitamin C also plays an important role in animal health as antioxidants by inactivating damaging free radicals produced through normal cellular activity and from various stress (Chew, 1995). It has been suggested that the antioxidant function of these micronutrients could enhance immunity by preserving the functional and structural integrity of immune cells. In this respect, the need for specific nutrients may be increased during infection which could require the feeding on diets formulated for optimal immune competence rather than growth and survival. Supplementation of vitamin C enhanced antibody production against *Edwarsiella ictaluri* in channel cat fish (Li and Lovell, 1985).

Aphanomyces invadans is a peronosporomyces fungus associated with the serious fish disease epizootic ulcerative syndrome (EUS) known as mitotic granulomatosis. The pathogen invades the dermis presenting initially as patches but rapidly invading to cause small circular lesions that continue to develop into large necrotic ulcers (Lilley and Roberts, 1997).

The present study is aimed to evaluate the Immunostimulant potential of Vitamin C in the fish *Cirrhinus mrigala* post infected with *A.invadans* by analyzing the haematological parameters.

MATERIALS AND METHODS

The experimental fish *Cirrhinus mrigala* (weight 45±5g) were purchased from local fish farm and allowed to acclimate to laboratory conditions for 15 days. During acclimatization they were fed with rice bran and groundnut oil cake *ad libitum.* During the experimental period the water quality variables: temperature (28±1^{o}C), pH (7.4±0.2), salinity (10±2) and dissolved Oxygen (>5mg-^{l}) were recorded. The water was changed daily in order to maintain the fishes in healthy state.

Feed Preparation

The basic diet (Control diet) was prepared by mixing Rice bran 10g, Wheat bran 10g, Soya flour 23g, dry fish meal 24g, Ground nut oilcake 23g and Tapioca flour 10g made as a dove, sterilized in pressure cooker for 30 minutes, cooled and made in the form of noodles by adding a little amount of sunflower oil which are then shade dried, broken into small desirable sized pieces. The Immunostimulant diet was prepared using the same composition of ingredients to which vitamin C (100mg/100g) was added after sterilization process.

The fishes were primarily divided into three experimental groups. The experimental group I was kept as control group which were fed with control diet and were not infected. The experimental group II was also fed with control diet further subdivided into two groups one received intraperitonial injection of 10^2 dilution of *A.invadans* and the other group received 10^5 dilution of *A.invadans*. Similarly the experimental group III fishes which were fed with Immunostimulant diet were also further subdivided into two groups one received 10^2 dilution of *A.invadans* and the other group received 10^5 dilution of *A.invadans*. The fishes were fed for 40 days with their respective feeds and then post challenged with selected doses of (10^2 and 10^5) dilution of *A.invadans*. The haematological analyses were carried out 24hours, 72 hours and 7th day after infection. The experiment was carried out in triplicate.

Haematological Analysis

After the experimental period blood was collected from the fishes by cutting the caudal peduncle and the blood was collected in heparinized tubes. All analysis was performed on pooled blood samples. Total Erythrocyte Counts (TEC), Total Leucocyte Counts (TLC) were counted using Haemocytometer with improved Neubaurer ruling chamber (Weber & sons, England), Blood smears stained with May-Grunewald's Giemsa's stain was used for differential leucocytes count. The data were analyzed statistically and students "t" test was used to test their significance.

RESULTS AND DISCUSSION

The Total Erythrocyte count (TEC) of the experimental group II &III (i.e., infected) decreased when compared to control uninfected fishes. The decrease was highly significant in control diet fed infected fishes (Group II) than Immunostimulant diet fed fishes in all the experimental duration and in both concentration of infection (Table 1).

Table 1: Haematological parameters of *C.mrigala* administered with control and Immunostimulant diet post challenged with *A.invadans.* (Values are Mean±SD of three samples).

Parameter	Duration	Uninfected	Infected			
			Control Diet		Immunostimulant Diet	
			C-1	C-2	I-1	I-2
TEC [x 10^{6}/mm^3]	24 hrs	0.78±0.1	0.56±0.3	0.69±0.4	0.67±0.5	0.71±0.5
	72 hrs	0.74±0.3	0.59±0.6	0.63±0.5	0.71±0.3	0.81±0.4
	7th day	0.76±0.4	0.14±0.4**	0.12±0.3**	0.44±0.2*	0.59±0.3*
TLC [x10^{4}/mm^3]	24 hrs	1.42±0.03	1.18±0.02	1.36±0.05	1.22±0.03	1.53±0.02
	72 hrs	1.38±0.02	1.29±0.04	1.58±0.03	1.88±0.04*	1.99±0.04*
	7th day	1.39±0.04	1.59±0.03	1.67±0.02	1.94±0.02*	2.07±0.06*
Lymphocytes[%]	24 hrs	45±3	43±4.2	42±5.3	43±4.2	44±3.5
	72 hrs	43±4	38±3.3*	37±3.2*	40±3.8	41±3.8
	7th day	47±6	43±4.5	42±4.2	40±4.1	36±4.1*
Neutrophils [%]	24 hrs	23±3	30±2.2*	33±3.4**	35±3.2**	31±3.2*
	72 hrs	25±2	31±3.4*	29±3.3	22±2.2	23±2.8
	7th day	21±3	32±4.2*	30±4.2*	31±2.3*	27±3.1
Monocytes[%]	24 hrs	19±2	12±2.1*	14±2.2	12±2.1*	14±2.1
	72 hrs	17±3	21±2.2*	23±3.1*	18±3.2	16±1.8
	7th day	18±2	15±1.4	16±2.3	17±2.4	14±1.4
Basophils[%]	24 hrs	9±1	6±1.2	4±1.4*	4±1.3*	7±0.4
	72 hrs	10±2	6±2.0	8±2.1	10±1.2	11±0.6
	7th day	9±2	7±1.2	9±2.2	8±1.3	13±1.2*
Eosinophils[%]	24 hrs	4±1	9±2.1*	7±1.4*	6±0.6	4±0.4
	72 hrs	5±2	4±1.0	3±1.0	9±0.4*	10±1.4**
	7th day	6±2	3±1.0	3±1.0	4±0.3	10±2.2**

* = Significant, **=Highly significant
C-1 = Control diet fed fishes infected with 10^2 dilution of *A.invadans*
C-2 = Control diet fed fishes infected with 10^5 dilution of *A.invadans*
I-1 = Immunostimulant diet fed fishes infected with 10^2 dilution of A.invadans
I-2 = Immunostimulant diet fed fishes infected with 10^5 dilution of A.invadans

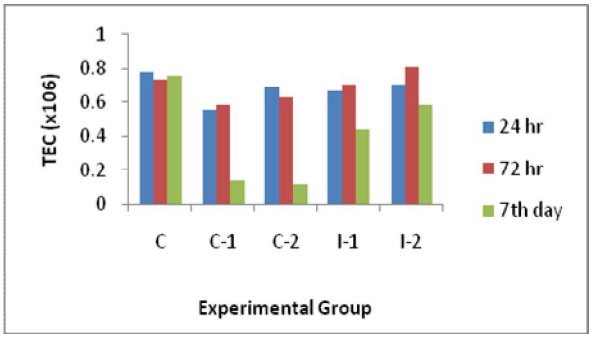

Fig. 1: Comparison of TEC.

Further in Immunostimulant diet fed fishes after 24 hours of infection the decrease was milder and during 72 hours of exposure recovery was observed, however during 7th day significant decrease was observed (Fig.1). Mousa and Khattab (2003) found a decrease in RBCs, Hb and Hct in the blood of the African catfish (*Clarias gariepinus*) after ochratoxin intoxication. Similarly Nile Tilapia (*Oreochromis niloticus*) when fed with Ochratoxin (400 and 600µg/kg diet) exhibited significant reduction in all blood parameters including RBC counts whereas there was no significant decrease in the fishes fed with ochratoxin+vitamin C(Adel M.E Shalaby) . In the present experiment decrease in the RBC counts in infected fishes fed with control diet may be attributed to destruction of mature RBCs and inhibition of erythrocyte production due to reduction of haem synthesis by fungal infection or it may be due to elimination of RBCs from circulation as a result of fungal infection induced extravasation of the blood. These results are in agreement with those of Ramadevi et al (1998) who found a decrease in RBCs, Hb and Hct in the blood of broiler chicks after ochratoxin intoxication. Fabiana et al (2007) observed that Vitamin C and E is essential for the protection of erythrocytes.

In the Total Leucocyte count (TLC) it was observed that a phenomenal decrease in control diet fed infected fishes during 24 hours, recovery during 72 hours and a marginal increase on 7th day. Whereas in Immunostimulant fed fishes minimal decrease in higher concentration of pathogen infection (I-1) that too only during 24 hour of exposure and there after increased steadily in both concentrations of the pathogen (Fig.2). The results agree with the works of Innocent et al (2004), Pandey et al (2000) and Rauthan et al (1995) which indicate improved resistance to infection.

In differential leucocyte count it was observed that the lymphocytes decreased in all the infected fishes but the decrease was minimal in Immunostimulant diet fed fishes. On the other hand highly significant increase in neutrophil population has been observed in all the infected groups during 24 hours after infection which remained high on 7th day also in control diet fed infected fishes but recovery to normalcy during 72 hour after infection itself in Immunostimulant diet fed fishes, thus enabling to recover faster from stress.

The monocytes population exhibited significant decrease in all the infected fishes after 24 hours of infection, thereafter fluctuated erratically in control feed fed fishes whereas recovered to normalcy in Immunostimulant fed fishes. The basophils decreased in control diet fed fishes whereas minimal increase was observed in Immunostimulant diet fed fishes. No much change was observed in the eosinophil counts of control diet fed fishes except during 24 hour of infection. Whereas the population remained high in Immunostimulant diet fed fishes. When *Piaractus mesopotamicus* was fed with diets supplemented with vitamin C and E, challenged by *Aeromonas hydrophila* it was observed that the numbers of total leukocyte counts, lymphocytes and eosinophils decreased, while the numbers of neutrophils and monocytes increased (Fabiana et al 2007). This is in agreement with the present experiment. Neutrophilia and monocytosis can be attributed to acute inflammatory response due to infection. Vitamin C is a potent Immunostimulant that enhances lymphocyte function (Head 1998). Monocytes undergo transformation into macrophages and may be involved in phagocytosis and killing of pathogens upon first recognition and subsequent infections. Similar observation has been reported by Shoemaker et al (1997). Granulocytes are the primary cells involved in initial stages of inflammation considered by Manning (1994). Our observation also shows an increase in the number of granulocytes due to inflammatory response in infected fishes.

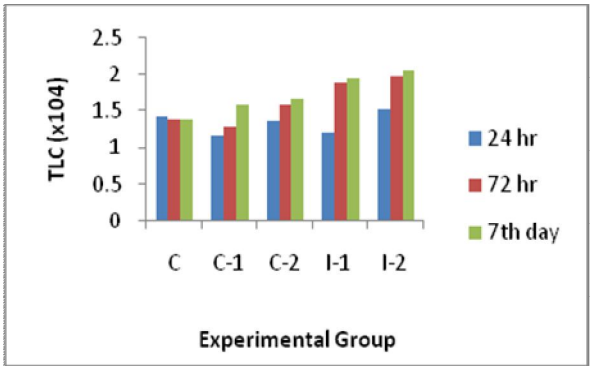

Fig. 2: Comparison of TLC.

CONCLUSION

From the present study it was observed that supplementation of feed with Immunostimulant (Vitamin C) improved the Total leucocyte counts and granulocyte population which nonspecifically helps to minimize infection induced stress, improved resistance against infection and faster recovery from stress. As many fishes cannot synthesize vitamin C it can be incorporated in fish feed to enhance fish health.

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