

Original Article

Efficiency of Levamisole in Improving the Immune Response of Catfish (*Clarias gariepenus*) to *Aeromonas hydrophila* Vaccine: Clinico-Pathological Studies

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Abstract

The effect of Levamisole on the immune response of 200 catfish (*Clarias gariepenus*) was evaluated. Fish were divided into 4 equal groups, reared in glass aquaria and fed on a basal diet. The 1st group served as a control. Fish of the 2nd group were fed Levamisole incorporated with diet (150mg/kg diet fed-1) for 2 months. The 3rd and 4th groups were vaccinated using *Aeromonas hydrophila* bacterin, at the first day of experiment, but fish of 4th group were fed on diet containing same dose of levamisole throughout the 2 months of experiment. At 1st, 3rd and 5th week, blood samples were collected from all groups for hematological, immunological and serum biochemistry studies while fish of 2nd group were subjected to histopathological investigations. Challenge was done to all groups, at 30th day of experiment, through immersion of fish in virulent *Aeromonas hydrophila* (1 g bacterial cells/liter) for 10 min, the mortalities recorded and the dead fish used for the bacterial re-isolation. Catfish of 1st group showed normal values throughout the period of the experiment. Fish of 2nd group showed activation of melanomacrophages. Hyperplasia of hematopoietic tissue was evident at 3rd-4th week. Degenerative changes were mild at 1st-2nd week and became prominent at 5th week. Fish of 3rd group showed a significant gradual increase of leucocytes, phagocytosis, antibody titer, total protein and globulin, at 1st-5th week post-vaccination. Fish of 4th group showed higher hematological and immunological values than those of 3rd group. The relative level of protection after the challenge infection was 30, 85 and 90% for the 2nd, 3rd and 4th groups; respectively. The histopathological finding of this experiment showed the safety of the selected dose of levamisole on catfish. The other findings indicate its efficiency as immunostimulant in improving the immune response of catfish to *Aeromonas hydrophila* vaccine.

Key Words: Levamisole, Cat fish (*Clarias gariepenus*), Hematological, Immunological, Serum biochemistry.

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Introduction

Fish culture is an important industry where the production of aquacultured fish worldwide increases every year. Diseases outbreak are encountered in the rapidly developing aquaculture industry and affecting the economic development of this sector (Yunxia, et al. 2001). Various chemotherapeutics have been used to treat bacterial infections in cultured fish during the last 20 years. However, the incidence of drug-resistant bacteria has become a major problem in fish culture (Aoki, 1992). Vaccination is a useful prophylaxis for infectious diseases of fish but the development of vaccine against intracellular pathogens has not so far been successful. Therefore, the immediate control of all fish diseases using only vaccines is impossible (Sakai, 1999). The most effective method, in our opinion, may be the development of natural disease resistance in fish, where, the use of immunostimulants may be an effective mean of increasing the immune competency and disease resistance of fish.

Levamisole hydrochloride corrects the immunologic imbalance by modifying activities of T-lymphocytes and phagocytes. It stimulates cell-mediated immune reactivity by potentate the rate of T-lymphocytes differentiation, responsiveness to antigens and mitogens, and activity of effectors lymphocytes (Booth and McDonald, 1982).

The present work was designed to study the effect of Levamisole on the health status and immune response of catfish (*Clarias gariepenus*) through vaccine administration together with histopathological and clinicopathological investigations.

Materials and Methods

Two hundred catfish (*Clarias gariepenus*), each of 150±15g body weight, were divided into 4 equal groups (each of 50 fish and 5 replicates). Fish reared equally in a 20 glass aquaria (50x60x70cm each) with water temperature maintained at 22+2°C and the fish fed on a

basal diet of 25% protein along the period of experiment. Water was partially renewed daily and monitored for water quality weekly. The 1st group served as a control. Fish of the 2nd group were fed levamisole incorporated with diet (150 mg/kg diet fed-1) for 2 months. The 3rd and 4th groups were vaccinated at the first day of experiment, but fish of 4th group were fed on diet containing same dose of Levamisole throughout the 2 months of experiment. Vaccination of fish in 3rd and 4th groups was carried out using *Aeromonas hydrophila* bacterin, in a dose of 0.1 ml formalin-killed bacterial cells/fish, via intraperitoneally (I/P) route.

Levamisole Hydrochloride: (Sigma chemical company) is a synthetic imidazothiazole derivative. It is the L-isomer of DI-tetramisole. The *Aeromonas hydrophila* was supplied kindly from The World Fish Center, Egypt while *E. coli* kindly supplied from the Department of Microbiology, Animal Health Research Institute, Dokki, Giza, Egypt and used for the evaluation of phagocytic percentage.

Vaccine prepared by cultivation of *Aeromonas hydrophila* strain in Trypticase soya broth (TSB) that incubated at 35°C for 48 hours. The bacterial cultures were inactivated by the addition of formalin to give a final concentration of 0.3% and were held at room temperature overnight. The broth culture was centrifuged at 6000 rpm for 15 min and washed three times with sterile saline solution. The preparation was held at 4°C until used. Safety test was performed according to Cardella and Eimers (1990). The safety test was performed by the intra-peritoneal (I/P) inoculation of 20 susceptible catfish with bacterin. The fish were kept under observation for 2 weeks post-injection. Sterility test was done by cultivation of the prepared vaccine on Rimler-Shotts agar and Trypticase soya agar and then incubated at 37°C for 24 hrs. The cultures were examined for bacterial growth.

The following values were estimated, at the end of 1st, 3rd and 5th weeks, in the blood and serum

of experimental fish; total red and white blood cells (Natt and Herrick, 1952 and Miller and Seward, 1971), blood hemoglobin (Drabkin, 1948), packed cell volume (Wintrobe, 1967), blood indices (Latimer, et al. 2003), the percentage and absolute values for different leukocytic cells (Jain, 1986), specific antibody titers (Baba, et al. 1993), phagocytosis (Torky and Diallo, 1983 and Leiboled, 1981), fractionation of serum proteins (Karcher and Landers, 2006), serum total proteins (Weichselbaum, 1946), serum level of aspartate aminotransferase (AST) and alanine aminotransferase ALT (Bergmeyer, et al. 1986), serum creatinine (Bartels, et al. 1972), serum uric acid (Caraway, 1963). Also, tissue specimens were collected from fish of group 3 and histopathological technique was done according to Drury and Wallington (1980) to evaluate the safety of the used levamisole dose.

Based on the recorded immune parameters of the first experiment the best response was seen after one month of levamisole application, same experiment repeated one more time using same number of catfish and artificial infection of all groups was done at 30th day of the experiment by immersion of 20 catfish from each group in diluted broth culture of virulent *Aeromonas hydrophila* (1:5 in 0.5% sodium chloride solution to give 1 g bacterial cells/liter) for 10 min. The challenged fish were kept under observation for 4 weeks and the dead ones were used for *Aeromonas hydrophila* re-isolation, meantime and the relative level of protection (RLP) among the challenged fish was estimated (Newman and Majnarich, 1982).

RLP= 100- percent of immunized mortality÷percent of control mortalityx100.

Statistical Analysis:

This was performed using one way and two ways analyses of variance (ANOVA). Duncan's

Multiple Range Test (Latimer, et al. 2003) was used to determine differences among treatments (mean at significance level of P <0.05). Standard errors were also estimated. Analysis was carried out using the SAS package (SAS, 1996).

Results

The experimented catfish showed no significant differences in the total erythrocytic count and other erythrocytic parameters between different groups (Table 1). The total leukocytic count was not significantly increased in levamisole group and significantly increased in both vaccinated and levamisole vaccinated groups, the increase was referred to the increase in lymphocytes (Table 2). A significant increase in the total protein and globulin was noticed in the vaccinated and levamisole vaccinated groups, the increase was varied with the period of experiment (Table 3). A significant increase in the AST, ALT, urea and creatinine was seen during some times of experiment in the levamisole vaccinated group (Table 4). The antibody titers was significantly increased in vaccinated and levamisole vaccinated groups while the phagocytic percentage was significantly increased in the levamisole vaccinated group, the degree of significance was varied with the time of experiment (Table 5).

The histopathological studies, in the group treated with levamisole, revealed activation of melanomacrophages and hyperplasia of hematopoietic tissue and tubular nephrosis mainly vacuolar degeneration in the kidney. The liver showed congestion and marked activation of melanomacrophages. Some of the hepatic cells exhibited pyknotic nuclei. Spleen showed activation of melanomacrophages centers and proliferation of lymphocytes within the lymphoid follicles (Figures 1-4).

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Table 1: Erythrocytic values in experimented catfish groups (mean+S.E):

Group	Period (week)	RBC (10 ⁶ /μL)	Hb (g/dL)	PCV (%)	MCV (fL)	MCH (Pg)	MCHC (%)
1.Control	1 st	2.562±0.025	9.825±0.035	28.850±0.050	112.47±3.40	38.304±2.1	34.055±0.21
	3 rd	2.505±0.036	9.685±0.015	28.250±0.018	112.77±1.5	38.66±0.97	34.28±0.52
	5 th	2.805±0.018	10.205±0.015	29.07±0.035	103.63±2.11	36.38±0.41	35.10±0.75
2.Levamisole	1 st	2.621±0.26	9.895±0.31	28.980±0.43	110.56±1.24	37.75±0.39	34.14±0.14
	3 rd	2.485±0.26	9.630±0.34	28.326±0.46	113.987±1.3	38.75±0.41	33.40±0.51
	5 th	2.840±0.31	10.190±0.29	28.99±0.48	102.08±1.2	35.88±0.52	35.15±0.64
3.Vaccinated	1 st	2.502±0.16	9.585±0.48	27.45±0.72	112.77±5.3	38.204±3.2	34.755±0.21
	3 rd	2.595±0.15	9.745±0.39	29.55±0.46	113.97±2.8	37.46±1.2	33.68±0.26
	5 th	3.005±0.33	10.475±0.01	30.87±0.17	102.04±1.4	34.38±0.16	34.255±0.31
4.Levamisole and Vaccinated	1 st	2.567±0.46	9.672±0.81	27.632±0.51	107.64±2.10	37.67±0.37	35.00±0.43
	3 rd	2.625±0.17	9.764±0.38	29.380±0.16	111.92±1.2	37.19±0.93	33.23±0.49
	5 th	3.059±0.28	10.450±0.34	30.52±0.27	99.77±1.1	34.16±0.69	34.23±0.48

*Significant at P <0.05, **Highly Significant at P <0.01

Table 2: Total and differential leukocytic count in experimented catfish groups (mean+S.E):

Group	Period (week)	T.L.C. (10 ³ /μL)	Neutro. (10 ³ /μL)	Lymph. (10 ³ /μL)	Monocy. (10 ³ /μL)	Eosinoph. (10 ³ /μL)	Basoph. (10 ³ /μL)
1.Control	1 st	24.05±0.050	8.041±0.017	14.241±0.026	0.552±0.002	1.116±0.005	0.101±0.005
	3 rd	25.75±0.023	9.541±0.018	14.421±0.050	0.582±0.001	1.156±0.003	0.111±0.004
	5 th	26.05±0.10	9.741±0.28	13.871±0.13	0.982±0.17	1.346±0.21	0.111±0.02
2.Levamisole	1 st	25.027±0.024	8.334±0.016	14.962±0.037	0.438±0.002	1.192±0.002	0.101±0.001
	3 rd	27.751±0.13	10.067±0.026	15.524±0.11	0.528±0.002	1.352±0.005	0.104±0.002
	5 th	26.215±0.21	10.162±0.37	13.690±0.29	0.960±0.15	1.295±0.11	0.108±0.02
3.Vaccinated	1 st	32.27±0.038*	10.501±0.025	20.041±0.017*	0.472±0.006	1.056±0.002	0.101±0.003
	3 rd	31.18±0.019*	10.501±0.031	18.731±0.036*	0.642±0.006*	1.156±0.001	0.111±0.002
	5 th	29.641±0.23*	9.701±0.40	17.751±0.11*	0.902±0.26	1.176±0.19	0.111±0.03
4.Levamisole and Vaccinated	1 st	36.58±0.12*	11.034±0.019	22.368±0.11*	0.418±0.027	1.659±0.001	0.101±0.002
	3 rd	37.12±0.024**	10.130±0.021	24.852±0.038**	0.823±0.002*	1.214±0.004	0.101±0.001
	5 th	32.451±0.42 *	10.117±0.21	19.810±0.29*	1.251±0.15	1.172±0.03	0.101±0.02

*Significant at P <0.05, **Highly Significant at P <0.01

Table 3: Proteinogram in experimented catfish groups (mean+S.E):

Group	Period (week)	T.P. (gm/dl)	Albu. (gm/dl)	Glob. (gm/dl)	A/G Ratio	α	β	δ
1.Control	1 st	5.980±0.010	2.54±0.01	3.44±0.011	0.738±0.05	1.4±0.02	1.3±0.01	0.7±0.02
	3 rd	6.14±0.12	2.75±0.12	3.39±0.10	0.811±0.09	1.4±0.02	1.29±0.01	0.7±0.02
	5 th	6.38±0.11	2.84±0.12	3.54±0.11	0.802±0.35	1.4±0.01	1.39±0.01	0.75±0.02
2.Levamisole	1 st	6.24±0.028	2.54±0.31	3.70±0.27	0.686±0.15	1.5±0.01	1.45±0.01	0.75±0.02
	3 rd	7.43±0.21	2.74±0.16	4.69±0.13	0.584±0.18	1.84±0.01*	1.65±0.02	1.2±0.01*
	5 th	6.56±0.13	2.83±0.18	3.73±0.11	0.758±0.16	1.41±0.02	1.31±0.01	1.01±0.01
3.Vaccinated	1 st	7.35±0.07*	2.38±0.04	4.97±0.05*	0.478±0.18	2.1±0.02*	1.9±0.02	0.97±0.01*
	3 rd	7.44±0.15*	2.71±0.09	4.73±0.13	0.572±0.05	1.8±0.02*	1.53±0.01	1.4±0.01*
	5 th	7.13±0.09	2.75±0.21	4.38±0.18	0.627±0.15	1.74±0.03*	1.39±0.03	1.25±0.02*
4.Levamisole and accinated	1 st	7.41±.16*	2.58±0.27	4.830±0.38*	0.528±0.13	1.8±0.02	1.77±0.01	1.26±0.01*
	3 rd	8.63±0.26**	2.76±0.29	5.87±0.34**	0.470±0.24	2.50±0.02**	1.77±0.01*	1.60±0.02**
	5 th	7.50±0.29*	2.73±0.20	4.77±0.37*	0.572±0.32		1.68±0.01	1.28±0.02*

*Significant at P <0.05, **Highly Significant at P <0.01

Table 4: Liver enzymes and renal function in experimented catfish along the period of study (mean+S.E):

Group	Period (week)	AST (u/L)	ALT (u/L)	Uric acid (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
1.Control	1 st	21.40±0.30	19.950±0.05	2.02±0.14	3.20±0.017	0.55±0.07
	3 rd	23.41±0.24	17.83±0.38	2.18±0.06	3.20±0.02	0.54±0.03
	5 th	24.30±0.29	18.37±0.42	2.20±0.11	3.19±0.01	0.55±0.03
2.Levamisole	1 st	24.11±0.28	22.43±0.31	2.05±0.03	3.63±0.01	0.58±0.05
	3 rd	24.09±0.21	18.40±0.31	2.19±0.10	3.24±0.04	0.56±0.03
	5 th	24.72±0.31	19.89±0.41	2.31±0.17	3.22±0.01	0.56±0.10
3.Vaccinated	1 st	29.50±0.12*	25.63±0.16*	2.08±0.09	3.42±0.04	0.56±0.01
	3 rd	27.31±0.25*	19.47±0.29	2.29±0.09	3.26±0.05	0.56±0.03
	5 th	25.61±0.37	19.81±0.17	2.29±0.24	3.23±0.01	0.57±0.03
4.Levamisole and accinated	1 st	31.18±0.25*	26.13±0.34*	2.17±0.04	4.27±0.02*	0.59±0.03
	3 rd	26.23±0.26*	19.34±0.24	2.31±0.9	4.26±0.01	0.58±0.02*
	5 th	26.49±0.13	20.07±0.25	2.33±0.22	3.23±0.03	0.57±0.04

*Significant at P <0.05, **Highly Significant at P <0.01

Table 5: Antibody titer and phagocytic percentage in experimented catfish along the period of study (mean+S.E):

Group	Period (week)	Antibody titers	Phagocytic %
1.Control	1 st	2.00±0.02	40.41±0.93
	3 rd	2.00±0.02	41.97±1.07
	5 th	2.00±0.02	40.27±0.92
2.Levamisole	1 st	2.01±0.02	41.17±1.16
	3 rd	2.39±0.04	49.19±1.14
	5 th	2.95±0.01	42.56±0.92
3.Vaccinated	1 st	3.11±0.04	44.20±0.66
	3 rd	8.13±0.03**	47.42±1.03
	5 th	6.72±0.06*	40.27±0.92
4.Levamisole and accinated	1 st	3.21±0.21	51.69±1.07
	3 rd	8.94±0.03*	72.57±1.08**
	5 th	6.83±0.04*	63.25±1.04*

*Significant at P <0.05, **Highly Significant at P <0.01

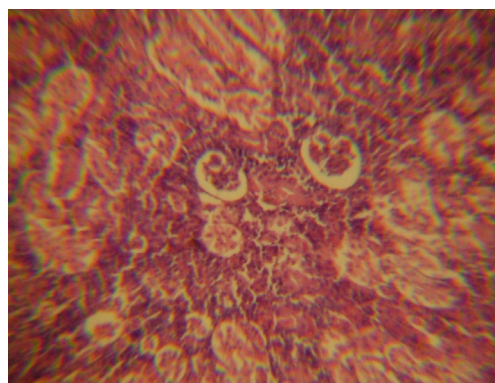


Fig. 1: Kidney, of catfish treated with levamisole, showing hyperplasia of hematopoietic tissue. H&E stain, x250.

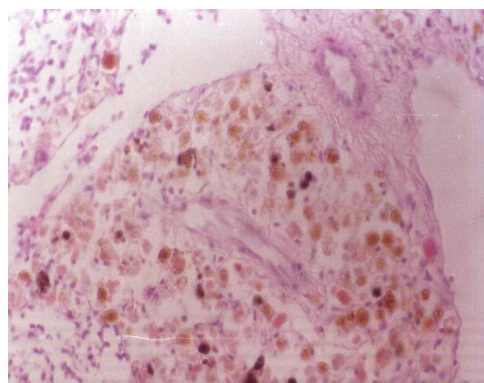


Fig. 3: Liver, of catfish treated with levamisole, showing congestion and marked activation of melanomacrophages. H&E stain, x100.

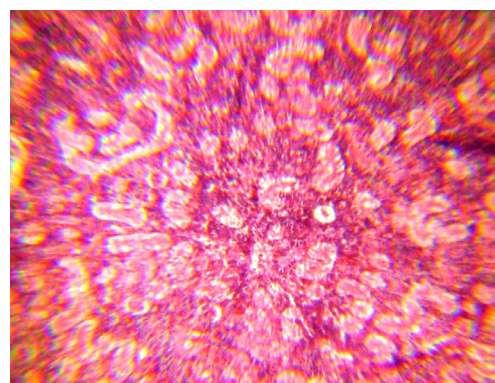


Fig. 2: Kidney, of catfish treated with levamisole, showing tubular nephrosis mainly vacuolar degeneration in the kidney. H&E stain, x100.

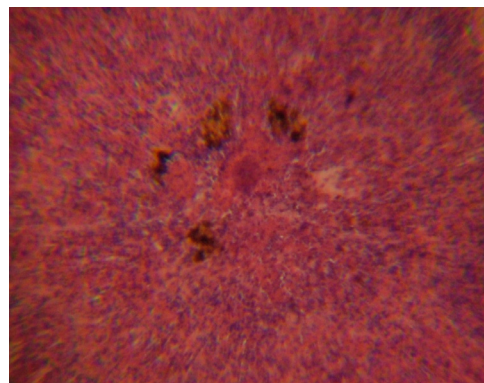


Fig. 4: Spleen, of catfish treated with levamisole, showing activation of melanomacrophages centers and proliferation of lymphocytes. H&E stain, x100.

Discussion:

Levamisole hydrochloride is a water soluble antinematodal drug of broad spectrum activity in various animal species (Jones, et al. 1977). Recently Schmahl and Taraschewski (1987) reported that, levamisole is effective in treating fish parasitized by monogenea. One year later, Taraschewski et al. (1988) stated that, levamisole is a highly potent drug treating *Anguillicola crassus* of eels. Also Hartmann (1989) and Fontaine et al. (1990) reported that, levamisole cleared the parasite (*Anguillicola crassus*) from swim bladder of eel.

Levamisole modulates the immune system and it has the greatest benefit in the immunologically depressed animal (Booth and McDonald, 1982). Levamisole, in the current study, induced no significant changes in the erythrocyte values that may show the safety of levamisole but a significant increase on the total leucocytic count in vaccinated fish was seen (Tables 1, 2). The increase in the total leukocytic count could be due to the stimulating effect of levamisole.

In our results, the addition of levamisole to the diet of catfish at small doses 150mg/kg of diet stimulated the humoral immune response against *Aeromonas hydrophila* bacterin, the increase was significant at the first week and highly significant at the third week of experiment (Table 3). These results are parallel to those reported by Siwicki et al. (1990) who recognized enhancement of specific immune response of rainbow trout indicated by significant increase of the plaque forming cells after injection of *Yersinia ruckeri* O-antigen in combination with small dose of levamisole (10µg/ml). Also in a parallel studies, the application of levamisole to carp diet in small doses enhanced the phagocytic activity in neutrophils and their myeloperoxidase activity, increase the leucocytes number and serum lysozyme levels. (Siwicki, 1987 and 1989). The immune stimulation of levamisole at small doses may be attributed to the activation of

the non-specific immune response particularly macrophages (Fischer, et al. 1975), this activation could enhance the antigen trapping and processing.

The present investigation dealt with the study of the effects of levamisole on fresh water fish as immunostimulant. Among the adverse effects of levamisole HCL are those reported in this work on some liver and kidney function tests as well as on histopathological examination. Levamisole HCL induced a non-significant increase in serum Alanine transaminase enzyme (S.ALT) and Aspartate aminotransferase (S.AST) of catfish treated with levamisole (150mg/kg diet) during the experiment (Table 4). These findings are supported by the reported histopathological alterations of the hepatic tissue. These alterations included perivascular aggregation of lymphocytes and hydropic degeneration of the hepatocytes. These findings are confirmed by the findings reported by Gammaz et al. (1993). El-Bouhi and El-Qelsh (1993) observed aggregation of melanomacrophages in the hepatoportal area and hydropic degeneration in the liver of *Tilapia nilotica* treated with levamisole as a medical bath. Our results revealed that, levamisole induced also a non-significant increase in the serum levels of urea, uric acid and creatinine (Table 4). These findings indicated that the urea, uric acid and creatinine clearance were decreased, the later was found to be due to the renal impairment (Benjamin, 1961). The increased plasma urea in this study was in accordance with Kaneko (1980) who mentioned that, increased urea production can occur in a variety of conditions such as renal shutdown resulting in insufficient urea excretion. A hypothesis appears to be accepted in our case since the kidney suffered from some degenerative changes as mentioned before. This suggestion was supported by the reported histopathological changes in this work on the kidney of catfish exposed to levamisole where tubular nephrosis was evident.

Levamisole induced non-significant increase of the antibody titer (Table 5). This agreed with the findings expressed by Zhang et al. (1999) who noticed that levamisole enhanced B-lymphocyte differentiation, and supported by Sun et al. (2003), they suggested that levamisole may modulate serum interleukin-6 (IL-6) level which is according to Sheehan (1997), secreted by TH2 (T-helper-2) and can promote B-cell activation, proliferation and differentiation into antibody producing plasma cells.

El-Bouhi and El-Qelsh (1993) revealed that, a medical bath treatment of fish (*Tilapia nilotica*) with levamisole caused interstitial aggregation of round cells and moderate activation of melanomacrophages of kidneys. Also, levamisole, in the current study, induced non-significant increase in the phagocytosis percentage of levamisole treated groups and significant increase in the phagocytosis of vaccinated group to a level comparable with that of the corresponding control (Table 5). These results were confirmed by those obtained by Drews (1990) and Afifi (1990) in which they suggested that levamisole can enhance production and secretion of IL-2 (Interleukin-2) and interferon. According to Sheehan (1997), IL-2 and interferon-gamma are secreted by TH1 (T-helper-1) and function to promote activation of Tc (T-cytotoxic) cells, NK (Natural Killer) cells and macrophage, and consequently phagocytic activity.

The histopathological findings in the group treated with levamisole revealed activation of melanomacrophages and immune tissue in different organs. Mild degenerative changes were seen in the liver and kidneys mainly vacuolar degeneration. The histopathological pictures support the recorded hematological and immunological values and show the safety of the selected dose of levamisole. These results are on line with that obtained by El-Bouhi and El-Qelsh (1993).

The challenge with *Aeromonas hydrophila* resulted a 85, 30 and 80% relative levels of

protection in groups 2, 3 and 4 respectively, these results nearly similar with Baba et al. (1993) results, they reported that carp immersed in a levamisole bath (10mg/ml, 24 h) showed enhanced resistance against *Aeromonas hydrophila* and their phagocytes revealed increase in chemotactic ability, phagocytic activity and chemiluminescence. The observation of Siwicki (1989) about the enhancing effect of levamisole on the non-specific immune response for 3 months is of great importance especially as a possible prophylactic measures. This observation was supported by the results obtained in the present study where catfish fed levamisole were protected against challenge with *Aeromonas hydrophila*. This protection was significant in catfish fed levamisole before challenge than those fed levamisole as therapy because the previously activation of phagocytic cells was more effective. Unfortunately, no available literature explained the therapeutic effect of levamisole against bacterial diseases.

It could be concluded that, levamisole may help to enhance the immune response of catfish to some vaccines and against infection.

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