IMMUNOSTIMULATING EFFECT OF LEVAMISOLE ON NONSPECIFIC IMMUNITY OF AFRICAN CATFISH (*CLARIAS GARIEPINUS*) AFTER IMMUNOSUPPRESSION INDUCED BY MALATHION

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ABSTRACT

One hundred and sixty African catfish, obtained from Abbassa fish farm were distributed into four equal groups (40 each) in aerated glass aquaria (100 L capacity) and given balanced pellets at 2% of body weight twice daily. The fish were allowed 2 weeks for acclimatization. Gp. (1) was the control. Gps. (2-4) were kept in 0.01 ppm sublethal concentration of Malathion for one week. Gps. (3&4) were then given levamisole 2-hour bath at 2.5 and 5 mg/Liter respectively. Blood samples were collected from 5 fish (gps. 1-4) at 0, 7, 14 and 21 days after the application of levamisole to study nonspecific defense mechanisms (total and differential leukocytic count, macrophage chemotaxis, lymphocytic proliferation index, serum and mucus lysozymes and serum immunoelectrophoresis).

The total leukocyte and lymphocyte counts, macrophage chemotaxis and globulin were significantly increased from the 1st to 3rd weeks of levamisole treatment when compared with gp. (2). The serum and mucus lysozyme and lymphocyte blast transformation were significantly increased from the 1st to 2nd weeks only when compared with gp. (2). These results indicate that levamisole is effective in augmenting the nonspecific defense system.

INTRODUCTION

Great attention allover the world, specially in the developing countries including Egypt, has been directed towards the supply of edible proteins, rich or at least sufficient in the essential amino acids, that are not easily synthesized in the mammalian body. The amino acid pattern of fish is closely related to that found in the animal protein, so fish is an essential source of cheap

animal protein facing the continuous increase of population.

Investigations on the effects of environmental contamination on fish health have increased in reeent years. In particular, chronic exposure to sublethal concentrations of heavy metals, pesticide or other chemicals has been suspected of increasing the sensitivity of fish to infectious diseases **(Siwicki and Studnicka, 1992)**. Therefore, the study of sublethal and chronic effects of pollutants on the immune system in fish has become important.

Organophosphorus insecticides are widely used in agriculture, and in fisheries for the control of plankton invertebrates and for the treatment and prevention of parasite infestation. Previous studies on the effects of organophosphorus compounds on fish health have concentrated on factors affeeting their toxiclty to fish, and the kinetics of elimination from fish and the determination of withdrawal periods **(Saudinka and Sopinska, 1983; Jeney and Jeney, 1986 and Demael et al., 1990)**.

Malathion is the organophosphrous insecticide most frequently used on crops and fields as an insecticide in addition to pest control in agriculture area in Egypt. Malathion has been known to eause a specific inhibition of acetylcholinesterase, which in some cases is accompanied, hy the inhibition of neuron target esterase **(Repetto et al., 1988)**. Malathion depressed the nonspecific immune response of fish **(Siwicki and Studnicka, 1992)**.

Studies on human and animals have demonstrated the effect of levamisole on the activity of immune funetion. Levamisole has been documented as potential immunostimulator; thus is used in the treatment and prophylaxis of fish diseases. **Siwicki (1987 and 1989)** recorded enhaneement of phagocytic activity, leukocyte migration, myeloperoxidase activity and increased lysozyme levels in fish treated with levamisole. Application of levamisole in food or bath enhanced the phagocytic activity of neutrophils and macrophages in fish **(Siwicki, 1989; Siwicki et al, 1989 and Findlay and Munday, 2000)**. An in vitro study showed an immunostimulatory effect of levamisole on the lymphocyte proliferation, macrophage and neutrophils aetivity in carp and rainbow trout **(Siwicki and Cossarini-Dunier, 1990 and Siwicki et al., 1992)**.

Findlay and Munday (2000) reported the first record of the use and effieacy of levamisole as an immunomodulator in Atlantic salmon fish, however there is no available literature about the effect of levamisole as immunostimulant in catfish *Clarias gariepinus*. This study was performed to investigate the immunomodulation effect of levamisole on the nonspecific immunity in African catfish *Clarias gariepinus* after immunosuppression induced by Malathion.

MATERIAL AND METHODS

Fish :

One hundred and sixty African *Catfish Clarias* gariepinus weighing 150-200 gm were transferred from Abbassa fish farm to the laboratory where they were acclimatized in a 100-L glass aquaria filled with dechlorinated tap-water, supplied with continuous aeration for 2 weeks for acclimatization. Fish were fed twice daily with nutritionally balanced pellets at 2% of body weight.

Experimental Design :

The fish were divided into four equal groups (40 each). Gp. (1) acted as a control. Gps. (2-4) were subjected to 0.01 ppm sublethal concentration of Malathion for one week (LC_{50} of Malathion in catfish *Clarias gariepinus* body weight range 90-120 gm was 0.044 ppm by *El-Bagori, 2000*). Gps. (3&4) were then treated with levamisole 2-hours bath at a rate of 2.5 and 5 mg/ Liter respectively. Following bath, all groups were held in full sanitary fresh-water system. Blood samples were collected from 5 fish of each group at 0, 7, 14 and 21 days after the first application of levamisole to study the nonspecific defense mechanisms.

Assay of nonspecific defenses

The total and differential leukocytic counts, macrophage chemotaxis, lymphocytic proliferation index, serum and mucus-lysozymes and serum immunoelectrophoresis were determined.

The total and differential leukocytic counts were determined according to **(Stoskoph, 1993)**. Peritoneal macrophage chemotaxis were assayed according to the method described by **Klesius and Sealey (1998)**. The lymphocytic proliferation index was determined according to **Ota (1984)**. Serum and mucus-lysozymes were determined turbidimetric assay according to **Sankaran and Gurnani (1972)**. Immunoelectrophoresis of serum protein was done using cellulose acetate according to **(Henry et al., 1974)**.

Statistical Analysis:

The data were analyzed by analysis of variance (ANOVA) using **State View 4.01** (1993) followed by Dun's multiple range test to indicate the groups, which were significantly different at ($P< 0.05$).

RESULTS & DISCUSSION

The results from gps. (3&4) were closely similar. Our study revealed increased nonspecific immune response in levamisole treated fish (tables, 1, 2 & 3).

The leukogram profile showed an increase in both the total leukocyte and lymphocyte counts in levamisole treated fish at the end of the 1st to 3rd weeks when compared with Malathion group, and at the end of the 2nd week only when compared with the control group (table, 1).

The macrophage chemotaxis assay gps. (3&4) revealed a significant increase from the end of the 1st to 3rd weeks when compared with gp. (2) and from the end of the 2nd week when compared with the control group (table, 2).

There was a significant increase in the lymphocyte transformation index (gps. 3&4) at the end of the 1st and 2nd weeks when compared with gp. (2), and at the end of the 2nd week only when compared with the control group (table, 2).

The serum and mucus (gps. 3&4) displayed a significantly increased lysozyme activity when compared with gp. (2) at the end of the 1st and 2nd weeks and at the end of the 2nd week only when compared with the control group (table2).

The serum electrophoresis patterns showed that the γ-globulin (gps. 3&4) was significantly increased from the 1st to 3rd week in comparison with gp. (2) and at the end of the 2nd week when compared with the control group (table, 3).

The specific immune response takes days to develop because it involves complex pathways of selection and synthesis of specific molecules such as antibodies. However the nonspecific defense mechanisms are ready and often need to be activated **(Douglas et al., 1991)**. Thus the nonspecific defense mechanism reacts faster after immunization, injury or infection by microorganisms. Then nonspecific defense mechanism assays, done with fish, include demonstrating activities of phagocytosis and chemotaxis of macrophages and monocytes **(Weeks and Warinner, 1986)**.

An alternative approach to vaccines and antibiotics would be the use of immunostimulating agents. During the last decade there has been an increasing interest in the modulation of the nonspecific immune system of fish for treatment and prophylaxis measure against diseases. Immunostimulants, as levamisole, have shown to be powerful activators of nonspecific defense mechanism which is the first line of defense against microbial infections, thereby preventing infectious diseases **(Sahoo and Mukherjee, 2001 & 2002)**.

Granulocytes and mononuclear phagocytes play a central role in the cell-mediated immunity of the nonspecific defense of fish **(Dalmo et al., 1996)**. The progressive increase of the total leu-

kocytic count in levamisole treated groups could be a result of stimulation of leukopoiesis by levamisole. Our results are in-agreement with **Siwicki and Studnicka (1992)** who reported elevated total leukocytic count in *Cyprinus carpio* administerated 5 mg/Kg.B.W. every three days for 28 days after induced immunosupression with trichlorfon pesticide. On the other hand **Findlay and Munday (2000)** reported insignificant differences in leucocrit value of levamisole treated fish with 2.5 mg/L for 2 hours when compared with the control group.

Lymphocyte blast transformation is a relatively simple, rapid and reproducible test, utilized in clinical and experimental immunology **(Ota, 1984)**. Lymphocyte transformation is the means for monitoring the effect of immunoenhancing or suppressive therapy where lymphocyte transformation may be defective even without detectable lymphopenia **(Lopez et al., 1975)**. Our study shows that levamisole treated fish showed a significantly augmented proliferation of T-lymphocyte. Levamisole as an immunomodulating of lymphocyte transformation has been documented with **Hajnzic et al., (1999) and Abdalla et al., (1995)** in children, suffering brain tumor and pre or postoperative medication patients respectively. **Johnkoski et al., (1996)** attributed the increased proliferation of mice T-lymphocyte treated with levamisole to enhanced interleukine-6 production with T-lymphocytes. Meanwhile **Cabaj et al., (1995)** recorded a decrease in lymphocyte transformation in lambs drenched with levamisole.

Macrophages represent the host s second line of defense to infectious agents, therefore determination of macrophage functions including chemotaxis is important in the evaluation and diagnosis of the defense disorder pathways **(Ammann and Fudenberg, 1980)**. Fish macrophages have potent bactericidal and larvicidal activities, in addition to possessing both intracellular and extracellular killing mechanisms **(Secombes and Fletcher, 1992)**. Our work revealed an increased macrophage chemotaxis response in immunocompromised fish treated with levamisole. **Sahoo and Mukherjee (2001)** recorded that feeding of levamisole to immunocompromised *Labeo rohita* fish, with aflatoxin, could restore neutrophil oxidative radical release and phagocytic activity. Similarly the macrophage phagocytic ability has been enhanced in Atlantic salmon fish treated with levamisole **(Findlay and Munday, 2000)**.

Lysozymes are widespread enzymes with antibacterial role, in many teleost tissues and secretions **(Lindsay, 1986)**. The lysozymes are distributed in fish tissues rich in leukocytes, and at sites where the risk of invasion is high (skin, gills and gastrointestinal tract) where lysozymes provide a protective function against viruses, neoplasms, bacteria, fungi and insects **(Dobson et al., 1984)**. In the recent years there has been an increase in the number of studies reflecting the importance of lysozymes as nonspecific immune system in fish. Our studies confirm the efficacy of levamisole in increasing the activities of both serum and mucus lysozymes. **Findlay and Munday (2000)** recorded a significant increase in both mucus and serum lysozymes in Atlantic sal-

mon fish after 2-weeks of levamisole bath 2.5 mg/L for 2-hours. Also **Sahoo and Mukherjee (2001)** reported elevated serum lysozyme activities in *Labeo rohita* fish fed on dietary intake of levamisole (5mg/ kg body weight at 3 day interval for 60 days).

Although the electrophoresis technique did not directly detect the immune defects, it is valuable and relatively simple technique that may help the diagnosis of a possible immunologic defect, also it is a valuable tool in assessing humoral immunity and in differential diagnosis **(Kaneko et al., 1997)**. Our studies revealed that the levamisole restored the total protein and gammaglobulin levels in the immunosuppressed fish. Similarly **Sahoo and Mukherjee (2001 and 2002)** reported increased total protein, albumin, globulin and antibody titer level by feeding levamisole to immunosuppressed fish with aflatoxin B1. In contrary **Morrison et al. (2000)** mentioned that the levamisole as adjuvant has a narrow range of efficacy when used by bath or intraperitoneal injection in Atlantic salmon fish.

As a point of view levamisole is potent immunostimulant with both doses 2.5 and 5.0 mg/ Liter through enhancement of the nonspecific immune system. This finding agrees with **Ziberg et al., (2000)** who recorded insignifleant difference of immune response in *Salmo salar* fish exposed to 1.25, 2.5 and 5.0 mg/liter levamisole.

This study provides strong evidence that bath treatment with levamisole enhances the nonspeelfic immune system. Also the present results suggest that the bath treatment of immunocompromised fish with levamisole may increase their resistance to infection, reduce fish mortality and could be of economic benefit.

Table (1): Total and differential leukocytic count in African catfish *Clarias gariepinus* treated with levamisole after immunosuppression induced by Malathion.

Group	1 (Control) a						2 (Malathion) b						3 (Levamisole) c						4 (Levamisole) d					
	TLC	Lymp	Neut	Esin	Baso	Mono	TLC	Lymp	Neut	Esin	Baso	Mono	TLC	Lymp	Neut	Esin	Baso	Mono	TLC	Lymp	Neut	Esin	Baso	Mono
0 day	25.24 ± 2.01bcd	15.41 ± 1.04bcd	8.02 ± 0.91	0.36 ± 0.04	0.01 ± 0.01	1.44 ± 0.15	19.99 ± 1.42a	9.71 ± 1.08a	8.69 ± 0.94	0.31 ± 0.03	0	1.28 ± 0.16	19.53 ± 1.14a	9.25 ± 1.05a	8.62 ± 0.92	0.34 ± 0.04	0.01 ± 0.01	1.31 ± 0.14	19.71 ± 1.12a	9.31 ± 1.06a	8.58 ± 0.91	0.43 ± 0.04	0.01 ± 0.01	1.38 ± 0.12
7 day	26.04 ± 2.18b	15.92 ± 1.11b	8.79 ± 0.88	0.41 ± 0.03	0	1.52 ± 0.18	20.16 ± 1.59acd	9.91 ± 1.06acd	8.65 ± 0.84	0.34 ± 0.04	0.02 ± 0.01	1.24 ± 0.13	25.70 ± 1.64b	15.12 ± 1.78b	8.79 ± 0.79	0.38 ± 0.03	0	1.41 ± 0.15	26.10 ± 1.65b	15.31 ± 1.75b	8.83 ± 0.78	0.44 ± 0.03	0.01 ± 0.01	1.51 ± 0.17
14 day	25.46 ± 2.18bcd	15.18 ± 1.04bcd	8.41 ± 0.78	0.39 ± 0.04	0	1.48 ± 0.16	20.45 ± 1.61acd	10.28 ± 1.12acd	8.45 ± 0.81	0.36 ± 0.03	0.01 ± 0.01	1.34 ± 0.15	33.37 ± 2.11ab	22.94 ± 1.68ab	8.51 ± 0.68	0.44 ± 0.04	0	1.48 ± 0.16	34.38 ± 2.15ab	23.75 ± 1.71ab	8.65 ± 0.66	0.49 ± 0.04	0	1.49 ± 0.16
21 day	25.86 ± 2.41	15.28 ± 1.83	8.59 ± 0.81	0.47 ± 0.04	0.01 ± 0.01	1.51 ± 0.16	23.55 ± 2.27cd	13.01 ± 1.42	8.61 ± 0.85	0.44 ± 0.03	0	1.49 ± 0.12	29.00 ± 2.62b	18.42 ± 1.92b	8.58 ± 0.87	0.45 ± 0.03	0.01 ± 0.01	1.54 ± 0.19	29.07 ± 2.65b	18.51 ± 1.95b	8.62 ± 0.75	0.48 ± 0.04	0.01 ± 0.01	1.45 ± 0.15

Significant at a, b, c, d < 0.05

TLC = Total leukocyte count.

Lymp = Lymphocyte.

Neut = Neutrophil.

Esin = Esinophil.

Baso = Basophil

Mono = Monocyte

Table (2): Macrophage chemotaxis, lymphocyte transformation and serum and mucus lysozymes in African catfish, *Clarias gariepinus*, treated with levamisole after immunosupression by Malathion.

Group	1 (Control) a				2 (Malathion) b				3 (Levamisole) c				4 (Levamisole) d			
	Macrophage	Lymphoc.	Lysozyme µg/ml		Macrophage	Lymphoc.	Lysozyme µg/ml		Macrophage	Lymphoc.	Lysozyme µg/ml		Macrophage	Lymphoc.	Lysozyme µg/ml	
	Chemotaxis	Transform.	Serum	Mucus	Chemotaxsis	Transform.	Serum	Mucus	Chemotaxis	Transform.	Serum	Mucus	Chemotaxis	Transform.	Serum	Mucus
0 day	9.91 ± 0.59bcd	1.22 ± 0.06bcd	6.89 ± 0.81bcd	14.4 ± 1.21bcd	5.81 ± 0.42a	0.82 ± 0.01a	3.89 ± 0.41a	8.14 ± 1.11a	5.92 ± 0.52a	0.84 ± 0.02a	3.48 ± 0.44a	8.49 ± 1.28a	5.84 ± 0.51a	0.85 ± 0.02a	3.45 ± 0.42a	8.46 ± 1.27a
7 day	9.28 ± 0.51b	1.21 ± 0.05b	6.24 ± 0.57b	15.3 ± 1.28b	6.15 ± 0.49acd	0.88 ± 0.02acd	4.41 ± 0.40acd	9.48 ± 1.13acd	9.54 ± 0.54b	1.37 ± 0.06b	6.85 ± 0.42b	15.91 ± 1.35b	9.39 ± 0.52b	1.32 ± 0.05b	6.78 ± 0.41b	15.74 ± 1.32b
14 day	9.45 ± 0.58cd	1.11 ± 0.04cd	6.45 ± 0.64cd	15.89 ± 1.35cd	7.01 ± 0.59cd	1.01 ± 0.05cd	5.74 ± 0.68cd	13.18 ± 1.41cd	14.21 ± 0.74ab	1.68 ± 0.07ab	10.81 ± 0.88ab	21.46 ± 1.85ab	14.53 ± 0.76ab	1.62 ± 0.06ab	10.91 ± 0.89ab	22.51 ± 1.91ab
21 day	9.86 ± 0.61	1.19 ± 0.06cd	7.82 ± 0.78	15.74 ± 1.32	8.04 ± 0.69cd	1.12 ± 0.05	6.21 ± 0.67cd	13.86 ± 1.48cd	12.49 ± 0.88b	1.31 ± 0.04	9.78 ± 0.69b	17.12 ± 1.82	12.41 ± 0.79b	1.34 ± 0.05	9.81 ± 0.74b	17.01 ± 1.79

Significant at a, b, c, d < 0.05

Table (3): Serum electrophoresis of African catfish *Clarias gariepinus* treated with levamisole after immunosuppression induced by Malathion.

Group	1 (Control) a					2 (Malathion) b					3 (Levamisole) c					4 (Levamisole) d				
	TP	Album	α glob	β glob	γ glob	TP	Album	α glob	β glob	γ glob	TP	Album	α glob	β glob	γ glob	TP	Album	α glob	β glob	γ glob
0 day	4.35 ± 0.22bcd	1.42 ± 0.19	0.98 ± 0.15	1.46 ± 0.15	0.49 ± 0.05bcd	3.19 ± 0.20a	1.11 ± 0.18	0.77 ± 0.14	1.09 ± 0.18	0.22 ± 0.03a	3.16 ± 0.22a	1.09 ± 0.19	0.72 ± 0.15	1.15 ± 0.16	0.20 ± 0.02a	3.12 ± 0.21a	1.08 ± 0.18	0.74 ± 0.16	1.12 ± 0.15	0.18 ± 0.02a
7 day	4.39 ± 0.23b	1.49 ± 0.17	0.96 ± 0.17	1.42 ± 0.16	0.52 ± 0.04b	3.38 ± 0.19a	1.18 ± 0.15	0.82 ± 0.15	1.13 ± 0.17	0.25 ± 0.02acd	3.70 ± 0.24	1.26 ± 0.14	0.79 ± 0.14	1.19 ± 0.15	0.46 ± 0.03b	3.74 ± 0.25	1.28 ± 0.15	0.80 ± 0.14	1.17 ± 0.16	0.49 ± 0.04b
14 day	4.41 ± 0.25	1.47 ± 0.18	0.99 ± 0.14	1.48 ± 0.18	0.47 ± 0.03bcd	3.55 ± 0.25	1.23 ± 0.14	0.84 ± 0.13	1.21 ± 0.17	0.27 ± 0.03acd	4.01 ± 0.21	1.29 ± 0.14	0.83 ± 0.15	1.20 ± 0.14	0.60 ± 0.04ab	4.1 ± 0.22	1.31 ± 0.15	0.86 ± 0.16	1.26 ± 0.18	0.67 ± 0.04ab
21 day	4.39 ± 0.21	1.48 ± 0.18	0.96 ± 0.16	1.44 ± 0.17	0.51 ± 0.03	3.81 ± 0.22	1.29 ± 0.15	0.88 ± 0.15	1.28 ± 0.15	0.36 ± 0.04acd	4.12 ± 0.25	1.36 ± 0.16	0.87 ± 0.16	1.31 ± 0.15	0.58 ± 0.04b	4.14 ± 0.24	1.32 ± 0.15	0.88 ± 0.16	1.35 ± 0.18	0.59 ± 0.04b

Significant at a, b, c, d < 0.05

TP = Total Protein **Album** = Albumin **glob** = globulin

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الملخص العربى

تأثير الليفاميزول كمنشط للمناعة الغير متخصصة فى أسماك القرموط الإفريقى بعد تثبيط مناعتها بالملاثيون

المشتركون فى البحث

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أجريت هذه الدراسة لتقييم كفاءة الليفاميزول كمنشط للمناعة فى سمكة القرموط الإفريقى بعد تثبيط مناعتها بالملاثيون. تم تقسيم الأسماك إلى أربعة مجاميع (٤٠ سمكة فى المجموعة) بعد أن تم أقلمتها تحت الظروف المعملية فى أحواض زجاجية سعة ١٠٠ لتر لمدة إسبوعين وتغذيتها بنسبة ٢٪ من وزن جسم السمكة. المجموعة الأولى كانت ضابطة أما المجموعات الثانية والثالثة والرابعة فقد تم تعريضهم للملاثيون بتركيز ٠,١ جزء فى المليون لمدة إسبوع ثم تغيير المياه وتعريض المجموعتين الثالثة والرابعة لحمام مائى لمادة ليفاميزول بتركيز ٢,٥ ، ٥ ملجم / لتر على التوالى لمدة ساعتين.

أظهرت النتائج مايلى :-

- إرتفاع معنوى فى عدد كرات الدم البيضاء والخلايا اللمفاوية فى الأسماك التى تم معاملتها بالليفاميزول مقارنة بمجموعة الملاثيون عند نهاية الإسبوع الأول إلى نهاية الثالث بينما كانت معنوية عند نهاية الإسبوع الثانى فقط بمقارنتها بالمجموعة الضابطة.
- أوضحت نتائج الانجذاب الكيميائى للخلايا الأكولة للأسماك التى تم معاملتها بالليفاميزول إرتفاعاً معنوياً من نهاية الإسبوع الأول إلى نهاية الثالث بالمقارنة بمجموعة الملاثيون، فى حين كانت معنوية فى نهاية الإسبوع الثانى بالمقارنة بمجموعة الكنترول.
- وجد أن هناك زيادة معنوية فى معامل تحويل الخلايا اللمفاوية للأسماك التى حصلت على حمام مائى بالليفاميزول فى نهاية الإسبوع الأول والثانى بالمقارنة بمجموعة الملاثيون بينما كانت معنوية فى نهاية الإسبوع الثانى بمقارنتها بالكنترول.
- أظهرت النتائج أن هناك فروق معنوية فى نتائج إختبار المواد المحللة فى المصل والمخاط بالنسبة للأسماك التى تم

معالجتها بالليفاميزول عن المجموعات الأخرى.

- إرتفاع معنوى ملحوظ فى الجاما جلوبيلين للأسماك المعالجة بالليفاميزول عند نهاية الإسبوع الأول إلى نهاية الثالث بالمقارنة بمجموعة الملاثيون وعند الثانى فقط بالمقارنة بالكنترول.

ويتضح من هذه الدراسة أن الليفاميزول له تأثير قوى وفعال فى رفع المناعة لدى أسماك القرموط الإفريقى وذلك للوقاية من الأمراض التى تصيبها وعوامل الإجهاد المختلفة.