EFFECTS OF BIOGEN® AND LEVAMISOL HCL® ON THE IMMUNE RESPONSE OF CULTURED *OREOCHROMIS NILOTICUS* TO *AEROMONAS HYDROPHILA* VACCINE

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ABSTRACT

The effect of Biogen (2 g/kg feed) and levamisol HCl (1 g/kg feed) on phagocytic assay, activities of serum enzymes (S.GOT, S.GPT, ALP), cholesterol, creatinine, glucose, total proteins, albumin, globulin and antibody titers before and after immunization of *Oreochromis niloticus* (*O. niloticus*) by intramuscular injection (IM) with formalin killed *Aeromonas hydrophila* vaccine were studied. The results proved that Biogen and levamisol HCl causes a marked immunopotentiating effect more than control after IM vaccination. The immunopotentiating effect of Biogen and levamisol HCl covered both, the humoral and cell-mediated immune response and a marked rise in the antibody titers at day 70 after vaccination which reached to 9.0 ± 0.0 and 9.0 ± 0.0 after IM vaccination respectively, compared to the vaccinated control group (6.5 ± 0.5). Also, phagocytic activity obtained from fish, fed with ration supplemented with Biogen and levamisol HCl was significantly increased than ration supplemented with basal diet (control group). The results of enzyme activities (ALP, S.GOT, S.GPT) and creatinine, cholesterol, glucose as well as total proteins, albumin and globulin prove that the group fed with ration containing Biogen and Levamisol HCl were similar to control group in enzyme activities. The total proteins also, appeared to be high in group fed on ration containing Biogen and levamisol HCl than group fed basal diet (control group). The relative survival percentage after IM vaccination was 69.80% and 59.80% in Biogen and levamisol HCl supplemented groups respectively compared to the vaccinated non-treated group where it was 23.20%.

INTRODUCTION

In recent years, the need for an increase in the world's food supply is generally acknowledged. The serious shortage of animal proteins, manifested by the poor health condition of people in many regions of the world, together with the relative high price of animal proteins created a great demand towards fish, which provides protein of high digestibility and nutritive value.

To day, much effort has been made to harvest fish from natural water resources. However, the development of artificial fish breeding farms, based on modern and scientific techniques to intensify fish production is being attempted.

Oreochromis niloticus are important food fish in many tropical and subtropical countries. More than 20 species of the genus Tilapia have been cultivated in developing countries due to their high tolerance to adverse environmental conditions, their relatively fast growth and the easy handling for breeding (**Guerrero, 1982**).

Few data are available on the use of Biogen and levamisol HCl as immunostimulants in fish, broilers and large animals (**Siwicki, 1987; Zeinab and Alkelch, 1993; Yoshida *et al.*, 1995** and **Saffinaze, 2001**). The shortage of literature concerning the effect of these immunostimulants enforced us to study their effects on phagocytic assay, some serum enzymes, total proteins, albumin, globulin in *Oreochromis niloticus* as well as their effects on the immune response to *Aeromonas hydrophila* vaccine.

MATERIAL AND METHODS

Biogen was supplied from China Way-Tawan, a new trade name for probiotic. This product was added to the *O. niloticus* food at a rate of 2 g/kg food. Levamisol HCl was supplied from Chemical Industries Development (CID), Egypt. This product was added to the *O. niloticus* food by 1 g/kg food. *Aeromonas hydrophila* was isolated from internal organs of diseased cultured *Oreochromis niloticus* obtained from Barseek Fish Farm.

Experimental fish:

A total number of 360 *Oreochromis niloticus* (50 ± 5 g body weight) were obtained from Barseek Fish Farm, El-Behera, Egypt. Each forty fish were kept in an artificial aeriated glass aquarium (measuring 100 x 75 x 50 cm) containing dechlorinated tap water at a temperature of 20 – 23 °C, pH 7.1 – 7.3. The fish were fed with a 30% protein diet.

Experimental procedure:

The fish were divided into 3 equal groups (120 each). Those of group 1 were fed on free basal diet and kept as control, whereas those of group II and III were fed on rations well mixed with Biogen and levamisol HCl 2 g/kg feed

and 1 g/kg feed, respectively. The fish were fed once a day at a feeding rate of 5% of the body weight till the end of the experiment (12 weeks) (**Coche, 1977**).

Blood samples:

At the end of the 2nd, 4th, 6th, 8th, 10th and 12th week during the experimental period, approximately 2 ml blood sample were collected from different groups via the caudal vessels from 3 fish using disposable syringe.

A portion of blood was collected containing anticoagulant (0.1 ml of 4% sodium citrate solution/1 ml blood) for determination of phagocytic assay (activity and index) according to **Kawahara *et al.*, (1991)**.

The blood serum was separated by centrifugation at 3000 rpm for 15 minutes and kept at – 20 °C until assayed. Serum transaminase (glutamic-pyruvic transaminase, GPT, and glutamic-oxalacetic transaminase, GOT) were determined according to **Reitman and Frankel, (1957)**, while serum alkaline phosphatase was estimated according to the modified method of **Kind and King, (1954)**. Serum cholesterol, creatinine and glucose were determined according to **Schettler *et al.*, (1975)**, **Bartels, (1971)** and **Trinder, (1969)**, respectively. Serum total proteins and albumin were determined according to **Doumas *et al.*, (1981)** and **Reinhold, (1953)**, while serum globulin was calculated as the difference between total proteins and albumin (**Coles, 1974**), also albumin to globulin ratio (A/G) was calculated.

Determination of phagocytic activity and phagocytic index:

Phagocytic activity was determined according to **Kawahara *et al.*, (1991)**. 50 µg *Candida albicans* culture were added to 1 ml of citrated blood and shaken in water bath at 23 – 25 °C for 3 – 5 hours. Smears of blood were then stained with Giemsa solution.

Phagocytic activity (PA) = percentage of phagocytic cells containing yeast cells.

$$\text{Phagocytic index (PI)} = \frac{\text{Number of yeast cells phagocytosed}}{\text{Number of phagocytic cells}}$$

Immunostimulant measurement, and challenge test:

The initial immunization with formalin killed *Aeromonas hydrophila* vaccine was followed by a challenge with the same antigen at the end of the experiment (day 70 after vaccination) in the three groups (120 each) according to **Ellis, (1988)**. The relative percent survival (RPS) was calculated according to **Amend (1981)**.

A virulent strain of *A. hydrophila* was inactivated by formalin according to **Sakai *et al.*, (1984)**. The inactivated *A. hydrophila* was tested for

safety and sterility according to **Anderson *et al.*, (1970)**. The formalin inactivated *A. hydrophila* preparation was mixed with an equal volume of sterile saline (**Badran, 1990**). The bacterial number was adjusted at Macfarland's tube No. 2 (6 x 10^8 cells/ml). 0.2 ml of formalin-inactivated bacterial suspension was injected IM into fish. Seven days post-injection with inactivated bacteria and on weekly intervals throughout 10 weeks, 2 fish were taken from each group for blood collection from the caudal vessels and serum was then separated. The immune response to *A. hydrophila* was detected by microagglutination test after preparation of stained antigen according to **Eurell *et al.*, (1979)** and **Collins *et al.*, (1976)** respectively. After the antibody titration, the survival fish were intraperitoneally (IP) challenged with 0.1 ml/fish containing 9 x 10^7 cells of the virulent *A. hydrophila*. Daily morbidity and mortality were recorded.

$$RPS = 1 - \frac{\text{Vaccinated mortality \%}}{\text{Control mortality}} \times 100$$

Statistical analysis:

Statistical analysis of the obtained data was performed using the Statistical Analysis System (**SAS, 1987**).

RESULTS

The effect of Biogen and levamisol HCl supplementation on the cell-mediated immunity of *O. niloticus* was evaluated by measuring the phagocytic activity and index (Table 1). The mean percentage of phagocytic activity was higher in the *O. niloticus* supplemented with Biogen and levamisol HCl than and control groups.

No significant differences of ALP, S.GOT and S.GPT in Biogen and levamisol HCl supplemented groups as compared to control (Table 2). Also, from Table (3), noted that no significant differences in creatinine, cholesterol and glucose in Biogen and levamisol HCl supplemented groups, except, glucose in levamisol HCl supplemented groups. However, a significant increase of total proteins and globulin especially in the last 4 weeks of Biogen and levamisol HCl supplemented groups, while, no significant differences of albumin in all treated groups (Table 4).

Injection vaccination (IM) in Biogen and levamisol HCl supplemented groups have a significant immunopotentiating effect on the antibody production of *O. niloticus* fish. In the Biogen and the levamisol HCl supplemented groups, the IM application of *Aeromonas hydrophila* vaccine elicited higher mean of antibody titers (9.0 ± 0.0, 9.0 ± 0.0) respectively, Table (5).

It is worthy to be noted that the injection vaccination (IM) in Biogen and levamisol HCl supplemented groups has been proved to be more efficacious to immunize fish against *Aeromonas hydrophila* than control vaccinated groups. The relative survival percentage was 23.2%, 69.8% and 59.8% vaccinated (control, Biogen and levamisol HCl supplemented groups) respectively. While, non-vaccinated (control, Biogen and levamisol HCl supplemented groups) were 0%, 49.8% and 39.8% respectively as shown in Table (6).

DISCUSSION

Immunostimulants have been extensively studied in fish species both at whole animal and on a cellular level. The immunostimulants attach to specific receptors on the cell surface of the phagocytes and lymphocytes. This activates the cell resulting in increased production of enzymes that can destroy pathogens, chemical messengers (interferon, interleukins and complement proteins) that stimulate other arms of the immune system and increased activity of T and B lymphocytes (**Raa *et al.*, 1992; Matsuo and Miyazono, 1993**).

In the present study, the results revealed that Biogen and levamisol HCl potentiates the immune response stronger than basal diet supplemented groups. The immunopotentiating effect covers both the humoral and cell-mediated immune response. The immunopotentiating effects of Biogen and levamisol HCl are based on the use of naturally arising metabolites as active substances for a nutritional performance promotion. As it has been shown previously in higher animals such as swine, cows, poultry and fish (**Ramadan *et al.*, 1991** and **Glawischnig, 1991**), these biogenic performance promoters have a positive effect on the intestinal flora, thereby improving the digestion, availability of natural feed, supply of nutrients and utilization of energy which influence the growth of animals and fish.

Biogen and levamisol HCl increased phagocytic activity and index in tested fish. Nearly similar results were reported by **Siwicki, (1987)** and **Yoshida *et al.*, (1995)** who used levamisol for stimulation of phagocytic activities in *Cyprinus carpio L* and African catfish. The effects of Biogen and levamisol HCl may be due to the activation of immune memory and macrophages by lymphokines which exist in fish and play an important part in the cell-mediated immunity as stated by **Roberts, (1989)**.

In the present work, the results cleared that Biogen and levamisol HCl potentiate the immune response after intramuscular injection (IM) with formalin killed *Aeromonas hydrophila* vaccine. Following IM injection of *Aeromonas hydrophila* vaccine, Biogen and levamisol HCl elicited higher antibody titers which are maintained for a longer period and led to higher protection than control groups. This phenomenon corresponds to those of **Johnson *et al.*, (1982); Plumb, (1984)** and **Roberts, (1989)**. They found that

the IM injection of the vaccine stimulates the antibody production and leads therefore to a better protection. This phenomenon is supported by increase of total proteins, globulin and phagocytic activity.

Studies as performed and presented will lead to improvements in fish diets. Such improved diets can lead to improved disease resistance in vaccinated as well as non-vaccinated fish. Thus, immunostimulants are particularly suitable for boosting immature immune systems. Effective against a number of opportunistic pathogens. Useful at times of known stress such as transportation and vaccination. Enhances immune response to conventional vaccines.

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Table (1): Phagocytic assay in different experimental groups (means ± standard error).

Periods	Phagocytic activity			Phagocytic index		
	GP1	GP2	GP3	GP1	GP2	GP3
2nd week	12.68 ± 1.33 a	14.50 ± 0.73 b	13.22 ± 0.17 a	4.60 ± 0.31 a	5.91 ± 0.11 b	4.80 ± 0.12 a
4th week	13.11 ± 1.24 a	14.73 ± 0.17 a	13.15 ± 0.33 a	4.90 ± 0.51 a	5.77 ± 0.03 b	4.90 ± 0.14 a
6th week	12.73 ± 1.22 a	16.22 ± 1.34 b	15.12 ± 0.34 b	4.63 ± 0.41 a	6.10 ± 0.71 b	5.40 ± 0.15 b
8th week	13.74 ± 1.09 a	19.41 ± 1.71 b	18.90 ± 0.43 b	4.80 ± 0.33 a	7.20 ± 0.34 b	6.80 ± 0.17 b
10th week	14.53 ± 1.37 a	18.55 ± 1.54 b	18.24 ± 0.51 b	5.11 ± 0.54 a	7.10 ± 0.24 b	6.80 ± 0.31 b
12th week	13.25 ± 1.12 a	19.34 ± 1.20 b	18.59 ± 0.34 b	4.90 ± 0.29 a	7.20 ± 0.09 b	6.50 ± 0.32 b

GP 1 = control; GP 2 = Biogen; GP 3 = levamisol.
Letters on GP2 or GP3 other than that on the control (GP1) indicates significant difference (P<0.05).

Table (2): Activities of serum alkaline phosphatase (ALP), glutamic pyruvic transaminase (GPT) and glutamic oxalacetic transaminase in different experimental groups (means ± SE).

Periods	ALP (IU/100 ml)			S.GPT (U/L)			S.GOT (U/L)		
	GP1	GP2	GP3	GP1	GP2	GP3	GP1	GP2	GP3
2nd week	14.21 ± 0.71 a	14.50 ± 0.31 a	14.65 ± 0.35 a	74.22 ± 1.31 a	72.11 ± 0.13 a	71.15 ± 1.21 a	73.18 ± 0.77 a	72.44 ± 0.78 a	73.55 ± 1.31 a
4th week	14.27 ± 0.39 a	14.30 ± 0.11 a	14.78 ± 0.27 a	73.51 ± 1.37 a	73.15 ± 0.17 a	70.33 ± 0.78 a	74.13 ± 0.45 a	73.43 ± 1.37 a	72.48 ± 0.93 a
6th week	14.55 ± 0.22 a	14.20 ± 0.27 a	14.73 ± 0.13 a	74.50 ± 1.34 a	73.73 ± 1.22 a	72.21 ± 1.34 a	73.51 ± 0.16 a	72.51 ± 1.81 a	73.81 ± 1.37 a
8th week	15.31 ± 0.39 a	14.28 ± 0.22 a	14.89 ± 0.31 a	74.71 ± 1.39 a	72.41 ± 0.78 a	68.73 ± 1.23 b	73.13 ± 0.77 a	74.34 ± 1.38 a	72.78 ± 1.23 a
10th week	14.43 ± 0.71 a	14.91 ± 0.37 a	14.97 ± 0.12 a	73.81 ± 1.31 a	73.31 ± 1.22 a	69.11 ± 0.93 b	75.14 ± 0.93 a	73.51 ± 0.81 a	73.41 ± 0.83 a
12th week	14.39 ± 0.28 a	14.83 ± 0.24 a	14.93 ± 0.13 a	74.81 ± 1.39 a	72.43 ± 1.31 a	71.13 ± 0.17 a	74.24 ± 0.53 a	72.13 ± 0.39 a	73.78 ± 1.39 a

GP 1 = control; GP 2 = Biogen; GP 3 = levamisol.
Letters on GP2 or GP3 other than that on the control (GP1) indicates significant difference (P<0.05).

Table (3): Serum creatinine, cholesterol and glucose in different experimental groups (means ± SE).

Periods	Creatinine (mg %)			Cholesterol (mg %)			Glucose (mg %)		
	GP1	GP2	GP3	GP1	GP2	GP3	GP1	GP2	GP3
2nd week	0.67 ± 0.07 a	0.65 ± 0.05 a	0.67 ± 0.08 a	189.0 ± 3.73 a	169.33± 2.14 b	181.34± 2.78 b	72.81± 2.09 a	68.91 ± 1.87 a	71.83 ± 1.39 a
4th week	0.81 ± 0.09 a	0.69 ± 0.01 b	0.71 ± 0.08 b	194.0 ± 4.11 a	189.31± 2.41 a	183.71± 1.98 b	72.11± 1.39 a	70.31 ± 1.91a	74.91 ± 1.73 a
6th week	0.73 ± 0.08 a	0.71 ± 0.09 a	0.72 ± 0.09 a	187.23 ± 3.74 a	185.35± 2.33 a	188.71± 2.78 a	71.31± 1.31 a	71.31 ± 1.71 a	78.55 ± 1.22 a
8th week	0.77 ± 0.07 a	0.81 ± 0.01 a	0.83 ± 0.09 a	194.11 ± 3.93 a	189.31± 2.74 a	187.38± 2.78 a	73.71± 1.28 a	73.71 ± 1.89 a	81.71 ± 1.91 b
10th week	0.73 ± 0.05 a	0.69 ± 0.02 a	0.71 ± 0.08 a	187.41 ± 3.71 a	190.74± 1.87 a	189.78± 2.91 a	73.81± 1.39 a	70.89 ± 1.37 a	84.38 ± 1.83 b
12th week	0.69 ± 0.03 a	0.68 ± 0.03 a	0.69 ± 0.08 a	191.50 ± 3.13 a	184.89± 1.39 a	188.91± 1.94 a	72.88± 1.37 a	79.89 ± 2.81 a	82.78 ± 1.33 b

GP 1 = control; GP 2 = Biogen; GP 3 = levamisol.
Letters on GP2 or GP3 other than that on the control (GP1) indicates significant difference (P<0.05).

Table (4): Serum total proteins, albumin and globulin in different experimental groups (means ± SE).

Periods	Total proteins (g %)			Albumin (g %)			Globulin (g %)		
	GP1	GP2	GP3	GP1	GP2	GP3	GP1	GP2	GP3
2nd week	5.84 ± 0.17 a	5.84 ± 0.18 a	5.76 ± 0.19 a	3.48 ± 0.18 a	3.16 ± 0.15 a	3.17 ± 0.25 a	2.36 ± 0.14 a	2.68 ± 0.14 a	2.59 ± 0.11 a
4th week	6.34 ± 0.15 a	6.19 ± 0.93 a	5.62 ± 0.30 a	3.83 ± 0.22 a	3.21 ± 0.15 a	2.85 ± 0.18 b	2.51 ± 0.31 a	2.98 ± 0.17 a	2.77 ± 0.13 a
6th week	6.38 ± 0.04 a	6.81 ± 0.13 a	6.48 ± 0.09 a	3.71 ± 0.27 a	3.74 ± 0.21 a	3.52 ± 0.18 a	2.67 ± 0.16 a	3.07 ± 0.21 a	2.96 ± 0.18 a
8th week	6.35 ± 0.12 a	7.42 ± 0.11 b	7.03 ± 0.11 a	3.93 ± 0.22 a	3.79 ± 0.25 a	4.07 ± 0.09 a	2.42 ± 0.07 a	3.63 ± 0.12 b	2.96 ± 0.12 b
10th week	6.36 ± 0.15 a	7.89 ± 0.22 b	7.30 ± 0.13 b	3.53 ± 0.13 a	3.98 ± 0.03 a	4.18 ± 0.17 a	2.83 ± 0.31 a	3.91 ± 0.15 b	3.12 ± 0.13 b
12th week	6.66 ± 0.11 a	8.23 ± 0.14 b	7.50 ± 0.15 b	3.95 ± 0.13 a	3.85 ± 0.09 a	3.96 ± 0.20 a	2.71 ± 0.41 a	4.38 ± 0.19 b	3.54 ± 0.16 b

GP 1 = control; GP 2 = Biogen; GP 3 = levamisol.
Letters on GP2 or GP3 other than that on the control (GP1) indicates significant difference (P<0.05).

Table (5): Antibody titers (log_{10}) in different experimental groups (means ± standard error).

Weeks post-vaccination	Vaccinated Control	Vaccinated + Biogen	Vaccinated + Levamisol
1	2.0±0.0	3.5±0.0	4.0±0.0
2	2.0±0.0	4.5±0.5	4.5±0.0
3	2.5±0.0	5.5±0.0	4.5±0.0
4	3.0±0.0	7.5±0.5	5.5±0.5
5	4.5±0.5	8.0±0.0	6.0±0.0
6	4.5±0.5	9.0±0.5	6.5±0.5
7	6.5±0.5	9.5±0.5	8.0±0.5
8	6.5±0.0	10.0±0.5	8.5±0.0
9	7.5±0.0	10.0±0.5	9.0±0.0
10	6.5±0.5	9.0±0.0	9.0±0.0

Control non-vaccinated serum samples were antibody negative.

Table (6): Effect of Biogen and levamisol HCl on protection of *Oreochromis niloticus* against a virulent strain of *Aeromonas hydrophila* after vaccination by intramuscular injection of *Aeromonas hydrophila* vaccine (n = 30).

Weeks post-vaccination	Control		Biogen		Levamisol	
	Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated
Dead	30	23	15	9	18	12
Survival	0	7	15	21	12	18
Mortality %	100	76.66	50	30	60	40
Relative percent survival (RPS)	0	23.20	49.80	69.80	39.80	59.80

الملخص العربي

تأثير البيوجين و الليفاميزول على الاستجابة المناعية في اسماك البلطي النيلي للقاح الأيروموناس هيدروفيلا

رياض حسن خليل نادية محفوظ مجدى خليل سليمان

صممت هذه الدراسة لاستبيان تأثير إضافة مركبات البيوجين (2 جم/كجم عليقة) و الليفاميزول (1 جم/كجم عليقة) إلى علائق أسماك البلطي النيلي من ناحية تأثيرهم على نشاط الخلايا الأكولة، نشاط بعض الإنزيمات المختلفة (SGOT, SGPT, ALP)، الكوليسترول، الكرياتينين، الجلوكوز، البروتين الكلى، الألبيومين، الجلوبيولين و كذلك قياس منسوب الأجسام المضادة في سيرم الأسماك قبل و بعد حقن لقاح الأيروموناس هيدروفيلا.

و قد أوضحت النتائج أن إضافة البيوجين و الليفاميزول أدت إلى نشاط ملحوظ في الجهاز المناعي من خلال ارتفاع منسوب الأجسام المناعية في الأسماك المعالجة عنها في المجموعة الضابطة. أيضاً كان هناك نشاط في الخلايا الأكولة في المجموعة التي تغذت على علائق بها مركبات البيوجين و الليفاميزول. و أوضحت الدراسة أيضاً أن نشاط الإنزيمات (SGOT, SGPT, ALP) و الكوليسترول، الكرياتينين، الجلوكوز، البروتين الكلى، الألبيومين و الجلوبيولين كانت جميعها متشابهة بين المجاميع التي غذيت على علائق بها البيوجين و الليفاميزول و المجموعة الضابطة ما عدا نسبة البروتين هي التي كانت عالية عن المجموعة الضابطة.

و أوضحت الدراسة أن نسبة الأسماك المتبقية (relative survival percentage) بعد إجراء اختبار التحدي في الأسماك المحصنة في المجموعات التي تناولت علائق بها مركبات البيوجين و الليفاميزول هي 69.80 و 59.80% على التوالي بينما في الأسماك الضابطة التي غذيت على علائق عادية هي 23.20%.