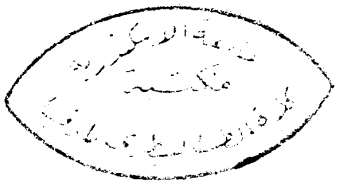
SOME STUDIES ON THE EFFECTS OF SOME IMMUNOSTIMULANTS ON CULTURED FRESHWATER FISH

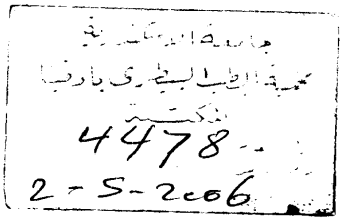


A Thesis

Presented to the Graduate School
Faculty of Veterinary Medicine, Alexandria University
In Partial fulfillment of the
Requirements for the Degree

Of

Master of Veterinary Sciences



In

(Fish Diseases and Hygiene)

By

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March 2006

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قرار لجنة الحكم والمناقشة

قررت لجنة الحكم والمناقشة بجلستها المنعقدة يوم الأربعاء الموافق ٢٠٠٦/٣/٨ السيد **ط . ب / أسامة السيد السنهورى** للحصول على درجة الماجستير فى العلوم الطبية تخصص (صحة وأمراض الأسماك) .

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بسم الله الرحمن الرحيم

وما أوتيتم من العلم إلا قليلاً

صدق الله العظيم

سورة الإسراء الآية رقم ٨٥

Dedicated to
My family and to my Wife

ACKNOWLEDGEMENT

First of all prayerful thanks to ***our GOD*** *who gives me every thing I have.*

I am greatly indebted to ***Dr. Magdy Khalil Soliman****, Professor of Fish Diseases and Hygiene, Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University for his stimulating supervision, and for valuable and continuous share and interest encouragement.*

Special thanks and full appreciation are due to ***Dr.Riead Hassan Khalil****, Associate Professor of fish Diseases and Hygiene, Faculty of Veterinary Medicine, Alexandria University for post-graduate studies researches , their direct and effective advises during the course of this study.*

Special thanks and full appreciation are due to ***Dr.El-Said El-Mankhly*** *, Professor of pathology, Faculty of Veterinary Medicine, Alexandria University for post-graduate studies researches , their direct and effective advises during the course of this study.*

Also , special thanks for ***Dr . Talaat Talaat Saad****, Lecturer of fish Disease and Hygiene, Faculty of Veterinary Medicine, Alexandria University for direct effective help during the fulfillment of this study.*

Thanks are also due to ***the Staff members of Department of Avian and Aquatic Animal Medicine*** *Faculty of Veterinary Medicine, Alexandria University, for their great support and encouragement during this work.*

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I- INTRODUCTION

Utilization of immunostimulants in fish culture offers a wide range of attractive methods for including and building protection against diseases.

A poor growth rate implies that the immune responsiveness would be markedly impaired ***(Peters and Hang, 1985).*** Few studies, however, have been concerned with the effect of nutrients on the immune response. A dietary deficiency leads to immune suppression in fish ***(Blazer and Wolke, 1984; Li and Lovell, 1985 and Soltan et al., 2000).*** Inducing a protective immune response to a pathogenic organism before the individual fish becomes naturally exposed to it (Prophylaxis) seems at first sight an eminently sensible way of preventing an infection ***(Ellis, 1988).*** The extensive use of antibiotics for treatment and prophylaxis has the serious disadvantage of increasing plasmid-encoded antibiotic resistance especially ***Aeromonas hydrophila*** which affects humans as well as fish ***(Ramadan et al., 1991).***

These antibiotic-resistance strains may become a human health problem, particularly when fish is eaten raw ***(Aoki et al., 1971)*** or improperly cooked ***(Rahim et al., 1984).***

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.

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

It was stated that the interaction between the microbiota, including probiotics, and the host is not limited to the intestinal tract. Probiotic bacteria could also be active on the gills or the skin of the host but also in its ambient environment. ***(Geert et al., 2002).***

Those modes of probiotics are as follows: protection of inhibitory compounds, competition for chemicals or available energy; competition for adhesion sites; enhancement of the immune response; improvement of water quality; interaction with phytoplankton; source of macro- and micronutrients; and enzymatic contribution to digestion ***(Geert et al., 2002).***

Immunostimulants are a group of chemicals or biological substances given to the human, animals, birds and fish to enhance the protection against pathogens via increasing the specific and non-specific defenses of the body. ***(Anderson and Jeney, 1992)***

Also immunostimulants represent a modern and promising tool in aquaculture, enhancing the resistance of cultured fish to disease and stress. ***(Bangi et al., 2000)***.

In several studies, water quality has been recorded during the addition of the probiotics, especially Bacillus Spp.

The rationale is that gram-positive Bacillus Spp. are generally more efficient in converting organic matter back to CO_2 than are gram-negative bacteria, which would convert a greater percentage of organic carbon to bacterial biomass or slime, and a lot of bacterial cultures containing nitrifying bacteria to control the ammonia level in culture water are available ***(Geert et al., 2002).***

Sandlheath Industrial State (2003), reported that, vaccination and treatment have been able to control many diseases that affect farmed fish. However, circumstances change and new diseases appear for which specific treatments may not be available . This coupled with consumer concerns over food safety and protection of the environment has meant that new strategies for disease control have to be developed through the introduction of immunostimulant in aquaculture industry.

Use of ***Biogen*** ® and ***Levamisole*** HCl® one know to use as immunostimulants in fish, broilers and large animals ***(Siwicki, etal., 1990; Zeinab and Alkelch, 1993; Yoshida et al., 1995 and Safinaze, 2001).*** Due to the importance of fish Industry in our country the effect of these immunostimulants on fish enforced us to study their effects.

Therefore, the present work was designed to investigate the effects of some immunostimulant on the immune responses of both ***Monosex tilapia*** and ***O. niloticus***.

II- REVIEW OF LITERATURE

The success of modern aquaculture has been the direct result of good management, good nutrition and good disease control.

Smith et al. (2003) reported that, there is a growing need to control, prevent or minimize the devastating effects of disease in fish culture resources to toxic chemicals or antibiotics. In keeping up with approaches to disease control in fish and higher mammals . Interest is developing in compounds that confer protection and / or enhance immune reactivity to likely pathogens in shellfish (Sometimes, erroneously, referred to as "Shellfish vaccines".

II-1-Types of immunostimulant:-

Olivier et al. (1985) injected ***Coho salmon (Onchorhynchus kistutch)*** with ***levamisole*** mixed with modified freund's complete adjuvant (MFCA). They decided that, the resistance against ***A. Salmonicida*** was increased.

Taraschewski et al. (1988) indicated that, the effects of live nematocidal drugs (***Levamisole HCL, metrifonate, fenbendazole, mebendazole, and ivermectin***) on the nematode ***Angillicola crassus,*** pathogenic in eels and recently introduced in Europe, were tested under in vivo conditions.

Siwicki et al. (1989) reported that, Oxolinic acid, a promising drug for the treatment of bacterial fish disease, was tested for possible immunomodulatory effects on fish. ***Levamisole*** , a known immunostimulator for higher vertebrates, were also compared for causing changes in the nonspecific defense compartment and the specific immune system in ***Rainbow trout.***

Siwicki and Cossarini-Dunier (1990) **reported that, the** *levamisole* **is an anthelmintic drug which is often used in cattle, is known for its immunomodulatory effect in several species.**

Anderson and Jeney (1992) reported that, three drugs, ***levamisole*** (an approved veterinary drug in USA), a quaternary ammonium compound (QAC), and a short-chain polypetide (ISK) were found to affect the non-specific defense mechanism activities.

Siwicki et al. (1994) stated that, the immunostimulant preparations as *Macrogard,* ***Candida utilis, Saccharomyces cervisiae, Evetsel, Chitosan and Finnstim*** were the most important immunostimulant in aquaculture.

Hamre and Lie (1995) indicated that there are higher growth rate of Atlantic salmon, (***Salmo salar L***) in supplemented α- tocopherol group (300 mg/kg diet) than un-supplemented group.

Baker and Davies (1996) reported that supplemental alpha tocopherol acetate in diet of catfish would increase growth above the levels associated with un-supplemented diets.

Merchie et al. (1996) showed that, the feed efficiency, growth rate and calculated biomass in European sea bass fed control ***ascorbic acid*** free diet lower than in the ascorbic acid supplemented diet.

Biologicals and Seattle (1997) mentioned that, as in other animals, substances such as Freund's complete and incomplete adjuvant, light oils and bacterial lipopolysaccharides have been shown to induce elevated antibody production when added to bacterines and administered to fish. Also, they reported that, substances such as beta-1, 3 glucans, chitosan, ***levamisol*** and other inflammatory agents shows enhancing effects on the specific immune response when added to immunogens and administered by injection, bath or by feeding. These substances may also act to elevate non-specific defense mechanisms against disease agents as most of them are active even when given alone.

Wang and Wang (1997) demonstrated, the efficiencies of eleven immunostimulant polysaccharides including Bar (***glucan*** extracted from barley), curldan, Dex (dextran sulfate), inulin, krestin, laminara, levan PO (glycogen extracted from pleurotus ostreatus), sclerglucan, YG (***yeast glucan***), and zymosan in increasing the resistance of fish against different diseases.

ICRAM et al. (2000) reported that, ***glucans*** alpha-tocopherol and ascorbic acid have a great immunostimulatory effect on fish via its effect on the innate immunity.

Cook et al. (2001) they investigated that, the effects of prolonged administration of a commercial ***beta-glucan*** based immunostimulant preparation, Eco Activa, in the form of a feed supplement, on non-specific immune parameters and the growth rate of ***Snapper (Pargus auratus).***

Ortuno et al. (2002) reported that, the yeast, ***Saccharomyces cervisiae***, enhances the cellular innate immune response.

Safinaz (2001) found that addition of ***Biogen*** ® at rate of 0.2% (1kg / ton) improve both of innate and humoral immunity of ***O. niloticus***.

Saad (2002) reported that, in general the addition of ***Biogen*** ® as a fish immunostimulant causes increase of WBCs counts in acute and chronic Ochratoxicosis affected ***O. niloticus***.

Sahoo and Mukherjee (2002) used four dietary immunomodulators viz., beta - 1, 3 ***glucan, levamisol*** , vitamins C and E, in Indian major carp species, ***rhou (Labeo rohita Ham)*** for increasing the immunity of this fish.

Villamil et al. (2002) indicated that, lactic acid bacteria have a great immunostimulatory effect via activation of macrophages in vitro and in vivo. They also, reported that, out of six lactic acid bacterial strain tested only heat-killed ***Lactococcus lactis*** significantly increased the macrophage activity.

Smith et al. (2003) reported that, the agents currently under scrutiny for crustaceans include, lipopolysaccharides and killed bacterial cells. They are thought to act as "immuno-stimulants" because of their known effects on the crustacean immune system.

II-2. Action of immunostimulants in fish:-

Semons and Rosenthal (1977) approved that ***levamisole*** enhances the metabolic and phagocytic activities of neutrophils. The number of phagocytes, leucocytes and the level of lysozyme in serum were also increased.

AWT (1978) stated that vitamin E act as a lipid-soluble antioxidant protecting biological membranes and lipoproteins against oxidation. It has stimulating effect on antibody synthesis, antitoxic effect in the cell metabolism and it has been demonstrated to be an essential dietary nutrient for all fish studied.

Johnson et al. (1982 b) compared between active immunization and uses of immunostimulants and they decided that uses of immunostimulants give immunity for a short time compared with active immunization.

Siwicki et al. (1987) injected ***Carp*** with ***levamisole*** and found that, phagocytic activity , yellow peroxides activity in neutrophils were enhanced. Also, increase leucocytic number and serum lysozyme levels were increased. The injected ***Carp*** with various diseases doses of ***levamisol*** e to determine the dose that give the best result, they found the dose equal to, 5 mg producing the strongest stimulatory effect when injected intra peritoneal in carp spawners, while the doses 15 & 20 mg suppresses the immune response and immuno-status was investigated till the end of the experiment.

Swicki et al. (1989) reported that ***levamisle*** injection increased protection against stress related diseases by enhancing non specific immune response such as PA and PI chemilumenes responses of leucocytes and natural killer cells activity.

Sandel and Daniel, (1988) indicated that vitamin C acts as a metabolic antioxidant, detoxifying numerous peroxide metabolites, thus protecting cell membranes and other intracellular components and processes that are sensitive to oxidation.

Siwicki et al. (1989), bathing carp fingerling in ***levamisole*** at level of 1 mg /ml found that after , the growth was stimulated and the natural mortality losses decreased.

Kajita et al. (1990) injected Rainbow trout with high dose of ***levamisole*** 5mg / Kg and subsequently exposed to ***Vibro anguillarum.*** They, found that, no stimulating response occurred. But when injected with 0.1 mg / kg - 0.5 mg/kg. They, found increases in serum antibacterial activity, complement activation, phagocyte elevation and natural killer cell enhancement.

Siwicki et al. (1990) cultivated spleen cells with ***levamisole*** in vitro and found that, the phagocytic activity enhanced and the number of plaque forming cell increased . Against ***Y. Ruckeri*** O-antigen also lymphocyte proliferation increased. But, when the dose of ***levamisole*** increased to 50 ml, the antibodies forming cells in the spleen and antibody production was completely suppressed.

Anderson and Jeney (1992) reported that the immunostimulants when given alone, elevated the non-specific factors. On the other hand when they injected in combination

with an ***A. salmonicida*** O-antigen bacterine, the non-specific factors were further elevated, and the specific response was raised over samples taken from fish given the bacterine without the immunostimulants.

Doglas and Anderson (1992) studied the effect of immunostimulants, adjuvant and vaccine carriers in fish culture. They found that, a wide range of protection and reduce uses by increasing the specific and non specific immuno-response and can be administered before, with or after vaccine.

Siwicki et al. (1992) reported that when ***levamisole*** was injected with antigens caused a synergistic nonspecific defense stimulators.

Anderson and Jeney (1992) injected ***levamisole*** and ***A. salmonicida*** bacterine then challenge all fish (salmon and trout). Other group put fish in bath containing ***levamisole*** and bacterine then challenged. They found that, the degree of immune activation and protection was clearly evident in both types of fish . The fish given ***levamisole*** only all dead by days , whereas in group given ***levamisole*** and bacterine companion the mortality was delayed until 21 days. The survival rate after challenge in fish given ***levamisole*** only was 57% compared to 66% for fish given ***levamisole*** bacterine compensation.

Baba et al. (1993) immersed carp fish in a ***levamisole*** bath (10 mg / ml, 24 h) and detected the immune status against ***A. hydrophila.*** They found that, ***levamisole*** increased phagocytes .This effect lasts for 2 weeks.

Brattgjer et al. (1994) reported that, treatment of ***Atlantic salmon*** with ***M-glucan*** resulted in enhanced serum lysozyme activity in 3rd week of the experimental period. The results indicate that ***M-glucan*** elevates the activity of the non-specific part of the immune system.

Engstad and Robertson (1994) reported that ***Atlantic salmon*** macrophages express a receptor that rapidly recognizes and mediates uptake of nonopsinized ***beta-glucan*** particles. The ingestion of particles was shown to be inhibited by preincubating the macrophages with ***glucans*** containing beta -1, 3-linkages, but not by ***glucans*** containing other linkages.

Siwicki et al. (1994) reported that total plasma protein and total immunoglobulin levels were elevated by the dietary immunostimulants. A challenge with the virulent pathogen that causes ***Furunculosis***, ***Aeromonas salmonicida***, showed that the immunostimulated groups of fish were resistant to the disease. This confirmed the potential use of these substances in fish culture for the prevention of diseases.

Jorgensen and Robertson (1995) reported that, despite the stimulatory effect of ***glucan*** on the respiratory burst activity of salmon macrophages, these cells did not increased bactericidal activity against the a virulent and virulent strain of *A.* ***salmonicida*** . Up regulation of burst activity alone is thus apparently not sufficient to enhance bactericidal activity against this pathogen .

Olva Vadestein (1997) found that immunostimulations of fish have potential roles for reducing losses in aquaculture during both larval and on-growing stages.

Biologicals and Seattle (1997) reported that, immunostimulants defined as adjuvant combined with immunogenes are effective enhancers of the immune response in fish.

Biologicals and Seattle (1997) reported that, light oil adjuvant are now used successfully with injectable bacterines of ***Aeromonas salmonicida***, and in multivalent fish vaccines where ***A. salmonicida*** is combined with ***Yersinia Sp., and/or Vibrio Spp***. antigen preparations. The oils are thought to act as depots or reservoirs, holding the antigens in globules at the site of injection, thus allowing prolonged dosage in additions to the oils such as muramyl dipeptide, bacterial lipopolysaccharides and other populations such as T-cells, neutrophils and other phagocytic cells important in the cellular mediated response. Conjugation of antigens with alum is another traditional approach which has been used for ***A. salmonicida and Y. ruckeri*** bacterines with varying results. The process enables antigen to be held in a reservoir and also may detoxify harmful substances.

Figueras et al. (1998) they reported that the highest activity of all immune parameters was obtained when ***glucans*** were injected after the bacterine. It is concluded that the sequence of ***glucan*** administration is critical when used as a vaccine adjuvant.

AlicJa et al. (2000) indicated that ***levamisole*** increases the metabolic and phagocytic activity of neutrophils and leucocytic number and phagocytes.

ICRAM et al. (2000) from their investigations on the effect of a combination of dietary glucans, alpha-tocopherol and ascorbic acid on the innate response of ***seabass (Dicentrachus labrax)*** they observed that, no significant differences were observed in protein content or in albumin/globulin ratio. Compared to lysozyme activity, which showed marked individual variation, complement-mediated haemolytic activity has been shown to be a more reliable indicator of sea bass immunocompetence.

Cook et al. (2001) reported that, ***EcoActiva*** is able to stimulate non-specific immunity in ***Snapper*** through increased respiratory burst activity of macrophages which considered as an important component of the host defense network.

Sahoo and Mukherjee (2001) reported that a single intraperitoneal injection of Aflatoxin B_1 (AFB_1) at 1.25 mg/ g (- 1) body weight caused a significant ($P< 0.05$) reduction in non-specific immunity as measured through neutrophil phagocytic indexes. Serum bactericidal activity , and specific immunity as measured through bacterial agglutination titer against ***Edwardsiella tarda***, as well as reduced protection against ***Aeromonas hydrophila*** challenge in comparison to control fish which were exposed neither to aflatoxin nor to ***glucan***. Feeding of ***glucon*** to healthy fish raised the non-specific and specific immunity level and protection against bacterial infection compared with the control. Feeding of ***glucan*** to AFB1-induced immunocompromised fish for 7 days significantly raised the degree of resistance against ***A. hydrophila*** challenge and the non-specific immunity level in comparison to non-treated AFB1 exposed fish.

Shahat (2001) noticed that increasing dietary level of vitamin E to 2000 mg/kg may be an easy and effective means of increasing immune function and increasing disease resistance in ***Monosex*** and ***O. niloticus***.

Also he noticed that the anterior kidney concentration of ascorbic acid (A.A) in tilapia fed diet supplemented with ascorbic acid (400 mg/kg) and α-tocopherol (α -TOH)

2000 mg/kg diet together was 166+4 mg/kg and higher than those fed A.A and α-TOH deficient diet (1.3 mg/kg). Also he reported that the supplementation of A.A and α-TOH together increased the whole body level of A.A to 1.3 fold compared by those fish supplemented with A.A only, and increased α-TOH in the body to .05 fold compared by those fish supplemented with α-TOH only.

Cuesta et al. (2002) concluded that, ***levamisole*** enhanced ***seabream*** natural cytotoxic cell activity both in vitro and in vivo and had a great and lasting action when administered by feeding.

Ortuno et al. (2002) reported that the enhancement action of ***Sacharomyces cervisiae*** was dose-dependent except for the cytotoxic activity that was only stimulated by the lower dose of yeast assayed. As yeast cell walls are able to enhance the ***Seabream*** cellular innate immune response, these results support the possible use of whole yeast as natural immunostimulants in common fish diets.

Saad (2002) noticed that, the addition of ***Biogen*** ® with the ***OTA*** causes reduction of the effect of ***OTA*** on blood enzymes, body weight, body weight gain, feed conversion and feed efficiency. This attributed to the ***Biogen*** ® which contain the extract of Ginseng plant that increase the vitality and activity of the fish. Also, the ***Biogen*** ® increase and activate the antibodies and immunity of the fish against any stress that the fish exposed to it. Also contain some useful bacteria that help in destruction of the ***OTA*** and hindered its effects, also the ***Biogen*** ® activate the liver to get ride of the OTA, and considered as a stomachic to the fish that improve the fish appetite.

Sahoo and Mukherjee (2002) studied the effect of vitamin E supplementation on an ***Indian major carp*** subjected to Aflatoxin B. They noticed that vitamin E is capable of significantly ($P<0.05$) increasing specific immunity and reducing mortality in immunocompromised fish but failed to enhance specific immunity and protection in healthy fish.

Sealey and Gatlin (2002) fed ***Juvenile hybrid*** striped bass nine semi purified diets of (0, 25, 2500 mg vitamin C/kg diet and/or vitamin E at 0, 30, 300 mg/kg diet. They observed that plasma protein and total immunoglobulin levels in exposed fish were not affected by dietary vitamin levels, whereas respiratory burst activity increased with vitamin E supplementation.

Sahoo and Mukherjee (2002) suggested that, the introduction of immunostimulants into the diet of fish grown in farms under immunosuppressive / stressful conditions could increase their resistance to infection by reducing mortality rates and offer economic benefits.

Also they reported that immunostimulators were capable of significantly ($P< 0.05$) increasing specific immunity and reducing mortality in immunocompromised fish but failed to enhance specific immunity and reduce mortality in healthy fish. The increased bacterial agglutination titer by ***beta-1, 3 glucan***, and reduced mortality losses by both beta - 1, 3 ***glucan*** and ***levamisole*** were marked in healthy vaccinated fish compared with their controls. Similarly, all four substances significantly reduced the mortality rates in immunocompromised and healthy unvaccinated fish . Out of these four substances, ***glucan*** was recorded to be the most effective immunomodulator in ***rohu***.

Villamil et al. (2002) indicated that ***L. lactis*** (***lypolytic lacyis***) proved to be the strain with more effects on the host immune function, further in vivo and in vitro experiments were conducted with this bacterium. The in vitro capacity of ***L. lactis*** to adhere to turbot intestinal mucous was positively confirmed. When orally administered, ***L. lactis*** significantly increased the macrophage CL response and the serum NO concentration after 7 days of daily administration. The antibacterial effect of the extracellular products from the six LAB strains against the fish-pathogenic bacterium ***Vibrio anguillarum*** was also demonstrated in vitro.

Cook et al. (2003) suggested that routine incorporation of beta-***glucan*** preparations like ***EcoActiva*** during Winter may enhance macrophage function and growth rates at a time of increased disease susceptibility and little or no growth.

Also they reported that, fish fed on a diet supplemented with ***EcoActiva*** and held at the winter temperature had a significant enhancement of macrophage superoxide anion production upon stimulation with phorbol myristate (PMA), and this increased activity was maintained throughout the trial. Macrophage activity in fish fed the supplemented diet and held at the summer temperature was also increased. However, ***EcoActiva*** failed to increase either classical or alternate complement activity. Most significantly ***EcoActiva*** resulted in an increase in growth rates of the fish held at the winter temperature as to the control fish , although no difference was seen between the groups held at the summer temperature.

Lee and Dabrowski (2003) demonstrated that total ***Ascorbate*** concentration of liver of ***Yellow perch (Perca flavescens)*** were significantly higher in those fed the vitamin C supplemented diet than in those fed the vitamin C deficient diet. The liver alpha tocopherol concentration was increased by supplemental vitamin E deficient dietary groups.

Ortuno et al. (2003) reported that ***Gilthead*** under stress, fed diet with vitamin C at 3g/kg have lower blood glucose concentration and enhanced hemolytic complement activity than other fish fed commercial (control) diet.

The same authors noticed that ***Gilthead*** under stress fed diet with vitamin E at 1.2g/kg diet, suffer from lower blood glucose concentration with enhanced hemolytic complement activity than fish fed on control diet.

Rodriguez et al. (2003) reported that, among the humoral responses, serum peroxidases and complement activity were significantly decreased after 6 weeks of feeding with the ***Sacchromyces cervisiae.***

The non specific immune system is responsible for initiating a rapid and general response to an invading disease organism. Once the animals physical barriers, such as the skin and enzymes on mucous membranes have been overcome, phagocytic cells (e.g. macrophage and neutrophils) within the body tissues and fluids locate, engulf and destroy the invading organisms. This is the role of immunostimulants in fish protection against diseases ***(Sandlheath Industrial State, 2003).***

Naglaa-Mangoad (2004) reported that the uses of ***levamisole*** increases the resistance to stress conditions by increasing the non specific defense mechanisms by increasing Phagocytic activity and Phagocytic index, leucocytic count, myeloperoxidase activity in neutrophiles activity in neutrophil and serum lysozyme activity.

II.3.Uses of immunostimulants in different fish species:

Swicki et al. (1989) used oxolinic acid, oxytetracycline and ***levamisole*** in salmonids to increase the non-specific and specific activity.

Swicki et al. (1989) used ***levamisol*** as an immunostimulating effect on non-specific immunity of ***Carp (Cyprinus carpio)***.

Siwicki et al. (1990) used ***levamisole*** to increase the stimulatory activity of spleen cells of ***Rainbow trout (Onchrhynchus mykiss)*** and increase the effect of lymphocyte and macrophage in ***Carp (Cyprinus carpio).***

The same authors used ***levamisole*** to increase the neutrophil activity in rainbow trout ***(Oncorhynchus mykiss)***.

Brattgierd et al. (1994) used the yeast ***glucan*** to increase and stimulate the activity of macrophages in ***Atlantic salmon, (Salmo salar L.)***

Jorgensen and Robertson (1995) used yeast ***beta-glucan*** to stimulates respiratory burst activity of ***Atlantic salmon (Salmo Salar L.)*** macrphages.

Wang and wang (1997) enhanced the resistance of ***Tiapia and Grass carp to Aeromonas hydrophila and Edwardsiella tarda*** infections by several polysaccharides as (Glucose namely, Bar, Krestin, ***scleroglucan*** and zymosan).

Figueras et al. (1998) used ***beta-glucans*** and a ***Vibrio damsela*** vaccine as useful tool for immunostimulation in ***Turbot (Scaphthalmus maximus L.)***

Bangi et al. (2000) used glucogan as long-term oral administration of ***Seabass (Dicentrachus labrax)*** to increase the immune status of fish .

Paulsen et al. (2001) used yeast ***beta-glucans*** and bacterial lipopolysaccharide for the immunostimulation in ***Atlantic salmon (Salmo Salar L.)*** through enhancement of lysozyme production in macrophages .

Sahoo and Mukherjee (2001) used the beta- 1, 3 ***glucan*** in diet to increase diseases resistance in healthy and aflatoxin B1- induced immunocompromised ***rhou (Labeorohita Hamilton).***

Cuesta et al. (2002) reported that ***levamisole*** is a potent enhancer of ***Gilthead seabream*** natural cytotoxic activity.

Ortuno et al. (2002) reported that, oral administration of yeast, ***Saccharomyces cervisiae*** in ***Gilthead seabbream (Sparus aurtal.),*** can enhances the cellular innat immune response.

Sahoo and Mukherjee (2002) reported that dietary immunomodulation were most effective immunostimulant in ***Indian major carp (Labeorhita).***

Villamil et al. (2002) used lactic acid bacteria in ***turboot*** as immunostimulant for prevention of infection.

Cook et al. (2003) reported that, using a commercial immunostimulant preparation, ***EcoActiva*** as a feed supplement enhances macrophage respiratory burst and the growth rate of ***Snapper (Pagrus auratus, Sparidae Bloch and Schneider).***

Rodriguez et al. (2003) used ***Saccharomyces cervisiae*** strain as immunostimulant to ***Seabream (Sparus aurata L.).***

Smith et al. (2003) reported that immunostimulant usage in crustaceans have a positive effect against infection.

Shewita (2004) used the Vitamin C and/ or Vit. E for improving the immunity, weight gain, food conversion and body weight of ***Oreochromis niloticus***.

-Period of action:-

Siwicki et al. (1990) reported that spleen and kidney macrophages must be in contact with ***levamisol*** for 2 h before phagocytosis of opsonised ***Yersinia ruckeri.***

Anderson and Jeney (1992) observed from the challenge tests, with the virulent pathogen, ***A. salmonicida,*** showed 5 - 6 day delay in the onset of mortalities in the fish given the immunostimulants alone, and a 12 - 14 day delay when immunostimulants given were combined with the bacterine. In the groups given the Quaternary Ammonium Chloride (QAC) or short chain polypeptide (ISK) with the bacterine, there was a 20% and 40% survival rate, respectively.

Siwicki et al. (1992) reported that, ***levamisole*** gave its maximum effects after 10 days from its administration to fish via increasing the non-defense reactions .

Brattgjerd et al. (1994) reported that ***M-glucan*** affect the phagocytic assay with macrophage and ***Vibrio salmonicida*** the initial uptake of bacteria was elevated at both 3 and 6 weeks after ***glucan*** treatment. The same author found that the ***Saccharomyces cervisiae, evetsel, chitosan or finnstim*** gave its maximum effect after one week from its administration to Rainbow trout.

Jorgensen and Robertsen (1995) investigated the in vitro effects of ***glucan*** on the respiratory burst and bactericidal potential of Atlantic salmon head kidney macrophages. ***Salmon*** macrophages were incubated for 1 - 7 days with various concentrations of yeast ***beta-glucan(MacroGard)*** and tested for respiratory burst activity by the reduction of nitroblue-tetrazolium (NBT) after exposure to phorbol myristate acetate (PMA) or opsonized zymosan. The macrophages showed a marked increase in respiratory burst activity 4 to 7 days after addition of ***glucan***, macrophages treated with 0.1 - 1 microgram ml-1 gave a maximum respiratory burst response. Whereas 10 micrograms ml-1 had no effect and 50 micrograms ml-1 was inhibitory . Also, they reported that, the ***glucan*** also triggered respiratory burst activity directly, but this occurred only at relative high concentrations with a maximal effect at > or = 200 micrograms ml-1. The validity of using NBT-assay as a measure of respiratory burst activity was confirmed by using inhibitors of O2-production (superoxide dismutase, trifluoperzine and diphenylene iodonium).

Sahoo and Mukherjee (2001) used beta - 1, 3 ***glucan*** at 0.1% in fish feed at 7 days to healthy fish and Aflatoxin B1 (AFB1)-induced immunocompromised fish, ***Labeo rohita*** (one of the major tropical carp species), in 60 day trial, and they gave its maximum effects on non-specific and specific immunity of the fish at this day.

Cuesta et al. (2002) found that, the action of ***levamisole*** was slightly increased after 24 h of incubation and its cytotoxic activity was found to increase along the period of experiment and remained greatly enhanced till to the end of the experiment which extended to about 6- weeks post-administration.

Ortuno et al. (2002) give a lypholised whole yeast, ***Saccharomyces cervisiae***, in the diet on the ***Seabream*** innate immune response were investigated. ***Gilthead seabream (Sparus aurata L.)*** specimens were fed four different diets for 4 weeks: a commercial diet as control and the same diet supplemented with 1, 5 or 10 g/Kg yeast. After 1, 2 and 4 weeks, and give a perfect effect at this periods.

Rodriguez et al. (2003) reported that the ***Saccharomyces cervisiae*** give its maximum effects as an immunostimulator perfectly at 2, 4 or 6 weeks after administration. The lysozyme activity increased after 2 and 4 weeks of feeding the ***Saccharomyces*** strain. In the cellular responses studied, phagocytosis was increased to a significant degree at all the assayed times but only the ***Saccharomyces*** strain-supplemented diet, while respiratory burst activity (after 4 weeks) and natural cytotoxicity (after 4 and 6 weeks) increased with either yeast strain . The intra-cellular peroxidase content was not affected by the dietary supplementation of either yeast strain.

Sahoo and Mukherjee (2002) used immunomodulation against ***E. tarda*** and it gave its maximum effect after 30 day of administration.

Villamil et al. (2002) reported that, out of six lactic acid bacterial strains tested, only heat-killed ***Lactococcus lactis*** significantly increased the turbot kidney macrophage chemiluminescent (CL) response after 24 h of incubation. Nitric oxide (NO) was also significantly enhanced by this bacterium after 72 h of incubation with either viable (10^3 and 10^6 cells/ml) or heat-killed (10^6 cells/ml) bacteria . Viable Leusenteroides (10^6 cells /ml) was also capable of significantly increasing NO production.

-Method of administration.

Figueras et al. (1998) used Yeast ***(Saccharomyces cervisae)*** beta-1, 3 glucans as adjuvant in a ***Vibrio damsela*** vaccine for turbot ***(Scophthalmus maximus L.)*** and injected the vaccine to turbot.

Cuesta et al. (2002) administered the ***levamisole*** via feed.

Sahoo and Mukherjee (2002) adminstered the immunomodulation via feed to increase the resistance to diseases .

Rodriguez et al. (2003) used ***Saccharomyces cervisae*** as immunostimulator via the feed of ***Gilthead seabream (Sparus aurata L.)***.

Bangi et al. (2000) and Sahoo and Mukherjee (2001) applied the glucans to fish via the feed.

-Effect of stress on immune responses:

Snieszko (19 stated that three factors must occur concurrently for an infectious sease outbreak tc elop pathogen organism, susceptible fish and a predisp ing ressful) conditior e fish stress associated with environmental changes result in reased susceptibilit to disease. He reported that, fish that were fed well, un-crowded

and put in a good environment were less likely to develop a disease. He stated also that fish pathogens were common in stocked ponds under natural water system but caused problems only when the fish had been weakened by some outside or pre-disposing environmental factors. The environmental factors were frequently referred as "stressors". He added that fish can adapt to some of the changes in their environment particularly those that develop slowly. The magnitude and speed of change or the nature of the introduced factors may be great enough to exceed the normal range of adaptation. He added that, when fish were unable to adjust to their environment they become stressed and an infectious disease condition often followed.

Gratzek and Reinert (1984) mentioned that, common stress factors in the laboratory can include extremes of pH, temperature, or other factors, such as the accumulation of intermediates of nitrification. Each aberration from optimal conditions has a direct physiological effect on fish that can cause death in a variety of ways. The stress effect in fish leading to infectious diseases is a common physiological manifestation of some common biochemical change, probably hormonal, which may compromise the immune system and lead to a decreased disease resistance and ultimately death. Minimization of stress by careful attention to basic fish culture practices is perhaps the best way one can ensure successful laboratory experiments.

Barry et al. (1993) and Pottinger et al. (1994) mentioned that , the stress responses vary between fish species, the developmental stage of the fish, as well as between individuals within the same species.

Wester et al. (1994) declared that many fish diseases are related to environmental quality, various environmental pollutants have immunotoxic potential and many fish diseases have an immunological component.

Siwicki et al. (1994) reported that fish have short life span and cold water environment make slowing the development of specific immune response .

Iger et al. (1995) stated that, a number of effects of stress, however, are primarily related to aquatic environment such as an increasing permeability of the surface epithelia including the gills and thus hydromineral disturbances.

Wendelaar Bonga (1997) concluded that, the stress response in teleost fish shows many similarities to that of the terrestrial vertebrates. These concern the principle messengers of the brain-sympathetic-chromaffin cell axis (equivalent of the brain-sympathetic-adrenal medulla axis) and the brain -pituitary-interrenal axis (equivalent of the brain-pituitary-adrenal axis), as well as their functions, involving stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and mainly suppressive effects on immune functions. There is also growing evidence for intensive interaction between the neuroendocrine system and the immune system in fish. Conspicuous differences, however, are present , and these are primarily related to the aquatic environment of fishes. For example, stressors increase the permeability of the surface epithelia, including the gills, to water and ions, and thus induce systemic hydromineral disturbances. High circulating catecholamine levels as wells as structural damage to the gills and perhaps the skin are prime causal factors. This is associated with increased cellular turnover in these organs. In fish, cortisol combines glucocorticoid and mineralocorticoid actions, with the latter being essential for the restoration of hydromineral homestasis, in concret with hormones such as prolactin (in

freshwater) and growth hormone (in seawater). Toxic stressors are part of the stress literature in fish more so than in mammals. This is mainly related to the fact that fish are exposed to aquatic pollutions via the extensive and delicate respiratory surface of the gills and, in seawater, also via drinking. The high bioavailability of many chemicals in water is an additional factor. Together with the variety of highly sensitive perceptive mechanisms in the integument, this may explain why so many pollutants evoke an integrated stress response in fish in addition to their toxic effects at the cell and tissue levels.

-Dose of immunostimulants:-

Schmahl and Taraschewski (1987) indicated that, praziquantel caused irreversible lesions in the parasite tegument (beginning with 1 microgram / ml for 90 min 0.075 - 0.1 microgram / ml (90 min); ***Levamisole*** -HCl was effective in a concentration range of 20 - 50 micrograms / ml for 120 min. Like Praziquantel, Niclosamide and ***Levamisole*** -HCl led to damage of the parasite tegument. The opisthaptor region and the openings of the cephalic glands were most severely affected. ***Levamisole*** -HCl (10 micrograms / ml) and Niclosamide (0.2 microgram / ml) were effective against ***Diplozoon paradoxum*** after an exposure of 90 min and 45 min in vitro. The parasites were severely affected along the midbody. Metrifonate caused a lysis of the tegument and a strong secretion of slime. It is suggested that chemotherapy against Gyrodactylus Sp. may be accomplished with 10 mg Praziquantel / 1 for 3 h during storage in smaller tanks. Niclosamide (0.1 mg/1 for 90 min), or ***Levamisole*** -HCl (50 mg/1 for 120 min) may be used alternatively. Concentrations of Niclosamide and ***Levamisole*** -HCl have to be calculated with accuracy, since fish only tolerate a very narrow range of these drugs (Niclosamide, 0.1 mg / 1 for 120 min; ***Levamisole*** - HCl, 50 mg/1 for 120 min).

Siwicki et al. (1989) found that, ***levamisole*** and metrifonate were most effective in a freshwater bath (1 mg/l) for 24 h. The 50% lethal dose of ***levamisole*** was 250 mg/1 per 24 hours, whereas that of metrifonate was only 10 mg/1 per 2 hours.

Siwicki et al. (1989) showed that, oxolinic acid, used at recommended doses for the treatment of bacterial diseases, did not cause immunosuppression in either the nonspecific defense or specific immune system compartments, whereas tetracycline at 10 mg/kg caused reduced activity in both. Fish given ***levamisole*** injections before the antigen injection showed a stimulated nonspecific defense but a much reduced specific immune response.

Siwicki et al. (1990) found that, elevations in nonspecific defense and specific immune response in the 5 micrograms / ml, levels of ***levamisole*** . The 25 micrograms / ml levels investigated a slight elevation in some nonspecific levels but showed suppression in the specific immune response . The 50 micrograms/ ml levels suppressed all indicators whether ***levamisole*** was given alone or combined with ***Yersinia ruckeri*** O-antigen or DNP-Ficoll. These antigens are themselves nonspecific defense stimulates, and a synergestic effect was evident when combined with 25 micrograms/ml and 5 micrograms/ml levels of ***levamisole*** .

Siwicki et al. (1990) reported that, at low concentrations, i.e. 12.5 to 0.1 micrograms ***levamisole*** stimulated proliferation with phytohemagglutinin P (PHA-P) and Con A, no effect at medium concentrations and acted as a suppressor at high concentrations, 100 micrograms / ml PHA-P and 100 to 10 micrograms / ml for con A. Doses of 200 and 100 micrograms / ml were found to be suppressive, whereas 6.2 - 0.8 micrograms / ml were stimulatory, as observed by chemiluminescence.

Siwicki et al. (1992) reported that, after 10 days of culture with either 50 micrograms / ml, 25 micrograms / ml, 5 micrograms / ml or no ***levamisole*** in the media, the nonspecific defense reactions were measured by determining the metabolic activity of neutrophils, and the elevations in nonspecific cellular defense in the 5 micrograms / ml levels of ***levamisole***. The 25 micrograms/ml levels instigated a slight elevation in nonspecific cellular defense and the 50 micrograms/ml levels suppressed all indicators, whether ***levamisole*** was given alone or combined with ***Yersinia ruckeri*** O-antigen or DNP-Ficoll.

Jorgensen and Robertson (1995) reported that, the macrophages showed a marked increase in respiratory burst activity 4 to 7 days after addition of ***glucan***. Macrophages treated with 0.1 - 1 microgram mL-1 gave a maximum respiratory burst response, whereas 10 micrograms ml-1 had no effect and 50 micrograms ml-1 was inhibitory. The ***glucan*** also triggered respiratory burst activity directly, but this occurred only at relative high concentrations with a maximal effect at > or = 200 micrograms ml-1.

Bangi et al. (2000) investigated the effect of a combination of dietary ***glucans***, alpha-tocopherol and ascorbic acid on the innate immune response of cultured sea ***bass (Dicentrachus labrax)***. After 5 weeks of adaptation on a commercial diet containing 100 P.P.m. ascorbic acid and 200 P.P.m. alpha-tocopherol. Sea bass were switched to a diet supplemented with 2% beta-1.3 / beta- 1.6 ***glucans*** and ascorbic acid and alpha-tocopherol at 500 p.p.m. The supplemented diet was given at 2% of body weight per day over a 2-week period, every 3 months.

Safinaz (2001) used biogen in a dose of 0.1 % of diet (1kg/ton).

Sahoo and Mukherjee (2001) reported that, the immunostimulant beta- 1, 3 ***glucan*** was fed at 0.1% in feed for 7 days to healthy and alflatoxin B1 (AFB1)-induced immunocompromised fish, ***Labeo rohita*** (one of the major tropical ***Carp*** species), in a 60 day trial.

Cuesta et al. (2002) used the ***levamisole*** in the feed by about 0, 75, 150 or 300 mg ***levamisole*** / Kg diet for 10 consecuative days.

Ortuno et al. (2002) used ***Saccharomyces cervisiae*** in the diet of ***Gilthead seabream (Sparus aurata L.)*** to detect its effect on innate immune response . Specimens of the fish were fed four different diets for 4 weeks, a commercial diet as control and the same diet supplemented with 1, 5 or 10 g / Kg yeast for 1, 2 and 4 weeks.

Huang et al. (2003) fed hybrid ***tilapia*** diets supplemented with 0, 50, 100, 200, 450, and 700 mg α-tocopherol acetate/kg. They noticed that protein content of fish fed diet containing the lowest vitamin E level was the lowest ($p > 0.05$) among all groups, no difference was found in other body constituents among test fish ($P>0.05$).

Lee and Dabroweski (2003) fed ***Yellow perch (Perca flavescens)*** semi purified diets containing vitamin E at levels (0 or 160 mg/kg) with or without vitamin C supplementation (250 mg/kg). They noticed that the fish fed 250 mg/kg vitamin C and 160 mg/kg vitamin E showed significantly higher weight gain, feed intake and feed efficiency than the groups fed vitamin C deficient diets.

Lee and Dabrowski (2003) pointed out that total ***Ascorbate*** concentration of liver were significantly higher in fish ***(Yellow perch)*** fed vitamin C supplemented diets (250 mg/kg diet)than in fish fed the vitamin C deficient diet.

Shewita (2004) concluded that diets supplemented with vitamin C/E at levels of 400/300, 400/150, 400/75, 200/300 and 200/150 mg/kg diet were more efficiently utilized by fish than those fed the control diet or diets supplemented with other levels of vitamin C and/or vitamin E. Economically the best groups that, fed basal diet supplemented with Vitamin C/E 200/150 mg/kg followed by the group fed basal diet supplemented with Vitamin C/E 400/300 mg/kg as the net profit for them were 57 and 51piaster/20 fish. respectively. Also he found that, the fish fed the diet supplemented with vitamin C/E at rates of 1000/750, 1000/75 mg/kg diet showed highest growth performance, highest antibody response and body protein retention.

II.4. Methods of detection the effects of immunostimulant:

The immunostimulant in its action can be demonstrated by cellular and some tests that must be applied:-

a-Cells used for detection of the effect of immunostimulant:-

Siwicki et al. (1990) for in vitro immunization and cultivation method used spleen organ sections to investigate the effects of ***levamisole*** on the immune response and the main effect of ***levamisole*** was found in spleen and kidney macrophages.

Siwcki et al. (1992) reported that an in vitro immunization and cultivation method for fish spleen organ section was used to investigate the effects of ***levamisole*** on the neutrophil activity.

Brattgjerd et al. (1994) reported that, a prepared polysaccharide from the cell wall of yeast , ***M-glucan***, has previously been demonstrated to have immunostimulatory effects in ***Salmonids*** as observed by enhanced in vivo non-specific disease resistance in ***Atlantic salmon, Salmo salar L. and*** increased in vitro bactericidal activity of rainbow trout, ***Oncorhynchus mykiss (Walbaum),*** macrophages.

Jorgensen and Robertsen (1995) investigated the in vitro effects of ***glucan*** on the respiratory burst and bactericidal potential of ***Atlantic salmon*** head kidney macrophages after incubation of salmon macrophages for about 1 - 7 days with various concentrations of yeast. And they showed that, head kidney macrophages isolated from ***glucan*** injected ***Rainbow trout (Oncorhynchus mykiss) and Atlantic Salmon (Salmo salar L.)*** have increased ability to kill ***Aeromonas salmonicida***.

Cook et al. (2001) investigated the in vitro effects of a commercial beta-***glucan*** preparation, ***EcoActiva***, on the respiratory burst activity of head-kidney macrophages isolated from ***Pink snapper (Pagrus auratus),*** a marine fish cultured in Australia. Macrophages incubated with ***EcoActiva*** displayed morphological characteristics of activation, and were stimulated to produce superoxide. Pre-incubation with low levels of ***EcoActiva*** significantly increased the resposne to phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), indicating that ***EcoActiva*** could prime these macrophages to culturing macrophages with both LPS and PMA, or ***EcoActiva***, and PMA, increased burst activity compared with the response to PMA alone, however, this increase was additive and not synergestic.

Paulsen et al. (2001) reported that, ***Atlantic salmon*** head kidney macrophages grown in the presence of particulate yeast ***beta-glucan*** and bacterial lipopolysaccharide (LPS) showed increased production of lysozyme in the culture supernatants compared to non-treated controls.

Cuesta et al. (2002) used ***Gilthead seabream (Sparus aurata L.)*** head-kidney (HK) leucocytes were incubated with 10 (3) to 10 (- 4) ng ***levamisole*** / ml for 4, 24 or 48 h and then assayed for their natural cytotoxic activity against xenogenic tumor cells.

Rodriguez et al. (2003) for detection of the effect of ***Saccharomyces*** strain-supplemented diet, they found that lysozyme activity increased after 2 and 4 weeks of feeding ***Saccharomyces*** strain. They found that of cellular responses studied, phagocytosis was increased to a significant degree at all the assayed times but only by the ***Saccharomyces*** mutant strains.

b-Tests used for detection the effects of immunostimulants :-

Taraschewski et al. (1988) Studied the tissue alterations resulted from ***levamisol*** by means of light and electron microscopy.

Siwicki et al. (1989) measured the non-specific defense activity by demonstrating neutrophil metabolic activity by the nitroblue tetrazolium assay, by counting engulfed bacterial cells for a phagocytic index and by counting leukocytes with adherent bacterial cells for the adherence index. The specific immune response was monitored by the passive hemolytic plaque assay demonstrating the numbers of antibody-producing cells.

Anderson and Jeney (1992) reported that, the changes in circulatory neutrophil and phagocytic activity levels, and the specific immune response factors, measured by numbers of plaque-forming cells, and circulating antibody levels.

Siwicki et al. (1992) measured the ***levamisole*** effect by nitro-blue tetrazolium test, and phagocytic and adherence indexes by incubating the fish cells with suspensions of formalin-killed ***Staphylococcus aureus***. The specific immune system activity was shown by the passive hemolytic plaque assay demonstrating the numbers of antibody-producing cells.

Brattgjerd et al. (1994) reported that the hydrogen peroxide (H2O2) production of isolated head kidney macrophages from ***glucan***-injected fish was measured 3 and 6 weeks after ***M-glucan*** treatment and was increased at both time-points upon phorbol myristate acetate - (PMA) triggering. Without PMA triggering the difference was only significant 3 weeks after ***glucan*** injection when compared to a control group injected with saline.

Siwicki et al. (1994) measured the immunostimulant activity in fish blood using hematocrite test and lymphocyte counts.

Wang and wang (1997) mesured the activity of immunostimulants as Bar, Curdlan, Krestin, ***Scleroglucan*** and zymosan by using the number NBT-positive staining cells. Which might indicate that to activate non-specific phagocytes in fish is one of the antibacterial mechanism of polysaccharides.

Figueras et al. (1998) measured the activity of different immunostimulants using several immune parameters like (index and rate of phagocytosis, passive haemolytic plaque numbers, and agglutinating titers) at different times post-inoculation.

Bangi et al. (2000) for detection of the effect of ***glucans***, alpha-tocopherol and ascorbic acid on the innate immune response of cultured ***Seabass (Dicentrachus labraux)*** measured plasma lysozyme concentration, content and distribution of major plasma and complement activity prior to feeding supplemented diet and after 40 weeks.

ICRAM et al. (2002) measured the activity of ***glucans*** and elevated doses of vitamins using complement activation and lysozyme activity tests.

Ortuno et al. (2002) reported that for judging the immunostimulant activity the following tests must be determined which include, serum complement titers, as a humoral parameter, and phagocytic, respiratory burst, myeloperoxidase and natural cytotoxic activities of head-kidney leucocytes, as cellular parameters.

Sahoo and Mukherjee (2002) the imunostimulant modulators can be determined by bacterial agglutination titer and disease resistance against microorganisms.

Rodriguez et al. (2003) reported that, for detection the activity of ***Saccharomyces cervisiae*** strains as immunostimulants lysozyme activity must be determined.

Smith et al. (2003) reported that the activity and judgment of immunostimulant must be determined according to the cost-effective control of infection in aquaculture, especially for long lasting protection in both adults and juveniles.

II.5. Characteristics of a good immunostimulant:

Sandleheath (2003) reported that the good immunostimulant must be characterized by, Particularly suitable for boostering 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 vaccine, safe and non-toxic-no risk of overdose, no resistance problems and environmentally friendly & fully biodegradable.

III- MATERIAL AND METHODS

III. I. Materials:

1 Fish:

A total number of 240 healthy fish (average weight 30 ± 5 gm) from them about 120 ***Monosex*** Tilapia and 120 ***Oreochromis niloticus***, were collected from private fish farm at Behera Province. Fish were transported alive to the laboratory in plastic bags containing water enriched by air (2/3).

2. Aquaria:

Fish were kept into prepared glass aquaria (90 X 50 X 35 Cm). These aquaria were used for holding the experimental fish throughout the period of the present study, supplied with chlorine free tap water according to ***Innes (1966).*** The continuous aeration was maintained in each aquarium using an electric air pumping compressors. Water temperature was kept at 22 ± 1 °C.

All fish were acclimatized for at least 2 weeks prior to the experiment.

3. Fish diets:

Fish were fed on commercial fish food containing 25% crude protein . The diet was daily provided at 3% of body weight as described by ***Eurell et al. (1978)***. The daily amount of food was offered on two occasions over the day at 9 AM and 3 PM.

4- Microbial strains :

a- Yeast strains:-

The ***Candida albicans*** strain was kindly supplied by the Dept. of Poultry & Fish Diseases, Fac. of Vet. Med., Alexandria University.

b- Aeromonas hydrophila:-

Strain N.5 (supplied by the Dept. of Poultry & Fish Diseases, Fac. of Vet. Med., Alexandria University.)

5- Media:-

a-Media used for bacterial isolation:-

-Trypticase soya agar (TSA), BBL, Cat. No. 11764.
- Trypticase soya broth (TSB) , RS media ***(Rimler&Shotts 1973)***

b-Media used for *Candida albicans* preparation:

-Sabaroud's dextrose broth ***(Oxoid, 1982)***.

6-Kits for clinico-biochemical analysis:-

-Kits for GOT, GPT, Alkaline phosphates, total protein and serum albumin (Pasteur, Lab, France).

7- Solutions, buffers, reagents and stains:

Phosphate buffer saline (PBS pH 7.4) consists of:-

Sod. chloride	8.0 g
Pot. chloride	0.2 g
Di-sodium hydrogen phosphate	1.15 g
Pot. dihydrogen phosphate	0.2 g
Distilled water	up to 1000 ml.

According to ***Cruickshank et al. (1982).***

- Normal saline (0.85% sodium chloride).
- Formalin 40 W/V, (Chemajit).
- Giemsa and methylene blue stains were prepared according to ***(Cruickshank et al., 1982).***
- Chloroform, Methyl alcohol and Xylene (Adwic).
- Sodium Citrate 4 %.
- stained antigen

8-Apparatus used:

-Spectronic 2000 "Bausch and Lomb".
-Binocular microscope "Bausch and Lomb".
-Shaker water both.
-Slides , glasses, disposable syringes .
-Haemocytometer.
- Micro – titer plates .

9-Immunostimulants used:-

Biogen: It is a kind of feed additives (Growth promotor) consists of **Allicin** (Aged garlic extract) not less than 0.247 Mmole/g, ***Bacillus subtilis*** Natto (6 X 10^7 cells/g), high unit **hydrolytic enzymes** not less than 3690 units/g (Amylotic, lipolytic, proteolytic and cell separating enzymes), germanium (Gensen) 4188 ppm of Ge-elemnr/g and organic selenium ***(China Way Corporation, 1999).***

***Levamisole*:**

Levamisole HCl 10 % (Memphese) used as immunostimulants.

III.2. Methods:

1-Experiment I:-

A total of 120 ***Oreochromis niloticus*** (30 ± 5 g body weight) 60 from ***Monosex*** and other 60 ***O. niloticus*** were used. Each ten fish were kept in one aquarium containing dechlorinated tap water at a temperature of 22 ± 1 C, pH 7.1 - 7.3. The fish were fed with a 25% protein diet at ratio of 3 % from total body weight, the daily amount given at 9A.M. and 3 P.M.

2-Experimental procedure:-

The fish were divided into 3 equal groups from each type of fish (40 fish each) 20 *iloticus* and 20 ***Monosex tilapia***.

Group I:- Supplemented w ***Biogen*** ®. (1 Kg. / Ton) . Saad (2002)
Group II: Supplemented w *Levamisole* HCl. (1/4 Kg / Ton). Naglaa (20
Group III: Is served as contr and fed regular diet.

Table (1) : Design of the experiment I.

Fish group	Fish species			
	Monosex tilapia		*Oreochromis niloticus*	
I	*Biogen®*		*Biogen®*	
	10 fish	10 fish	10 fish	10 fish
II	*Levamisole*		*Levamisole*	
	10 fish	10 fish	10 fish	10 fish
III	Control		Control	
	10 fish	10 fish	10 fish	10 fish

III.2.2.1. Blood sampling:

At zero day, 2nd, 4th, 6th and 8th week during the experimental period. Blood samples were collected from different groups via the caudal vessels. ***(Hawk et al., 1965).***

One ml of blood was collected with syringe containing anticoagulant (0.1 ml of 4% sodium citrate solution / 1 ml blood) and used for phagocytic assay according to ***Kawahara et al. (1991)*** and differential leucocytic count and the other ml was used for collection of the serum, by putting in refrigerator, then centrifuged to get the serum. The collected serum was used for biochemical determination ***(Lied et al., 1975).***

3. Differential leucocytic count:

Blood film was taken and prepared according the method described by ***Lucky (1977)***. A drop of citrated blood put on slide at one end and by another slide make distribution of it and then make drying in air for about 2 minutes, fixation by methyle alcohol for about 5 ml then stained by diluted Giemsa's solution. The stain solution (10 drops of the dye were added to 10 ml of distilled water) was poured over the film and left for 20 minutes, then rinsed with water current, dried and examined by oil immersion lens. The percentage and absolute value for each type of cells were calculated according to ***Schalm (1986).***

4. Determination of phagocytic activity and phagocytic index:

Phagocytic activity was determined according to ***Kawahara et al. (1991).*** Fifty µg ***Candida albicans*** (previously adjusted to be 100 mg/ml W/V) was added to 1 ml of citrated blood collected from exposed and control fish and shaken in water bath at 25 C for 5 hours. Smears of the blood were then stained with Giemsa solution. Phagocytosis was estimated by determining the proportion of macrophages which contained intracellular yeast cells in a random count of 300 macrophages divided by 3 and expressed as percentage of phagocytic activity (PA). The number of phagocytized organisms was counted in the phagocytic cells and called phagocytic index (PI). Results were expressed as means ± S.E. and differences were evaluated by Student's t-test (S.A.S. 1987) .

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

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

5. Biochemical analysis :

5.1. Determination of serum glutamic oxalacetic transaminase (S. GOT) and serum glutamic pyruvic transaminase (S. GPT).

The activity of serum asparate aminotransferase (S. AST, commonly known as glutamic oxalacetic transaminase (S. GOT), and serum alanine aminotransferase (S. ALT), commonly known as glutamic pyruvic transaminase (S. GPT), were estimated according to ***Reitman and Frankle (1957)*** using commercial kits produced by Pasteur Lab.

Principle of the producer:

The GOT uses asparate and 2-oxoglutarate to yield glutamate and oxalacetate. The GPT uses alanine and 2-oxoglutrate and yield glutamate and pyruvate.

$$\text{L-Asparate} + \text{2-Oxoglutarate} \xrightarrow{\text{GOT}} \text{Glutamate} + \text{Oxaloacetate}$$

$$\text{L-Alanine} + \text{2-Oxoglutarate} \xrightarrow{\text{GPT}} \text{Glutamate} + \text{Pyruvate}$$

The oxalacetate or pyruvate formed in the above reactions react with 2-4-dinitrophenyl hydrazine to form phenyl hydrazones. These products give an increase colour in alkaline media which is measured at wave length 505 nm. The color intensity can be related to enzyme activity by a reference to standard curve.

5.2. Determination of serum alkaline phosphatase:

Serum alkaline phosphatase was estimated according to modified method of ***Kind and King (1954)*** using commercial kits produced by Pasteur Lab.

Principle of the procedure:

The procedure is based on the hydrolysis of thymolphthalein monophosphate by alkaline phosphatase there by liberating thymophthalein. Then addition of alkali terminates the enzymatic activity and converts thymoolphthalein, an alkaline indicator, to a blue green chromophore, the absorbance of which is measured at wave length 590 nm.

5.3. Determination of serum total protein:

Serum total protein was determination according to ***Doumas et al. (1981)*** using commercial kits produced by Pasteur Lab.

Principle of the procedure:

In this, the biuret reaction is used to quantitative serum total proteins. The method depends on the formation of coloured complex (purple) between peptide chains and cupric ions in strongly alkaline. The amount of complex formed, determined spectrophotometrically at wave length 540 nm is proportional to the protein concentration.

$$\text{Protein} + Cu^{++} \xrightarrow{OH} \text{Coloured complex}$$

5.4. Determination of serum albumin:

Serum albumin was determined according to ***Reinhold (1953)*** using commercially available kits of Chemroy.

Principle of the procedure:

Albumin + Bromocresol purple ----------→ BCP-albumin complex

The amount of complex formed is measured photometrically at wave length 605 nm. is proportional to the albumin concentration.

5.5. Determination of serum globulin:

Serum globulin was determined by subtract the total serum albumin from total serum protein according to ***(Coles, 1974 and Khalil, 2000)***.

5.6. Determination of serum albumin / globulin ratio:

Determined by devision of serum albumin vaule on serum globulin value. According to the method implied by ***(Saffinaz, 2001).***

6-Experiment II:

The experiment II were carried out for determination of antibody titer and challenge against ***Aeromonas hydrophila***. (Local isolate).

The initial immunization with formalin killed ***Aeromonas hydrophila*** vaccine was followed by a challenge with the same antigen at the end of the experiment (8^{th} week) in the 3 groups (40 each) according to ***(Ellis, 1988 and Safinaz, 2001).***

6.1. Bacterine preparation:

A.hydrophila virulent isolate (N 5) was used for bacterine preparation according to the method described by ***Sakai et al. (1984) and Badran (1990).*** The organism was inoculated in Tryptacase Soya Broth (TSB) and incubated at 30 C for 48 hrs.

The bacterial growth was inactivated by formalin to a final concentration of 0.3% and held at room temperature overnight. The inactivated bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes, washed three times with sterile saline, re-suspended in 10 ml sterile saline and kept at 4 C. Safety tests of the prepared bacterine were carried out according to ***Anderson and Conroy (1970)*** by inoculation in TSB and incubated at 30 C for 24 hrs.

The formalin inactivated bacterial cells were mixed with an equal volume of 0.85% sterile saline . Bacterial number was adjusted to MacFalan's No. 2 (6 X 10^{8} cells / ml).

The experimental fish (5 fish from each species) were submitted for bacterial isolation, for verification of absence of tested bacteria ***(A. hydrophila)*** by inoculation on RS media.

Fourty ***Oreochromis niloticus*** and fourty ***Monosex*** fish (which fed diet with ***Biogen*** ® and ***levamisole***) were inoculated intraperitoneally (IP) with 0.2 ml/fish of formalin inactivated bacterine. Twenty control ***O. niloticus*** and twenty control ***Monosex tilapia*** were similarly injected (IP) with 0.2 ml /fish sterile saline. (fish fed normal diet) After 2 weeks, the injected fish received booster dose from bacterine and control fish injected with 0.2 ml/fish sterile saline (same dose). Blood collection was carried out from the caudal vessels of inoculated fish, every week for 4 weeks. Prior to blood collection, the fish were dried properly using special absorbent paper. Collected blood was kept overnight in the refrigerator. Serum was separated aseptically gently and centrifuged at 6000 rpm for

10 minutes. Aspiration of supernatant serum using sterile pipette was carefully done and stored at - 20 C ***(Lucky, 1977)*** until use.

6.2. Preparation of stained antigen:

To 10 ml of the bacterine suspension in sterile saline solution one drop of Lofeller's alkaline methylene blue (10 %) was added to increase the visibility of the serological reaction ***(Collins et al., 1976).*** The prepared antigen was used for serum antibody detection.

6.3. Antibody titration against *A. hydrophila*:

Detection of immune response to ***A. hydrophyla*** was evaluated by microagglutination (MA) test according to the method described by ***Badran (1990) and Khalil (2000).*** In a standard micro-titer plate (U-shaped wells), serial two fold dilutions of serum were made in sterile saline, using a 0.025 ml pipette dropper and 0.025 ml microdiluter. ***A. hydrophyla*** antigen (0.025 ml) was added to the diluted serum. The suspensions were mixed and incubated overnight in the refrigerator. A positive serological reaction was indicated by bacterial agglutination.

Agglutination titers were expressed as log $_2$ of the highest serum dilution still giving a clear agglutination ***(Badran, 1990).*** The negative controls consisted of :

i) One drop of sterile physiological saline and one drop of tested serum.
ii) One drop of sterile physiological saline and one drop of stained antigen. The positive controls were carried out using collected positive antisera

6.4. Challenge test:

After 28 days post-immunization both of injected with bacterine and control groups were infected with 0.2 ml of virulent strain of ***A. hydrophila*** previously adjusted to 10^4 cells Specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation. (One week) according to ***Soliman (1988).***

The potency of bacterine was examined by calculating the relative level of protection (RLP) by the following formula:

$$RLP = 1 - \frac{\text{\% mortality of vaccinated fish}}{\text{\% mortality of control}}$$

According to ***Newman and Majnarich (1982).***

7. Pathological studies

Following complete necropsy in the first experiment, fresh specimens from liver, spleen and kidneys of both ***O. niloticus*** and ***Monosex tilapia*** were collected and rapidly fixed in 10% neutral buffered formalin. Thereafter, these fixed specimens were processed through the conventional paraffin embedding technique (dehydration in ascending grades of ethyl alcohol, clearing in chloroform and embedding in melted Paraffin wax at 60 C). Paraffin blocks were cut at 5 microns thick sections which stained with hematoxylin and eosin (H & E) according to the method described by ***Culling (1983).***

8. Statistical analysis:

The data of hematological and biochemical examinations of exposed fish were statistically analyzed using t-test, Duncan-test after ANOVA and simple correlation according to ***(SAS, 1987)*** to examine the significant effect of the main variables on the studied parameters. After that the results presented in the form of figures according to Harvard graphics (HGW-4) computer program.

IV- RESULTS

IV.1. The Effect of different immunostimulants on blood constituents:

IV.1.1. serum enzymes:-

The effects of immunostimulants on serum enzymes are summarized in table (1,2) and Fig (1,2). From the results we can concluded that there were slight significant ($p< 0.01$) differences between different immunostimulant on ***O.niloticus & Monosex Tilapia*** serum enzymes compared with control and the effects on ***Monosex Tilapia*** more than ***O.niloticus*** .

The serum enzymes ALT, AST and AP levels were increased in case of ***Biogen*** ® treatments with the time, followed by ***levamisole*** treated group compared with control one.

IV.1.2. Serum proteins:

The level of serum proteins (total protein, albumin, globulin and albumin /globulin ratio) gradually increased with the time in case of groups received diet with ***Biogen*** ® followed by that received ***levamisole*** in ***O. niloticus*** and ***Monosex tilapia*** (Table 3, 4) and (Fig. 3,4).

The globulin level increased from 6th week till to the end of experiment especially in ***Biogen*** ® . In case of ***Monosex tilapia*** the globulin level was higher than that of ***O. niloticus***. (Table 3,4 and Fig 3,4) in both types of immunostimulants.

IV.1.3. Differential leucocytic counts:-

The lymphocyte number increased progressively from 0-day to 4th week and slightly decreased in 6th week .

Table (5) and Fig. (5) clarify that, the level of lymphocyte increased in ***Monosex tilapia*** than in ***Oreochromis niloticus*** received ***Biogen*** ® and finally the groups fed diet with ***levamisole*** . The results indicated that the lymphocyte increased in the ***Biogen*** ® treated group than the ***levamisole*** treated group. But in the last weeks of experiment the number of lymphocytes decreased progressively, especially in groups received ***levamisole*** .

From the results it was clear that, the number of monocytes increased progressively by increasing the time among the different treatments. The groups received ***Biogen*** ® characterized by a high level of monocytes followed by the groups received ***levamisole*** compared with control group.

Moreover, there were increasing in levels of basophilis with progression of time especially in ***Monosex tilapia*** than the other fish.

The eosinophils level significantly increased, with progress of time. The fish got diet that contained ***Biogen*** ® was characterized by high eosinophils level than the other treatments at the 1st , 2nd and 4th weeks, Generally in case of ***Monosex tilapia*** the eosinophils level were higher than the ***O. niloticus***.

The neutrophils level also, decreased at different weeks than the control , but increased progressively till reach it's maximum level at 8th week and the ***levamisole*** caused higher neutrophils level, than ***Biogen*** ® .

IV.1.4. Phagocytic activity and phagocytic index:-

Table (6) and Fig. (6,7,8,9&10) summarized the effect of immunostimulants on phagocytic activity and index. From the Table & Figs we can notice that, the phagocytic activity and index differ significantly among different treatment groups. ($P< 0.01$).

The phagocytic activities were higher in the groups fed diet containing, ***Biogen*** ® and ***levamisole*** especially in the ***O. niloticus*** than the ***Monosex*** tilapia. While the phagocytic index was higher at the 2nd weeks in ***Biogen*** ® , and ***levamisole*** treatments especially in ***O. niloticus***

IV.1.5. Antibody titration:-

The results of the effect of immunostimulants on antibody titration are illustrated in Table (7) and Fig (11) and clarify that, the antibody titration differ significantly among different groups at different weeks ($P< 0.01$).

The highest antibody titers were observed in, ***Monosex*** fish fed ***Biogen*** ® containing diet and ***levamisole*** treated group compared to the control.

It was cleared that, the control groups were positive to the presence of antibody against tested ***A. hydrophila.*** These fish may be previously exposed to natural infection to ***A. hydrophila*** , which subsequently gave +ve antibody against ***A. hydrophila.***

IV.1.6. Relative level of protection upon challenge with *A.hydrophila*:

Results of RLP are summarized in Table (8). From the table we can notice that RLP of ***Monosex tilapia*** was higher than ***O. niloticus*** in case of ***Biogen*** ® treated group higher than that of ***levamisole*** group.

The results of antigen sterility proved negative bactriological isolation. while injected ***A. hydrophila*** was successfully isolated from dead fish after challenge.

IV.1.7. Histopathological alterations:-

Generally, from histopathologic point of view, the effects of the two tested immunostimulants (***Biogen*** ® and ***levamisole***) were obvious on haematopoitic organs in both species (***O.niloticus*** and ***Monosex tilapia***). In addition, this effect was more marked in ***Monosex tilapia*** than in ***O.niloticus***. Histopathologically , the immuno-promoting effect was similar in nature either in both species upon the use of any drug, but, it was variable in degree and distribution (The splenic reaction in all experimental groups was hyperactivation and enlargement of the melanomacrophage centers. This reaction was the greatest in ***O.niloticus*** treated with ***Biogen*** ® and it was the least in ***Monosex*** treated with ***levamisole***).

1-Kidney:-

The immunostimulant effect on the kidney tissue was manifested by moderate to severe (according to species and/or drug) proliferation of the lymphoid elements in the anterior kidney (Fig. 12). Furthermore, the posterior kidney showed focal to multifocal

proliferation of the interstitial lymphoid elements (Fig. 13, 14). In addition, the renal melano-macrophage centers were variably enlarged and hyper-activated (Figs. 15, 16).

2- Spleen:

The lymphoid elements of the splenic paranchyma had increased in size upon the extent of the stromal tissue (Fig. 17, 18). Additionally, the splenic melanomacrophage centers were markedly proliferated, enlarged and hyperactivated to the extent that the melanin pigment itself was concentrated inside the macrophages and appeared dark brown to black granules (Fig.19,20).

3-Hepatopancrease:-

It was the least reacted organ against the tested immunostimulants. The microscopic examination revealed that the hepatic melano-macrophage centers appeared slightly activated (Fig. 21). However, the livers of ***O.niloticus*** treated with ***Biogen*** ® exhibited more lymphocytic cell proliferation within the hepatic parenchyma (Fig. 22).

The specimen from control group organs revealed normal histological structures.(more or less)

V- TABLES AND FIGURES

Table (1): Effect of different treatments on serum enzymes (ALT, AST and AP) of *Oreochromis niloticus*

Time	Treatment	ALT	AST	AP
		$\bar{X}$±SE	$\bar{X}$±SE	$\bar{X}$±SE
Zero day	No treatment	66.72±1.43	51.75±1.56	13.04±0.40
2nd week	OB *	64.25±0.83 ^{B}	52.64±1.28 ^{B}	13.86±0.32 ^{B}
	OL **	64.88±0.53 ^{B}	51.88±0.69 ^{B}	13.88±0.21 ^{B}
	O ***	67.84±0.58 ^{A}	56.83±3.18 ^{A}	14.20±0.07 ^{A}
Total mean		65.65±1.75	53.78±5.09	13.98±0.94
4th week	OB	77.53±0.56 ^{A}	62.50±2.12 ^{B}	15.44±0.24 ^{A}
	OL	71.49±1.09 ^{B}	63.79±2.89 ^{A}	15.63±0.34 ^{A}
	O	65.93±0.68 ^{C}	55.18±2.56 ^{C}	14.13±0.03 ^{B}
Total mean		71.65±3.66	60.49±4.30	15.06±0.80
6th week	OB	64.84±2.04 ^{B}	55.10±0.85 ^{B}	14.60±0.33 ^{A}
	OL	68.13±1.00 ^{A}	53.15±1.03 ^{C}	14.32±0.26 ^{B}
	O	68.46±0.53 ^{A}	60.17±0.05 ^{A}	14.05±0.22 ^{C}
Total mean		67.14±3.66	56.14±2.88	14.32±1.41
8th week	OB	67.95±1.57 ^{A}	53.13±2.66 ^{A}	13.66±0.34 ^{C}
	OL	67.31±1.06 ^{A}	53.50±1.47 ^{A}	14.28±0.21 ^{A}
	O	67.33±0.73 ^{A}	53.14±0.98 ^{A}	13.89±0.20 ^{B}
Total mean		67.53±1.60	53.25±4.80	13.94±0.66

For each time means within the same column carrying different letters are significantly different at ($P< 0.01$)

* OB = ***Oreochromis niloticus*** + ***Biogen®***
** OL = ***Oreochromis niloticus*** + ***Levamisole***
*** O = ***Oreochromis niloticus*** control

Table (2): Effect of different treatments on serum enzymes (ALT, AST and AP) of *Monosex tilapia*.

Time	Treatment	ALT	AST	AP
		$\bar{X}$±SE	$\bar{X}$±SE	$\bar{X}$±SE
Zero day	No treatment	68.87±0.77	54.22±1.18	13.58±0.21
2nd week	MB *	65.02±2.25 ^{C}	56.45±0.91 ^{B}	13.98±1.13 ^{B}
	ML **	66.02±0.21 ^{B}	54.38±4.33 ^{C}	14.26±0.42 ^{A}
	M ***	69.07±0.73 ^{A}	58.12±1.78 ^{A}	15.38±0.47 ^{A}
Total mean		66.70±2.07	56.31±3.20	14.54±0.87
4th week	MB	65.49±1.52 ^{A}	58.70±0.45 ^{A}	14.66±0.46 ^{B}
	ML	69.02±1.65 ^{B}	59.41±1.08 ^{A}	14.25±0.08 ^{B}
	M	68.78±0.87 ^{B}	57.86±2.81 ^{B}	15.39±0.20 ^{A}
Total mean		67.76±5.17	58.65±3.88	14.76±0.62
6th week	MB	72.14±1.08 ^{B}	57.86±1.50 ^{A}	14.53±0.55 ^{A}
	ML	67.47±0.59 ^{A}	58.16±1.72 ^{A}	14.09±0.18 ^{B}
	M	67.90±0.63 ^{A}	58.63±0.43 ^{A}	14.80±0.54 ^{A}
Total mean		69.17±3.77	58.21±2.45	14.47±0.58
8th week	MB	67.84±2.42 ^{A}	53.79±0.59 ^{A}	13.16±0.06 ^{A}
	ML	67.29±1.59 ^{A}	52.85±0.59 ^{A}	13.34±0.15 ^{B}
	M	68.05±0.24 ^{A}	51.77±1.45	13.16±0.98 ^{B}
Total mean		67.72±1.87	52.80±2.20	13.22±0.71

For each time means within the same column carrying different letters are significantly different at ($P< 0.01$)

* MB = ***Monosex*** + ***Biogen***®

** ML = ***Monosex*** + ***Levamisole***.

*** M = Control ***Monosex***.

Table (3): Effect of different treatments on serum proteins (total protein, globulin and albumin/ globulin ratio) of *Oreochromis niloticus* fish.

Time	Treatment	Total protein	Albumin	Globulin	Albumin / Globulin ratio
		$\bar{X}$±SE	$\bar{X}$±SE	$\bar{X}$±SE	$\bar{X}$±SE
Zero day	No treatment	7.62±0.53	3.53±0.19	4.09±53	0.87±0.13
2nd week	OB *	7.17±0.13 ^{A}	4.37±0.42 ^{A}	2.80±0.30 ^{B}	1.58±0.34 ^{A}
	OL **	6.87±0.86 ^{B}	3.55±0.07 ^{B}	3.31±0.89 ^{A}	1.13±0.37 ^{B}
	O ***	6.84±0.56 ^{B}	3.67±0.50 ^{B}	2.79±0.34 ^{B}	1.33±0.26 ^{C}
Total mean		6.84±0.76	3.86±0.52	2.96±0.87	1.34±0.40
4th week	OB	5.04±0.18 ^{B}	3.19±0.81 ^{A}	3.84±0.63 ^{C}	1.94±0.96 ^{A}
	OL	5.19±0.03 ^{B}	3.15±0.07 ^{A}	2.04±0.09 ^{B}	1.54±0.10 ^{B}
	O	6.46±0.30 ^{A}	3.17±0.02 ^{A}	1.29±0.31 ^{A}	0.96±0.10 ^{C}
Total mean		5.56±1.59	3.17±0.39	2.39±0.64	1.48±0.54
6th week	OB	6.80±0.07 ^{A}	3.70±0.14 ^{A}	3.10±0.07 ^{A}	1.19±0.07 ^{B}
	OL	6.31±0.27 ^{A}	3.57±0.26 ^{C}	3.05±1.04 ^{A}	1.34±0.33 ^{A}
	O	6.75±0.58 ^{A}	3.69±0.52 ^{B}	2.73±0.50 ^{B}	1.32±0.54 ^{A}
Total mean		6.62±1.51	3.65±0.33	2.96±0.54	1.28±0.33
8th week	OB	8.16±1.07 ^{A}	3.87±0.16 ^{A}	4.28±1.09 ^{A}	0.95±0.28 ^{C}
	OL	6.09±0.04 ^{B}	3.57±0.35 ^{B}	3.52±0.31 ^{B}	1.44±0.30 ^{A}
	O	5.77±0.54 ^{C}	3.33±0.43 ^{C}	2.44±0.41 ^{B}	1.38±0.29 ^{B}
Total mean		6.67±1.12	3.59±0.40	3.08±0.96	1.25±0.37

For each time means within the same column carrying different letters are significantly different at ($P< 0.01$)

* OB = ***Oreochromis niloticus*** + ***Biogen®***
** OL = ***Oreochromis niloticus*** + ***Levamisole***
*** O = ***Oreochromis niloticus*** control

Table (4): Effect of different treatments on serum proteins (total protein, globulin and albumin/ globulin ratio) of *Monosex tilapia*.

Time	Treatment	Total protein	Albumin	Globulin	Albumin / Globulin ratio
		$\bar{X}$±SE	$\bar{X}$±SE	$\bar{X}$±SE	$\bar{X}$±SE
Zero day	No treatment	6.96±0.35	2.69±0.13	3.27±0.37	1.13±0.15
2nd week	MB *	7.11±0.42 ^{A}	3.37±0.30 ^{A}	3.74±0.45 ^{A}	0.91±0.15 ^{B}
	ML **	6.66±0.34 ^{B}	3.97±0.22 ^{A}	2.69±0.20 ^{B}	1.48±0.12 ^{A}
	M ***	6.43±0.35 ^{B}	3.84±0.47 ^{A}	2.59±0.57 ^{C}	1.56±0.57 ^{A}
Total mean		6.73±0.61	3.72±0.37	3.00±0.79	1.31±0.38
4th week	MB	6.48±0.26 ^{B}	3.31±0.18 ^{B}	3.17±0.17 ^{A}	1.04±0.08 ^{A}
	ML	6.07±0.22 ^{A}	3.27±0.07 ^{B}	2.80±0.18 ^{B}	1.17±0.06 ^{B}
	M	6.43±0.26 ^{A}	3.75±0.11 ^{A}	2.67±0.16 ^{C}	1.40±0.05 ^{C}
Total mean		6.32±0.75	3.44±0.45	2.88±0.74	1.20±1.04
6th week	MB	6.62±0.28 ^{A}	3.16±0.07 ^{B}	3.57±0.37 ^{A}	1.24±0.17 ^{A}
	ML	6.29±0.23 ^{A}	3.11±0.05 ^{C}	3.18±0.22 ^{B}	0.97±0.07 ^{B}
	M	5.74±0.43 ^{B}	3.59±0.22 ^{A}	3.03±0.15 ^{C}	1.18±0.08 ^{A}
Total mean		6.21±0.67	3.28±0.26	2.92±0.72	1.13±0.55
8th week	MB	8.30±0.18 ^{A}	4.26±0.10 ^{A}	4.04±0.15 ^{A}	1.05±0.05 ^{A}
	ML	7.09±0.62 ^{B}	3.52±0.29 ^{C}	3.57±0.33 ^{B}	0.98±0.03 ^{A}
	M	6.82±0.08 ^{C}	3.78±0.18 ^{B}	3.03±0.14 ^{C}	1.25±0.11 ^{A}
Total mean		7.40±0.88	3.85±0.33	3.54±0.74	1.09±0.36

For each time means within the same column carrying different letters are significantly different at ($P< 0.01$)

* MB = ***Monosex*** + ***Biogen***®
** ML = ***Monosex*** + ***Levamisol***e.
*** M = Control ***Monosex***.

Table (5): Effect of different treatments on differential leucocytic count of *Monosex tilapia and Oreochromis niloticus.*

Week		Group *	N	Lymphocyte	Monocyte	Basophils	Eosinophils	Neutrophils
				$\bar{X}\pm SE$	$\bar{X}\pm SE$	$\bar{X}\pm SE$	$\bar{X}\pm SE$	$\bar{X}\pm SE$
0-day	Ore	1	3	53.3 ± 2.52^{A}	1.33 ± 0.58^{A}	9.33 ± 2.52^{A}	10.33 ± 0.58^{A}	25.00 ± 5.00^{D}
		2	3	52.66 ± 0.58^{B}	1.33 ± 0.58^{A}	7.67 ± 0.58^{C}	9.33 ± 0.58^{C}	28.33 ± 0.58^{C}
	Mo	3	3	53.33 ± 2.08^{A}	0.66 ± 0.57^{B}	8.00 ± 1.00^{B}	8.66 ± 0.57^{B}	29.66 ± 0.57^{B}
		4	3	50.00 ± 1.00^{C}	1.33 ± 0.57^{A}	7.66 ± 0.75^{C}	11.00 ± 1.00^{C}	30.00 ± 2.64^{A}
Total Mean			12	52.33±2.05	1.16±0.57	8.16±1.40	9.83±1.11	28.25±3.19
2nd week	Ore	OB	3	55.00 ± 1.00^{D}	1.67 ± 0.58^{B}	9.33 ± 2.08^{B}	11.67 ± 0.58^{B}	22.33 ± 0.58^{E}
		OL	3	54.67 ± 0.58^{D}	1.67 ± 0.58^{B}	7.67 ± 0.58^{D}	10.67 ± 0.58^{C}	25.33 ± 0.58^{D}
		O.C	3	52.00 ± 1.00^{C}	2.33 ± 1.53^{C}	10.33 ± 0.58^{A}	10.00 ± 1.00^{C}	24.67 ± 3.06^{F}
	Mo	MB	3	57.67 ± 3.51^{B}	0.67 ± 0.58^{D}	8.67 ± 0.58^{C}	7.67 ± 0.58^{E}	26.00 ± 2.00^{C}
		ML	3	57.33 ± 4.04^{B}	1.33 ± 0.58^{B}	8.33 ± 0.58^{C}	12.00 ± 1.00^{A}	22.00 ± 3.61^{F}
		M.C	3	59.00 ± 1.00^{A}	3.00 ± 1.00^{A}	8.67 ± 0.58^{C}	8.00 ± 2.00^{D}	21.00 ± 2.00^{G}
Total mean			18	55.94±3.44	1.77±1.03	8.83±1.24	10.00±1.71	23.55±3.92
4th week	Ore	OB	3	57.67 ± 0.58^{E}	1.33 ± 0.58^{B}	6.33 ± 0.58^{D}	11.67 ± 0.58^{B}	22.67 ± 1.15^{D}
		OL	3	56.00 ± 1.00^{D}	1.33 ± 0.58^{A}	7.33 ± 0.58^{C}	11.00 ± 1.73^{B}	25.67 ± 2.31^{B}
		O.Co	3	55.00 ± 1.00^{C}	1.67 ± 1.53^{A}	7.67 ± 1.15^{C}	10.67 ± 0.58^{C}	25.33 ± 1.15^{B}
	Mo	MB	3	61.67 ± 2.89^{A}	1.33 ± 0.58^{A}	7.67 ± 1.15^{C}	12.33 ± 2.89^{A}	16.67 ± 1.15^{H}
		ML	3	59.33 ± 2.31^{B}	0.67 ± 0.58^{B}	9.00 ± 1.00^{B}	12.00 ± 1.73^{A}	18.33 ± 0.58^{G}
		M.Co	3	55.67 ± 1.15^{C}	2.00 ± 2.00^{B}	10.00 ± 1.00^{A}	11.67 ± 1.15^{B}	20.00 ± 1.00^{F}
Total mean			18	57.55±3.13	1.38±1.06	8.00±1.37	11.55±1.74	21.44±3.71
6th Week	Ore	OB	3	53.00 ± 1.73^{D}	1.33 ± 0.58^{C}	6.67 ± 0.58^{D}	10.67 ± 0.58^{B}	28.67 ± 2.08^{B}
		OL	3	54.00 ± 1.73^{E}	1.33 ± 0.58^{C}	9.33 ± 2.08^{A}	11.67 ± 1.15^{A}	24.00 ± 4.36^{E}
		O.Co	3	52.00 ± 1.73^{F}	1.33 ± 0.58^{C}	8.00 ± 0.00^{B}	10.00 ± 3.46^{B}	24.67 ± 5.86^{E}
	Mo	MB	3	61.00 ± 1.73^{A}	4.33 ± 0.58^{A}	9.00 ± 0.00^{A}	11.67 ± 1.15^{A}	17.67 ± 3.06^{J}
		ML	3	53.00 ± 3.46^{C}	3.33 ± 2.08^{B}	9.67 ± 2.31^{A}	9.33 ± 0.58^{C}	23.67 ± 2.08^{I}
		M.Co	3	57.67 ± 2.89^{B}	3.00 ± 2.65^{B}	6.00 ± 0.00^{C}	7.00 ± 1.73^{D}	25.67 ± 2.31^{D}
Total mean			18	55.11±3.19	2.44±1.42	8.11±1.72	10.05±1.76	24.05±5.31
8th week	Ore	OB	3	55.67 ± 2.31^{A}	1.67 ± 1.53^{C}	9.33 ± 0.58^{B}	7.33 ± 0.58^{D}	31.00 ± 3.46^{F}
		OL	3	51.00 ± 1.73^{B}	1.67 ± 1.53^{C}	9.33 ± 0.58^{B}	8.00 ± 1.73^{C}	34.00 ± 1.73^{C}
		O.Co	3	48.33 ± 1.15^{C}	2.33 ± 1.53^{B}	11.33 ± 2.08^{A}	8.67 ± 2.31^{C}	23.33 ± 0.58^{K}
	Mo	MB	3	51.00 ± 3.46^{B}	3.67 ± 1.53^{A}	8.67 ± 0.58^{C}	8.33 ± 0.58^{C}	32.67 ± 4.62^{F}
		ML	3	44.00 ± 5.20^{D}	3.00 ± 2.00^{A}	9.67 ± 0.58^{B}	9.67 ± 0.58^{B}	35.33 ± 0.58^{E}
		M.Co	3	42.33 ± 0.58^{E}	1.67 ± 1.53^{C}	11.00 ± 1.00^{A}	11.33 ± 1.15^{A}	24.33 ± 6.51^{J}
Total mean		18		48.72±5.16	2.33±1.65	9.88±1.30	8.88±1.57	30.46±6.29
Overall mean		84		53.93±4.97	1.81±1.31	8.59±1.61	10.06±1.95	25.48 ± 5.67

For each time means within the same column carrying different letters are significantly different at ($P< 0.01$)

Ore. = ***Orechromis niloticus*** M = ***Monosex***

OB = ***Oreochromis niloticus + Biogen*** OL = ***O. niloticus + Levamisole*** O. Co = ***O.niloticus control***

MB =***Monosex + Biogen*** ML = ***Monosex + Levamisole*** M. Co = ***Monosex control***

N = No. of fish

Table (6): Effect of different treatments on phagocytic activity and index of *Monosex tilapia and O. niloticus*.

Week		Group *	N	Phagocytic activity $\bar{X}$±SE	Phagocytic index $\bar{X}$±SE
0-day	Ore	1	3	21.33±2.31^{C}	1.97±0.12^{C}
		2	3	27.33±0.58^{B}	2.67±0.64^{B}
	Mo	3	3	27.67±4.04^{B}	2.83±1.10^{B}
		4	3	32.33±1.15^{A}	3.77±0.23^{A}
Total Mean			12	27.16±4.56	2.80±0.86
2nd – Week	Ore	OB	3	35.33±2.31^{A}	4.67±0.12^{A}
		OL	3	30.33±0.58^{B}	4.43±0.29^{A}
		O.C	3	30.33±1.15^{B}	4.20±0.17^{A}
	Mo	MB	3	29.00±1.73^{C}	3.78±0.59^{B}
		ML	3	23.00±1.73^{E}	3.43±0.06^{B}
		M.C	3	28.33±2.89^{D}	2.73±0.06^{C}
Total mean			18	29.38±4.69	3.87±0.82
4th week	Ore	OB	3	30.33±4.62^{A}	3.00±0.20^{A}
		OL	3	23.67±0.58^{C}	3.03±0.12^{A}
		O.Co	3	22.33±1.15^{D}	2.83±0.12^{A}
	Mo	MB	3	29.67±1.15^{A}	2.27±0.12^{B}
		ML	3	25.33±0.58^{B}	3.27±0.23^{A}
		M.Co	3	21.67±1.15^{E}	3.00±0.26^{A}
Total mean			18	25.15±14.12	2.90±0.49
6th Week	Ore	OB	3	30.00±1.73^{C}	2.90±0.49^{A}
		OL	3	32.00±1.73^{B}	3.00±0.17^{A}
		O.Co	3	29.33±1.15^{D}	2.17±0.46^{C}
	Mo	MB	3	35.67±4.04^{A}	3.27±0.12^{A}
		ML	3	30.33±0.58^{C}	2.97±0.12^{B}
		M.Co	3	29.00±1.73^{D}	2.30±0.29^{B}
Total mean			18	29.38±2.57	2.86±0.66
8th week	Ore	OB	3	25.67±0.58^{A}	2.23±0.12^{C}
		OL	3	24.33±2.31^{B}	2.43±0.06^{C}
		O.Co	3	21.00±1.73^{D}	3.20±0.17^{B}
	Mo	MB	3	25.33±7.51^{A}	4.33±0.06^{A}
		ML	3	23.67±2.89^{C}	3.73±0.06^{B}
		M.Co	3	23.67±4.62^{C}	3.20±0.87^{B}
Total mean		18		24.61±4.62	3.18±0.78
Overall mean		84		27.13±4.30	3.12±3.46

For each time means within the same column carrying different letters are significantly different at (P< 0.01)

Ore. = ***Orechromis niloticus***
OB = ***Oreochromis niloticus + Biogen***
O. Co = ***O.niloticus control***
MB =***Monosex + Biogen***
M. Co = ***Monosex control***
N = ***No. of fish***
M = ***Monosex***
OL = ***O. niloticus + Levamisole***
ML = ***Monosex + Levamisole***

Table (7): Antibody titers (Log 2) in different experimental groups (Means ± Standard error) in *Monosex* tilapia and *Oreochromis niloticus*.

Group		N	Antibody titration
			$\bar{X}$ ±SE
Oreochromis niloticus	***Biogen***	3	6 ± 1.3^{D}
	Levamisole	3	5 ± 1.5^{C}
	Control	3	3 ± 1.4^{E}
Monosex tilapia	***Biogen***	3	8 ± 2.5^{A}
	Levamisole	3	7 ± 3.4^{B}
	Control	3	3 ± 2.5^{E}
Total mean		18	5.16±2.5

- Control non-vaccinated serum samples were antibody negative.

Means within the same column carrying different letters are significantly different at ($P < 0.01$).

Table (8): Effect of *Biogen* and *levamisole* HCl suplementation on protection of *Monosex* tilapia an *Oreochromis niloticus* against virulent strain of *Aeromonas hydrophila* after vaccination b intramuscular injection with *Aeromonas hydrophila* bacterine (n = 10).

Parameters	*Biogen*				*Levamisol*				Control			
	M		ON		M		ON		M		ON	
	1	2	1	2	1	2	1	2	1	2	1	2
Survival	6	7	5	6	6	5	5	4	2	2	0	0
Dead	4	3	5	4	4	5	5	6	8	8	10	10
Mort %	40%	30%	50%	40%	40%	50%	50%	60%	80%	80%	100 %	100 %
R.P.L	4/8	5/8	5/10	6/10	4/8	3/8	5/10	4/10				
R.P.L%	50%	62.5%	50%	60%	50%	37.5%	50%	40%				

$$R.P.L.= 1 - \frac{\text{Percent of mortality in injected fish}}{\text{Percent of mortality in control fish}}$$

Key:
M: ***Monosex*** **ON:** ***Oreochromis niloticus***

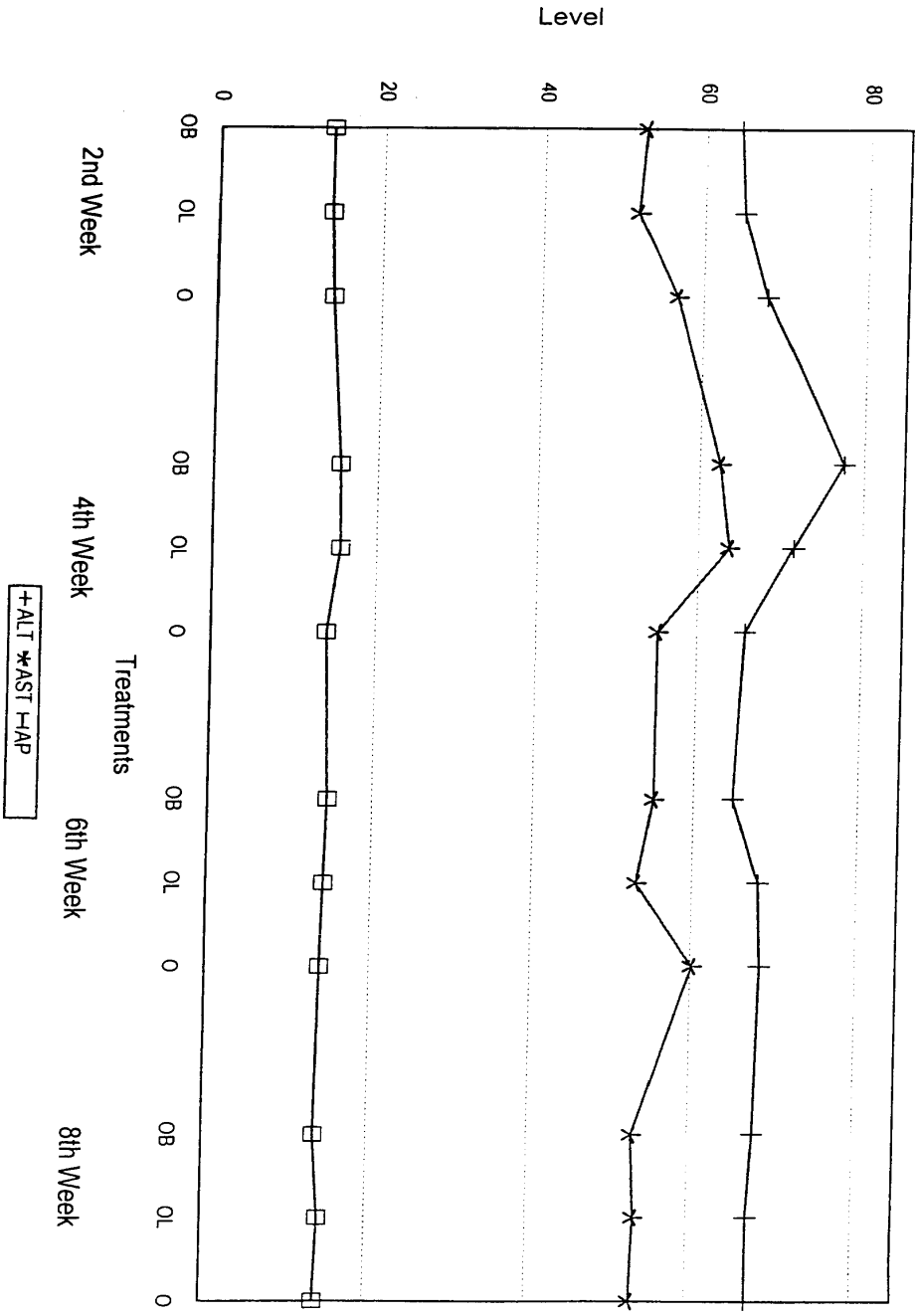

Fig. (1): Effect of different treatments on serum enzymes ALT, AST and AP of ***Oreochromis niloticus*** fish.

OB= O. niloticus with biogen **OL = O. niloticus with levamisole** **O= O. niloticus control**

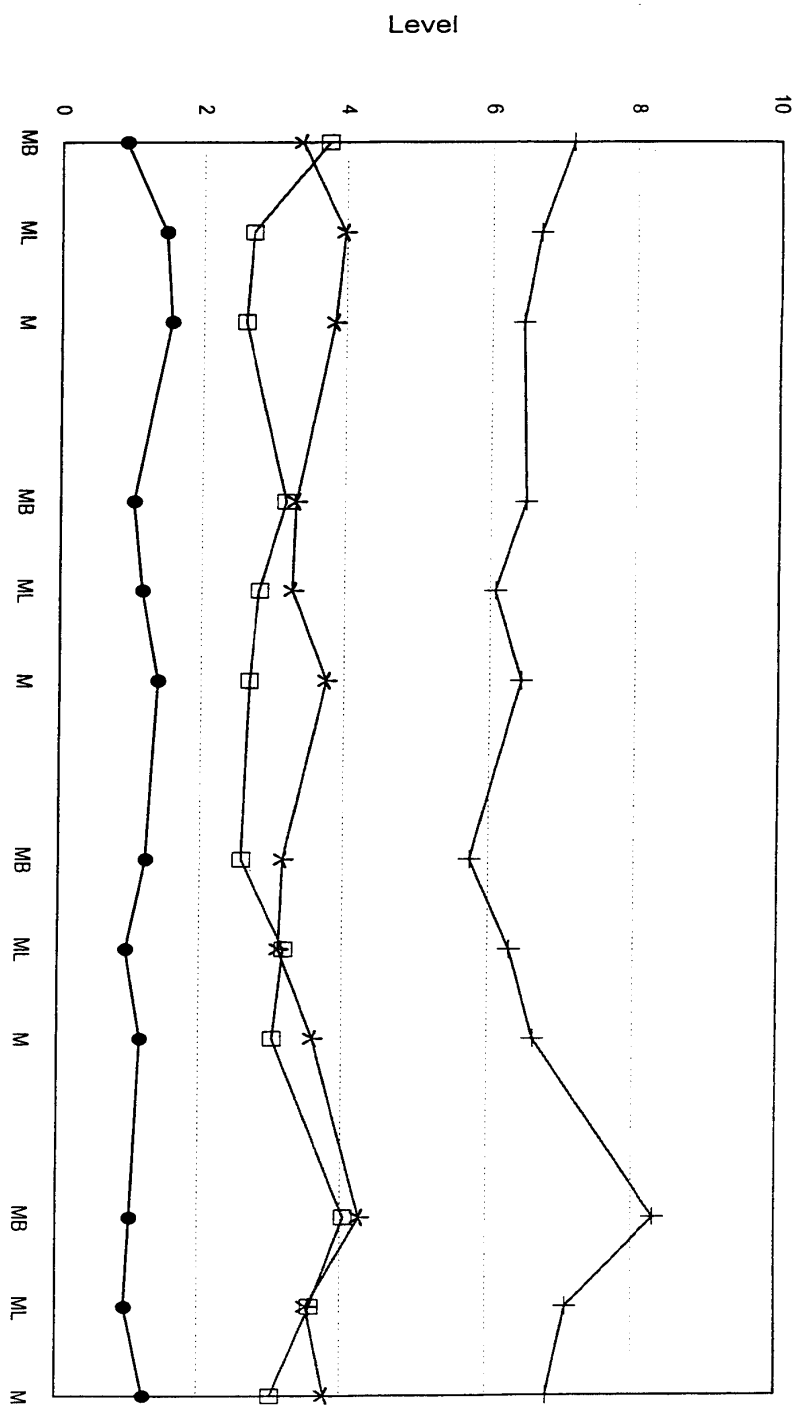

Fig. (2): Effect of different treatments on serum proteins (Total protein , Albumin, Globulin as well as Albumin/ Globulin ration) in blood serum of ***Monosex tilapia.***

MB=Monosex with biogen ML = Monosex with levamisole M= Monosex control

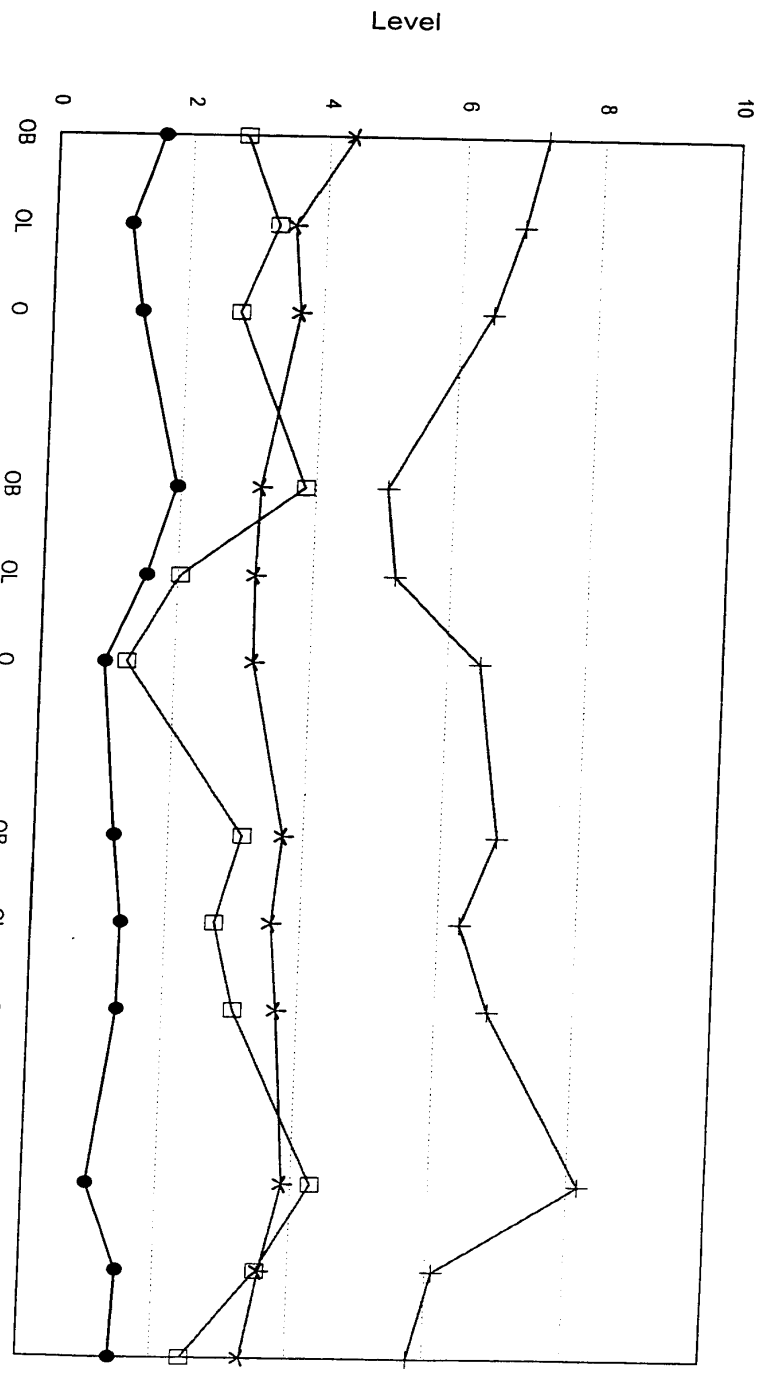

Fig. (3): Effect of different treatments on serum proteins (Total protein , Albumin, Globulin as well as Albumin/ Globulin ration) in blood serum of ***Oreochromis niloticus*** fish.

OB= O. niloticus with biogen **OL = O. niloticus with levamisole** **O= O. niloticus control**

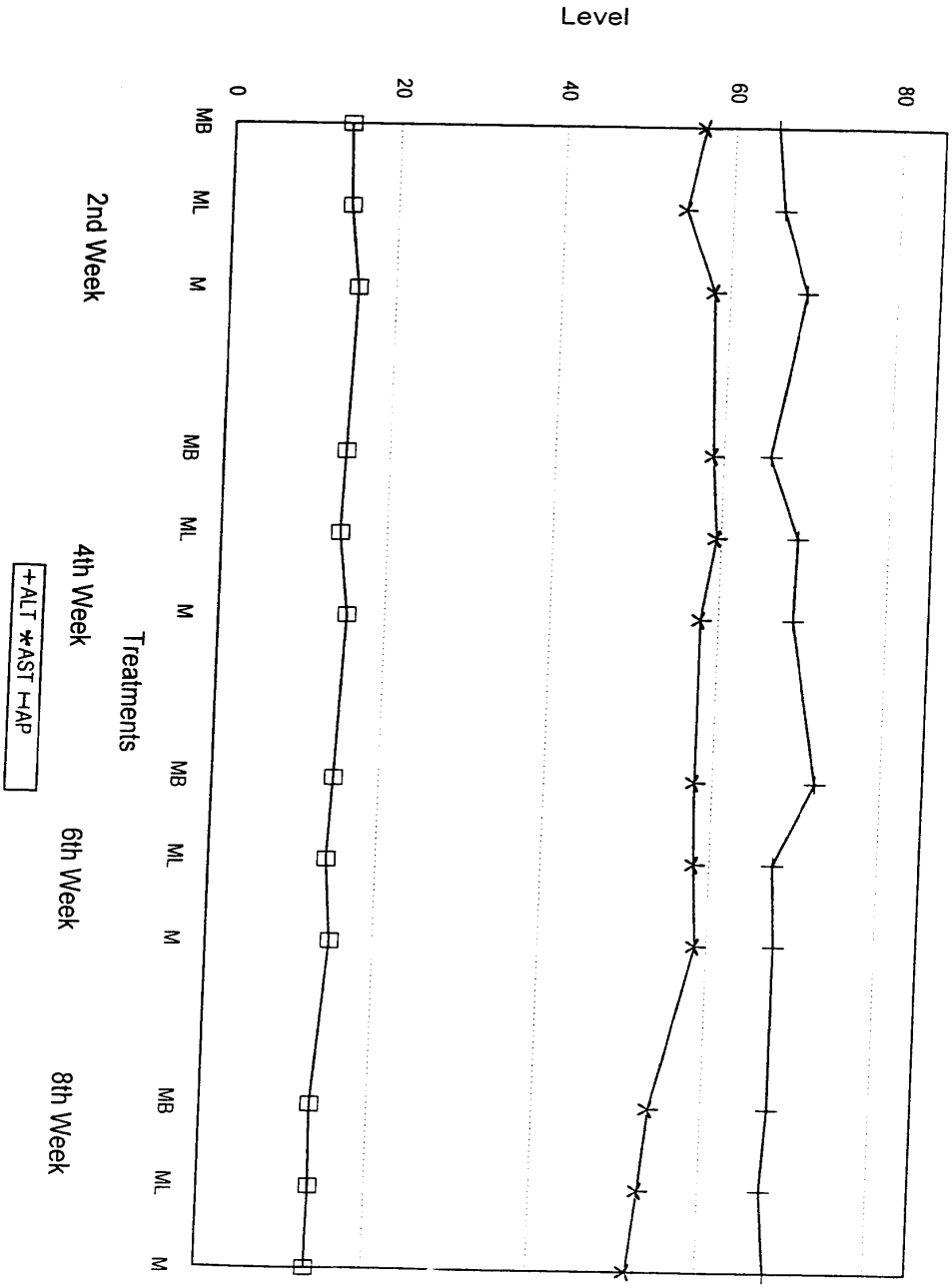

Fig. (4): Effect of different treatments on serum enzymes ALT, AST and AP of ***Monosex tilapia.***

MB= Monosex with biogen ML = Monosex with levamisole M= Monosex control

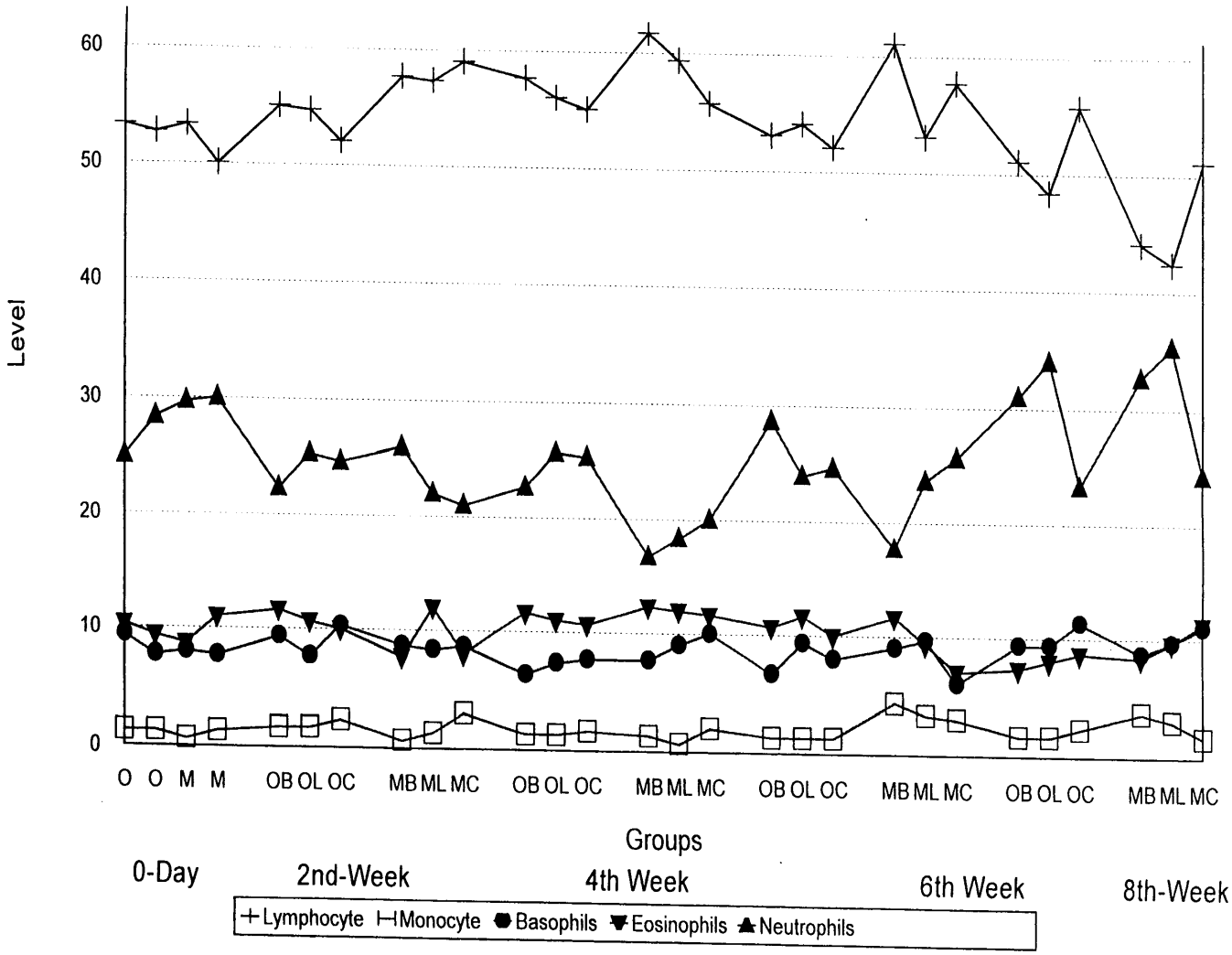

Fig. (5): Effect of different treatments on differential leucocytic count of *O. niloticus and monosex tilapia.*

O= O. niloticus at zero day M= Monosex tilapia at 0 day OB = O. niloticus with biogen OL = O. niloticus with levamisole MB = Monosex with biogen ML = Monosex with levamisol OC= O. niloticus control MC = Monosex control

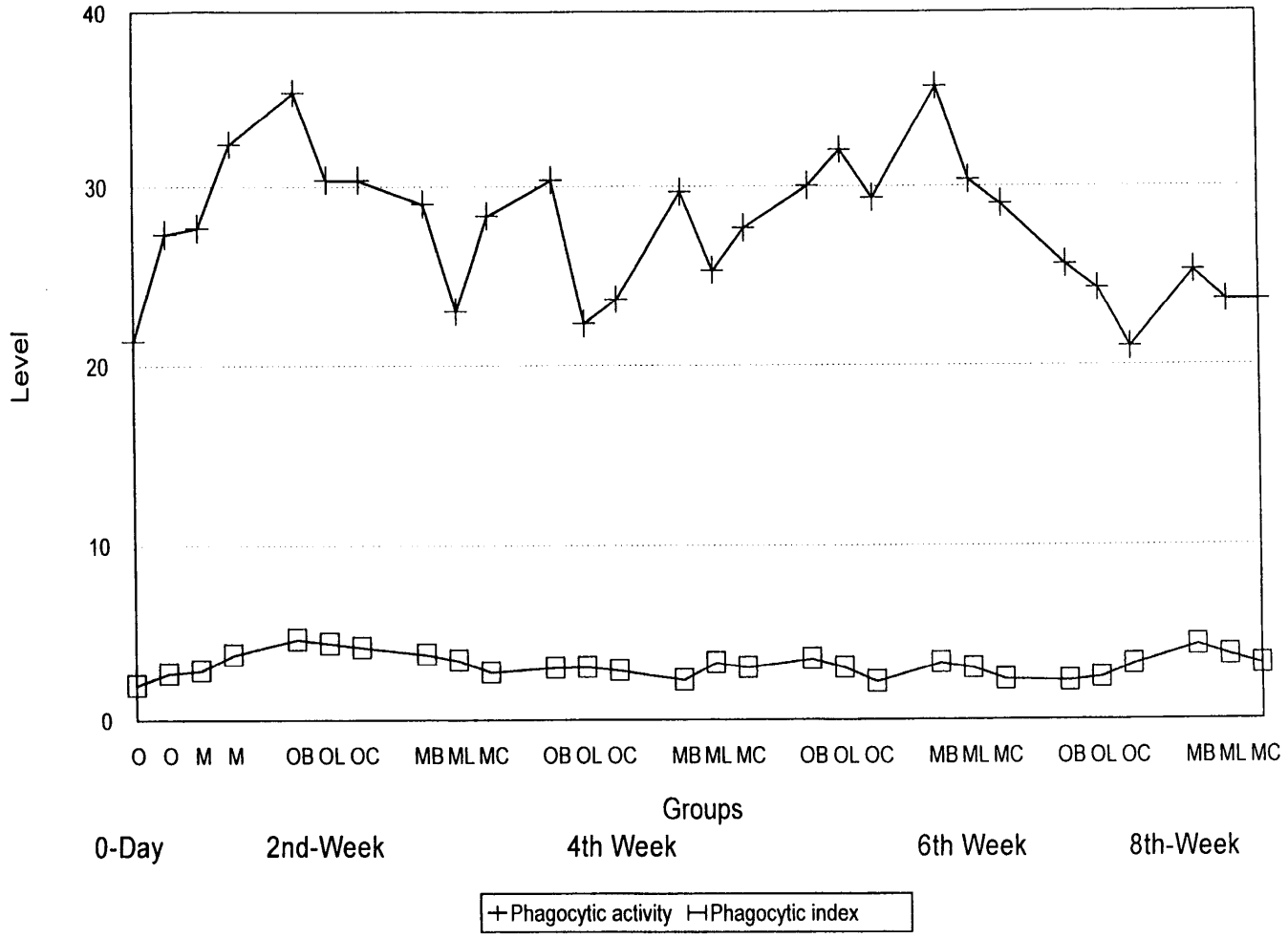

Fig. (6): Effect of different treatments on phagocytic activity and phagocytic index.

cus at zero day M= Monosex tilapia at 0 day OB = O. niloticus with biogen OL = O. niloticus with levamisole MB = Monosex biogen ML = Monosex with levamisol OC= O. niloticus control MC = Monosex control

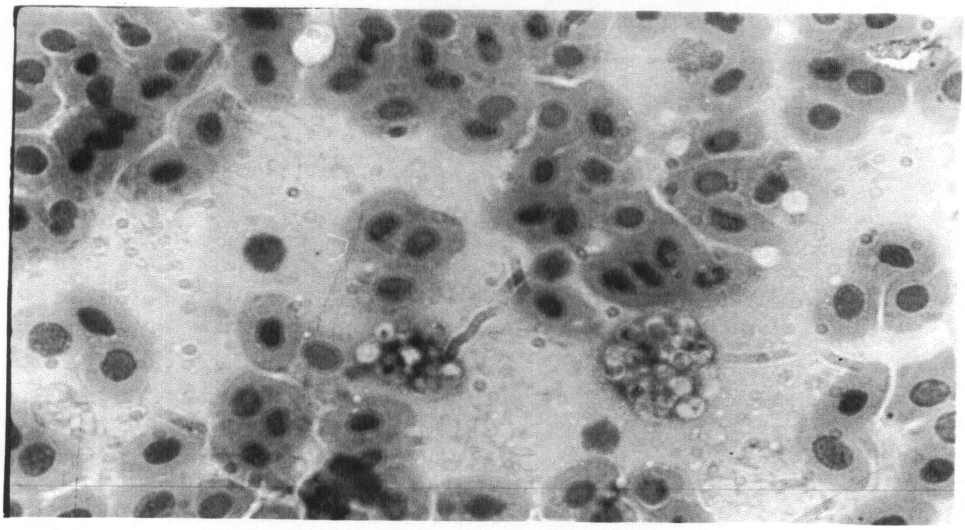

Fig. (7): ***O. niloticus*** fed with ***Biogen*** ® showing attachment and engulphment stage of phagocytosis (arrows).

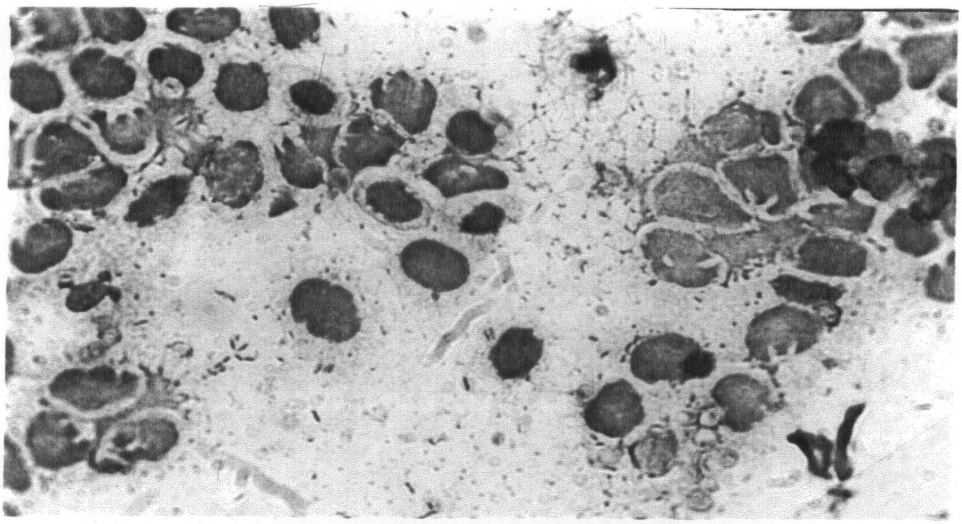

Fig. (8): ***O. niloticus*** fed with ***levamisole*** showing engulphment stage of phagocytosis (arrows).

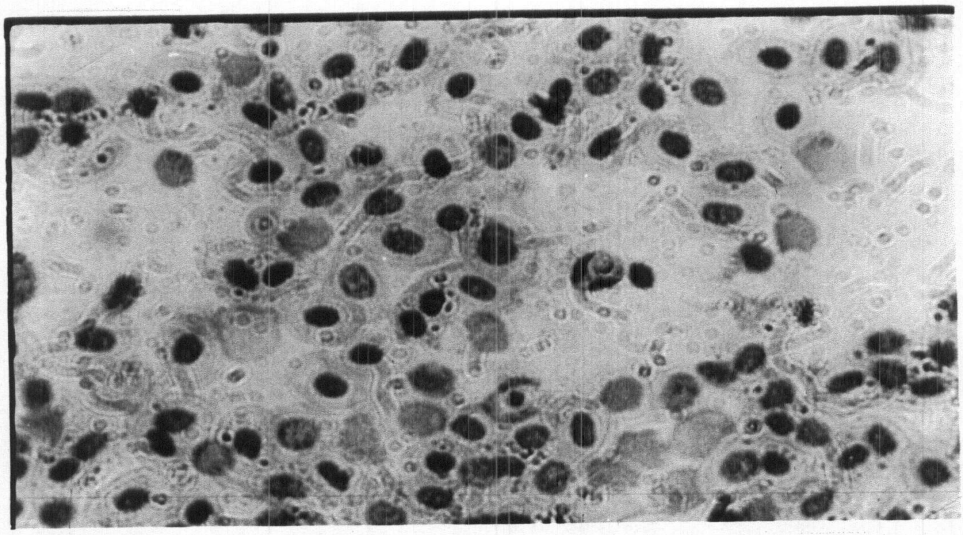

Fig.(9): ***Monosex tilapia*** fed with ***levamisole*** showing digestion stage of phagocytosis (arrows) (Control).

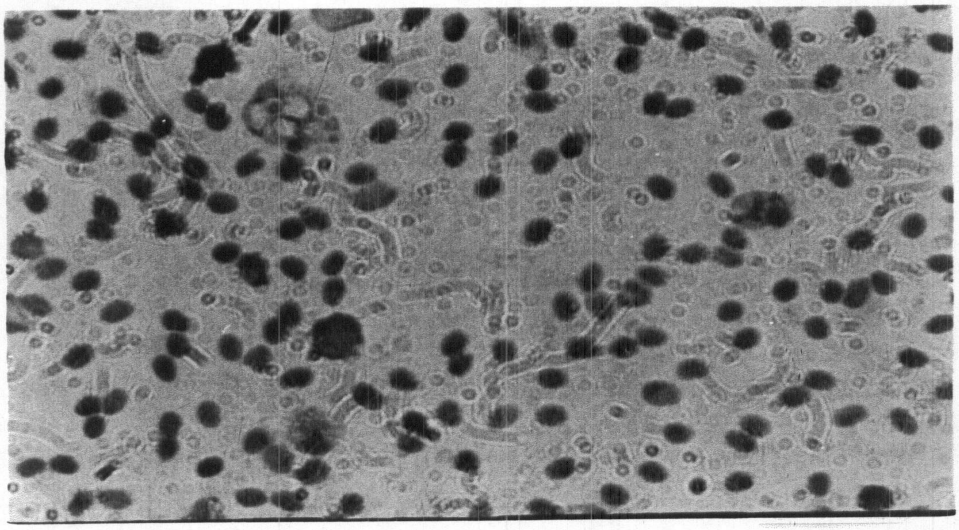

Fig. (10): ***Monosex tilapia*** fed with ***Biogen*** ® showing all stages of phagocytosis (arrows).

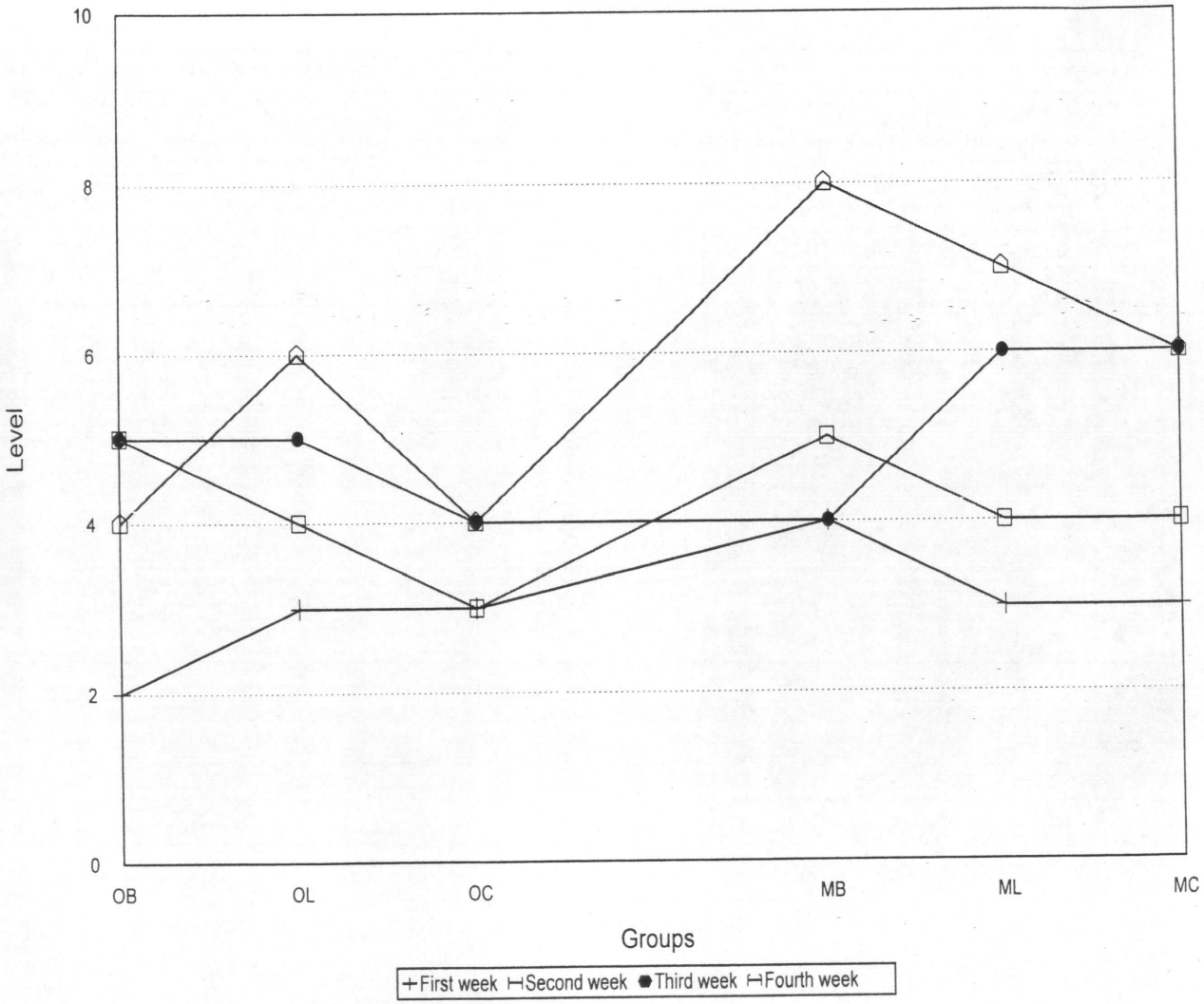

Fig. (11): Antibody titration levels at different weeks.

OB = O. niloticus with biogen **OL = O. niloticus with levamisole** **OC = O. niloticus control** **MB = Monosex with biogen**
ML = Monosex with levamisol **MC = Monosex control**

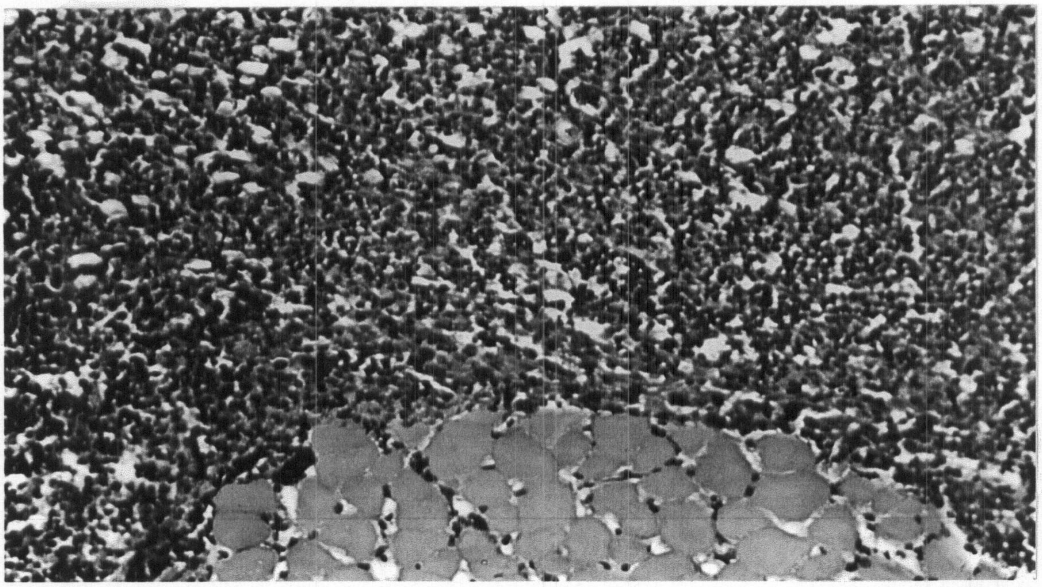

Fig. (12): Anterior kidney of ***O.niloticus*** treated with ***Biogen*** ® : showing marked proliferation of the lymphoid elements. H, E. (X 250).

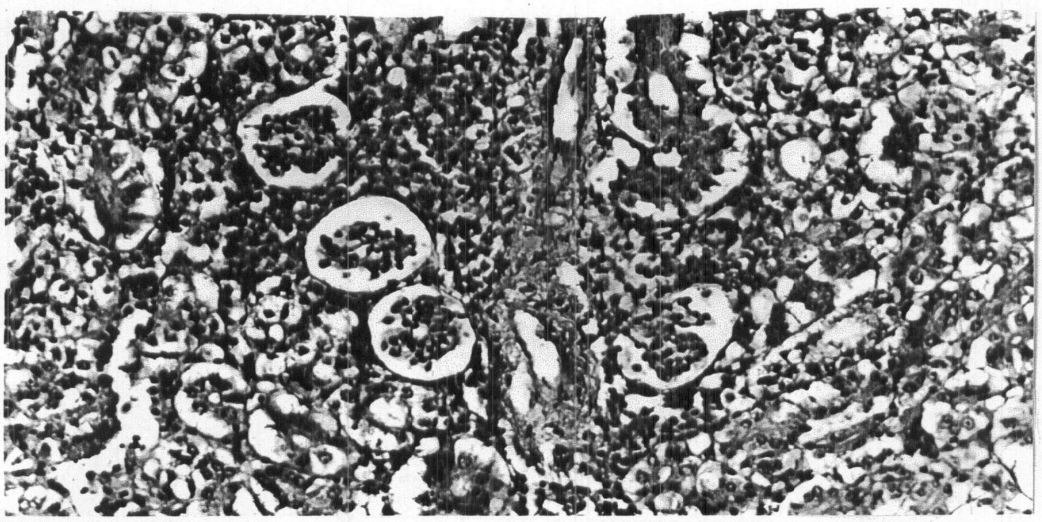

Fig. (13): Posterior kidney of ***O.niloticus*** treated with ***Levamisole :*** showing Proliferation of the interstitial lymphoid elements. H, E. (X 160).

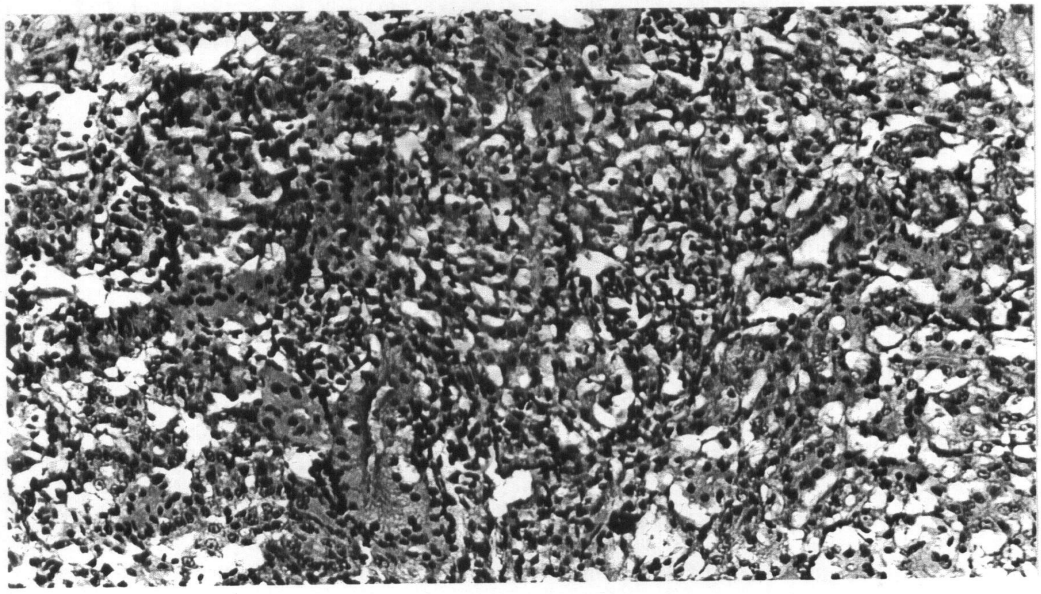

Fig. (14): Posterior kidney of ***Monosex tilapia*** treated with ***Biogen***: showing slight proliferation of the interstatial lymphoid elements. H, E. (X 160).

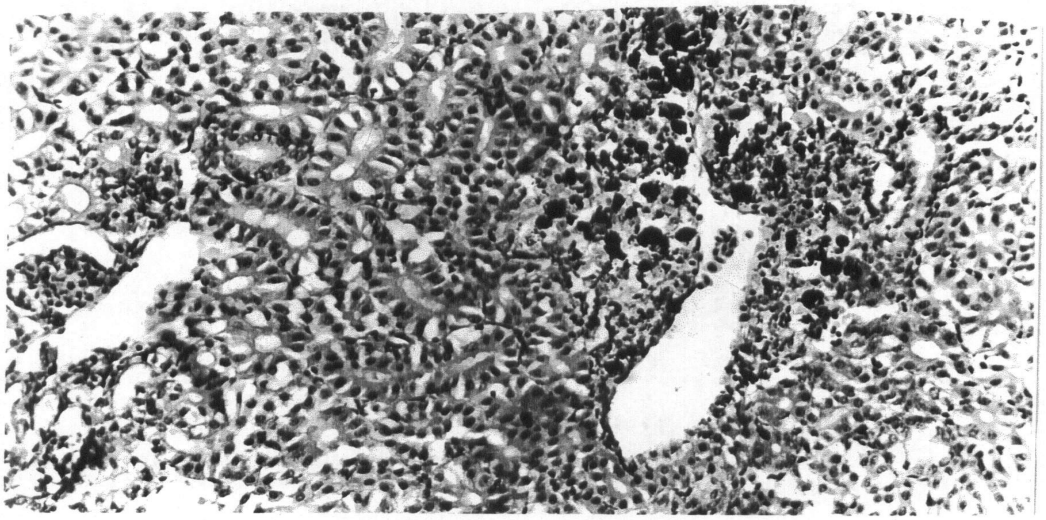

Fig. (15): Posterior kidney of ***O. niloticus*** treated with ***Biogen***: showing hyperactivation of the melanomacrophage centers. Note that the melanin pigment was dark black due to great concentration of the pigment.H, E. (arrows). (X 160).

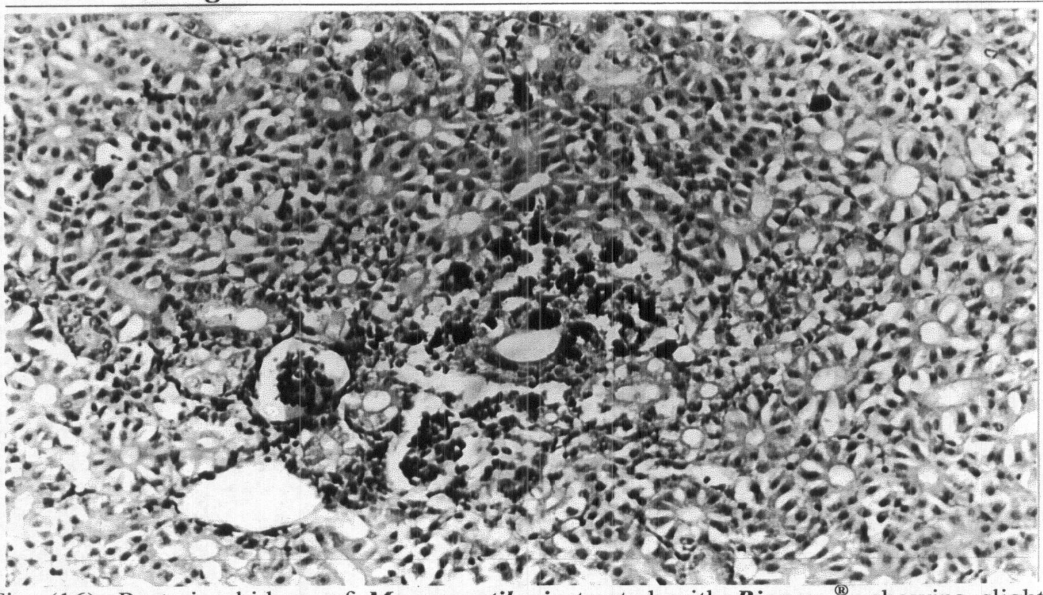

Fig. (16): Posterior kidney of ***Monosex tilapia*** treated with ***Biogen*** ®: showing slight hyperactivation of the melanomacrophage centers. H, E. (arrows). (X 160)

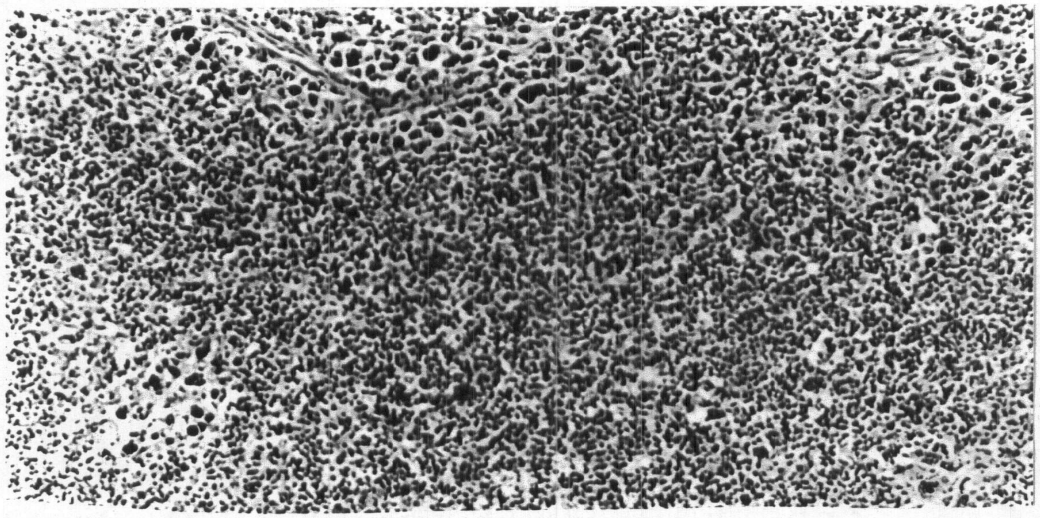

Fig. (17): Spleen of ***O.niloticus*** treated with ***Biogen*** ® : The splenic paranchyma was impacted with lymphoid elements with relative absence of stromal elements. H, E. (X 160).

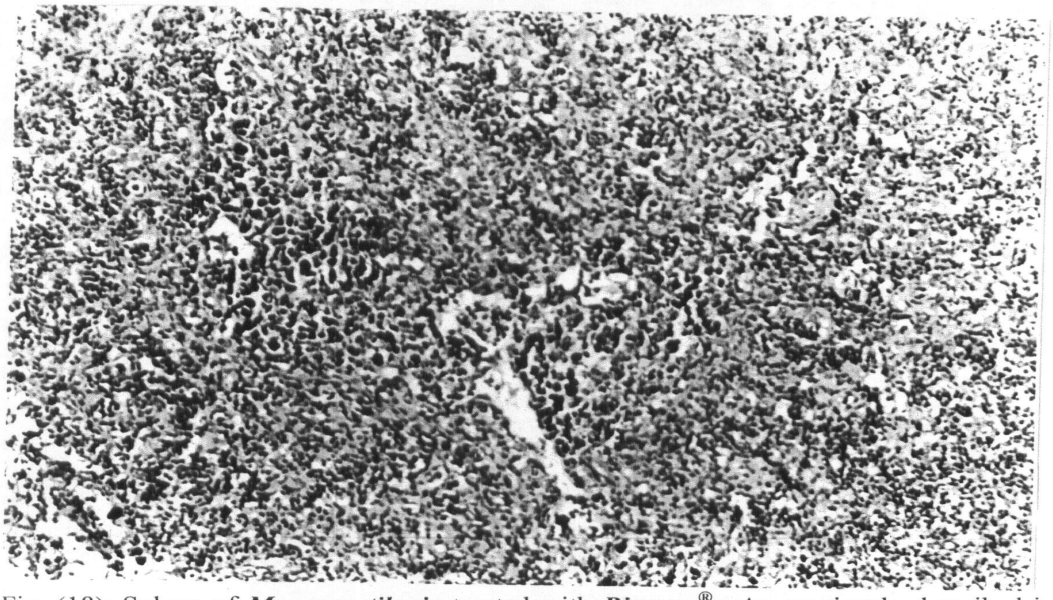

Fig. (18): Spleen of ***Monosex tilapia*** treated with ***Biogen*** ® : As previously described in Fig. (15); but, the stromal tissue was more evident. H, E. (X 160).

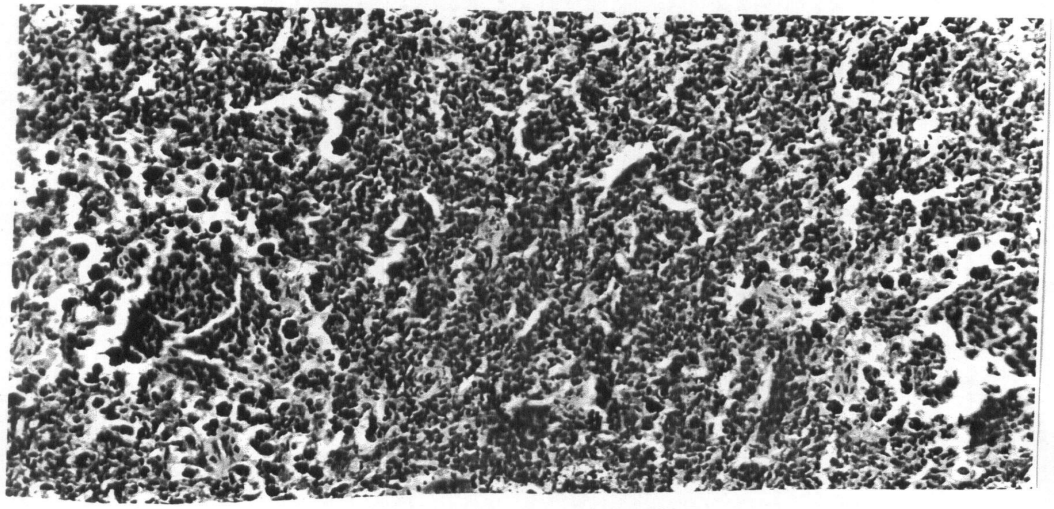

Fig. (19): Spleen of ***O.niloticus*** treated with ***Biogen*** ® : showing marked hyperactivation of melanomacrophage center with intensive concentration of melanin (black) pigment. H, E. (X 160).

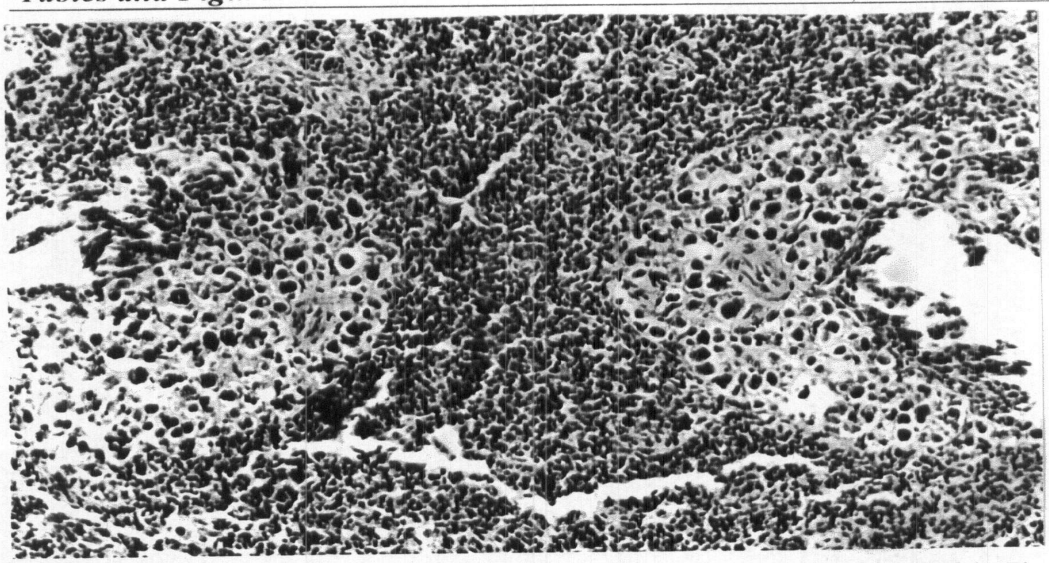

Fig. (20): Spleen of ***O.niloticus*** treated with ***levamisole***: As previously described in Fig. (17); but, the melanin pigment was slightly less concentrated (brown). H, E. (X 160)

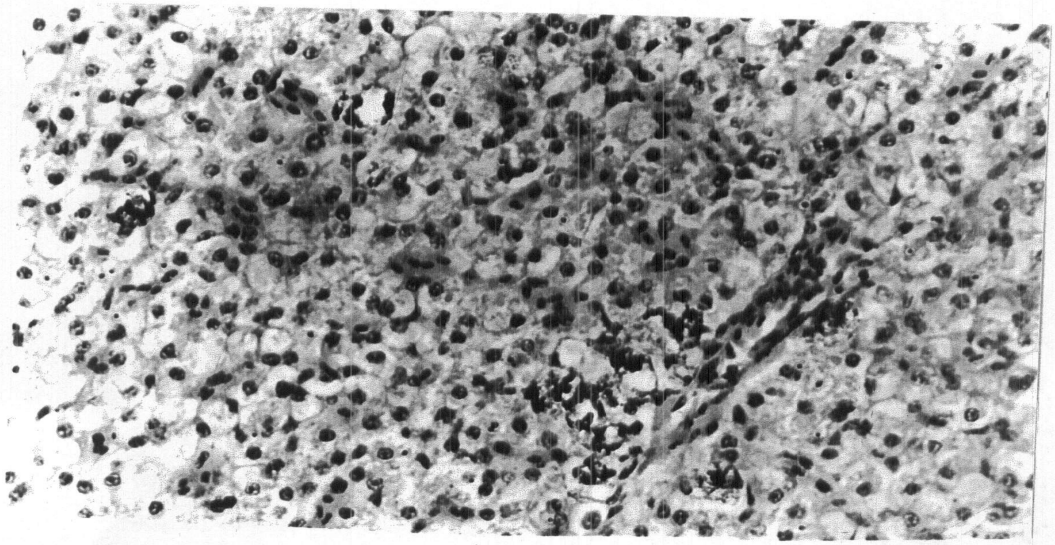

Fig. (21): Hepatopancrease of ***O.niloticus*** treated with ***Biogen*** ® : there is activated melanomacrophage centers. H, E. (X 160).

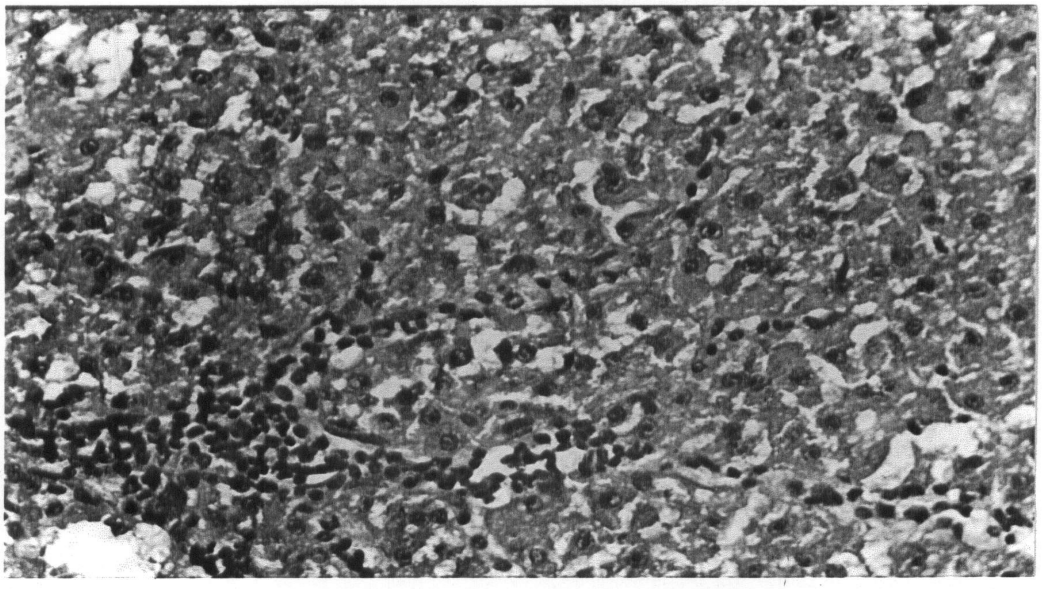

Fig. (22): Hepatopancrease of ***O.niloticus*** treated with ***Biogen*** ® : showing lymphocytic cell proliferation (arrow). H, E. (X 250)

VI- DISCUSSION

The importance of immunostimulants originated from that, the immunostimulants are a group of chemicals or biologicals giving to the human, animals, birds and fish to give them the protection against pathogens via increasing and enhancing the specific and non-specific defenses of the body ***(Anderson and Jeney, 1992; Biologicals and Seattle, 1997; Bangi et al., 2000 and Smith et al., 2003).***

Public awareness is currently focused on the wide-spread of fish diseases and the ability of immunostimulants in prevention of the fish diseases that may be transmitted to human ***(Smith et al., 2003)***, as well as which cause economic losses in fish.

Utilization of immunostimulants in fish culture offers a wide range of attractive methods for including and building protection against diseases. Also, ***Saad (2002)*** reported that the addition of immunostimulants to fish diet can inhibit the action of the pathogenic bacteria or pathogenic antigens and reduce the level of serum enzymes.

In the present investigation, the two tilapia species of fish either ***Oreochromis niloticus*** and ***Monosex tilapia*** treated with two types of immunostimulants for 8 weeks. The effects of the two immunostimulants on serum enzymes, serum proteins and deferential leucocytic counts of WBCs (lymphocyte, monocyte, eosinophils, Neutrophils as well as phagocytic activity , Index and the level of antibody titer and RPL, were determined .

Before fish treatments with immunostimulants there was no significant ($P< 0.05$) differences between ***O. niloticus*** and ***Monosex*** fish in its serum enzymes (ALT, AST and AP) . The levels of these enzymes were ALT (66.72), ALT (68.87) ; ALT (51.75), AST (54.22) and AP (13.04), AP (13.58) in case of ***O. niloticus*** and ***Monosex fish***, respectively.

Also, the results indicated that the total protein, Albumin, Globulin and Albumin/Globulin ratio were higher in serum of ***Monosex*** tilapia than ***O. niloticus*** ($P < 0.05$).

This results indicated that, under the experimental conditions, there is no any changes in the serum enzymes and serum protein among the two types of fish. This results agreed with those of ***Smith et al.(2003),*** who reported that, the stress conditions causes elevation of the serum enzymes levels and serum proteins than the fish live under the normal conditions or not treated with any treatments

1- Serum enzymes:-

The significant increase of ALT, AST and ALP enzymes in the groups received ***Biogen*** ® and ***levamisole*** was mainly due to the activity of immunostimulant that causes increasing of serum Alkaline phosphatase activity ***(Gupta et al., 1979 a, b, c, d. e and Gupta and Chatterjee, 1980).*** Also, the increase of serum transaminases (ALT and AST) may reflect myocardial and hepatic action of immunostimulant that leading to extensive liberation of the enzymes into blood circulation ***(Kachmor, 1970; Shotwell et al., 1975; Vermu et al., 1981; Richardi and Huff, 1983; Fuchs et al., 1986)***. Moreover, serum ALT and AST activities are considered as a sensitive indicator to evaluate (hepatocellular and myocardial activity) of immunostimulant ***(Raa, 1984 and Abo-hegab et al., 1992).***

Concerning the decrease of liver enzymes with the time can be explained by that . The addition of immunostimulants causes increase of the action of immune system and increase healthy status of fish and fish defense that leads to decreasing of the level of serum enzymes. This results attributed to that, the immunostimulants causes increasing of non-specific factors that causes decreasing of the serum enzymes levels. These results also agreed with those of ***(Anderson and Jeney, 1992; Siwicki et al., 1992; Brattgjerd et al., 1994; Bangi et al., 2000 and Smith et al., 2003)***, they reported that the immunostimulants r uce the level of activity of the serum enzymes at the period of 3 weeks of the perimental period, and they attributed this results to that the immunostimulants activate t.e non-specific part of the immune system.

The increasing level of serum enzymes in ***O. niloticus*** groups than ***Monosex*** groups and groups treated with ***levamisole*** than the groups treated with ***Biogen*** ® may be attributed to that the species differences in its sensitivity to different immunostimulants and differences between immunostimulants in its effect on serum enzymes ***(Cook et al., 2003; Rodriguez et al., 2003; Smith et al., 2003 and Shewita, 2004).***

The immunostimulants causes decreasing the level of serum enzymes and so overcome the action of stress conditions or the action of bacterial infections ***Naglaa Mangwood (2004).***

2-Serum proteins:-

The total serum proteins were useful in diagnosis of fish diseases ***(Mulcathy, 1967)***. In the present work, the significant increase in albumin, globulin, total protein and albumin/globulin (A/G) ratio was higher in the groups received ***Biogen*** than that taken ***levamisole*** and control group .

The levels of albumin, globulin and protein were higher in case of ***Biogen*** ® treated group than ***levamisole*** one while the levels in both treatment were higher than control group . This result attributed to the effect of immunostimulants on liver that causes increasing of serum protein concentration ***(Manning and Wyatt, 1984 a, b; Galtier, 1979 and Edvington et al., 1994)*** . Moreover, fish without immunostimulant diet and under stress has the greatest decrease in total protein due to liver affection ***(Saffinaz, 2001).***

Our results showed that decreasing in globulin level in groups taken ***levamisole*** than the groups taken ***Biogen*** ®, but the both higher than the control.

Meanwhile the albumin fraction increased with addition of immunostimulants (***Biogen*** ® or ***levamisole***) than the control groups which not treated with any immunostimulants.

The increasing of all serum proteins in case of ***Biogen*** ® treated groups was higher than that of the ***levamisol*** treated groups. Moreover, the increasing of serum proteins in the ***Monosex tilapia*** was higher than that of ***O. niloticus*** may be due to higher sensitivity of ***Monosex tilapia*** immune system to immunostimulant than that of ***O. niloticus*** .

Increase albumin el was also observed in the groups expose o nostimulant *Kar* ***(1989)*** stated that chronic liver disorder is u panied h lbuminaemia. Both hypogammaglobulinaemia d ouminaem. nfi the recorded hypoproteinaemia, which was associated with l. r damage ***(Manning and Wyatt, 1984 a, b).***

The same authors, also indicated that the immunostimulant causes increasing of albumin level than groups not take any immunostimulants. This result attributed to the using of immunostimulants in fish farms causes regeneration of the liver cells and increasing the immune status of fish body with increasing of serum proteins. ***(Manning and Wyatte, 1984 a, b).***

Stressor has a drastic effect on immune system of ***O. niloticus*** or ***Monosex tilapia*** in challenge experiments. This stress may activate the hypothalamus hypophysis-adrenal endocrine system and stimulate corticosteroids and catecholamines in fish blood which negatively affect the process of lymphobiosis and interfere with the synthesis of ascorbic acid, thus lowering the resistance of fish and induce immuno-suppression ***(Pickering, 1981; ICRAM et al., 2000; Cook et al., 2001; Montero et al., 2001 and Paulsen et al., 2001 and Sealey and Galtin, 2002).***

The immunostimulants causes increasing the level of serum proteins and so overcome the action of stress conditions or the action of bacterial infections which causes decreasing of the serum proteins level due to liver damage resulted from the bacterial infection. ***(Naglaa Mangwood,2004).***

3-Differential leucocytic counts:-

The blood parameters as leucocytic counts and differential leucocytic count have diagnostic importance and usually readily respond to identical factors such as physical, chemical and biological stressors ***(Hickey, 1976 and Soliman, 1996).***

The results indicated that, the differential leucocytic count in control groups was lower in neutrophils and basophils than groups take immunostimulant, while the groups received ***Biogen*** ® showed increase in lymphocytes than groups treated with ***levamisole*** and both (***levamisole & Biogen*** ®) revealed increases of D.L.C. than the control fish. These results agreed with those of ***(Gupta et al., 1979 a, b, c, d, e and Edvington et al. , 1994)*** which reported that fish not take any immunostimulants or live under stress conditions show decrease D.L.C. no. and increase susceptibility to infection .

Also there was increasing in lymphocyte, monocyte, basophils, eosinophils and neutrophils in groups taken immunostimulant (***Biogen*** ® and ***Levamisole***) than the control groups.

This increasing mainly in ***Biogen*** ® than that of ***levamisole*** treated groups due to its effect on haemobiotic organs ***(Forgacs and Carll, 1962 and Change et al., 1979).***

4-Phagocytic activity and phagocytic index:

Group fed ***Biogen*** ® characterized by increasing of Phagocytic activity and phagocytic index than the group treated with ***levamisole*** or the groups fed regular diet without immunostimulants. This result, attributed to the action of immunostimulants on liver, kidney, spleen and other haemopiotic organs as it activate this organs ***(Smith and Hamilton, 1970 and Huff et al., 1974; Fuchs et al., 1986 and Gekle et al., 1998),*** so it causes increases of D.L.C. and increases phagocytic activity and phagocytic index than control groups.

This results may suggests that, the action of ***Biogen*** ® and ***levamisole*** on fish leads to decreasing level of serum cortisol and increase fish resistance . The decrease of cortisol level may lead in turn to increase the phagocytosis process.

This results may be due to that ***Biogen*** ® containing allicin and gensing which increase the physiological function of fish and immune response with increasing resistance of fish to different stress related disease. ***(Safinaz 2001).***

Gensing active principle increasing phagocytic activity and phagocytic index with increasing of mitogenesis of T and B lymphocytes, which causes reduction of cell damage via its act directly on body cell promoting DNA , protein synthesis and increasing T-cell mediated immunity with increasing of body resistance due to increasing antibody producing cells. ***(Naglaa-Mangood, 2004).***

5-Antibody titers:-

The antibody titers increased progressively from the 2nd week to 4th week in ***Biogen*** ® and ***levamisole***. While, the titers were higher in ***Biogen*** ® group . The addition of immunostimulants causes raising of the antibody titers than the groups that not received immunostimulants. Also the antibody titer level was higher in ***Monosex tilapia*** than that of ***O. niloticus*** .

This results attributed to the immunostimulants contain some factors that enhancing the activity of the fish and stimulating the antibody secretion leads to increasing the antibody titers.

The Relative Level of Protection (RLP) was higher in the groups treated with ***Biogen*** ® than the groups treated with ***levamisole*** and all of them higher in its RLP than the control groups.

This results attributed to the action of immunostimulants on liver, kidney, spleen and other haemopiotic organs as it activate this organs ***(Smith and Hamilton, 1970 and Huff et al., 1974; Fuchs et al., 1986 and Gekle et al., 1998),*** which causes increases of D.L.C. counts and the relative level of protection of the fish against any stress conditions, its level in ***Monosex*** was higher than ***O. niloticus*** may be due to hormonal treatment, this results need more study to be clarify).

By bacterine injection and addition of immunostimulants , that affecting lymphoid tissue and increasing the fish immunity and increasing the activity of haemopiotic tissue like spleen and kidney mesonephrons. The immunostimulant causes increasing of serum protein and increasing immunoglobulin formation, also increasing humeral immunity . Moreover, the increasing of RLP was proved by higher titer of antibody in case of groups received immmunostimulant.

Mirkriakov (1969 and 1975); Collins et al. (1976) and Faisal et al. (1988), reported that immunostimulant increasing antibody titer in fish exposed to different environmental stressors than fish without immunostimulant.

From the previous results we noticed that the addition of immunostimulants improve the fish immunity and survival than the other groups that not taken any immunostimulants. This results agreed with those of ***Saad (2002)*** who reported that, the immunostimulants

causes reduction of the effect of stress condition on blood enzymes, body weight, body weight gain, feed conversion and feed efficiency.

Safinaz (2001) reported that , the gensing active material found in the ***Biogen*** ® immunostimulant causes decreasing of the cell damage, decrease of gama rays on DNA molecules and increasing the re-generation process of different cells also, it act on body cells promoting DNA and protein synthesis, with increasing natural body resistance.

Moreover, this results agreed with those of ***(Saad, 2002)*** who reported that, the ***Biogen*** ® immunostimulant contain the active material called allicin which activate and coordinate the function of various glands in body and enable them to work normally with high efficiency.

The allicin active principle increase antibody activity and inhibit RNA synthesis . Also the allicin has antibacterial and antifungal characteristics due to specific interference with sulphohydryl group so, it will increase the fish immunity ***(Barone and Tensey, 1977; Yang and Yu, 1990,; Kim et al., 1993; Nikitina et al., 1995; Angelo et al., 1998 and Feldberge et al., 1998).***

All the previous results explain why fish that fed on the immunostimulants supplemented diets of a good characters on blood enzymes , PA, PI and higher level of RLP than groups take diet free from immunostimulants .

Also results indicated that ***Monosex tilapia*** characterized by higher immunogenic characteristics than ***Oreochromis niloticus*** and gave a good response to the used immunostimulant this point need more research to be clarified.

6-Histpathlogicaly:

The Histopathological findings indicated that there was hyperactivation of melanomacrophage center in kidney, liver & spleen in case of fish received immunostimulant than control and this results agreed with those of ***(Easa, 1997 and Saad, 2002)*** which confirmed this results histopathologically by activation of melanomacrophage centers of spleen.

This results indicated that the immunostimulants induced hyperactivation of haemopiotic organs and MMCs especially in case of ***Monosex tilapia***. This results agreed with those of ***Agrawala et al. (2001***).

This results indicated that the ***Biogen*** ® and ***levamisole*** induced hyperactivation of haemopiotic organs and MMCs especially in case of ***Monosex tilapia***.

VII- CONCLUSION

We can included that ,

- The use of immunostimulant proved to be useful under culture condition , since it increased the PA , PI, globulin , antibody titers and RLP

- ***Biogen*** ® gave a better results than ***Levamisole***.

- ***Monosex tilapia*** gave a good response to the used immunostimulant than ***O.niloticus***

- From the results obtained we recommend the use of immunostimulant for at least 8 weeks to improve both cellular & humeral immunity as well as the health status of fish during the breeding period.

VIII- SUMMARY

A total number of 240 healthy fish (average weight 30 ± 5 gm) from them 120 ***Monosex tilapia*** and 120 ***Oreochromis niloticus***, were collected from private fish farm at Behera Province and used in this study.

The present work was designed to investigate the effects of immunostimulants(***Biogen*** ® and ***Levamisole*** HCl ®) on differential leucocytic counts, phagocytic activity, some serum enzymes, total proteins, albumin, globulin and vaccine potency .

There was a significant differences (P< 0.01) of the effect of the used immunostimulants on ***O. niloticus*** and ***Monosex*** fish serum enzymes.

The serum enzymes (ALT, AST and AP) levels were increased with the ***Biogen*** ® treatment , followed by ***levamisole*** than control group.

The levels of serum proteins (total protein, Albumin, globulin and albumin /globulin ratio)were increased in case of groups received diet with ***Biogen*** ® followed by that received diet with ***levamisole*** in both types of fish.

There was no-significant differences among serum enzymes (ALT, AST and AP) and serum proteins (total proteins, albumin, globulin and albumin/globulin ratio) between ***Oreochromis niloticus*** and ***Monosex tilapia***.

The lymphocyte number increased progressively from 0-day to 8th week in ***Monosex tilapia*** more than in ***Oreochromis niloticus*** and commonly the groups received ***Biogen*** ® and finally the groups of ***levamisole.***

The number of monocytes increased progressively by increasing the time among the different treatments. The groups taken ***Biogen*** ® characterized by a high level of monocytes followed by the groups received ***levamisol*** (similary), the eosinophil level increased significantly (P< 0.01), also, the neutrophils count increased progressively till reach it's a maximum level at 8th and the ***levamisole*** causes a higher neutrophils level, followed by ***Biogen*** ® immunostimulants.

The phagocytic activity and index differ significantly among different treatment groups. The phagocytic activity was higher in the groups fed on diets containing, ***Biogen*** ® and ***levamisole*** than control ones . Activation of phagocytosis was more prominent in the ***Monosex tilapia*** than ***O. niloticus*** .

The antibody titration differ significantly among different groups and different weeks (P< 0.01). The higher antibody titers observed in the first weeks in the groups of ***Monosex*** fed ***Biogen*** ® and ***Monosex tilapia*** fed ***levamisole*** .
In the 4th week the higher antibody titer was observed in case of ***Monosex tilapia*** fed ***Biogen*** ® .

The effects of the two tested immunostimulants (***Biogen***, ***levamisole***) were obvious in both species (***O.niloticus*** and ***Monosex tilapia***). In addition, the immunostimulation was more marked in ***O.niloticus*** than in ***Monosex tilapia***. Moreover, upon comparing this

immunostimulant effect among the two tested immunostimulants, ***Biogen*** was the best followed by ***Levamisole***.

The RLP was higher in group treated with ***Biogen***® followed by ***Levamisole*** than control groups especially in ***Monosex tilapia***.

Histopathologically , the immunopromoting effect on heamopiotic organs were similar in nature either in both species upon the use of any drug, but, it was variable in degree and distribution (i.e. the splenic reaction in all experimental groups was hyperactivation and enlargement of the melanomacrophage cnters,

The immunostimulant effect in the renal tissue was manifested by moderate to severe (according to species and/or drug) proliferation of the lymphoid elements in the anterior kidney. Furthermore, the posterior kidney showed focal to multifocal proliferation of the interstitial lymphoid elements. In addition, the renal melanomacrophage centers were variably enlarged and hyperactivated.

The lymphoid elements of the splenic paranchyma had increased in size upon the extent of the stromal tissue. Additionally, the splenic melanomacrophage centers were markedly proliferated, enlarged and hyperactivated to the extent that the melanin pigment itself was concentrated inside the macrophages and appeared dark brown to black granules.

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الملخص العربى

بعض الدراسات على تاثيرات بعض المنشطات المناعية على الأسماك المستزرعة

استخدم فى هذه الدراسة عدد ٢٤٠ سمكة متوسط وزن كل سمكة (٣٠ ± ٥) ١٢٠ سمكة بلطى وحيد جنس ، ١٢٠ سمكة بلطى نيلى . تم الحصول على تلك الأسماك من مزرعة برسيق بمحافظة البحيرة .

وتم إجراء هذا البحث بهدف معرفة مدى تأثير منشطات المناعة البيوجين والليفاميزول على معدل النشاط اللالتهامى وانواع كرات الدم البيضاء وعلى مستوى إنزيمات السيرم كما تم دراسة دور هذه المنشطات فى تنشيط مناعة الأسماك ضد ميكروب الأيروموناس .

أوضحت نتائج هذه الدراسة ما يلى :

١) أن هناك اختلافات معنوية فى محتوى الإنزيمات بين البلطى النيلى والأسماك وحيدة الجنس . حيث لوحظ أن هناك زيادة فى مستوى إنزيمات السيرم فى الأسماك التى أعطيت البيوجين عنها فى الأسماك التى أعطيت الليفاميزول أو المجموعة الضابطة وخصوصاً مع تقدم عمر الأسماك .

٢) أن استخدام البيوجين يؤدى إلى زيادة مستوى البروتين الكلى والالبيومين والجلوبيولين عنه فى حالة استخدام الليفاميزول والمجموعة الضابطة وتتضح هذه النتائج أكثر مع تقدم عمر الأسماك .

٣) عند بداية التجربة وبدون أى علاجات لوحظ عدم اختلافات معنوية بين البلطى النيلى والبلطى وحيد الجنس فى محتوى إنزيمات السيرم وبروتينات السيرم (البروتين الكلى والالبيومين والجلوبيولين ونسبة الالبيومين للجلوبيولين)

٤) لوحظ أن نسبة الخلايا الليمفاوية تزيد باستمرار فى الفترة الأولى من (٠ - ٤) أيام ثم بعد ذلك يبدأ عدد الخلايا الليمفاوية فى النقصان وخصوصاً عند

الأسبوع السادس ويتضح ذلك الانخفاض فى نسبة الخلايا الليمفاوية عند الأسبوع الثامن . ولوحظ أن نسبة الزيادة فى الخلايا الليمفاوية فى الأسماك وحيدة الجنس أعلى من نسبة الزيادة فى اسماك البلطى النيلى وأن البيوجين يؤدى إلى زيادة نسبة الخلايا الليمفاوية عن الليفاميزول .

٥) البيوجين منشط مناعى يؤدى إلى زيادة فى نسبة الخلايا وحيدة الخلايا والخلايا القاعدية والحامضية عن الليفاميزول أيضاً نسبة الزيادة فى تلك الخلايا فى البلطى وحيد الجنس أعلى منها فى اسماك البلطى النيلى ويلاحظ أن معدل الزيادة يزداد مع تقدم عمر الأسماك تحت الدراسة .

٦) الخلايا المتعادلة تبدأ قليلة العدد ثم بعد ذلك تبدأ فى الزيادة حتى تصل قمة الزيادة عند الأسبوع الثامن ، وأن الليفاميزول يؤدى إلى زيادة فى تلك الخلايا عن البيوجين أو الأسماك فى المجموعة الضابطة .

٧) معدل النشاط الالتهامى ودليل النشاط الالتهامى يزيد مع استخدام البيوجين عن الليفاميزول ويزداد مع تقدم عمر الأسماك وخصوصاً فى نهاية التجربة .

٨) أوضحت الدراسة أن البيوجين يؤدى إلى زيادة المناعة فى الأسماك ضد الأمراض عن الليفاميزول وفى أسماك البلطى وحيد الجنس عنه فى البلطى النيلى .

٩) أوضحت الدراسة الهستوباثولوجية أن البيوجين يؤدى إلى زيادة نشاط الأجهزة المناعية يليه الليفاميزول .

١٠) كانت أهم التأثيرات التى وجدت فى أعضاء المناعة للأسماك هى زيادة فى معدل الخلايا الليمفاوية ووجود Melanomacrphage centers .

١١) كما أن الحماية المناعية فى أسماك وحيد الجنس أعلى منها فى حالة البلطى النيلى خصوصاً التى تغذت على البيوجين يليه الليفاميزول .

من هنا يتضح أن استخدام أنواع معينة من الـ Immunostimulant خصوصاً البيوجين والليفاميزول يزيد من الحالة المناعية للأسماك على مقاومة الأمراض خصوصاً بعد مدة استخدام تصل من ٦ ـ ٨ أسابيع .

قرار لجنة الحكم والمناقشة

قررت لجنة الحكم والمناقشة بجلستها المنعقدة يوم الأربعاء الموافق ٢٠٠٦/٣/٨ بترشيح السيد **ط . ب / أسامة السيد السنهورى** للحصول على درجة الماجستير فى العلوم الطبية البيطرية تخصص (صحة وأمراض الأسماك) .

أعضـــــاء اللجنـــــــة :

أ . د / أحمـــد فكـــرى بـــدران

أستاذ أمراض الأسماك ورعـايتها

كلية الطب البيطرى - جامعة قناة السويس

أ . د / منـال عـــادل عيســى

أستاذ أمراض الأسماك ورعايتها - كلية الطب البيطرى

جامعة القاهرة - فرع بنى سويف

. د / مجـــدى خليـــل سليمـــان

أستـاذ صحـة وأمـراض الأسمـاك

كليـة الطـب البيطـرى - جامعة الإسكندرية (مشرفاً)

لجنة الإشراف

الأستاذ الدكتور/ مجدى خليل سليمان

أستاذ صحة وأمراض الأسماك

قسم أمراض الداوجن والأسماك

كلية الطب البيطرى

جامعة الإسكندرية

الدكتور/ رياض حسن خليل

أستاذ صحة وأمراض الأسماك المساعد

قسم أمراض الداوجن والأسماك

كلية الطب البيطرى

جامعة الإسكندرية

(٥)

بعض الدراسات على تأثيرات بعض المنشطات المناعية على الأسماك المستزرعة

رسالة علمية

مقدمه إلى الدراسات العليا بكلية الطب البيطرى - جامعة الإسكندرية

استيفاء للدراسات المقررة للحصول على درجة

الماجستير فى العلوم الطبية البيطرية

فى

صحة وأمراض الأسماك

مقدمه من

ط.ب./ أسامة السيد سنهورى

مارس ٢٠٠٦