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SCREENING THE EFFECT OF WATER POLLUTION WITH SOME PESTICIDES ON THE IMMUNE RESPONSE IN *OREOCHROMIS NILOTICUS* (*TILAPIA NILOTICA*) FISH

By

S.S. KHALAF-ALLAH

Department of Hygiene, Preventive Medicine & Feeding
Faculty of Veterinary Medicine, Moshtohor
Zagazig University, Benha Branch

SUMMARY

Nine groups of *Oreochromis niloticus* fish each consisting of 50 fish were used to assess the effect of pesticides on immunological response in fish. Four groups were immunized with Staphylococcus aureus antigen plus complete Freund's adjuvant. Four other groups were not immunized, but injected with the adjuvant. Fish in all groups were exposed to 1/10 of LC50 of the tested pesticides namely, lindane, dialdrin, diazinon and malathion. One group was exposed to distilled water treatment and served as controls. The results revealed that the mean total RBCs, WBCs counts, PCV, Hb, MCV, MCH, MCHC values were lower in vaccinated groups than in control groups exposed to the tested pesticides. The total protein, globulin and serum enzymes ALAT, ASAT values were lower in vaccinated groups than in controls. Phagocytosis and antibody titer were lower in vaccinated as compared to the non-vaccinated groups.

INTRODUCTION

At present, several types of chemical pesticides including insecticides, fungicides, acaricides, herbicides, algaecides and molluscocides are used in agriculture in many parts of the world for the control of plant and animal pests .

The main source of pollution of water bodies with pesticides is the melt waters, rain waters and underground waters. Pesticides may reach water bodies through the air at the time of their application to objects located nearby. Insecticides are applied to water to prevent the development of aquatic phase of blood sucking insects. Pesticides reaching water sources can be subsequently included in the trophic chains and cycles of various substances. As a result of circulation of pesticides in a water body, they sometimes accumulate in fish, silt, bottom, zooplankton, algae and aquatic plants. Organochlorine pesticides accumulate in fish mainly in the visceral fat, whereas the gills and muscles retain a

lower amount. Subsequently, with an increase in fat consumption, for example at the time of migration and hibernation, pesticides may enter the more sensitive organs and induce poisoning (Metelev et al., 1971).

Risk to man and fish-eating carnivores from pesticides can occur through air and water pollution and through consumption of fish or other aquatic organism in which pesticides are bioaccumulated (Vighi and Funari, 1995).

It has been presumed for decades that environmental pollutants especially pesticides can affect one or more of the immunological functions in fish, for it is almost common knowledge that fish frequently then become more susceptible to various diseases (Snieskzo, 1974). Given the extreme variety of pesticides used, it is almost surprising so little is known about how pesticides affect the immune systems of fish (Plumb and Areechon, 1990). Zeeman and Brindley (1981) suggested a decreased disease resistance in fish exposed to various pesticides.

MATERIALS AND METHODS

Experimental fish

Oreochromis niloticus fish weighing 150-200 gm were obtained from the aqua culture department, Ministry of Agriculture in Egypt. Fish were acclimated to laboratory conditions at least three weeks prior to experiments. Fish were kept in glass tanks measuring 120 x 60 x 45 cm, water was aerated continuously to ensure water areation. Commercial fish ration was supplied to fish twice daily and withhold three days prior to bioassay.

Experimental design

Nine groups of *O. niloticus* fish each consisting of 50 fish were used to assess the effect of pesticides on immunological response in fish. Four groups were immunized with 0.05 ml of Staphylococcus aureus antigen plus complete Freund's adjuvant, the first group was exposed to lindane treatment, the second group was exposed to dialdrin, the third group was exposed to diazinon, the forth group was exposed to malathion. The four other groups were not immunized, but injected with the adjuvant, while the ninth group was exposed to distilled water treatment and served as control group.

Haematological examination

Blood samples from vaccinated and non-vaccinated groups were taken from the caudal vein of fish. Each sample was divided into two portions, the first one was heparinized, while the second was centrifugated at 3000 rpm for 5 minutes to obtain serum. The total erythrocytic count (RBCs) and haemogolbin concentration (Hb) were determined by using electronic blood cell counter (Cell Dyne, 300, Sequoi-tuner). Total leucocytic counts (WBCs) were determined according to Blaxhall and Daisley (1973). Packed cell volume (PCV) was determined by means of

micro-haematocrit centrifuge (Schalm, 1979). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to the method of Coles (1986). Blood smears were made, stained with Giemsa for differential counts. Aspartic aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined following the techniques described by Reitman and Frankel (1975). Total protein, albumin and globulin concentration of serum were estimated following the methods described by Weichsolbaum (1946) and Drupt (1974). Protein fraction horizontal zone electrophoresis was carried out following the technique described by Schalm (1979).

Determination of median lethal concentrations (LC50) of pesticides

Four toxicity bioassay experiments were conducted separately for each pesticides following the techniques described by the United States Department of Interior Fish and Wildlife (1964). Three replicate aquaria each containing 20 fish per tank were used for each pesticide that were prepared in distilled water and three tanks were used as controls. Treatments in the tests consisting in a series of eight concentrations ranging from 0% in the control to the lowest concentration that resulted in 100% mortality after 48 hours for each pesticide. Mortality in fish was statistically analyzed according to Hubert (1980) and regression lines were drawn by plotting the probits of mortality against the logarithms of the concentrations of the pesticides tested.

Determination of antibody titer

Enzyme linked immunosorebant assay (ELISA) was performed to determine antibody titer in serum of vaccinated and non-vaccinated groups exposed to acaricides treatment. Ninety-six well vinyl assay microtiter plates (Costar, Cambridge, MA) were coated overnight at 4°C with 5µg of *Staph. aureus* antigen per well in 100 µl of 0.1 M carbonated buffer. The plates were then washed with Dulbecco's phosphate-buffered saline (D-PBS) containing 0.05% Tween-20. Each well was blocked with 100 µl of 1% normal goat sera and 0.5% normal *O. niloticus* fish sera, incubated for 1 hour at 37°C and then washed three times with D-PBS + Tween-20. A 0.1 of fish serum was added to each well, incubated at 37°C for 1h. and then washed three times. Rabbit anti-fish serum was added to each well and incubated at 37°C for 1 h. Goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase was added to each well and incubated at 37°C for 1 h. After an additional 3 washes, 100 µl phosphatase substrate was added to each well and incubated at 37°C for 1 h. The resulting enzymatic reaction in each well was determined by ELISA Reader.

Macrophage phagocyosis assay

Macrophage phagocytosis was conducted in vitro in the Staph. aureus vaccinated and

non-vaccinated groups (50 fish each) exposed to acaricide treatment following the technique described by Cossarini-Dunier (1987). Two weeks after vaccination, 25 fish from each group were intraperitoneally (i..p.) injected with 0.05 ml thioglycolate and the remaining 25 fish were served as control to ensure that fish had no previous bacterial infection and to evaluate the macrophage elicitation. Thioglycolate was added to Staph. aureus culture and incubated for 24 hours, after incubation the culture was diluted to 1:10 and 0.05 ml of the diluted culture was i.p. injected into each of the 25 thioglycolate injected fish. Twenty four hours after injection, all fish were sacrificed and imprints from the peritoneal cavity were made on glass slides and stained with Giemsa. The number of macrophages containing Staph. aureus bacteria were counted under light microscope.

RESULTS AND DISCUSSION

In common with other vertebrates, the fish immune system is used to defend against invading harmful organisms. Haematological picture is an index of the health status of fish and other aquatic organisms. The results recorded in Table (1) revealed that the haematological picture in *O. niloticus* fish exposed to sublethal concentrations of diazinon and malathion pesticides indicated that there was a decrease in mean values of PCV, Hb, MCV, MCH, MCHC and total RBCs count. Anaemia is characterized by decreases in the total erythrocytes and haematocrit values (McLeay and Haward, 1977).

The mean total leukocyte counts in both organophosphate and organochlorine pesticides exposed fish groups were generally lower than that in controls. There was a general decrease in differential lymphocyte, monocyte and neutrophil counts, while there was an increase in the mean thrombocyte counts in all groups subjected to both types of pesticides. Lymphocytes were the most predominant cells of WBCs, followed by thrombocytes, neutrophils and monocytes. These results were supported by the results of by Ellsasser et al., (1985) who mentioned that lymphocytes are the dominant cells in blood of channel catfish. Leucopenia is mainly due to reduction in the number of circulating lymphocytes. The decrease in leukocyte counts in environmental stressed fish in comparison with controls was supported by the finding of Dheer et al., (1987). Both environmental stressors and exposure to sublethal concentrations of pollutants are known to cause lymphopenia in Colisa fish (Agrawal and Srivastava, 1976) as well as in other teleosts (Hickey, 1976 and McLeay and Howard, 1977).

Initial non-specific aspect of immune responsiveness is carried out by macrophages and neutrophils. Certain immune cells are attracted to the site of foreign antigen entry, these include tissue macrophages and monocytes in the blood which phagocytize the invading antigen. Macrophages may actually be monocytes hat have entered the tissues and differentiated. Macrophages are considered to be of greater immunological importance in fish than in the

Table (1): Haematological picture in vaccinated and non-vaccinated pesticides exposed and control *O. niloticus* fish .

Mean blood values (± S. E)	Lindane		Dialdrin		Diazinon		Malathion		Control
	Vaccinated *	Non - vaccinated	Vaccinated *	Non - vaccinated	Vaccinated **	Non - vaccinated	Vaccinated **	Non - vaccinated	
RBCs (x 106)	5.31±0.02	5.65±0.04	5.43±0.07	5.67±0.05	5.01±0.03	5.47±0.06	4.89±0.01	5.33±0.04	5.76±0.03
PCV%	19.8±0.33	20.7±0.46	19.9±0.23	20.9±0.37	18.4±0.29	20.1±0.37	19.3±0.26	20.7±0.39	22.6±0.40
Hb(gm%)	11.3±0.02	11.8±0.05	10.9±0.02	11.4±0.06	10.5±0.05	12.0±0.01	10.6±0.03	11.9±0.04	13.6±0.07
MCV	30.2±1.96	33.9±1.23	29.7±1.05	32.9±1.35	28.2±1.58	32.7±1.29	27.9±1.06	30.6±1.35	36.9±1.64
MCH	18.5±0.51	20.0±0.30	18.9±0.20	19.8±0.37	18.2±0.58	19.6±0.27	17.2±0.39	19.3±0.43	21.7±0.35
MCHC%	52.4±1.28	56.7±1.59	53.6±1.28	55.2±1.73	50.3±2.07	54.7±2.36	50.6±1.27	53.4±1.93	59.2±1.67
WBCs (x103)	9.61±0.53	9.93±0.74	9.70±0.32	9.89±0.48	9.06±0.36	9.63±0.41	9.45±0.36	9.79±0.55	10.69±0.32
Therombocytes	2.24±0.68	2.43±0.96	2.17±0.57	2.38±0.73	2.14±0.69	2.42±0.82	2.02±0.76	2.32±0.94	2.85±0.59
Neutrophils	1.07±0.83	1.13±0.79	1.01±0.63	1.05±0.46	0.93±0.33	0.99±0.25	0.95±0.47	1.02±0.39	1.10±0.16
Lymphocytes	5.32±0.73	5.76±0.59	5.42±0.69	5.74±0.91	5.19±0.57	5.58±0.68	5.21±0.69	6.01±0.63	6.28±0.50
Monocytes	0.37±0.01	0.39±0.03	0.37±0.04	0.40±0.01	0.33±0.04	0.44±0.08	0.36±0.06	0.42±0.09	0.44±0.04

* : Significant at P < 0.05
** : Significant at P <0.01

Table (2): Mean serum biochemical parameters and some enzyme levels in vaccinated and non-vaccinated pesticides exposed *O. niloticus* fish

Mean blood values (± S. E)	Lindane		Dialdrin		Diazinon		Malathion		Control
	Vaccinated *	Non - vaccinated	Vaccinated *	Non - vaccinated	Vaccinated **	Non - vaccinated	Vaccinated **	Non - vaccinated	
Total protein	5.07±0.68	5.47±0.54	5.28±0.49	5.69±0.36	4.49±0.67	4.97±0.74	5.01±0.43	5.53±0.52	6.02±0.79
Albumin	2.09±0.19	2.33±0.21	2.03±0.14	2.24±0.12	2.14±0.09	2.19±0.07	2.15±0.21	2.29±0.13	2.18±0.15
Globulin	2.46±0.83	2.97±0.62	2.13±0.43	2.33±0.39	2.78±0.05	2.98±0.49	2.83±0.54	2.93±0.67	3.84±0.54
Globulin fraction %									
Alpha 1	20.68±2.62	21.68±2.25	21.49±3.52	22.84±4.28	18.75±3.51	20.47±2.05	18.67±2.32	21.63±2.96	26.36±3.86
Alpha 2	5.43±0.68	5.81±0.85	5.46±0.76	5.97±0.57	5.24±0.68	5.74±0.78	5.17±0.83	5.96±0.72	6.74±0.58
Beta	9.83±1.24	10.44±0.97	10.01±1.21	10.43±1.08	9.01±0.94	9.96±1.25	9.03±1.35	9.72±1.21	12.78±1.63
Gama	10.37±1.58	10.99±1.18	10.15±1.42	10.95±1.62	11.09±1.79	11.92±1.79	11.54±1.02	12.73±0.97	14.06±1.37
Enzymes level ALAT (µ/l)	93.34±7.27	103.14±8.62	94.83±6.53	155.08±9.62	89.87±2.69	92.32±4.62	78.97±2.54	81.75±3.38	120.16±8.82
ASAT (µ/1)	60.02±0.27	65.90±3.67	59.58±3.14	67.58±2.21	54.47±3.05	59.31±4.66	54.05±3.21	61.05±1.54	89.66±2.53

* : Significant at $P < 0.05$
** : Significant at $P < 0.01$

other vertebrates, so their activity is frequently assayed as a test for immune function (Weeks et al., 1992). Neutrophils and thrombocytes are also attracted to the site of invasion, where they release enzymes including lysozymes that destroy harmful substances. Neutrophils and thrombocytes have been reported to be phagocytic (MacArthur and Fletcher, 1985).

The results displayed in Table (2) showed that there was a general decrease in the mean total protein, albumin and globulin values in serum samples collected from *O. niloticus* fish exposed to diazinon (4.49 ± 0.67, 2.14±0.99, 2.79 ±0.65, respectively) and malathion (5.01 ±0.43, 2.15 ±0.21, 2.83 ± 0.54, respectively) as compared to that of lindane (5.07±0.68, 2.09 ±0.19, 2.46 ±0.83, respectively) and dialdrin (5.28 ± 0.49, 2.03 ±0.14, 2.13 ±0.48, respectively). The mean values of those parameters were lower than that in controls. The results of electrophoretic pattern of serum protein revealed a decrease in the gamma globulin fraction. The mean globulin fractions were lower in the vaccinated groups exposed to organophosphorus in comparison to those exposed to organochlorine pesticides The mean value of alpha 1, alpha 2 and beta fractions of serum globulin was lower in vaccinated group exposed to malathion being 18.67 ± 2.32, 5.17 ±0.83 and 9.03 ± 1.35, respectively. Gamma globulin fraction was lower in vaccinated groups exposed to diazinon and malathion being (11.09 ± 1.79 & 11.54 ± 1.02, respectively). Gammaglobulins have been primarily associated with antibody production. In general, a decrease in gammaglobulin concentration accompanies a decrease in antibody level. Alterations in gammaglobulins are usually a response of the reticuloendothelial system to antigenic stimulation or invasion of the body by foreign materials (Coles, 1986).

The mean ALAT values in serum of fish exposed to organophosphorus pesticides (diazinon and malathion) were significantly lower (81.75± 4.62 & 92.32 ± 4.62) than those recorded in organochlorines (lindane & dialdrin) (103.14 ± 4.62) & 105.08 ± 4.62.. In addition, the mean serum ASAT values were lower in diazinon and malathion (59.31± 4.66 & 61.05 ±3.54) in comparison to dialdrin & lindane (67.58± 5.21 & 68.90 ±3.67). ALAT and ASAT enzymes are good indices for the health status of liver. Parenchymatous tissue necrosis is considered the main source of ASAT and the increased activity in serum of *O. niloticus* declared the necrotic necrobiotic changes. Exposure of fish to environmental pollutants might result in stimulation or depression of the enzyme activity depending on the concentration of pollutant and the duration of exposure (Venberg and Venberg, 1974).

Concerning the phagocytic activity in fish groups vaccinated and non-vaccinated with Staph. aureus antigen, it was evident from the results shown in Table (4) that there was a sgnificant ($P < 0.01$) decrease in phagocytic activity in vaccinated groups exposed to diazinon and malathion Monocytes actively phagocytize bacteria (Suzuki,

1984) and play an important role in the defense mechanism without specific antisera (Rijkers, 1982). Moreover, McLeay and Howard, (1977) suggested that a decrease in WBCs count might indicate lower phagocytic activity, which would facilitate the survival of any invading organisms. Cossarini-Dunier, (1987) and Coassarini - Dunier *et al.*, (1987) had tested Atrazine and lindane for their direct effect on carp macrophage phagocytosis in vitro. No effect was found at concentration up to the limit of their solubility in water.

From the results recorded in Table (5), it was clear that there was a significant ($P < 0.05$) decrease in the mean antibody titer in vaccinated fish groups and exposed to diazinon (7.65 ± 1.28) and malathion (8.01 ±1.89). However, the decrease in the mean antibody titer was insignificant in lindane (11.44± 1.37) and dialdrin (10.29 ± 2.05) exposed fish groups. The specific immune response must be included in reactions to individual antigens. Humoral immunity is essentially the production of antibodies from lymphocytes. Production of lymphocytes in fish is apparently in the head of kidney, gut-associated tissues and spleen. In fish, however, only the IgM antibody class has been found. Antibodies do not destroy antigen-bearing invaders, they instead inactivate antigens and mark them for destruction by macrophages and complement (Jurd, 1990). From the results obtained in this study, it was evident that there was a decrease in antibody titer in vaccinated groups of fish exposed to the tested pesticides in comparison to non-vaccinated groups. It was obvious from this study that Organochlorine insecticides (lindane and dialdrin) may have relatively little effect on the fish immune system, a finding that was supported by the work of Bennett and Wolke (1987a & b) who mentioned that endrin may has little effect on the immune responsiveness in trout fish at least when exposures are at low levels, even for long times, but, if exposures produce a generalized stress response reflected in an elevated serum cortisol, then immunosupression may occur.

Moreover, Cleland and Sonstegard, (1987) and Cleland et al., (1988) have tested Mirex, an organochlorine insecticide in trout fish via inclusion in the diet at 50 ppm. This dietary exposure caused little effect on humoral immune expression. Similar negative results were found when humoral immune expression was measured in carp given food contaminated with lindane (up to 1000 ppm) for 2-3 months (Cossarini-Dunier, 1987). When trout fish were given a daily dose of 1mg/kg of lindane in their diet for 30 days, a decrease in chemiluminescent response was observed but there was no effect on lymphocytic proliferation and on the number of circulating B-lymphocytes (Dunier et al., 1994). From the very limited work that has been done, it appears that organophosphorus insecticides may also exhibit relatively little immunotoxicity. Dunier et al., (1991) tested the effect of trichlorofon and dichlorvos on lymphocytic proliferation and phagocytosis in vitro using carp. This caused suppression of these two functions. This may have relevance when these pesticides are used for

Table (3): Median lethal concentrations (LC50) of the tested pesticides .

Pesticide	LC_{50} (%)	Confidence limits		Criterion x^2	
		Upper	Lower	Factual	Tabular
Lindane	0.250	0.310	0.190	1.352	3.186
Dialdrin	0.052	0.062	0.043	2.796	5.284
Diazinon	0.572	0.684	0.461	3.118	4.899
Malathion	1.160	1.430	0.891	1.456	6.718

Table (4): Macrophage phagocytic activity in Staph. aureus antigen vaccinated and non-vaccinated *O. niloticus* fish exposed to pesticides

Treatment	Mean macrophage phagocytic index				
	Lindane	Dialdrin	Diazinon	Malathion	Control
Vaccinated	28.58	25.46*	19.75**	16.35**	32.39
Non-vaccinated	31.89	28.57	26.23	27.64	40.21

* : Significant at $P < 0.05$ ** : Significant at $P < 0.01$

Macrophage phagocytic index = Number of macrophages containing Staph. aureus organisms / 100 macrophages

Table (5): Antibody titer in Staph. aureus antigen vaccinated and non-vaccinated T. nilotica fish exposed to pesticides

Treatment	Mean macrophage phagocytic index				
	Lindane	Dialdrin	Diazinon	Malathion	Control
Vaccinated	11.44±1.37	10.29*±2.05	7.65*±1.28	8.41**±1.89	12.63±2.58
Non-vaccinated	0.0	0.0	0.0	0.0	0.0

* : Significant at $P < 0.05$
** : Significant at $P < 0.01$

ectoparasite treatments of fish in aquaculture. However, when dichlorvos was administered in water, no effect was detected on the humoral response to Yersinia ruckeri. Channel catfish exposed to malathion for 30 days exhibited suppression of antibody agglutination (Plumb and Areechon, 1990). These results supported the results obtained in this study suggesting that organophosphorus pesticides are relatively more potent than organochlorines on immunosupression of fish.

The other major arm of the specific immune response is the cell mediated type which depends on the function of T lymphocytes. The presence of T lymphocytes in fish was for some time controversial but now they are generally considered to be present (Anderson, 1990 and Sachell, 1991).

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