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IMMUNOSUPPRESSIVE EFFECTS OF METRIFONATE ON *OREOCHROMIS NILOTICUS*

EL-GOHARY, M .S[1]., SAFINAZ, G. MOHAMED[2], KHALIL , R.H[3]
EL- BANNA. S[4] AND SOLIMAN, M.K[3]

[1]Animal Health Research Institute , Kafr El Sheikh .
[2]Institute of Ochanography and Fisheries , Alex . Branch .
[3]Dept . of Avian Aquatic Med . Fac . of Vet . Med . Alex. Univ.
[4]Dept. Aquatic Aimal Med. Agric.Rec.Center, Animal Health Res. Inst. Alex. Laboratory

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ABSTRACT

The effect of metrifonate on the immunity of the exposed fish are evaluated through the determination of the antibody titer and mortality rate during the challenge trial. In the chronic toxic exposure to metrifonate by dose 0.17mg/liter for 8 weeks, the more clear signs were darkness of fish color with hemorrhagic patches & loosing of scale at end of the trial the color return to normal. Determination of some blood parameters revealed significant decrease of white blood cell count, as there were lymphopenia and nueutrophilia during the chronic exposure to metrifonate. Phagocytic assay revealed a significant decrease in both phagocytic activity (PA) and phagocytic index(PI).Hypoproteinami hypoglobulinaemia and hypoalbuminaemia were observed. The challenge trial indicted that, the metrifonate decrease the immunity of exposed fish.. The using of biogen"R" as a growth promoter in fish diet minimized the toxicity of metrifonate.

INTRODUCTION

Metrifonate is a broad spectrum insecticide, that is particularly effective against Dipetra. It is used mainly against insectipests in field and fruit crops, and also used to control forest insects. Another application of metrifonate includes the control of endo and ectoparasites in domestic animals and fish (World Health Organization, 1984). Also, in human being metrifonate is used in treatment of Alzheimer disease (Moriearty *et al.,* 1991) and in treatment of *Schistosoma heamatobium* primarily in Africa (Yakoub, 1990 and Nhachi *et al.,* 1991).

Moreover, to the best of our knowledge, the researches about the effect of metrifonate on immune responses of fish is more or less quite lack. This behaved us to initiate this study to qualify its immunosuppressive effects.

The aim of the present work is to study the following:

(1) The effects of chronic toxicity of metrifonate on some blood parameters and serum chemistry constitute of intoxicated *O.niloticus*; and

(2) Evaluation of the effect of metrifonate on the immune response with or without the use of some immunostimulation.

MATERIALS & METHODS

Fish :

A total No. of 50 apparently healthy *Oreochromis niloticus* were obtained from Barseek fish farm at Behera governorate. Fish were transported alive to the Laboratory of Fish Disease in the Dept. of Avian and Aquatic Animal Med., Fac. of Vet. Med., Alex. Univ. in plastic bags containing water enriched by oxygen (2/3). Average body weights of fish were 30± 5g.

*Corresponding author

Fish were kept in prepared glass aquaria (90x50 x 35 cm). These aquaria were supplied with chlorine free tap water according to (Innes, 1966), and used for holding the experimental fish throughout the period of the pres ent study. Gas support of oxygen was maintained in each aquarium using an electric air pumping compressors. Water temperature was kept at 22±1 ^{o}C.
All fish were acclimatized for 2 weeks prior to the experiment.

Metrifonate:

($C_4H_8Cl_3O_4P$) was obtained from Adwia Pharm. Co., Egypt.

Biogen:

It is a kind of feed additive (growth promoter) from allicin (aged garlic extract) not less than 0.247 micromil/g, *Bacillus subtiles* Natto (6 X 10^7 cells/g), high unit hydrolytic enzymes not less than 3690 units/g. (amylolytic, lipolytic, proteolytic and cell separating enzymes), germanium (genseng) 4188 ppm of Ge-element/g. and organic selenium (Chinaway Corporation manual, 1999).

Effect of chronic exposure of metrifonate:

Chronic toxicity of metrifonate:

A total of 90 apparently healthy *O. niloticus* were used in this experiment and divided into 3 groups, contain 3 aquaria) Ten fishes were kept in 50 liters/aquarium according to Table (1).

Blood samples:

Blood samples were collected from the caudal artery using disposable tuberculin syringe at 0 day, 2 weeks, 4 weeks and 8 weeks for determination of differential leucocytic count according to (Lucky, 1977) and phagocytic activity. Moreover, blood samples were mixed with citrated solution and used for detection of phagocytic activity (0.1 ml of 3.8% sodium citrate solution /1ml of blood). (Hawak *et al.,* 1965). Serum preparation was done for biochemical determination (Lied *et al.,* 1975).

Table 1: Experimental design for the chronic toxicity of metrifonate on *O. niloticus.*

Type of treatments	***A***			***B***			***C***		
	Met.* + commercial diet			**Met.+ diet with 0.3% Biogen**			**Control fed on commercial diet**		
No. of aquaria (replicate)	1	2	3	4	5	6	7	8	9

* *Met .:* metrifonate (0.17 mg/L) have dose of LC_{50} (El Gohary , 2004).
The diet was offered twice daily at 3% from fish body weight. The fish were kept for 8 weeks

Determination of phagocytic activity and phagocytic index :

Phagocytic activity was determined according to Kawahara *et al.* (1991) and Soliman (1997). 50mg *Candida albicans* culture 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. Smear of the blood were then stained Gimsa stain solution.

Phagocytosis was estimated by determining the proportion of macrophages which contained intracellular yeast cells in a random count of 300 macrophages and expressed as percentage of phagocytic activity (PA). The number of phagocytized organisms was counted in each phagocytic cells and called phagocytic index (Pl). Results were expressed as means ± S.E and differences were evaluated by Student's F-test.

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

$$\text{Phagocytic index (P1)} = \frac{\text{Total number of yeast cells phagocytized}}{\text{Number of phagocytic cells}}$$

Determination of serum total protein:

Serum proteins was determined according to Doumas *et al.* (1981). Using commercial kits produced by Pasteur labs.

Determination of serum albumin and globulin:

Serum albumin was determined according to Reinhold (1953) using commercial available kits of Chemroy. Moreover, the serum globulin was calculated by subtract the total serum protein from total serum albumin according to Cloes (1974).

Evaluation of potency of prepared vaccine:

The preparation of bacterin from virulent strain of *Aeromonas hydrophila* (strain No. 17) for injection was carried out according to the method of Soliman (1988). The formalin inactivated cells were mixed with an equal volume of 0.85% sterile saline. Bacterial number was adjusted to MacFarlan's No. 2 (6 X 10^8 cells / ml). Equal volumes of the inactivated bacterial cells suspension and incompleted Freund's adjuvant were mixed together. Forty *Oreochromis niloticus* were divided into 4 groups each group contain 10 fishes. The *first group* treated with metrifonate only. The dose of metrifonate as in chronic exposure (0.17 mg/L) for 28 days, the *second group* treated with metrifonate plus biogen and the *third group* fed on regular diet. Each were inoculated intraperitoneally (lP) with 0.2 ml/fish of formalin inactivated and adjuvant bacterial suspension. The control group contain 10 fishes were similarly injected (lP). With 0.2 ml/fish sterile saline. After 2 weeks, the injected fish received booster dose from bacterin (the same dose). Blood collection were carried out from the caudal vessels of the fish before injection and each week for 28 days. Collected blood was kept overnight in the refrigerator. Serum was separated aseptically gently and centrifuged at 6000 rpm from 10 minutes. Aspiration of supernatant serum using sterile pipette was carefully done and stored at – 20^{o}C until use (lucky, 1977).

The preparation of steamed antigen was done according to Collins et al. (1976) by using *Aeromonas hydroplila* strain N. 17.

Antibody titration against *A. hydrophila* bacterin:

Detection of immune response to *A. hydrophila* was evaluated by microagglutination (MA) tested according to the method described by Badran (1990).

Challenge test:

After 28 days post immunization , the immunized fish, as well as, the control one were injected i/p with 0.2 ml of virulent strain of *A. hydrophila* (the same strain used for preparation of bacterin) previously adjusted (10^4 cells/ml).

The fish were daily observed for any clinical signs and mortalities for 7 days. Bacterial isolations were done from freshly dead for specificity of death.

The potency of bacterin was determined by calculation of the relative level of protection (RLP) by the following formula:

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

According to the procedure of Newman and Majnarich (1982).

RESULTS

Differential leucocytic count:

The results of differential leucocytic count were summarized in Table (2). These results indicated that, after 2 weeks: significant reduction in lymphocytic and basophil count was observed and after 4 weeks, there were significant reduction in lymphocytes were recorded Also, after 8 weeks, the results were similar to those noticed after 4 weeks.

Table (2): Effect of metrifonate on the differential leucocytic counts

Different periods	*Treatments*	*Lymphocytes*	*Monocytes*	*Basophils*	*Eosionophils*	*Neutrophils*
0- day	Control	65.33±1.53 C	3.66±0.58 A	8.66±0.58B	11.33± 0.58A	11.66±1.53 C
2 weeks	Metrifonate only	60.00 ±1.00 A	3.00±1.00 A	7.00±1.00 B	11.33 ±0.58C	17.00±1.00 A
	Metrifonate + B.	67.00 ±1.00 B	4.33±0.58 A	7.33±0.58 B	9.66 ±0.58 B	17.66±1.53 A
	control	67.66 ±0.58 A	3.33±0.58 B	8.66±0.58 A	11.00±1.73 A	17.33±1.53 A
4 weeks	Metrifonate only	59.66±0.58 A	4.33±0.58 A	6.33±0.58 A	8.00±0.58 A	16.00±1.00 B
	Metrifonate + B.	69.00±1.00 B	5.00±1.00 B	8.00±0.58 A	8.00±1.00 A	17.33±2.52 A
	control	67.00±1.00 B	3.66±0.58 C	7.00±1.00 B	8.00±1.00 A	19.00±1.00 A
8 weeks	Metrifonate only	58.33 ±2.52 A	3.33±0.58 A	7.00 ±1.73 A	9.33 ±0.58 C	19.33±0.58 C
	Metrifonate + B.	68.33 ±0.58 B	5.33±0.58 B	8.33 ±1.53 A	6.66 ±0.58 B	15.33±0.58 B
	control	64.33 ±0.58 B	3.00±1.00 A	8.00 ±2.00 A	9.00 ±1.00 A	21.00±1.00 A

Phagocytic activity & phagocytic index:

Phagocytic activity and phagocytic index in case of fish exposed to metrifonate only showed a significant decrease as compared to control group Table (3).

Serum protein:

Albumin, globulin and total proteins decreased significantly from 2 weeks of exposure till the end of experiment compared to the control (Table 4).

Second exposure in which *O.niloticus* were exposed to metrifonate and biogen for 8 weeks

Differential leucocytic count:

After 2 weeks of exposure significant reduction in basophils and eosinophils, and significant increase in monocytes were noticed (Table 2). While after 4 weeks of exposure, there were significant increase in lymphocytes, monocytes and basophils and significant decrease in neutrophils were observed. At the end of exposure there was significant increase in lymphocytes and monocytes, and significant decrease in eosinophils and neutrophils Table (2).

Phagocytic activity and phagocytic index:

There was no significant change in phagocytic activity throughout the time of exposure, meanwhile, phagocytic index showed a significant decrease after 4 weeks and 8 weeks of exposure as compared with control group (Table 3).

Serum protein:

Albumin, globulin and total protein showed a significant decrease from 2 weeks of exposure, but, total protein and globulin values were returned to normal levels at the end of exposure (Table 4).

Table (3): Effect of metrifonte on the phagoocytic activity and phagocytic index of *O.niloticus* during chronic exposure.

Different periods	*Treatmnts*	*phagocytic. activity*	*Phagocytic. index*
0-day	Control	15.00 ± 1.00 a	10.33 ± 3.06 a
At the end of	Metrifonate only	15.66 ± 0.58 a	7.00 ± 1.00 a
2 weeks	Metrifonate + B.	18.00 ± 1.00 b	11.33 ± 0.58 b
	Control	18.33 ± 1.63 a	10.33 ± 1.53 a
After,	Metrifonate only	18.33 ± 1.53 a	7.66 ± 3.06 a
4 weeks	Metrifonate + B.	21.00 ± 2.00 a	10.66 ± 0.58 b
	Control	21.00 ± 1.00 a	8.66 ± 1.53 c
After,	Metrifonate only	16.33 ± 2.08 a	8.66 ± 1.15 a
8 weeks	Metrifonate + B.	18.33 ± 1.53 a	11.66 ± 1.15 b
	Control	18.00 ± 2.65 a	10.00 ± 1.00 b

For each week means within the same column having the different letters are significantly different at ($P<0.01$).

Table (4): Effect of metrifonate on serum proteins of *O.niloticus* during chronic exposure.

Periods	*Treatments*	*Proterin*	*Albumin(AL)*	*Globulin(GL)*	*AL/GL*	*AL/GL%*
Zero	Control	AB 7.91+0.15	ABC 3.51+0.38	AB 4.28+0.24	CB 0.81+0.13	CD 79.28+12.92
After 2 weeks	Metrifonate only	DE 6.46±0.25	BCD 3.14±0.59	CD 3.22±0.64	CD 0.99±0.33	CD 99.00±33.07
	Metrifonate + B.	C 7.23±0.31	ABCD 3.36±0.44	BC 3.87±0.34	CD 0.87±0.19	CD 87.90±18.67
	Control	AB 7.93±0.15	ABC 3.53±0.38	AB 4.39±0.24	CB 0.80±0.13	CD 80.90±12.86
After 4 weeks	Metrifonate only	F 5.64±0.23	ABC 3.47±0.24	E 2.17±0.38	A 1.63±0.35	A 163.73±35.20
	Metrifonate + B.	BC 7.60±0.44	ABC 3.42±0.34	AB 4.17±0.16	CD 0.82±0.15	CD 82.20±15.31
	Control	AB 8.00±0.30	BCD 3.33±0.35	A 4.66±0.57	D 0.72±0.15	D 72.59±14.87
After 8 weeks	Metrifonate only	G 4.71±0.23	D 2.73±0.11	E 1.98±0.26	AB 1.39±0.23	AB 139.73±23.02
	Metrifonate + B.	A 8.13±0.21	AB 3.75±0.15	AB 4.37±0.35	CD 0.86±0.10	CD 86.39±10.40
	Control	A 8.22±0.29	A 4.03±0.40	AB 4.18±0.12	C 0.96±0.12	CD 96.55±11.89

For each week, means within the same column having different letters are significant different at ($P<0.01$).

Determination of antibody titer and relative level of protection (RLP) of metrifonate treated fish :

From table 5 we can observe that the group treated with metrifonate and feed with biogen the antibody titer was high and came in the second rank after control injected with bacterin group.

Finally the metrifonate treated group only arranged in the end of list.

The relative level of protection is recorded in Table (6).

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

Positive control: Fish injected with bacterin and challenged .

Negative control: Fish injected with saline and not challenged.

The results of bacteriological and mycological examination achieved from freshly dead fish that showed clinical signs and PM lesion revealed negative isolation.

Table (5): Antibody titers in *O.niloticus* exposed to o.17mg/L of Metrifonate.

Different periods	*Treatments*	*Antibody titer (10 log)*
0-day	metrifonate only	2.33 + 0.57 A
	metrifonate + biogen	2.33 + 0.57 A
	Control + bacterien	2.33 + 0.57 A
	Control + saline	2.33 + 0.15 A
1 week	metrifonate only	2.66 + 0.57 D
	metrifonate + biogen	4.33 + 0.57 B
	Control + bacterien	5.33 + 0.57 A
	Control + saline	2.33 + 0. 5 D
2 weeks	metrifonate only	3.33 + 0.57 D
	metrifonate + biogen	5.00 + 0.00 B
	Control + bacterien	6.33 + 0.57 A
	Control + saline	2.66 + 1.15 E
3 weeks	metrifonate only	3.33 + 0.57 D
	metrifonate + biogen	5.66 + 0.57 B
	Control + bacterien	6.66 + 0.57 A
	Control + saline	2.66 + 0.57 E
4 weeks	metrifonate only	3.33 + 0.57 D
	metrifonate + biogen	6.66 + 0.57 B
	Control + bacterien	7.66 + 0.57 A
	Control + saline	3.33 + 0.15 A

Table (6): Mortality % of *O.niloticus* exposed to 0.17mg/L metrifonate and injected with *Aeromonas hydroohila* hot strain.

Items	*metrifonate only*	*metrifonate + biogen*	*Postivie control*	*Negative control*
Total No. of fish	10	10	10	10
Survival fish	3	6	0	10
Mortality (%)	70	40	100	0
Relative level of protection (RLP) (%)	30	60	0	100

DISCUSSION

The direct effect of chronic exposure of *O. niloticus* to sub lethal dose of metrifonate for 8 weeks on immune responses were examined with some trials to overcome these effect diagnostic importance and usually readily respond to identical factors such as physical, chemical and biological stressors (Hickey, 1976 and Soliman, 1996).

Differential leucocytic count in chronic exposure in the present investigation revealed lymphopenia, reduction on basophil, monocytosis, neutropenia followed by neutrophilia .The same results were reported in different fish by Eissa (1979); Siwicki *et al.* (1990); Patil (1993) and El-Boushy (1994).

Robert (1989) recorded lymohopenia and neutrophilia in actually stressed fish due to the toxicity by some pesticides. These finding may lead to decrease in resistance of fish and increase in susceptibility to infection. Meanwhile, Gill et al. (1991) noticed lymphocytosis, neutropenia and monocytosis in *Barbus conchnius* fish due to 4 weeks exposure to sublethal levels of endosulfan (organophosphrus compound) Sampath et al. (1993) recorded an increase in the total leucocytic count in *Oreochromis mosambicus* which exposed to Ekalux ® (organophosphrus Compound). The discrepancy in these results may by due to difference in fish species, dose and duration of exposure.The decrease in the above mentioned blood parameters specially leucopoenia, can be attributed to generalized stress response that cause increase pituitary–internal activity (Donaldson, 1981).

The significant reduction of phagocytic activity and phagocytic index in the present study after chronic administration of metrifonate come in agreement with the results described by Siwicki *et al.* (1990) who found a dose-dependent suppression of antibody response in fish treated with pesticides. These reduction on Phagocytic activity and Phagocytic index may be attributed to the action of organophosphorus compound on hematopoietic organs as will as prevention of uptake of macrophage arming factor which reflect badly on the phagocytic activity.

The results may suggest a stress effects of metrifonate on fish which leads to increase level of serum cortisol . The increase of cortisol level may lead in turn to suppression of the phagocytosis processes.

This suppression may be mediated directly via the corticosteroid receptors on macrophages or indirectly through the enhanced production of certain factors by the macrophages themselves which suppress the secretion of other macrophages products (Stephan & Susanne 1986and Jain, 1993).

Pickering (1981) stated that the suppressive effect of corticoids is due to enhanced production of certain factors by the macrophages themselves (e.g. α-2 macroglobulin) which suppress other macrophage products. Also, the release of macrophage arming factors (MAF) by antigen stimulated T-lymphocyte is unaffected by corticoids but there uptake by macrophage and their subsequent increased cytotoxic activity is inhibited (Dimitriu, 1976).

Concerning the little increase in both PA & Pl after one month exposure to metrifonate compared the early results of exposure can by explained by that exposed fish may developed some physiological adaptation to the toxicant . The same conclusion was recorded by Anderson (1990) and Safinaz (2001). Significant decrease in PA and Pl due to exposure to organophosphorus compound and chemical pollution bayluscide in catfish and *O. niloticus,* phenol in grass carp and *O. niloticus,* were also reported by Faisal et al . (1988) Khalil (1998), Soliman (1997) and Safinaz (2001) respectively.

The total serum proteins are useful in diagnosis of fish diseases (Mulcahy, 1977). In the present work, significant hypoproteinemia followed the chronic exposure were recorded.

These results are confirmed by Khalifa (1990), Gill et al. (1991) and Mallreoldy and Philip (1991).

The chronically exposed fish to metrifonate for 8 weeks and injected with prepared bacterin adjuvant *A. hydrophila* showed marked decrease in the antibody titer. This may by due to direct cytotoxic effect of Metrifonate on lymphoid tissue leading to immunosuppression in exposed fish. Faisal et al. (1988) found that the antibody titers in catfish exposed to different the environmental conditions or to field dose of metrifonate were significantly lower than the control fish.

Metrifonate also considered as a chemical stress or has drastic effect on immune system of *O. niloticus*. This stress may activate the hypothalamus–hypophysis-adrenal endocrine system and stimulate corticosteroids and catechohamines in fish blood which negatively affect the process of lymphopoiesis and interfere with the synthesis of ascorbic acid, thus lowering the resistance of fish and induce immunosppression (Pickering, 1981).

In the present findings, decrease of relative level of protection (RLP) induced by injected bacterin may by due to direct cytotoxic effect of metrifonate on lymphoid tissue leading to immunosppression in exposed fish (Faisal et al., 1988 and Safinaz 2001).

Regarding to the effect of biogen which used as a feed additives, in group 2 to overcome the drastic effect of metrifonate, a promising positive results were obtained in differential leucocytic count, phagocytic activity and phagocytic index serum enzymes and protein and increasing the immune status of fish leading to decrease the mortality rate among the treated fish. This finding were clear in case of biogen. The possible explanation for these encouraging results that both allicin and ginseng (active ingredient of biogen) improved the physiological function of fish and immune response which in turn increased the ability of exposed fish to resist the stress effect of metrifonate.

This explanation is discussed by Kim et al. (1993) who reported that, water fraction of panax ginseng cell may reduce damage caused by gamma–rays specially damage of DNA molecules, and a role in the repair or regeneration process of cell damage .

They also added that, ginseng extract acts directly on body cell promoting DNA and protein synthesis and enhance the natural body resistance. While Lun et al. (1994) reported that allicin can activate and coordinate the function of various glands of the body thus enable them to work normally with high efficient.

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