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Some Studies on the Effect of Copper (I) Nicotinate Complex on the Immune Response and Some Biochemical Parameters of *Mugil cephalus* Fish Vaccinated With *Yersinia Ruckeri* Bacterin

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ABSTRACT

The present study was designed to investigate the influence of dietary supplementation of Copper (I) nicotinate complex at different concentrations (30 and 60 mg/kg feed) for 4 weeks on the immune response and some biochemical parameters of ***Mugil cephalus*** fish vaccinated with *Yersinia ruckeri* bacterin. The relative level of protection against challenge with virulent strain of ***Yersinia ruckeri*** was also determined. The present findings indicated that, the using highest concentration of Copper (I) nicotinate (60mg/kg feed) was preferable for stimulation of hemopiosis which reflected on the significant increase of some tested hematological parameters. Both concentrations Copper (1) nicotinate complex had no adverse effect on liver cells which reflected on the unchanged activity of liver enzymes and increase the serum total protein, albumin and globulin. The increased lymphocytes percentage and serum globulin along the course of experiment was elevated phagocytic index and activity gave a strong evidence of the immunostimulant effect of copper (1) nicotinate complex in the levels of cell mediated and humeral immunity. This immunostimulant effects were dose dependant and

confirmed by the increase in both antibody titer and relative level of protection against the injected virulent strain of ***Yersinia ruckeri***.

Keywords: Copper (I) nicotinate complex; Immune response; Biochemical parameters; ***Mugil cephalus*** fish.

Abbreviations: ALT (Alanine aminotransferase); AST (Aspartate aminotransferase); ATP (Adenosine triphosphate); GTP (Guanosine triphosphate); Hb (Haemoglobin); NAD (Nicotinamide adenine dinucleotide); NADP (Nicotinamide adenine dinucleotide phosphate); NADPH (reduced form of NADP); PCV (Packed cell volume); RLP (Relative level of protection); SEM (Standard error of mean); TEC (Total erythrocytic count); TLC (Total leucocytic count); UTP (Uridin triphosphate).

INTRODUCTION

Immunostimulants represents an emerging class of chemicals that are designed to amplify immune responses against infectious diseases, pathogens and tumor cells. The field of essential metalloelement complexes has attracted many authors in the last decade. Biochemical mechanisms have been postulated concerning the mode of action of such vitamine metal complexes on enzyme reactivity (***Franklin and Richardson, 1980***). The same authors reported the medical benefits of the different copper complexes. ***Al-Mulla Hummadi et al. (2005 and 2006)*** reported that, an organic complex of copper chloride, ascorbic acid and nicotinamide has an immunomodulating effect similar to BCG and a direct antileishmanial effect resulting, partially or entirely from the inhibition of enzymes that are necessary for the parasites carbohydrate metabolism. Another copper complexes had been extensively studied as a potent antitumor, anti-inflammatory and antidiabetic (***Greenaway et al.,***

1999; Li et al., 1999; Tsuji and Sakurai, 1998). Copper (I) nicotinate complex reduced the adverse effects of 5-fluorouracil on patients with hepatocellular carcinoma and enhanced defense mechanisms against oxidative stress (***El-Saadani, 2004***) and produced antiulcerogenic activity (***El-Saadani et al., 1993***). Literatures concerning the effect of copper (I) nicotinate complexes in fish are so deficient. Our previous work on Big head carp fish (***El-Ashmawy, et al., 2007***) demonstrated that, fish supplemented with copper (I) nicotinate complex (either 30 or 60 mg/kg feed) showed a significant increase in the percentages of most hematological and biochemical parameters. The same authors demonstrated that both concentrations of copper (1) nicotinate complex had no adverse effect on liver functions, induced immunostimulant effect in tested Big head carp fish and increased the relative level of protection against the injected virulent strain of ***Yersinia ruckeri***. Therefore, the present study was planned to investigate the effect of dietary supplementation of different concentration of copper (I) nicotinate complex on the immune response and some biochemical parameters of another fish species (***Mugil cephalus***) vaccinated against the same bacterial strain used in the last work (***Yersinia ruckeri*** bacteria).

MATERIALS AND METHODS

2.1. copper (I) nicotinate complex

Copper nicotinic acid complex (Fig. 1) is a bright red pure crystalline compound [$CuCl(HNA)_2$]. The complex has been synthesized by the method of ***Gohar and Dratovisky (1975)*** and prepared by Research, Development and Quality Control Division Pharco Pharmaceuticals, Alexandria, Egypt.

2.2. Vaccines and Virulent strains

Yersinia ruckeri bacterin was prepared and evaluated according to ***Badran (1990).*** Stained ***Yersinia ruckeri*** bacterin was prepared according to ***Collins et al. (1976)*** to increase the visibility of the serological reaction.

2.3. Fish, Aquaria and Experimental Design

A total number of two hundred and ten apparently healthy ***Mugil cephalus*** fish with an average body weight of 60 ± 5 gm were obtained from private fish farm at Egypt and used in this study. Fish were acclimited for 2 weeks and fed on commercial fish food containing 25% crude protein. The diet was daily provided at a percentage of 3% of body weight as described by ***Eurell et al. (1979).*** No drugs were given to the fish along the course of the experiment except those under investigation. Glass aquaria measuring 90 × 60 × 50 cm (10 fish each) were used for holding the fish groups during the experiment. The aquaria were supplied with chlorine free tap water and supplemented with continuous aeration. Throughout the experiment 3 separate subgroups of 10 fish per each treated main group were used . Each subgroup of fish only represents one observation. The present study was divided into two main experiments each of 4 weeks duration . In the first experiment, 90 fish were used and divided into 3 groups (30 fish each). The first group served as a control without drug treatment. The second and the third groups were supplied daily with 30 and 60 mg copper (I) nicotinate complex /kg feed, respectively along the experimental period (4 weeks). However, in the second experiment, the remaining 120 fish were divided into 4 groups (30 fish) each. Fish of the first

group were injected intraperitonially (I/P) with 0.2 ml/fish sterile saline and given feed without medicament and served as non medicated control. The fish in the second group were injected I/P with 0.2 ml/fish of formalin inactivated bacterin from ***Yersinia ruckeri*** and given feed without medicaments and served as vaccinated control. The fish in the third and fourth groups were similarly inoculated with 0.2 ml/fish of formalin inactivated ***Yersinia ruckeri*** bacterin and received daily 30 and 60 mg copper (1) nicotinate complex/kg diet, respectively along the experimental period (4 weeks). The booster dose of bacterin was given 2 weeks after the first injection. Afterwards, the same fish were challenged by inoculation of 0.2 ml of virulent strain of ***Yersinia ruckeri*** of the same strain used for bacterin preparation in the second experiment and fish mortalities were recorded during one week duration period.

2.4. Sampling and the analytical methods

In the first experiment, fish were weighed weekly, and at the end of experiment, the body of fish were cleaned and blotted dry with adsorbent paper. Blood samples were collected from the caudal vessels using disposable tuberculin syringe (***Hawk et al., 1965***) for estimation of total erythrocytic count (TEC), total leucocytic count (TLC) , packed cell volume (PCV) ***Stoskopf (1993).*** Haemoglobin percentage (Hb %) was assessed according to ***Drubkin (1947),*** and differential leucocytic count was determined according to ***Schalm (1986).*** Both phagocytic activity and index were also determined according to ***Hawk et al. (1965).*** Similarly, blood was collected without anticoagulant for serum separation as described by ***Leid et***

al. (1975). The obtained sera were used for spectrophotometric determination of the activities of AST and ALT as directed by Reitman and ***Frankel (1957).*** Serum total protein, albumin, globulin and glucose values were determined spectrophotometrically as implied by the methods of ***Doumas et al. (1981), Reinhold (1953), Coles (1974) and Trinder (1969),*** respectively**.**

After end of the second experiment, blood samples were collected and the obtained sera were stored at –20ºC until used for detection of immune response against ***Yersinia ruckeri*** according to the method described by ***Badran (1990).*** Briefly, in a standard microtiter plate (U-shaped wells), serial two fold dilutions of serum were made in sterile saline solution using a 0.025 ml pipette dropper and 0.025 ml micro diluter. *Yersinia ruckeri* stained antigen (0.025 ml) was added to the diluted serum. The suspensions were kept in humidified clumber and incubated overnight in the refrigerator dilution still giving a clear agglutination. The negative controls consisted of: a) one drop of sterile physiological saline and one drop of tested serum. b) one drops of sterile physiological saline and one drop of stained antigen. The positive controls were carried out using collected positive antisera. A positive serological reaction was indicated by bacterial agglutination. Agglutination titers were expressed as logs of the highest serum . Specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation for one week after challenge. The potency of bacterin were evaluated by calculating the relative level of protection (RLP) by subtracting the value resulted from the division of percent of mortality of vaccinated fish group on percent of mortality of non

vaccinated control {RLP = 1 – (percent of mortality of vaccinated fish group/percent of mortality of non vaccinated control group); ***Newman and Majnarich(1982)***.

2.5. Statistical analysis

The obtained data were compared between groups within different periods by using student– *t*– test. All data are presented as mean ± standard error of mean (SEM) by using repeated ANOVA. All tests were performed using computer package of the statistical analysis system (***SAS, 1987***).

RESULTS

The present findings revealed that, highest concentration of copper (I) nicotinate (60mg/kg feed) only induced a significant increase in the body weight of ***Mugil cephalus*** throughout the experimental period when compared with the control. However, the average body weight was increased significantly ($p < 0.05$) within the time and reaching its maximum at 4 weeks in all groups (Table 1). The obtained results revealed also that, Total Leucocytic Count (TLC) was significantly increased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control throughout the experimental period (Table 2). This increase was more pronounced in fish supplemented with highest concentration of copper nicotinate (60 mg/kg diet) than the lowest concentration (30 mg/kg diet) throughout the experimental period (Table 2). However, the Total Erythrocytic Count (TEC) was not significantly changed ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control (Table 2).

Table 1: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on body weight (gm) in Mugil cephalus fish.*

Treatment	Duration of Treatment			
	1st week	2nd week	3rd week	4th week
Control	**55.2 ± 0.80Bd**	**56.8 ± 0.58Bc**	**58.8 ± 0.37Bb**	**61.6 ± 0.24Ba**
30 mg/kg diet	**54.4 ± 1.36Bd**	**57.4 ± 1.03Bc**	**59.2 ± 0.86Bb**	**62.4 ± 0.24Ba**
60 mg/kg diet	**58.6 ± 1.25Ad**	**60.2 ± 0.97Ac**	**62.0 ± 0.45Ab**	**64.6 ± 0.68Aa**

Capital letter: indicated that means within the same column carrying different letter are significantly differed at (P< 0.05). Small letter: indicated that means within the same row carrying different letter are significantly differed at (P< 0.05). Values are expressed as mean ± SEM, n = 30 fish (1st experiment).

In addition, TLC and TEC within the same group were not changed significantly ($p < 0.05$) throughout the experimental period (Table 2).

The data summarized in Table 2 indicated that, the percentages of Hb and PCV were significantly increased ($p < 0.05$) only in fish supplemented with highest copper nicotinate concentration (60 mg/kg diet) when compared with the control and lowest copper nicotinate concentration (30 mg/kg diet) which remained comparable throughout the experimental period. There were no significant changes in Hb and PCV values within the same group throughout the experimental period (Table 2).

The percentages of lymphocytes and esinophils were significantly increased ($P \leq 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. However, the percentage of monocytes was not significantly

Table 2: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on TLC ($10^3/mm^3$), TEC ($10^6/mm^3$), Hb (g/dl) and PCV(%) in Mugil cephalus fish.*

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
TLC	1st week	20.0 ± 0.58Cc	22.0 ± 0.33Ba	23.9 ± 0.58Aa
	2nd week	20.1 ± 0.58Cc	22.0 ± 0.58Ba	24.0 ± 0.33Aa
	3rd week	19.9 ± 0.58Cc	21.7 ± 0.33Ba	23.8 ± 0.33Aa
	4th week	20.0 ± 0.88Cc	21.9 ± 0.58Ba	24.0 ± 0.88Aa
TEC	1st week	2.0 ± 0.16Aa	1.9 ± 0.12Aa	1.9 ± 0.18Aa
	2nd week	2.1 ± 0.02Aa	2.1 ± 0.09Aa	2.1 ± 0.03Aa
	3rd week	2.0 ± 0.12Aa	2.1 ± 0.15Aa	2.2 ± 0.03Aa
	4th week	2.1 ± 0.03Aa	1.9 ± 0.03Aa	2.0 ± 0.09Aa
Hb	1st week	8.7 ± 0.88Ba	8.5 ± 0.67Ba	10.3 ± 0.33Aa
	2nd week	8.9 ± 0.33Ba	8.6 ± 0.58Ba	10.1 ± 0.33Aa
	3rd week	8.9 ± 0.33Ba	8.7 ± 0.88Ba	9.9 ± 0.88Aa
	4th week	8.5 ± 0.58Ba	8.3 ± 0.58Ba	10.0 ± 0.33Aa
PCV	1st week	31.7 ± 1.19Ba	31.5 ± 1.73Ba	33.9 ± 1.15Aa
	2nd week	32.3 ± 0.88Ba	32.0 ± 1.73Ba	34.1 ± 1.45Aa
	3rd week	32.7 ± 0.33Ba	31.9 ± 1.53Ba	34.3 ± 1.20Aa
	4th week	31.7 ± 0.67Ba	30.3 ± 1.20Ba	33.9 ± 0.88Aa

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changed in all group ($p < 0.05$; Table 3). In addition, the percentage of basophils was significantly increased ($p < 0.05$) only in fish supplemented with highest copper nicotinate concentration (60 mg/kg feed) when compared with the control and lowest copper nicotinate concentration (30 mg/kg diet) which remained comparable throughout the experimental period (Table 3). The percentage of neutrophils was significantly decreased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. This decrease was more observed in fish supplemented with highest concentration of copper nicotinate (60 mg/kg) than the lowest used concentration (30 mg/kg) throughout the experimental period (Table 3). There was no significant changes in all types of leucocytes within the same group throughout the experimental period (Table 3).

The values of phagocytic activity and index were significantly increased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. This increment was more marked in fish received highest concentration of copper nicotinate (60 mg) than the lowest used concentration (30 mg) throughout the experimental period (Table 4). However, the phagocytic index and activity values within the same group were significantly increased only at the fourth week of the experiment (Table 4).

In the present study, serum total proteins values were increased significantly ($p < 0.05$) in fish received copper (I) nicotinate when compared with the control group (Table 5). However, this increase was more pronounced in fish received highest concentration of copper nicotinate than the lowest used concentration (Table 5).

Table 3: *Effect of daily supplementation of copper (I) nicotinate for 4 weeks on the differential leucocytic count (%) in Mugil cephalus fish.*

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
Lymphocyte	1st week	48.3 ± 0.33Ba	52.0 ± 0.58Aa	53.2 ± 0.33Aa
	2nd week	48.3 ± 0.58Ba	52.9 ± 0.58Aa	53.0 ± 0.58Aa
	3rd week	49.0 ± 0.58Ba	52.1 ± 0.58Aa	52.3 ± 0.33Aa
	4th week	49.1 ± 0.03Ba	52.2 ± 0.88Aa	52.3 ± 0.33Aa
Monocyte	1st week	1.3 ± 0.33Aa	1.3 ± 0.33Aa	1.2 ± 0.33Aa
	2nd week	1.4 ± 0.33Aa	1.3 ± 0.33Aa	1.2 ± 0.88Aa
	3rd week	1.2 ± 0.58Aa	1.1 ± 0.33Aa	1.1 ± 0.33Aa
	4th week	1.2 ± 0.58Aa	1.3 ± 0.33Aa	1.3 ± 0.58Aa
Basophil	1st week	8.1 ± 0.58Ba	8.3 ± 0.88Ba	10.3 ± 0.33Aa
	2nd week	8.2 ± 0.58Ba	8.3 ± 0.88Ba	10.7 ± 0.33Aa
	3rd week	8.3 ± 0.33Ba	8.3 ± 0.33Ba	10.3 ± 0.88Aa
	4th week	8.0 ± 0.88Ba	7.9 ± 0.58Ba	10.3 ± 0.33Aa
Eosinophil	1st week	6.3 ± 0.33Ba	8.3 ± 0.33Aa	8.1 ± 0.33Aa
	2nd week	6.5 ± 0.33Ba	8.1 ± 0.33Aa	8.0 ± 0.33Aa
	3rd week	6.0 ± 0.33Ba	7.7 ± 0.33Aa	8.0 ± 0.58Aa
	4th week	5.9 ± 0.58Ba	7.8 ± 0.88Aa	8.0 ± 0.58Aa
Neutrophil	1st week	36.0 ± 0.00Aa	30.1 ± 0.58Ba	27.2 ± 1.15Ca
	2nd week	35.6 ± 2.33Aa	29.4 ± 1.15Ba	27.1 ± 0.33Ca
	3rd week	35.5 ± 0.58Aa	30.8 ± 0.33Ba	28.3 ± 0.58Ca
	4th week	35.8 ± 1.86Aa	30.8 ± 0.88Ba	28.1 ± 0.67Ca

Capital letter: indicated that means within the same row carrying different letter are significantly differed at (P< 0.05). Small letter: indicated that means within the same column carrying different letter are significantly differed at (P< 0.05). Values are expressed as mean ± SEM, n = 30 fish (1st experiment).

Table 4: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on phagocytic activity (%) and index in Mugil cephalus fish.*

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
Phagocytic activity	1st week	20.0 ± 0.58Cb	22.5 ± 0.33Bb	24.5 ± 0.88Ab
	2nd week	19.8 ± 0.58Cb	23.0 ± 0.58Bb	25.3 ± 0.33Ab
	3rd week	19.9 ± 0.58Cb	23.0 ± 0.58Bb	25.1 ± 0.33Ab
	4th week	22.0 ± 0.58Ca	25.3 ± 0.88Ba	27.3 ± 0.67Aa
Phagocytic index	1st week	2.0 ± 0.06Cb	2.9 ± 0.06Bb	4.2 ± 0.03Ab
	2nd week	1.8 ± 0.03Cb	2.7 ± 0.09Bb	4.0 ± 0.12Ab
	3rd week	1.9 ± 0.09Cb	2.8 ± 0.06Bb	3.9 ± 0.09Ab
	4th week	1.9 ± 0.21Ca	3.0 ± 0.06Ba	4.4 ± 0.12Aa

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The value of serum albumin of all fish supplemented with copper (I) nicotinate was significantly increased ($p \leq 0.05$) when compared with the control (Table 5). The value of serum globulin of all fish supplemented with copper (I) nicotinate was significantly increased ($p < 0.05$) when compared with the control. This increment was more recorded in fish fed highest concentration of copper nicotinate (60 mg/kg) than the lowest used concentration (30 mg/kg) throughout the experimental period (Table 5). The data presented

in Table 6 indicated that the activities of ALT and AST were not changed in all treated group when compared with the control group throughout the whole experimental period. In addition, total protein, Albumin, globulin, ALT and AST activities within the same group were not changed significantly ($p < 0.05$) throughout the experimental period (Table 5, 6). The present findings also indicated that, the values of glucose were significantly ($p < 0.05$) decreased in all fish supplemented with copper (I) nicotinate when compared with the control (Table 6). This decrease was more recorded in the highest concentration of copper nicotinate (60 mg) than the lowest used concentration (30 mg) throughout the experimental period (El-Ashmawy, et al., 2007; Table 6).

The values of antibody titer in fish vaccinated against *Yersinia ruckeri* bacterin was significantly increased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. This increment was more marked in the highest concentration of copper nicotinate (60 mg/kg) than the lowest used concentration (30 mg/kg) throughout the experimental period (4 weeks, Table 7). In addition, the values of antibody titer were time dependant reaching its maximum at fourth week of the experiment in all tested fish (Table 7). The percentage of RLP of fish challenged by *Yersinia ruckeri* virulent strain was significantly increased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. This increment was higher in fish fed highest concentration of copper nicotinate (60 mg/kg diet; 60%) than the lowest used concentration (30mg/kg diet; 40%) throughout the experimental period (one week; Table 8).

Table 5: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on total protein (g/dl), albumin (g/dl) and globulin (g/dl) in Mugil cephalus fish.*

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
Total proteins	1st week	3.0 ± 0.06Ca	4.6 ± 0.03Ba	6.0 ± 0.15Aa
	2nd week	3.0 ± 0.06Ca	4.8 ± 0.03Ba	5.8 ± 0.09Aa
	3rd week	3.2 ± 0.12Ca	4.8 ± 0.03Ba	5.8 ± 0.09Aa
	4th week	2.9 ± 0.06Ca	4.7 ± 0.03Ba	5.9 ± 0.09Aa
Albumin	1st week	2.1 ± 0.03Ca	3.1 ± 0.06Ba	3.9 ± 0.25Aa
	2nd week	2.2 ± 0.03Ca	3.2 ± 0.16Ba	3.9 ± 0.22Aa
	3rd week	2.3 ± 0.63Ca	3.2 ± 0.06Ba	4.0 ± 0.10Aa
	4th week	2.2 ± 0.15Ca	3.0 ± 0.19Ba	4.1 ± 0.06Aa
Globulin	1st week	0.9 ± 0.03Ca	1.5 ± 0.03Ba	2.1 ± 0.18Aa
	2nd week	0.8 ± 0.03Ca	1.6 ± 0.17Ba	1.9 ± 0.15Aa
	3rd week	0.9 ± 0.54Ca	1.6 ± 0.09Ba	1.8 ± 0.15Aa
	4th week	0.7 ± 0.10Ca	1.7 ± 0.20Ba	1.8 ± 0.03Aa

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Table 6: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on ALT (U/l), AST (U/l) and glucose (mg/dl) in Mugil cephalus fish.*

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
ALT	1st week	68.3 ± 0.33Aa	66.7 ± 1.45Aa	67.7 ± 0.88Aa
	2nd week	68.1 ± 1.45Aa	67.7 ± 0.33Aa	68.0 ± 0.58Aa
	3rd week	67.9 ± 0.88Aa	68.1 ± 0.33Aa	68.0 ± 0.58Aa
	4th week	67.8 ± 2.65Aa	67.3 ± 1.20Aa	67.0 ± 1.15Aa
AST	1st week	82.7 ± 0.33Aa	83.3 ± 0.33Aa	82.0 ± 0.58Aa
	2nd week	81.5 ± 1.53Aa	82.0 ± 0.58Aa	81.7 ± 1.45Aa
	3rd week	81.1 ± 1.20Aa	81.3 ± 0.33Aa	80.0 ± 0.58Aa
	4th week	82.3 ± 0.33Aa	81.5 ± 0.88Aa	82.0 ± 0.58Aa
Glucose	1st week	81.1 ± 0.88Aa	80.7 ± 1.86Ba	81.3 ± 0.88Ca
	2nd week	80.4 ± 1.20Aa	81.3 ± 0.33Ba	80.7 ± 0.33Ca
	3rd week	81.3 ± 1.76Aa	80.7 ± 0.33Ba	81.3 ± 0.88Ca
	4th week	81.3 ± 0.33Aa	80.5 ± 0.33Ba	81.0 ± 0.58Ca

Capital letter: indicated that means within the same row carrying different letter are significantly differed at (P< 0.05). Small letter: indicated that means within the same column carrying different letter are significantly differed at (P< 0.05). Values are expressed as mean ± SEM, n = 30 fish (1st experiment).

Table 7: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on antibody titer in Mugil cephalus fish vaccinated with Yersinia ruckeri bacterin. Values are expressed as mean ± SEM, n = 30 fish (2nd experiment).*

Period	Control (not vaccinated)	Control (vaccinated)	30 mg/kg diet	60 mg/kg diet
1st week	0.00	4 ± 0.05Cb	5 ± 0.03Bb	6 ± 0.05Ab
2nd week	0.00	4 ± 0.03Cb	5 ± 0.05Bb	6 ± 0.03Ab
3rd week	0.00	5 ± 0.03Ca	6 ± 0.05Ba	7 ± 0.03Aa
4th week	0.00	5 ± 0.03Ca	6 ± 0.03Ba	7 ± 0.05Aa

Capital letter: indicated that means within the same row carrying different letter are significantly differed at (P<0.05). Small letter: indicated that means within the same column carrying different letter are significantly differed at (P<0.05).

Table 8: *Effect of daily supplementation of copper (I) nicotinate complex for 4 weeks on protection of Mugil cephalus fish against virulent strain of Yersinia ruckeri after vaccination with Yersinia ruckeri bacterin. n = 30 fish (2nd experiment).*

Groups	Total number of tested fish	Number of dead fish	Mortality %	RLP (%)
Control (not vaccinated)	30	30	100	1- 100/100 = 0
Control (vaccinated)	30	21	70	1- 70/100 = 30
30mg/kg diet (vaccinated)	30	18	60	1- 60/100 = 40
60mg/kg diet (vaccinated)	30	12	40	1- 40/100 = 60

DISCUSSION

The medical benefits particularly the immunostimulant effect of the different copper complexes were reported (***Franklin and Richardson, 1980***). Our previous work (***El-Ashmawy, et al., 2007***) demonstrated the beneficial effect of dietary copper (I) nicotinate complex (either 30 or 60 mg/kg diet) on overall performance of Big head carp fish. Therefore we decided to investigate such effect in ***Mugil cephalus*** fish which considered as a highly consumed fish species in Egypt. The observed increase of TLC in fish kept on copper nicotinate complex may be attributed to the activation of lymphoid tissue. Copper nicotinate perhaps stimulate the haemopiotic tissues and subsequently led to production of extensive number of effective functional cells as a defense mechanism (***El-Ashmawy, et al., 2007***). This stimulation was dose dependant as the highest concentration of copper nicotinate was effective in the stimulation of lymphoid tissue than the lowest one. In addition, the increment of Hb and PCV in fish fed copper nicotinate indicated the direct effect of copper nicotinate on hemopiotic tissue. However, the increment of Hb percentage perhaps attributed either to increasing the synthesis of enzyme needed for biosynthesis of haem or increasing the size of red blood cells. ***Tanner et al. (1988)*** reported that, copper was involved in hemoglobin formation by simulation of ferro-oxidase I enzyme which catalyzes the oxidation of ferrous iron and plays a role in the transfer of iron from storage to sites of hemoglobin synthesis. This effect was done after the administration of the highest dose meaning that the lowest concentration failed to stimulate the haemopiotic tissue. Similar results were obtained in Big head carp fish when fed the same

concentrations of copper (I) nicotinate complex used in the present study (***El-Ashmawy, et al., 2007***).

Concerning the present hematological findings, the same findings were observed in our previous study in Big head carp fish using the same concentrations of copper nicotinate complex (***El-Ashmawy, et al., 2007***) except only for the value of monocytes which was increased in big head carp fish. Moreover, the increment of lymphocytes, basophils and esionphils in fish kept on copper nicotinate complex indicated the direct stimulation of copper nicotinate to lymphoid tissue (***El-Ashmawy, et al., 2007***). However, the efficiency of either highest or lowest concentrations of copper nicotinate for lymphocyte stimulation was the same while, that for the other cells was differed as the highest concentration only was able to induce such stimulation. Moreover, the highest concentration only able to stimulate the basophils after prolonged period of administration (2 weeks).

All leucocytes were calculated as a percentage of the whole leucocytic count which constitute 100 %. The significant decrease of the percentage of neutrophils in copper nicotinate received groups was simply attributed to the significant increase of other leucocytes. As the highest concentration of administered copper nicotinate induced significant elevation of other leucocytes in the expense of neutrophils than the lowest copper nicotinate concentration, the decrease in the percentage of neutrophils was more pronounced than that of the administered copper nicotinate lowest concentration.

Obviously, the increment of phagocytic index and activity in highest concentration copper nicotinate received group than the

lowest concentration received one introduced another evidence of the superiority of that concentration for stimulation of phagocytic cells than the lowest concentration. Similar results concerning the phagocytic index and activity were obtained in Big head carp fish when fed the same concentrations of copper (I) nicotinate complex used in the present study, the increase in phagocytic assay mainly due to increase in blood parameter espicially in lymphocytes and monocytes (***El-Ashmawy, et al., 2007***).

Hematological parameters of fish blood, are useful tools that aids in diagnosis of disease. It can also be used to study immnuopotentiators. Such tests are general but not conclusive and must be correlated with biochemical tests of the subject. The significant increase of total protein, albumin and globulin in fish administered copper nicotinate complex perhaps attributed to the role of copper in protein biosynthesis as it is vitally concerned in the growth process (***Underwood, 1977***). In addition, copper is involved in the formation of disulphide linkage of collagen and elastin proteins (***Tanner et al., 1988***). Moreover, the significant increase of serum globulin indicated the immunostimulant effect of copper nicotinate particularly for the highest concentration used. The role of copper in humeral and cell mediated immunity was reported by ***Radostits et al. (2000).*** The same author and others reported that, copper deficiency caused alteration in humeral response (***Prohaska and Failla, 1993***). In addition, the results concerning the effect of copper nicotinate on the activities of ALT and AST indicated that, both used concentrations of copper nicotinate were safe to the liver that it preserve liver enzymes at normal values (Table 6). In addition, the

significant increase ($p < 0.05$) of total protein, albumin and globulin indicated that, the administered copper nicotinate did not disturb the liver functions. However, the highest concentration was more preferable in performing the liver function than the lowest used concentration. Similar results of liver enzymes and protein patterns were obtained in Big head carp fish fed the same used copper nicotinate complex doses (***El-Ashmawy, et al., 2007***). In addition, the significant decrease of serum glucose in fish administered copper nicotinate complex indicated the hypoglycemic effect of copper nicotinate particularly for the highest concentration used. This decrease perhaps attributed either to decrease the absorption of glucose from the intestine or the stimulation of hypoglycemic hormone like insulin. This result is in accordance with those obtained by ***Tsuji and Sakurai (1998).***

The results concerning the effect of the administration of copper nicotinate complex on the values of antibody titer in fish vaccinated against ***Yersinia ruckeri*** bacterin are correlated with those obtained by ***El-Saadani (2004)*** and in big head carp kept in the same experimental situation (***El-Ashmawy, et al., 2007***). Moreover, the present results concerning the RLP of vaccinated control fish and fish fed copper nicotinat complex either highest or lowest concentrations and challenged by *Yersinia ruckeri* virulent strain are disagree with those obtained by ***El-Ashmawy, et al. (2007)*** which were 30, 67 and 44%, respectively. Furthermore, the RLP of the present vaccinated control fish challenged by *Yersinia ruckeri* virulent strain (30%) are disagree with those obtained by ***EL-Gamal (2005; 25%)*** and ***El-Ashmawy, et al. (2007; 22%).*** The confliction between our previous (***El-***

Ashmawy, et al., 2007) and our present findings perhaps attributed to species difference. The increment of antibody titer of copper nicotinate administered group against ***Yersinia ruckeri*** bacterin along with the significant increase of the percentage of RLP challenged by ***Yersinia ruckeri*** virulent strain confirmed the above mentioned immunostimulant effect of copper nicotinate. This immunostimulant effect of copper nicotinate was reported in big head carp fish exposed to the same experimental design (***EL-Ashmawy et al., 2007***). From the biochemical point of view, it is suggested that most of the absorbed copper (I) nicotinate complex was biologically utilized as such. This suggestion is based up on the structural stability of the complex with regard to competing ligands (***Gohar and Dratovsky 1975***). Moreover, the most probable modification of the ligand in this copper complex is reduction to the pentadienyl derivatives shown in Figure 1. Hence, this complex could be conjugated in hepatocytes with phosphoribose resembling that of nicotinic acid (***Petrack et al., 1963***). Accordingly, NAD and NADP like structures are suggested to be more reactive as hydrogen carriers. This is due to the electronegatively of the chloride ion. The ion pair of electrons on nitrogen in turn are attracted toward the copper atom as shown in Figure 1. This electron shift enhances stability of the complex. The predicted higher reactivity of NAD and NADP-like structures enhances the activity of oxido-reductases which catalyze anaerobic and aerobic oxidation that result in accumulation of ATP, GTP and UTP. These nucleotides are essential compounds for biosynthesis and production of immunoglobulin (***Haselkorn and Rothman-Denes, 1973***).

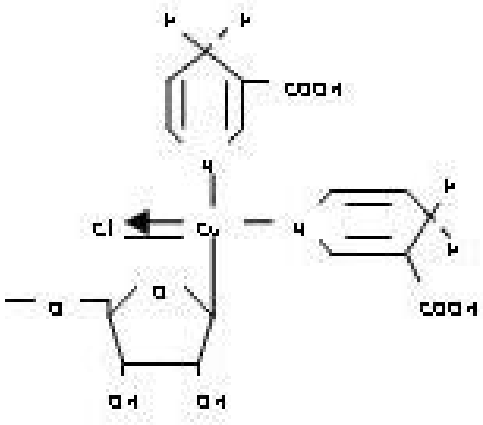

Fig. 1: *Probable Cu^{+} conjugation to ribose (redrawn after McDonald, et al., 1995)*

CONCLUSION

The present study can introduce a powerful evidence of the immunostimulant effect of copper nicotinate in the level of cell mediated and humeral immunity without adverse effect on the liver functions of *Mugil cephalus* fish. This immunostimulant effect was dose dependant as highest concentration of administered copper nicotinate (60 mg/kg of fish diet) was preferable for the stimulation of humeral and cell mediated immunity in the examined fish than the lowest used concentration (30 mg/kg diet).

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بعض الدراسات عن تأثير مركب الكابرنيكوتينيت على الاستجابة المناعية وبعض العناصر البيوكيميائية فى أسماك البورى المحصنة بمصل بكتيريا اليارسينيا روكارى

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فى هذه الدراسة تم تغذية الأسماك على عليقة تحتوى على مركب الكابرنيكوتينيت بتركيزين 30 ، 60 ملجم فى العليقة لمدة 4 أسابيع لدراسة تأثيرها على الجهاز المناعى وأجريت بعض التحاليل الكيميائية لأسماك البورى المحصنة ببكتيريا اليارسينا روكارى ، وتم أيضاً دراسة اختبار التحدى ضد بكتيريا اليارسينا روكارى وحساب معدلات المقارنة .

ولقد خلصت هذه الدراسة إلى :

1- أن استخدام التركيز العالى من مركب الكابرنيكوتينيت أدى إلى زيادة نسب خلايا الدم التى تم اختبارها أيضاً كلا التركيزين يبين أن هناك زيادة فى نشاط خلايا الكبد والتى ظهرت من خلال ثبات نشاط أنزيمات الكبد .

2- أيضاً أظهرت النتائج أن هناك زيادة فى كل من البروتين الكلى والزلال والجلوبيولين .

3- زيادة فى خلايا الليمفوسيت وزيادة فى نسبة النشاط الالتهامى ومعامل الالتهام والعد الكلى لكرات الدم البيضاء والذى يعكس التأثير المباشر لمادة الكابرنيكوتينيت على المناعة الخلوية للأسماك .

4- أيضاً وجدت زيادة فى معدلات الأجسام المضادة ضد بكتيريا اليارسينا روكارى المقاومة نتيجة التحصين .

5- فى النهاية أكدت الدراسة إلى أن هذه المادة تعتمد على الجرعة المستخدمة حيث بزيادة الجرعة تزيد الحالة المناعية للأسماك ضد البكتيريا المحقونة أثناء اختبار التحدى .