Vet. Med. J., Giza. Vol.55, No.2. (2007):507-522.

ASSESSMENT OF IMMUNE RESPONSE IN *BIG HEAD CARP* FED ON COPPER (I) NICOTINATE AND VACCINATED WITH YERSINIA RUCKERI BACTERIN

EL-ASHMAWY*. M., SAAD** T.T. El-BAHR*** S.M. M. SALAMA**** O.M.

Dept. of Pharmacology*, Aquatic and Avian Diseases**, Biochemistry***, Faculty of Veterinary Medicine, Alexandria Uni. and Research, Development and Quality Control Division Pharco Pharmaceuticals****, Alexandria, Egypt.

Received: 1. 3.2007.

Accepted: 17.3.2007.

SUMMARY

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 diet) for 4 weeks on the immune response and some biochemical parameters of *Big head carp* fish (Japanese Carp Puntius goionotus) (Bleeker) vaccinated against *Yersinia ruckeri* bacteria. The survival rate against challenge with virulent strain of *Yersinia ruckeri* was also determined. The present findings indicated that, fish supplemented with copper (I) nicotinate showed a significant increase in the percentages of Hb, PCV, lymphocytes, monocytes, basophils, eosinophils and total leucocytic count. However, the percentage of neutrophils was decreased significantly. Consequently, phagocytic activity and index were increased significantly.

Both concentrations of the used copper nicotinate had no adverse effect on liver functions which reflected on the unchanged activity of liver enzymes and the increase in both serum total protein and albumin. Moreover, the significant increase of serum globulin was also recorded. The increased lymphocyte percentage and serum globulin along with increased phagocytic index and activity gave a strong evidence of the immunostimulant effect of copper nicotinate in the level of cell mediated and humeral immunity. This immunostimulant effect was dose dependant and confirmed by the increase in both antibody titer and relative level of protection against the injected virulent strain of *Yersinia ruckeri*.

INTRODUCTION

Immunostimulants represents an emerging class of drugs that are designed to amplify naturally occurring immune responses against infectious diseases, pathogens and tumor cells. The field of essential metalloelement complexes has attracted many authors in this last decade. Biochemical mechanisms have been postulated concerning the mode of action of such vitamin 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. Therefore, the present study was planned to investigate the effect of dietary supplementation of copper (I) nicotinate complex on the immune response and some biochemical parameters of Big head carp fish vaccinated against Yersinia ruckeri bacteria.

MATERIALS AND METHODS

Copper nicotinate

Copper nicotinic acid complex 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.

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.

Fish, Aquaria and Experimental Design

A total number of hundred apparently healthy Bighead carp fish were obtained from Barseek fish farm at Behera province, Egypt with an Average body weight of 60 ± 5 gm were used in this study. Fish were 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 or vaccines were given to the fish along the course of the experiment except those under investigation. A total of 10 glass aquaria measuring 90 × 60 × 50 cm (one aquarium for each 10 fish) were used for holding the fish groups during the experiment. The aquaria were supplied with chlorine free tap water and continuous aeration.

The present study was divided into two main experiments each of 4 weeks duration time. In the first experiment, 60 fish were used and subdivided into 3 groups of 20 fish for 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/kg diet, respectively for the whole experimental period (4 weeks).

However, in the second experiment, the remaining 40 fish were used and subdivided also into 4 main groups of 10 fish for each. Fish of the first group were injected intraperitonially (I/P) with 0.2 ml/fish sterile saline and given feed without medicaments and served as non vaccinated control. The fish in the second group were injected I/P with 0.2 ml/fish of formalin inactivated bacterin from *Yersinia ruckeri* 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 nicotinate/kg diet, respectively for the whole experimental period (4 weeks). The booster dose of bacterin was given 2 weeks after the first injection.

Sampling and the Analytical Methods

In the first experiment, fish were weighed weekly. After the end of this experiment, the body surface 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) and packed cell volume (PCV) according to

Stoskopf (1993). Haemoglobin percentage (Hb %) was assessed according to Drubkin (1947), and differential leucocytic count (DLC) was determined according to Lucky (1977) and 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 colorimetric 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 colorimetrically as implied by the methods of Doumas et al. (1981), Reinhold (1953), Coles (1974) and Trinder (1969), *respectively.*

After the end of the second experiment, blood samples were collected and the obtained sera were stored at –20°C until used for detection of immune response to *Yersinia ruckeri* according to the method described by Badran (1990). 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. Specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation (one week).

The potency of bacterin was evaluated by calculating the relative level of protection (RLP) by the following formula which described by Newman and Majnarich (1982):

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

The brief description of the method implied for detection of immune response to Yersinia ruckeri (Badran, 1990) is that, 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 mixed and incubated overnight in the refrigerator. A positive serological reaction was indicated by bacterial agglutination. Agglutination titers were expressed as logs of the highest serum dilution still giving a clear agglutination. The negative controls consisted of; a) one drops 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.

Statistical analysis

The obtained data were analyzed by using computer package of the statistical analysis system (SAS, 1989).

RESULTS AND DISCUSSION

The results of the present study revealed that, copper (I) nicotinate induced a significant increase in the body weight of Big head carp only four weeks post administration. However, this increase was pronounced in fish received highest concentration of copper nicotinate (60 mg/kg diet) than in those received 30 mg/kg diet throughout the experimental period (4 weeks; Table 1). The obtained results revealed also that, TLC was significantly increased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. This increase was more pronounced in fish supplemented with highest concentration of copper nicotinate (60 mg/kg diet) than the lowest used concentration (30 mg/kg diet) throughout the experimental period (Table 2). However, the TEC was not significantly changed ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control (Table 2). The increase of TLC 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. This stimulation was dose dependant as the highest concentration of copper nicotinate was effective in stimulation of lymphoid tissue than the lowest one. It is well known that copper is necessary for formation of skeleton cells of fish.

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. The increment of Hb and PCV indicated the direct effect of copper nicotinate on hemopiotic tissue. 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 ferroxidase 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.

The present findings revealed also that, the percentage of lymphocyte was significantly increased ($p < 0.05$) in all fish supplemented with copper (I) nicotinate when compared with the control. However, the percentage of

monocytes was significantly increased ($p < 0.05$) only in fish supplemented with highest concentration of copper (I) nicotinate after third and fourth weeks when compared with

the control (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 diet) when compared with the control and lowest copper nicotinate concentration (30 mg/kg diet) which remained comparable throughout he experimental period (Table 3). Moreover, the percentage of eosinophils 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 fish received highest concentration of copper nicotinate (60mg/kg) than the lowest used concentrations (30mg/kg) 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). 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 4). The increment of lymphocytes, monocytes, basophils and esionphils indicated the direct stimulation of copper nicotinate to lymphoid tissue. 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.

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.

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 increase in serum total proteins values was more pronounced in fish received highest concentration of copper nicotinate than the lowered used concentration (Table 5). The value of serum albumin of all fish supplemented with copper (I) nicotinate was significantly increased when compared with the control. This increment was more recorded in the highest concentration of copper nicotinate (60 mg/kg diet) than the lowest used concentration (30 mg/kg diet) only after third and fourth weeks of the experiment (Table 5).

The value of serum globulin of all fish supplemented with copper (I) nicotinate was significantly increased 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) only after first and second weeks of the experiment (Table 5). The albumin/globulin ratio of all fish supplemented with copper (I) nicotinate was significantly increased after first, second and third weeks post experiment when compared with the control. This increment was inhibited after the fourth week of the experiment and remained comparable with the control group (Table 5).

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 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. The significant increase of total protein, albumin and globulin 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).

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 (Table 6). The significant decrease of serum glucose 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 values of antibody titer in fish vaccinated against Yersinia ruckeri bacterin was significantly increased ($p< 0.5$) 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).

These findings are correlated with those obtained by El-Saadani (2004). The percentage of relative level of protection of fish vaccinated against 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 (67%, 60 mg) than the lowest used concentration (44%, 30mg) throughout the experimental period (one week, Table 8). The present results concerning the relative level of protection of control vaccinated fish against Yersinia ruckeri virulent strain are similar to those obtained by EL-Gamal (2005). The increment of antibody titer of copper nicotinate administered group against Yersinia ruckeri bacterin along with the significant increase of the percentage of relative level of protection against Yersinia ruckeri virulent strain confirmed the above mentioned immunostimulant effect of copper nicotinate. Both antibody titer and percentage of relative level of protection were dose dependant. These findings are in agreement with those obtained by EL-Ashmawy et al. (2007) who reported that addition of copper (I) nicotinate complex to broiler diet significantly stimulated their humeral and cell mediated immunity vaccinated against Newcastle virus disease and elevated their survivals against challenge with virulent Newcastle virus strain. 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 HADP-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).

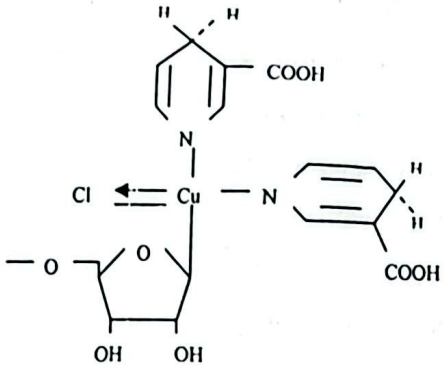

Fig. 1: Probable Cu^{+} conjugation to ribose

Table 1: Effect of daily supplementation of *copper nicotinate* for 4 weeks on body weight (gm) in *Big head carp.*

Treatment	Duration of Treatment			
	1st week	2nd week	3rd week	4th week
Control	55.6 ± 0.93 b	58.0 ± 1.00 b	60.0 ± 0.55 b	61.8 ± 0.58 c
30 mg/kg diet	56.0 ± 1.30 b	59.8 ± 0.49 b	61.6 ± 0.60 b	64.2 ± 0.58 b
60 mg/kg diet	59.8 ± 0.86 a	62.2 ± 0.58 a	64.6 ± 0.60 a	66.4 ± 0.68 a

Means within the same column with different letters are significantly differed ($P < 0.05$). Values are expressed as mean ± SE, n = 5 fish (1st experiment).

Table 2: Effect of daily supplementation of *copper nicotinate* for 4 weeks on WBCs (× 10^4cmm), RBCs (× 10^6cmm), Hb (g/dl) and PCV(%) in *Big head carp*.

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
WBCs (103/ml)	1st week	23.67 ± 0.33c	24.00 ± 0.58b	26.00 ± 0.58a
	2nd week	21.67 ± 1.20c	22.33 ± 0.33b	25.00 ± 0.58a
	3rd week	23.67 ± 0.33c	24.00 ± 0.58b	26.00 ± 0.58a
	4th week	22.30 ± 0.33c	23.67 ± 0.33b	24.60 ± 0.58a
RBCS (106/ml)	1st week	02.60 ± 0.06a	02.33 ± 0.22a	02.13 ± 0.03a
	2nd week	02.57 ± 0.03a	02.43 ± 0.09a	02.50 ± 0.06a
	3rd week	02.33 ± 0.03a	02.50 ± 0.06a	02.57 ± 0.09a
	4th week	02.50 ± 0.12a	02.50 ± 0.12a	02.40 ± 0.10a
Hb gm%	1st week	10.33 ± 0.33b	10.33 ± 0.33b	12.00 ± 0.58a
	2nd week	10.33 ± 0.33b	10.67 ± 1.20b	11.00 ± 0.58a
	3rd week	10.67 ± 0.33b	10.33 ± 0.33b	12.00 ± 0.58a
	4th week	09.33 ± 0.33b	09.33 ± 0.33b	11.67 ± 0.88a
PCV %	1st week	28.00 ± 0.58b	29.00 ± 0.58b	33.33 ± 2.96a
	2nd week	30.33 ± 0.88b	29.67 ± 0.58b	31.42 ± 1.12a
	3rd week	30.33 ± 0.33b	31.33 ± 0.33b	31.67 ± 1.76a
	4th week	29.33 ± 0.88b	29.33 ± 0.88b	32.67 ± 2.33a

For each week means within the same row with different letters are significantly differed ($P < 0.05$).
Values are expressed as mean ± SE, n = 5 fish (1st experiment).

Table 3: Effect of daily supplementation of copper nicotinate for 4 weeks on the differential Leucocytic count (%) in Big head carp.

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
Lymphocyte	1st week	50.00 ± 0.58c	51.33 ± 0.33b	52.00 ± 0.58a
	2nd week	49.33 ± 0.88c	51.67 ± 0.88b	52.33 ± 0.33a
	3rd week	48.00 ± 0.58c	54.00 ± 0.58b	56.00 ± 0.58a
	4th week	48.67 ± 0.33c	54.67 ± 0.88b	57.33 ± 0.33a
Monocyte	1st week	01.33 ± 0.33a	01.67 ± 0.88a	01.33 ± 0.33a
	2nd week	01.00 ± 0.58a	02.00 ± 0.58a	01.33 ± 0.33a
	3rd week	01.67 ± 0.33b	01.00 ± 0.58b	02.33 ± 0.33a
	4th week	01.33 ± 0.33b	01.33 ± 0.33b	03.33 ± 0.33a
Basophil	1st week	08.33 ± 0.33b	09.00 ± 0.33b	10.00 ± 0.58a
	2nd week	08.67 ± 0.33b	08.00 ± 0.58b	11.00 ± 0.58a
	3rd week	09.00 ± 0.58b	09.00 ± 0.33b	11.33 ± 0.33a
	4th week	09.33 ± 0.88b	10.00 ± 0.58b	11.33 ± 0.33a
Eosinophil	1st week	08.00 ± 0.58c	08.20 ± 0.33b	09.00 ± 0.58a
	2nd week	08.67 ± 0.33c	10.67 ± 0.33b	11.67 ± 0.33a
	3rd week	08.67 ± 0.33c	09.33 ± 0.33b	10.00 ± 0.58a
	4th week	09.33 ± 0.33c	10.33 ± 0.58b	11.33 ± 0.33a
Neutrophil	1st week	32.33 ± 0.33a	29.00 ± 0.58b	27.67 ± 1.45c
	2nd week	32.33 ± 1.67a	27.67 ± 0.88b	23.67 ± 1.20c
	3rd week	33.67 ± 0.67a	27.00 ± 0.58b	20.33 ± 0.88c
	4th week	31.00 ± 0.58a	23.00 ± 2.31b	17.67 ± 0.33c

For each week means within the same row with different letters are significantly differed ($P < 0.05$).
Values are expressed as mean ± SEM, n = 5 fish (1st experiment).

Table 4: Effect of daily supplementation of *copper nicotinate* for 4 weeks on phagocytic activity and index in *Big head carp*.

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
Phagocytic activity	1st week	25.00 ± 0.58b	25.33 ± 0.88b	27.00 ± 0.58a
	2nd week	23.33 ± 0.33b	23.67 ± 0.33b	24.33 ± 0.33a
	3rd week	26.33 ± 0.33b	26.33 ± 0.88b	27.33 ± 0.33a
	4th week	25.76 ± 0.33b	26.33 ± 0.33b	28.33 ± 0.33a
Phagocytic index	1st week	02.50 ± 0.06b	02.60 ± 0.06b	03.00 ± 0.17a
	2nd week	02.63 ± 0.47b	02.40 ± 0.17b	03.00 ± 0.06a
	3rd week	02.80 ± 0.06b	03.00 ± 0.06b	03.30 ± 0.06a
	4th week	02.10 ± 0.15b	02.63 ± 0.03b	03.00 ± 0.06a

For each week means within the same row with different letters are significantly differed ($P < 0.05$).
Values are expressed as mean ± SE, n = 5 fish (1st experiment).

5: Effect of daily supplementation of *copper nicotinate* for 4 weeks on total protein (g/dl), min (g/dl), globulin (g/dl) and albumin globulin ratio (g/dl) in *Big head carp*.

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
Total proteins	1st week	3.70 ± 0.06c	5.10 ± 0.03b	5.83 ± 0.15a
	2nd week	4.00 ± 0.06c	5.63 ± 0.03b	5.97 ± 0.09a
	3rd week	4.40 ± 0.12c	5.73 ± 0.03b	6.03 ± 0.09a
	4th week	4.00 ± 0.06c	5.03 ± 0.03b	6.03 ± 0.09a
Albumin (A)	1st week	1.87 ± 0.03b	3.70 ± 0.06a	3.90 ± 0.25a
	2nd week	2.73 ± 0.03b	4.07 ± 0.16a	4.03 ± 0.22a
	3rd week	3.17 ± 0.63c	3.80 ± 0.06b	4.20 ± 0.10a
	4th week	2.70 ± 0.15c	3.23 ± 0.19b	4.30 ± 0.06a
Globulin (G)	1st week	1.83 ± 0.03c	1.43 ± 0.03b	1.93 ± 0.18a
	2nd week	1.27 ± 0.03c	1.57 ± 0.17b	1.93 ± 0.15a
	3rd week	1.23 ± 0.54c	1.93 ± 0.09b	1.83 ± 0.15b
	4th week	1.30 ± 0.10b	1.80 ± 0.20a	1.73 ± 0.03a
A/G ratio	1st week	1.02 ± 0.12b	2.59 ± 0.10a	2.07 ± 0.31a
	2nd week	2.16 ± 0.05b	2.67 ± 0.36a	2.13 ± 0.26b
	3rd week	1.19 ± 0.46c	1.98 ± 0.12b	2.33 ± 0.23a
	4th week	2.12 ± 0.26a	2.45 ± 0.42a	2.48 ± 0.03a

each week means within the same row with different letters are significantly differed ($P < 0.05$).
lues are expressed as mean ± SE, n = 5 fish (1st experiment).

ble 6: Effect of daily supplementation of *copper nicotinate* for 4 weeks on ALT (U/l), AST (U/l) and ucose (mg/dl) in *Big head carp*.

Parameters	Period	Treatment		
		Control	30 mg/kg diet	60 mg/kg diet
ALT	1st week	69.00 ± 0.33a	71.00 ± 0.58a	71.67 ± 0.33a
	2nd week	70.67 ± 0.88a	70.00 ± 0.58a	70.33 ± 1.76a
	3rd week	71.76 ± 0.53a	72.67 ± 0.88a	72.33 ± 0.88a
	4th week	69.60 ± 0.88a	70.00 ± 0.58a	70.67 ± 0.33a
AST	1st week	75.33 ± 0.88a	76.00 ± 0.58a	76.33 ± 0.33a
	2nd week	76.67 ± 0.33a	76.00 ± 0.58a	76.00 ± 0.58a
	3rd week	78.00 ± 0.58a	77.67 ± 0.33a	76.67 ± 0.33a
	4th week	82.67 ± 1.20a	82.00 ± 1.15a	82.00 ± 0.58a
Glucose	1st week	89.67 ± 1.20a	85.67 ± 0.88b	83.00 ± 0.33c
	2nd week	87.67 ± 0.88a	85.67 ± 1.45b	83.00 ± 0.58c
	3rd week	88.67 ± 0.67a	86.33 ± 0.58b	84.67 ± 0.45c
	4th week	93.33 ± 0.33a	90.00 ± 0.58b	87.67 ± 0.33c

For each week means within the same row with different letters are significantly differed ($P < 0.05$).
Values are expressed as mean ± SE, n = 5 fish (1st experiment).

Table 7: Effect of daily supplementation of *copper nicotinate* for 4 weeks on antibody titer in *Big head carp* vaccinated with *Yersinia ruckeri* bacterin.

Treatment	Days after immunization			
	7 days	14 days	21 days	28 days
Control* Control Vaccinated	4 ± 0.93a	3 ± 1.00a	4 ± 0.55a	4 ± 0.58a
30 mg/kg diet	5 ± 1.30b	5 ± 0.49b	6 ± 0.60b	6 ± 0.58b
60 mg/kg diet	5 ± 0.86b	6 ± 0.58c	6 ± 0.60b	7 ± 0.68c

Means within the same column with different letters are significantly differed ($P < 0.05$).

*Control group has zero antibody titer.

Values are expressed as mean ± SE, n = 5 fish (1st experiment).

Table 8: Effect of daily supplementation of *copper nicotinate* for 4 weeks on mortality ratio and relative level of protection (%) of *Big head carp* against virulent strain of *Yersinia ruckeri* after vaccination with *Yersinia ruckeri* bacterin.

Treatment	Parameters	
	Mortality ratio	RLP
Control Control vaccinated	9/10 7/10	22 %
30mg/kg diet	5/10	44.4 %
60mg/kg diet	3/10	66.7 %

Means within the same column with different letters are significantly differed ($P < 0.05$).

n = 10 fish (2nd experiment).

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. 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 fish than the lowest used concentration (30 mg/kg diet).

REFERENCES

Al-Mulla Hummadi, Y.M., AL-Bashir, N.M. and Najim, R.A. (2006): Leishmania major and Leishmania tropica: II. Effect of an immunomodulator, S(2) complex on the enzymes of the parasites. Exp. Parasitol. 112, 85-91.

AL-Mulla Hummadi, Y.M., Najim, R.A. and AL-Bashir, N.M. (2005): Leishmania major and Leishmania tropica: I. The *in vitro* effects of an immunomodulator, S2-complex. Exp. Parasitol. 111, 47-54.

Badran, A. F. (1990) The role of adjuvants in the immune response of the fish. Zag. Vet. Med. J. 18: 126 – 136.

Coles, E.H. (1974): Veterinary Clinical Pathology. W.B. Saunders Company, Philadelphia and London, pp. 211-213.

Collins, M. T.; Dawe, D. L. and Gratzek, J. B. (1976) Immuno response of Channel catfish (Ictalurus punctatus) under different environmental conditions. J. Am. Vet. Med. Ass. 169 (a): 991 – 994

Doumas, B.T., Bayson, D.D., Carter, R.J., Peters, T. and Schaffer, R. (1981). Estimation of total serum protein. Clinic. Chem. 27, 1642-1643.

Drubkin, D. (1947): Spectrophotometric methods XIV. The crystographic and optical properities of the haemoglobin of man in comparison with those of other species. J. Biol. Chem. 164, 703 – 723.

EL-Ashmawy, I.M., EL-Lakany, H.F. and Salama, O.M. (2007): Effect of Copper (I) nicotinate complex on the immune response of broiler vaccinated against Newcastle virus vaccine. Alex. J. Vet. Sci. (In press).

EL-Gamal, H.L. (2005): Some studies on the effect of *Yersinia Ruckeri* in the cultured fresh water fish. Ph.D. Vet, Alex. Univ.

EL-Saadani, M.A. (2004): A combination therapy with copper nicotinate complex reduces the adverse effects of 5-fluorouracil on patient with hepatocellular carcinoma. J. Exp. Ther. Oncol. 4, 19-24.

EL-Saadani, M.A., Nassar, A.Y., and Abou el-Ela, S.H. (1993): The protective effect of copper complexes against gastric mucosal ulcer in rats. Biochem. Pharmacol. 46, 1011-1018.

Eurell. T. E., S. D. H, Lewis and L. C. Grumbles. (1979) : Comparison of selected diagnostic tests for detection of motile Aeromonas septicaemia in fish. Am. J. Vol. Res., 39, 1384-1386.

Franklin, T.A. and Richardson, M.F. (1980): Reactions of metal ions and vitamins: III.
Synthesis and infrared spectra of metal complexes with pyridoxamine and pyridoxine. Inorganic Chemica Acta, 46, 191-197.

Gohar, M. and Dratovisky, M. (1975): Perchlorates of Cu (I) complexes with some pyridine derivatives. Coll. Cerch. Chem. Commun. 40, 26.

Greenway, F.T., Riviere, E., and Girerd, J.J. (1999): Copper (II) complexes of a non steroidal anti- inflammatory drug niflumic acid. J. Inorg. Biochem. 76, 19-27.

Haselkorn, R. and Rothman-Denes, L. (1973): Protein synthesis. Annu. Rev. Biochem. 42, 397-438.

Hawk, P.B., Oscar, B.L. and Summerson, W. (1965): Hawk's Physiological Chemistry. London J. and A. Churchill Ltd. 14th Ed.

Li, C., Wu, J., Wang, L. (1999): Synthesis, characterization and antitumor activity of copper (II) complex with nicotinamid-4-bis (2-chloroethyl) aminobenzaldimine. J. Inorg. Biochem. 73, 195-202.

Leid, E., Gezrde, Z. and Braskhan, O.R. (1975): Simple and rapid technique for repeated blood sampling in rainbow trout. J. Fish Res. Board of Canada 32, 699-701.

Lucky, Z. (1977): Methods for the diagnosis of fish diseases. Amerno Publishing Co., PVT, Ltd. New Delhi, Bombay, New York.

McDonald, P., Edward, R.A. and Green balgh, J.F. (1995): Animal nutrition 5th ed. pp. 103-106, English language, Book society

Newman, S. G. and Majnarich, J. J. (1982): Direct immersion vaccination of Juvenile rainbow trout, Salmo gairdneri Richardison, and Juvenile coho salmon, Onchorhynchus kisutch (Walbaum), with a Yersinia ruckeri bacterin. Journal of Fish Diseases. 5, 339 – 341.

Petrack, B., Greengard, P., Craston, A. and Kalinsky, H. (1963): Nicotinamide deamidase in rat liver and biosynthesis of NAD. Biochem. Biophys. Res. Commun. 13, 472-477

Prohaska, J.R. and Failla, M.L. (1993): Copper and Immunity 1st ed. New York, Plenum Press, pp. 309-332.

Radostits, O.M., Blood, D.C., Cay, L.C. and Hinchcliff K W. (2000): Veterinary Medicine, Text book of the disease of cattle, pig, sheep, goat and horses 9th ed

Reinhold, R.P. (1953): Determination of serum albumin. Clinic. Chem., 21, 1370-1372.

Reitman S. and Frankel, S. (1957): A colorimetric method for determination of serum glutamate oxalo-acetic acid and pyruvic acid transaminases. Amer. J. Clin. Pathol., 29, 56-63.

SAS (1987): Statistical analysis system. User's Guide Statistics. SAS Institute Cary, North Carolina.

Schalm, O W. (1986): Veterinary Haematology, 4th Ed, Lea and Febiger, Philadelphia.

Stoskopf M. K. (1993): Fish Medicine W.B. Saunders Comp. Philadelphia.

Tanner, M.; Jaya Wardena, P. A.; Xusoff, F. M. and Subasing, R. (1988): Immunological parameters of Japanese Carp Puntius goionotus (Bleeker) exposed to copper and challenged with Aeromonas hydrophila. Shell Fish Immunol. May; 11 (4): 281 - 91.

Trinder, P (1969): Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. L. I. N. Biochem. 6, 24.

Tsuji, A. and Sakurai, H. (1998): Generation of nitric oxide from streptozotocin (STZ) in the presence of copper (II) plus ascorbate. Biochem. Biophys. Res. Commun. 245, 11-16.

Underwood, E.J. (1977): Trace elements in human and animal nutrition 3rd ed. pp. 57-127. Acad. Press New York.