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Histopathology and Biochemical Analysis of Common Carp (*Cyprinus carpio*) Exposed to Sublethal Concentrations of Carboxin-thiram (Vitavax Thiram)

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ABSTRACT

This study aimed to investigate histopathological, biochemical and behavioral changes of common carp (*Cyprinus carpio*) exposed to sublethal concentration of Carboxin-Thiram (Vitavax thiram) for one week in static conditions. Live specimens of *C. carpio* were obtained and exposed to 5, 15 and 30 mg L^{-1} carboxin-thiram for one week. There was one control group (no toxicant) and three replicates. The physicochemical properties of water and the following were constant: pH: 8±0.5, temperature: 19±0.4°C, hardness: 345±6 ppm and dissolved oxygen: 7.3±0.3. Results showed the kinds of pathologies such as secondary lamellae shrinking, lamellar fusion, lamellar aneurism, lamellar clubbing and hyperplasia in gills and lipidosis, pyknotic nuclei, atrophy, aggregation of blood cell, hemosiderin and biliary stagnation in liver. Biochemical analysis revealed that total protein and albumin serum decreased and glucose level increased significantly after 7 days exposer. This study revealed the adverse effect of carboxin-thiram in *C. carpio* for the first time.

Key words: Vitavax thiram, biochemical changes, histopathology, *Cyprinus carpio*

INTRODUCTION

Treating seeds with pesticides is an important and increasingly common practice in agriculture. According to FAO (2012), in the last twenty years we have seen a significant increase worldwide in the use of seed dressing pesticides. The residues from treating seeds with pesticides can cause poisoning and impacts on aquatic and soil communities and leave pesticide traces in food products (Paulsrud *et al*., 2001).

Carboxin-thiram is a new component fungicide which is widely used in agriculture to seed prior to planting for control of various fungi that cause seed and seedling diseases or to cure existing plant diseases (Teixeira *et al*., 2013). It manufactured form combination of Carboxin (5,6-dihydro-2-methyl-N-phenyl-1-4-oxathiin-3-Carboxanrnide), a member of the oxathiin class of systematic fungicides (Aydin *et al*., 2012), thiram (tetramethylthiuram disulfide), a dimethyl dithiocarbamate compound (Sharma *et al*., 2003), ethylene glycol and 9-(2-carboxyphenyl)-3,6-bis (diethylamino) xanthylium chloride (17-18, 17-18, 20-25 and 1% weight percent, respectively). Thiram and carboxin has been formulated for use as dusts, wettable powders and flowable

suspensions and also in combination with other pesticides and highly toxic to fish (Sharma *et al*., 2003; Aydin *et al*., 2012). According to the literature, thiram exhibit strong toxic effects and disrupt the inhibition of enzymes such as aldehyde dehydrogenase especially on teleost species in early life stages (Van Leeuwen *et al*., 1985a, b) and carboxin cause oxidative stress in fishes (Aydin *et al*., 2012). The synergistic interaction between these two chemicals can result in toxicity that is greater than would be expected by the addition of the toxicities of the individual components. No studies to date have examined the toxicological interaction among the components of carboxin+thiram in fish, although the toxicological mechanisms of the individual thiram and carboxin components are well known (Mochida and Fuji, 2009; Greene and Kocan, 1997).

Fish occupy a prominent position in the field of toxicology; in studies concerning both human and ecological health (Shwetha *et al*., 2012). Therefore, to increase the understanding of the ecotoxicological effects of seed dressing pesticides on aquatic organisms and survival with Common carp (*Cyprinus carpio*) were performed with carboxin-thiram. The usefulness of either survival or pathology for the evaluation of the potential environmental risks of these products was also discussed.

MATERIALS AND METHODS

Acute toxicity test: Live specimens of *C. carpio* were obtained from Sari aquaculture ponds, Mazandaran, Iran. Samples weighted 40±10 g. They were acclimatized randomly in 70 L aquariums for one week. Seven aquariums were treated with 40, 50, 60, 70, 80, 90 and 100 ppm of carboxin-thiram with one control group (no carboxin-thiram). No feeding occurred during the test (96 h). There were no significant differences between aquariums in water quality and the following were constant: pH: 8±0.5, temperature: 19±0.4°C; hardness: 345±6 ppm and dissolved oxygen: 7.3±0.3. The photoperiod was 12 h light and 12 h dark. . Static acute toxicity test was performed following guideline the Organization for Economic Co-operation and Development OECD standard method (OECD., 1993). Mortality rates were recorded after 24, 48, 72 and 96 h and dead fishes were quickly removed from the aquarium. The nominal concentration of toxin causing mortality (LC_1, LC_{10}, LC_{30}, LC_{50}, LC_{70}, LC_{90} and LC_{99}) were computed on the basis of probit analysis (Finney, 1971).

Hematological and pathological test: Based on acute toxicity test, fish were exposed to sublethal carboxin-thiram toxicity (10, 25 and 50% of LC_{50}) with one control group and three replicate for 7 days. Water quality was constant and fixed according to acute toxicity test. Histological examinations were performed as described in Bucke (1982). Fish were anaesthetized with 200 ppm eugenol concentration in 5 L tanks and tissues were collected in the following order: the second gill arch was taken from gills and the hind part of liver was taken by abdominal dissection. Collected tissues were fixed in formalin solution 1-10 and Dehydration with Ethanol 96%, Clearing with xylenol, impregnation with paraffin, embedding, sectioning, mounting and Staining with H and E were performed, respectively. All of these steps were conducted by tissue processor under defined program (Tissue processor, Triangle biomedical sciences USA) Histopathological changes induced by treatments in the tissues were photographed using Nikon photomicroscope. Quantitative histological measurements were made in several tissues of gills and livers.

Hematological analyses were carried out as described in (Tripathi *et al*., 2003) for *C. carpio*. For measuring, the blood samples was placed in tubes and allowed to clot at 22-24°C temperature for

30 min. Serum was removed from the clotted sample after centrifugation at 5,000 rpm for 5 min and frozen at -80°C until analysis. Glucose was measured using a spectrophotometric method (WPAS2000-UV/VIS, Cambridge, UK) with reagents provided in standard analyses kits (Pars Azmon, Iran). Total serum protein was determined by the biuret reaction. Albumin-like protein was quantified by Bromcresol Green (BCG) dye binding. Cholesterol was determined by Liebermann's method, an endpoint-coupled reaction in which cholesterol esters are hydrolysed into free cholesterol and fatty acids by a microbial cholesterol esterase. Direct calcium concentration was determined by the modified method of Sarkar and Chauhan.

Data was analyzed by using SPSS 20 one way analysis of variance (ANOVA), The Least Squares Difference (LSD) post hoc test ($p<0.05$) was used to identify treatment effects at the end of the experiment (day 7). To illustrate the intensity tissue damage following procedure scoring system was applied as described analogous in Mitchell *et al*. (2012): (-) was used for no observed injuries, (+) for 1-3, (++) for 3-5, (+++) for 5-11 and (++++) for 11 and more observed injuries in samples.

RESULTS

No mortality observed during acclimation. Result showed that within 24 h test, LC_{50} value declined with increasing toxin concentration and duration of exposure (Table 1). According to Table 1 LC_{50} 96 h of carboxin-thiram to common carp was calculated 64.28±5 mg L^{-1}.

Clinical observations: Fish exposed to toxicant showed abnormal behavior as faster opercular activity, swimming erratically with jerky movements, protrusion of the eyes and bruise in the caudal fin. Exposed fish incurred curvature in vertebra and their gill pigmentation was decreased. Control group showed normal behavior during experiment. The results of the present study suggest that exposure of common carp to a sublethal concentration of carboxin-thiram for one week showed different toxic effects on more than one biological system.

Histological observation on the gill and liver: No injuries were observed in control group. Exposure to carboxin-thiram caused various gill injury after 7 days. Histological examination showed areas of hyperplasia, oedema and lifting of the gill epithelium and lamellar fusion in gills and hemosidrosis, hemorrhage, hydropic swelling and pyknotic nuclei in liver (Fig. 1 and 2). The extent of these injuries was different in various concentrations (Table 2 and 3).

Hematological analysis: Effects of different concentrations of carboxin-thiram on the hematological indices of common carp were represented in Table 4. Table shows blood glucose was significantly increased by increasing in toxicant concentration. Increasing in total protein was significant between toxicant and control group while there was no significant differences between

Table 1: Mortality rate in acute toxicity rate of carboxin-thiram for *Cyprinus carpio*

Concentration (mg L^{-1})	Total	24 h	48 h	72 h	96 h
Control	21	0	0	0	0
40	21	0	0	3	3
50	21	0	0	3	6
60	21	0	0	3	9
70	21	0	0	3	12
80	21	0	3	9	15
90	21	0	3	12	18
100	21	0	6	15	21

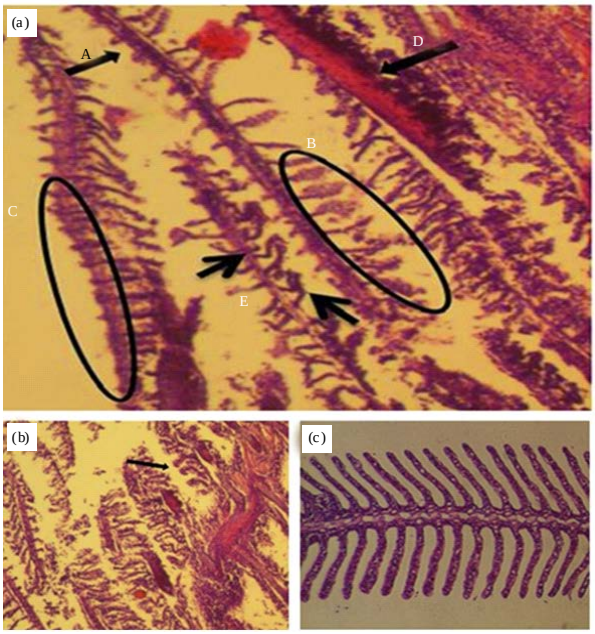

Fig. 1(a-c): Microphotographs of gill histopathological changes by Carboxin-Thiram in *Cyprinus carpio*, (a) Secondary lamellae shrinking (A and C), hyperplasia (B) and lamellar aneurism and destruction (D), (b) Lamellar clubbing and (c) Normal gill (control group). All pictures are magnified×100

Table 2: Index and scores for the *Cyprinus carpio* gills exposed to Carboxin-Thiram sub-lethal concentrations

	Lesions				
Concentration (ppm)	Secondary lamellae shrinking	Lamellar fusion	Lamellar aneurism	Lamellar clubbing	Hyperplasia
0	-	-	-	-	-
5	-	+	+	-	+
15	+	++	+	+	++
30	++	++	+	+	++

-: No observed lesions, +: 1-3 observed lesions, ++: 3-5 observed lesions, +++: 5-11 observed lesions, ++++: 11 and more observed lesions

Table 3: Index and scores for the *Cyprinus carpio* liver exposed to Carboxin-Thiram sub-lethal concentrations

	Lesions					
Concentration (ppm)	Lipidosis	Pyknotic nuclei	Atrophy	Aggregation of blood cell	Hemosiderin	Biliary stagnation
0	+	-	-	-	-	-
5	++	-	+	-	++	+
15	+++	+	++	++	++	++
30	++	+	++	++	+++	++

-: No observed lesions, +: 1-3 observed lesions, ++: 3-5 observed lesions, +++: 5-11 observed lesions, ++++: 11 and more observed lesions

Table 4: Hematological parameters of *Cyprinus carpio* under sublethal concentration of Carboxin-Thiram

	Treatments (mgr L^{-1})			
Factors	0	5	15	30
Glucose (mg dL^{-1})	63.50±5.42^{a}	119.17±18.02^{b}	133.79±15.15^{bc}	146.95±11.90^{c}
Total protein (g dL^{-1})	3.29±0.19^{a}	2.50±0.20^{b}	2.42±0.30^{b}	2.39±0.47^{b}
Albumin (g dL^{-1})	1.40±0.05^{a}	1.33±0.04^{a}	1.30±0.02^{a}	1.24±0.06^{b}
Cholesterol (mg dL^{-1})	144.08±14.2^{a}	137.47±10.34^{a}	134.31±16.91^{a}	130.29±13.66^{a}
Calcium (g dL^{-1})	5.10±0.4^{a}	4.74±0.84^{a}	4.63±0.36^{a}	4.82±0.72^{a}

Identical letters indicating no significant difference (ANOVA, $p<0.05$)

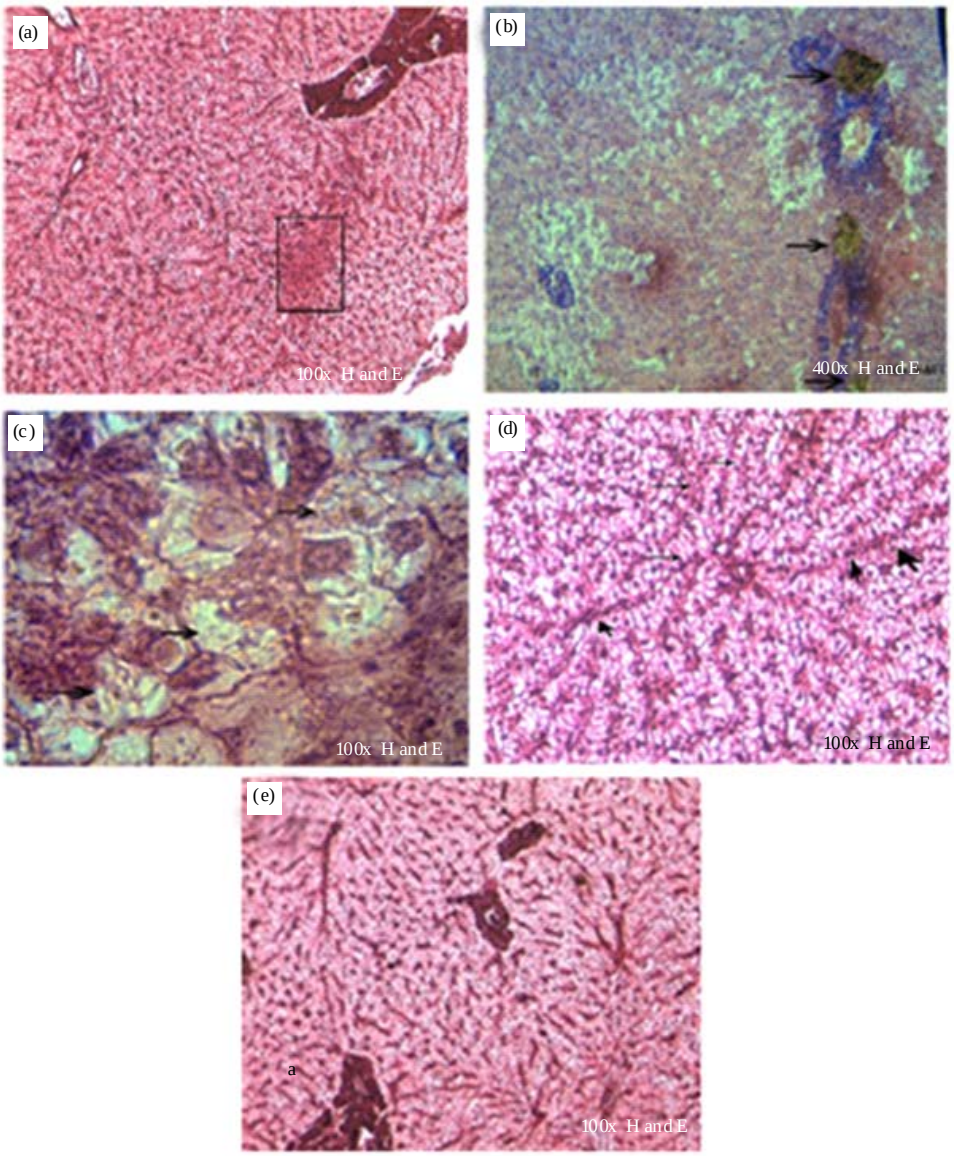

Fig. 2(a-e): Microphotographs of liver histopathological changes by Carboxin-Thiram in *Cyprinus carpio*, (a) Hemosiderin, (b) Biliary stagnation, (c) Vacuole formation, (d) Atrophy and (e) Normal liver

toxicant concentrations treatments ($p<0.05$). In addition, there was significant decrease in total protein by increasing toxicant ($p<0.05$). However, changes in blood cholesterol and calcium was not significant ($p>0.05$). Reduction in blood albumin was significant in 30 mg L^{-1} toxicant concentration.

DISCUSSION

The aim of the present study was to assess adverse effects of a sublethal exposure of carboxin-thiram to common carp by using selected biological and biochemical parameters. Contrasting results are limited on toxicity of carboxin-thiram on fish species. However, both thiram and

carboxin are highly toxic to fish by individually. According to Material Safety Data Sheet (2011), LC_{50} 96 h of thiram and carboxin were represented 0.13 and 2.3 mg L^{-1} for *Oncorhynchus mykiss*, respectively. In addition, LC_{50} 96 h of carboxin for *Lepomis macrochirus* was calculated 3.6 mg L^{-1}. Although, dissolved oxygen, pH, size and age, type of species, water quality, concentration and formulation of test chemicals are the major factors in affecting toxicity of chemicals to aquatic organisms (Nwani *et al*., 2010; Naserabad *et al*., 2015). Our study was conducted under static conditions and doses were constant during the experiment. Typical application rates for agricultural use range from 1-3 g L^{-1}, so we can easily speculate that the dose we chose could easily be found in the environment.

Gills are a vital organ in aquatic organisms because of transportation of respiratory gases and regulation of osmotic and ionic balance (Evans, 1993). They are the first organ that encountered to toxicants. Mallatt (1985) stated that gill lesions can be divided into two groups, one that reflects the direct effect of toxicants and another corresponding to defence responses of fishes. Pereira *et al*. (2013) declared that filament epithelium proliferation and necrosis were the biological responses that contribute most to discriminate the ecological status classification. In the present study, exposure of common carp to carboxin-thiram resulted in structural alterations of the gill lamellar fusion and swelling of the epithelial cells. Similar findings in this study (Fig. 1) were also observed by Boran *et al*. (2012) and Khanghah and Bozorgniya (2014) for other fungicides. The results of these studies clearly indicate that sublethal concentrations of fungicides have diverse effects on fish gills. Hyperplasia and lamellar fusion known to be induced by many gill tissue irritants, however, focal points of cellular hypertrophy and necrosis followed by epithelial rupture reflect the direct deleterious effects of fungicides in fish gills (Mazon *et al*., 2002).

The liver in fish is an organ that performs various functions associated with the metabolism of xenobiotics (Jimenez and Stegeman, 1990). These xenobiotics cause various degenerative alterations (general necrosis) in liver, which are irreversible and their persistence or progression may lead to a partial or total loss of organ function (Agamy, 2012). However, most proliferative alterations may not be reversible, depending on the severity and extent of the alteration. For this purpose, we classified the severity and level of injuries according to concentrations (Table 3). Results showed that level of injuries increased with carboxin-thiram concentrations. This suggests that toxicant higher concentrations increase the rate of accumulation of carboxin-thiram in liver. The types of injuries reported here (Fig. 2) for carboxin-thiram was coincided with other reports about histological changes in the hepatic tissue of fish (Gill *et al*., 1990; Martin *et al*., 2007; Boran *et al*., 2012). Biliary stagnation was the most observed histopathological changes in liver. This lesion resulted to an accumulation of bile inside hepatocytes, evidenced by the appearance of yellow cytoplasmic granules (Fig. 2) (Fanta *et al*., 2003; Simonato *et al*., 2008). Pacheco and Santos (2002) declared that lack of bile metabolism and excretion would cause manifestation of a physiopathological condition which consequently bile secreted by hepatocytes remains inside cells and is not released into the digestive tract. Fatty infiltration is a common response to chemical exposure in fish and reported by researchers (Metcalfe, 1998; Marty *et al*., 2003; Wolfe *et al*., 2001; Agamy, 2012). However, lipidosis also observed in control group but increased in toxicant concentrations (Table 3). Pyknotic nuclei were the minimum observed lesion in this study. This alteration appear to be commonly developed in fish exposed to toxicant (Do Carmo Langiano and Martinez, 2008). Increased cellular and nuclear volume of hepatocytes can be considered as responses to the stressor agent, since they indicate the activation of the liver functions and do not interfere with the hepatic performance but rather indicate the intensification of hepatocytes metabolic activity under adverse conditions (Takashima and Hibiya, 1995).

The blood of common carp showed significant increase in glucose during 7 days of carboxin-thiram intoxication. This might be due to the vulnerable stress induced by the toxicant resulted in hyperglycemia (Table 4). Hyperglycemia is a common response to stress that occurs as a result of the effects of catecholamines and cortisol (Barton, 2002). The increased plasma levels of glucose are consistent with those reported in Senegalese sole (*Solea senegalensis*) after exposure to 2-phenoxyethanol as an anesthetic (Weber *et al*., 2011) or in great sturgeon (*Huso huso*) (Shaluei *et al*., 2012). In addition declared that toxicant increase the glucose content in blood, because of intensive glycogenolysis and the synthesis of glucose from extra hepatic tissue proteins and aminoacids. In this study, blood glucose was increased significantly after exposure. ($p<0.05$). Insulin, is the main factor in the balance of glucose but this hormone is very low in fish (Velisek *et al*., 2005). Many factors affect blood glucose except of toxicological factor (such as nutrition, time of blood sampling, bloodletting from dead fish, manipulation stress, procrastination in serum remove and integration blood with serum) (Rabitto *et al*., 2005). Significantly, Authors tried to consider all these factors in this study. In order, feeding were stopped 24 before blood sampling, bloodletting was done quickly after anesthesia and serum was removed immediately.

Protein is the most important and abundant biochemical constituent present in the animal body. Both the protein degradation and synthesis are sensitive over a wide range of conditions and show changes to a variety of physical and chemical modulators (Shwetha *et al*., 2012). In this study, hypoproteinemia were occurred in toxicant treatments (Table 4). This reflects the decreased production or increased consumption of protein in cell. In fish, proteins are one of the main energy sources which play an important role in the maintenance of blood glucose (Shwetha *et al*., 2012). When fish exposed to toxicant with stress conditions, divercification of energy occurs to accomplish the impending energy demands and hence the protein level is depleted. The depletion of protein content may be due to break down of protein free amino acid under the effect of carboxin-thiram. In addition, continues to stress conditions and carbohydrates and lipids reduction; protein could be used as a source of energy and decreased. Decline in total protein after exposed to fungicides was also reported for *Puntius stigma* (Khiliare and Wagh, 1989), *Salmo trutta* (Egaas *et al*., 1999) and *Oncorhynchus mykiss* (Li *et al*., 2010).

Results showed decline in serum calcium and cholesterol level (Table 4). However, this reduction was not significant ($p>0.05$). A possible cause of depletion of calcium may be renal insufficiency and disrupted electrolyte balance (Srivastava *et al*., 1995). Bano (1982) also reported a depletion of serum calcium in *Clarias batrachus* under chemical stress of aldrin. In addition, Dhanapakiam and Ramasamy (2001) cited that serum cholesterol decreased in *Cyprinus carpio* after exposure to sublethal level of mixture heavy metals after 30 days.

In conclusion, exposure of common carp to sublethal concentration of carboxin-thiram alters biochemical and histopathological changes. Target tissues such as gills and livers could be used as biomarkers for assessing toxic effects of fungicide in environmental. The adverse effect of carboxin-thiram and the mechanism of action are reported for the first time.

Ethics statement: All experiments performed on fishes in this study complied with Society of Toxicology (code of ethics January 31, 1985; Revised June 1, 2005; Reviewed and Reaffirmed September 14, 2011; Revised November 5, 2012) and Canadian Council on Animal Care. All analyses and experiments were performed to minimize suffering. Fish were anaesthetized before tissue sampling. Study was conducted with minimal number of fish.

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