ARTICLE

Effect of Trichlorfon on Hepatic Lipid Accumulation in Crucian Carp Carassius auratus gibelio

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Abstract
This study evaluated the toxic effects of the organophosphate pesticide trichlorfon on hepatic lipid accumulation in crucian carp Carassius auratus gibelio. Seventy-five fish were divided into five groups (each group in triplicate), and then exposed to 0, 0.5, 1.0, 2.0, and 4.0 mg/L of trichlorfon and fed with commercial feed for 30 d. At the end of the experiment, plasma and hepatic lipid metabolic biochemical status were analyzed. Triglyceride contents were significantly (*P* < 0.05) increased in liver but decreased in plasma after 1.0, 2.0, and 4.0 mg/L trichlorfon treatments. Plasma insulin contents were markedly (*P* < 0.05) increased when trichlorfon concentrations were 0.5, 1.0, and 4.0 mg/L. There were no significant differences in hepatic hormone-sensitive lipase contents between the trichlorfon-treated fish and the controls. Hepatic cyclic adenosine 3′, 5′-monophosphate, very-low-density lipoprotein, and apolipoprotein B100 contents were decreased in the fish when trichlorfon concentration was 2.0 mg/L. Furthermore, electron microscope observations showed rough endoplasmic reticulum dilatation and mitochondrial vacuolization in hepatocytes with trichlorfon exposure. On the basis of morphological and physiological evidence, trichlorfon influenced crucian carp hepatic pathways of lipid metabolism and hepatocellular ultrastructure, which resulted in lipid accumulation in the liver.

Hepatic lipid accumulation in fish is a common problem in fish farming, and may have an impact on fish growth, resistance to environmental stress factors, disease susceptibility, and survival. Hepatic lipid accumulation is influenced by a variety of stimuli, such as parasites (Paperna 1991), toxins (Tanaka et al. 2002), antibiotics (He et al. 2006), environmental stress factors (Xu et al. 2009), and nutrient imbalance (Serrano et al. 1992; Shimeno et al. 1995; Russell et al. 2001). Research into lipid accumulation has focused on dietary nutrients imbalance and antifatty liver factor deficiency (Liang et al. 2002; Yang et al. 2003; Dias et al. 2004). Excess feeding with high-fat food is a major risk factor (Speare 2000). Lie et al. (1988) and Deplano et al. (1989) demonstrated that excessive fat in the diet was deposited as liver fat. Pulla-Reddy and Lokesh (1994) showed that dietary fat influenced the anti-oxidative system of the body, which resulted in hepatic lipid accumulation. There have been some reports on the effects of chemical pesticides on hepatic lipid accumulation (Rojik et al. 1983; Shakoori et al. 1996; Ibrahim and El-Gamal 2003). In a review Couch (1975) found the most commonly encountered nonspecific liver lesion after pesticide (lindane and methoxychlor) exposure was fatty change. In a previous study, Xu et al. (2009) showed that trichlorfon-induced hepatocyte apoptosis caused mitochondrial vacuolization and lipid droplet accumulation in the hepatocytes of crucian carp Carassius auratus gibelio (also known as C. carassius) in vitro.

Trichlorfon has been widely used as an organophosphorus pesticide in agriculture to control insect pests owing to its relatively low bioaccumulation and short-term persistence (Videira et al. 2001; Rao and Kavitha 2004). The dosage necessary to eradicate ectoparasites varies from 0.1 to 1 mg/L in ponds (Chang et al. 2006). Though trichlorfon half-life time in the water was short (57 h) (Lopes et al. 2006), excessive amounts are
often applied in fish and agriculture farm management. However, excessive amounts are often applied in fish and agriculture farm management. Residues of trichlorfon in the water have caused problems in nontargeted species such as fish, crabs, and shrimps (Soumis et al. 2003). The toxicity of trichlorfon has been studied extensively in both in vivo and in vitro conditions (Cukurcam et al. 2004; Yeh et al. 2005; Guimaraes et al. 2007; Ranaldi et al. 2008). Trichlorfon increased the production of lipid peroxidation (LPO) in animals (Chang et al. 2006; Feng et al. 2008; Giordano et al. 2007). It has been suggested that LPOs and their related compounds in biological tissues induced by oxidative stress influence various metabolic pathways (Tanaka et al. 2002).

Crucian carp is an omnivorous freshwater fish native to China. Aquaculture of this fish in China has expanded rapidly because of its fast growth, use of natural foods, tender flesh, and high resistance to disease (Hu et al. 2012). However, very few reports are available concerning hepatic lipid metabolism in this fish subjected to trichlorfon exposure. The aim of the present study was to determine the effect of trichlorfon on lipid metabolism and lipid transport in crucian carp. Plasma lipids and hepatic lipid metabolic status were investigated. In addition, hepatocellular ultrastructure was observed by electron microscopy. The results will help in gaining valuable information on fish hepatic lipid accumulation and hepatic physiology when fish are exposed to chemical pesticides.

METHODS

Chemicals and fish.—Trichlorfon (>90% pure) was purchased from Shanghai Biochemical Reagent, Shanghai, China. Age of the trichlorfon used was 6 months. Prior to the experiments, the crucian carp (weight, 74.56 ± 0.02 g [mean ± SE] length, 17.03 ± 0.13 cm) were acclimatized to laboratory conditions (0.85 × 0.55 × 0.45 m) for 2 weeks.

Experimental design.—After acclimation, 75 fish were divided into five groups and for each group the experiment was conducted in triplicate (three tanks per group). Each tank contained five fish. One of these groups was maintained in natural tap water and used as a control. The experimental aquaria were aerated, and chlorine concentrations in the water were measured daily and were below 0.05 mg/L. Fish in the other aquaria were exposed to 0.5, 1.0, 2.0, and 4.0 mg/L of trichlorfon. A concentration of 4.0 mg/L was 10% of the concentration lethal to 50% of a test population (LC50) in 96 h for crucian carp. The water was changed every 12 h during the first 24-h exposure period to avoid adsorption on exposed surfaces; the water was then changed daily until the end of the experiments (Tessier et al. 2000). The renewed water contained nominal exposure concentrations to ensure the desired concentrations of trichlorfon in the water. The half-life time of trichlorfon in water was 2.5 d, and the dissipation time of 95% of trichlorfon in water was 10.2 d (Lopes et al. 2006).

Fish were fed the designated diet at a ration of 3% wet body mass, twice daily for 30 d (Mattson et al. 1988; Shakoori et al. 1996; Ibrahim and El-Gamal 2003), and no fish mortality occurred during these exposures. Diet formulation and chemical composition are shown in Table 1. Remaining food was removed after feeding. During the feeding experiment, fish were reared under the following water quality conditions: pH of 7.0–7.4, photoperiod of 14 h dark : 10 h light, total ammonia nitrogen of 0.02–0.04 mg/L, and temperature of 22–24°C.

Sample preparation.—At the end of the experimental period, two fish from each tank were caught randomly. Blood was taken from the caudal vein with 2-mL heparinized syringes (sodium heparin) and centrifuged at 1,160 × g for 15 min at 4°C to separate the plasma. The hepatic sample for biochemical analysis was fully excised, washed with cold saline (0.85% NaCl), and homogenized in 0.1 M tris-HCl buffer (1:9 w/v) using a glass homogenizer at 4°C. The homogenate was centrifuged at 2,370 × g for 10 min at 4°C, and the resultant supernatant was used as the enzyme source for the estimation of all enzyme activities. The hepatic samples for triglyceride contents and enzyme activities were carefully weighed and homogenized (1:10 dilution) in ice-cold buffer with a teflon pestle attached to a motor-driven tissue-cell disrupter. The homogenization buffer solution was 0.02 M tris–0.01 M phosphate, pH 7.0, in 50% (v/v) glycerol (Moró et al. 2010). The extract was later centrifuged at 2,500 rpm at 4°C for 10 min, and the supernatant was used as the enzyme source. The hepatic samples for enzyme-linked immunosorbent assay (ELISA) analysis were homogenated with 0.2 mL of 20 mM HEPES buffer containing protease inhibitor.
(phenylmethanesulfonyl fluoride, 1 mmol/L) (1:4 w/v) and disrupted using a glass tissue grinder. Homogenates were centrifuged at 9,500 × g for 10 min at 4°C, and the resulting supernatants were transferred to 0.5-mL conical tubes. All the preparations were stored at −80°C until biochemical determination. The hepatic sample for electron microscope observation was fixed with 0.25% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.2).

Biochemical analysis.—Plasma insulin was measured by radioimmunoassay (RIA) using bonito (bluefin tuna Thunnus thynnus) insulin as the standard and rabbit anti-bonito insulin as antiserum (Gutierrez et al. 1984) and expressed as µIU/mL. Briefly, 100 µL aliquots of each sample and standard were added to tubes coated on the inner surface with an insulin antibody following the procedure similar to a triiodothyronine assay except that the tracer solution was 125I-labeled insulin and samples were incubated for 2 h. Sample insulin levels were later determined according to the standard curve generated after a parallelism test (Li et al. 2012). Hepatic hormone-sensitive lipase (HSL) activity was assayed continuously by pH-stat titration of free fatty acid (FFA) release (Nilsson and Belfrage 1979) and expressed as ng/mg protein. Plasma and hepatic triglyceride contents were assayed by enzymatic procedures using an automatic biochemical analyzer (Hitachi 7170, Tokyo, Japan) and expressed as µg/mL (Souza et al. 2007). Total proteins in liver and plasma were determined with Coomassie Brilliant Blue G-250 staining according to the classical Bradford method (Bradford 1976). All detection kits were purchased from Nanjing Jian Cheng Biology Company, Nanjing, China. Hepatic cyclic adenosine 3′,5′-monophosphate (cAMP), very-low-density lipoprotein (VLDL), apolipoprotein B100 (apo B100) and HSL contents were determined by ELISA (Xu et al. 2009) using a double-antibody sandwich method. Detection kits were purchased from R&D Systems China, Shanghai, China. According to the manufacturer’s protocol, the optical density (OD) of each well was determined by using an ELISA reader at 450 nm and a cytochrome c calibration curve, and was expressed as ng/mg protein.

Hepatocyte morphological assay.—For transmission electron microscope (TEM) observations after treatment of fish with trichlorfon, the hepatic samples were fixed with 0.25% glutaraldehyde in PBS (pH 7.2) for 4 h at 25°C, then washed in cacodylate buffer and postfixed with 1% osmium tetroxide solution, dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon. Ultrathin sections were prepared, counterstained with 4% uranyl acetate and lead citrate (Shirpoor et al. 2009), and observed using an H-7650 TEM (Hitachi High-Technologies, Tokyo, Japan).

Statistical analysis.—Data are expressed as means ± SE. After testing for homogeneity of variance, statistical differences between the treatment and control groups were determined by a single-factor one-way ANOVA followed by least-significant-difference multiple comparison tests when variances were homogeneous. When there was variance heterogeneity, multiple pairwise comparisons were made using Tamhane’s T2 test. Values were calculated using SPSS 16.0 software. For all tests, the level of significance was set at P < 0.05.

RESULTS

Plasma and Hepatic Triglyceride and Plasma Insulin Contents

The concentration–response relationship for plasma and hepatic triglycerides are shown in Figure 1A, B. Plasma triglyceride contents were significantly (P < 0.05) decreased in fish exposed to 1.0, 2.0, and 4.0 mg/L trichlorfon compared with the control and 0.5 mg/L trichlorfon. In contrast, hepatic triglyceride content was significantly (P < 0.05) increased. Plasma insulin content is presented in Figure 1C. Compared with the control, significant (P < 0.05) increases in insulin content occurred in fish after treatments of 0.5 and 1.0 mg/L trichlorfon. In 2.0-mg/L treatments, plasma insulin contents were increased slightly (P > 0.05).

Hepatic VLDL, Apo B100, and cAMP Contents

As shown in Table 2, the concentration–response relationships of hepatic VLDL, apo B100, and cAMP were reduced in fish exposed to trichlorfon. Hepatic VLDL content was markedly (P < 0.05) decreased at 2.0 mg/L trichlorfon compared with the control. No significant (P > 0.05) differences in liver content of VLDL in fish treated with 0.5 and 1.0 mg/L trichlorfon were observed. Compared with the control, hepatic apo B100 contents tended (P > 0.05) to decrease when

| Trichlorfon treatment (mg/L) | VLDL content (ng/mg protein) | Apo B100 content (ng/mg protein) | cAMP content (ng/mg protein) |
|-----------------------------|------------------------------|---------------------------------|-----------------------------|
| 0                           | 1.69 ± 0.19 z                | 85.84 ± 11.8 z                  | 14.50 ± 1.59 z              |
| 0.5                         | 2.04 ± 0.72 zy               | 64.10 ± 13.0 zy                 | 11.51 ± 1.79 z              |
| 1                           | 1.64 ± 0.52 zy               | 70.47 ± 12.2 zy                 | 11.90 ± 2.97 zy             |
| 2                           | 0.98 ± 0.08 y                | 37.03 ± 2.84 y                  | 6.44 ± 1.08 y               |
| 4                           | 0.67 ± 0.24 z                | 64.21 ± 3.75 z                  | 4.10 ± 0.65 x               |

Table 2. Effect of trichlorfon on hepatic very-low-density lipoprotein (VLDL), apolipoprotein B100 (apo B100), and cyclic adenosine 3′,5′-monophosphate (cAMP) contents in crucian carp. Values (mean ± SE) in the same column sharing different letters denote results significantly different from control (P < 0.05).
FIGURE 1. Effects of trichlorfon on crucian carp: (A) plasma triglyceride (TG) content; (B) hepatic TG content; (C) plasma insulin (INS) content. Values are mean ± SE.
trichlorfon concentrations were 0.5, 1.0, and 4.0 mg/L. No significant differences in the apo B100 contents were found in fish treated with 0.5, 1.0, and 4.0 mg/L trichlorfon. Hepatic apo B100 content was significantly ($P < 0.05$) decreased in fish exposed to 2.0 mg/L trichlorfon compared with the control. Hepatic cAMP content was significantly ($P < 0.05$) decreased at 2.0 and 4.0 mg/L trichlorfon compared with the control.

**Hepatic HSL Content and Activity**

The changes in hepatic HSL content and activity are shown in Figure 2A, B. There were no significant differences in hepatic HSL content in fish between the trichlorfon treatments and the control (Figure 2A). In the low trichlorfon concentration (0.5 mg/L), no significant difference in HSL activity was observed compared with the control. However, HSL activities in fish from the other treatment groups (1.0, 2.0 and 4.0 mg/L) were not detected (Figure 2B).

**Hepatocellular Ultrastructure**

The hepatocellular ultrastructure of all fish groups is shown in Figure 3. Hepatocytes in control fish had a normal ultrastructure as seen in Figure 3A. In normal hepatocytes, cells had a spherical nucleus in the center. The cytomembrane was intact, and mitochondria and rough endoplasmic reticulum (RER) were abundant in the cytoplasm. Rough endoplasmic reticulum was continuous with the external nuclear membrane and was concentrated in the perinuclear region. After treatment with 0.5 mg/L trichlorfon, dilatation of the mitochondrial matrix was observed (Figure 3B). In the 1.0-mg/L trichlorfon treatment, mitochondria were vacuolated and mitochondrial cristae were lost (Figure 3C). In the 2.0-mg/L trichlorfon treatment, swollen mitochondria and RER dilatation were present (Figure 3D). At 4.0 mg/L trichlorfon, broken mitochondrial membranes, mitochondrial vacuolization, loss of cytoplasm, and pyknotic nuclei were observed (Figure 3E).

**DISCUSSION**

Fish liver plays a vital role in lipid metabolism (Michalopoulos 2007). Nearly all the reactions related to lipid metabolism and lipid transport occur in the liver (Fabbrini et al. 2010). Hence, lipid accumulation occurs mainly in the liver. The aim of the present study was to determine the effect of trichlorfon on lipid metabolism and lipid transport in crucian carp. The results showed that crucian carp hepatic concentration of triglycerides increased, and hepatic HSL activity and hepatic cAMP, VLDL and apo B100 contents decreased in trichlorfon-exposure treatments. The results suggest that trichlorfon influenced hepatic pathways of lipid metabolism in crucian carp.

It is well known that the accumulation of lipid in hepatocytes represents a complex interaction, which includes a balance between triglyceride synthesis (lipogenesis), hydrolysis (lipolysis), and transport (Fabbrini et al. 2010). During these processes, both metabolic enzyme activities and the formation of lipoprotein influence lipid deposition. Hepatic lipolysis is a risk factor for lipid accumulation in liver. Westerbacka et al. (2007) found that gene expression of hepatic lipase and lipoprotein lipase (LPL) resulted in the release of FFAs from lipolysis of circulating triglyceride, which contributed to hepatoabdominal FFA accumulation. Triglycerides are hydrolyzed by cyclic AMP-regulated lipases in the mitochondrial matrix (Yeaman 1990) in liver. Hormone-sensitive lipase is a lipolysis rate-limiting enzyme in lipid metabolism and mobilizes triglyceride and cholesterol ester stores in several tissues (Yeaman 2004). In this experiment, HSL activities were inhibited significantly, and in the 1.0-, 2.0-, and 4.0-mg/L trichlorfon treatments, the activities were not detected in liver. However, there was no change in the HSL content in liver. The results illustrated that phosphorylation of HSL was inhibited, resulting in a decrease in HSL activity.

Hormone-sensitive lipase activity is triggered by the hormones epinephrine, norepinephrine, glucagon, and adrenocorticotropic hormone, and is transduced through the cAMP signaling pathway (Aboulach et al. 2006). An increased level of cAMP stimulates protein kinase A, which activates the HSL by phosphorylating it (Berg et al. 2002). In contrast, insulin can inhibit cAMP synthesis, which results in phosphorylation of HSL degradation (Aboulach et al. 2006). In addition to influencing HSL activity, insulin also activates lipoprotein lipases, promotes the entry of triglyceride into hepatocytes, and increases lipid deposition (Hillgartner et al. 1995; Capilla et al. 2003). Insulin may also induce zymoprotein synthesis, increase the related lipase activity, and increase lipid synthesis (Sessler et al. 1996). In our study, plasma insulin concentration increased in fish from all trichlorfon exposure treatments. This resulted in a decrease in hepatic cAMP concentration followed by inhibition of HSL activity. However, the precise biological action in trichlorfon-induced disorders of insulin is unclear. Hong et al. (2007) studied that trichlorfon was involved in hormonal disruption, inhibited cAMP, protein kinase (PKA), follicle-stimulating hormone, and subsequent reproductive dysfunctions.

Additionally, hepatic lipid accumulation is also caused by an inability to form the lipoproteins responsible for transporting lipids out of the liver. Lipid must be transported through the blood. Water-insoluble triglycerides depend on apolipoprotein to be packaged into lipoprotein transport particles, and then re-exported to other tissues or stored in adipose tissue (Fabbrini et al. 2010). Very-low-density lipoproteins are complex lipoprotein particles that are produced by the liver and secreted into the systemic circulation. These particles are mainly composed of triglyceride and apo B100 (Stryer 2002). Therefore, the quantities of apolipoprotein and VLDL present are associated with lipid transport (Liang et al. 2002). Related research has also indicated that fatty liver is associated with the change in apolipoprotein quantity (Fabbrini et al. 2008). Our results showed that hepatic apo B100 content decreased with increasing trichlorfon concentration, which resulted in a decrease in the hepatic VLDL content. Hepatic triglyceride cannot then be transported from the liver and is accumulated in the liver.
Ibrahim and El-Gamal (2003) determined that diazinon may interfere with lipid metabolism in mammals. They found that the high-density lipoprotein cholesterol (HDL-C) and phospholipids (PL) were decreased, but the low-density lipoprotein cholesterol (LDL-C) and triglyceride were increased.

A recent study showed that increased reactive oxygen species (ROS) led to DNA damage and generalized oxidative damage in all mitochondrial components, e.g., oxidative mitochondrial DNA (mtDNA) damage (Franco et al. 2009). In our previous study, trichlorfon induced hepatocyte apoptosis and caused...
FIGURE 3. Transmission electron microscope (TEM) images of crucian carp hepatocyte structural organization after trichlorfon treatment. (A) Control cells, nucleus (N), mitochondria (Mi), and rough endoplasmic reticulum (RER) in cytoplasm (TEM, bar = 1 μm); (B) 0.5 mg/L trichlorfon treatment, vacuolization of mitochondria (arrow up) (TEM, bar = 500 nm); (C) 1.0 mg/L trichlorfon treatment, vacuolization of mitochondria (arrow up) (TEM, bar = 1 μm); (D) 2.0 mg/L trichlorfon treatment, swelling of the mitochondria (arrow up), dilatation of RER (arrow right) (TEM, bar = 1 μm); (E) 4.0 mg/L trichlorfon treatment, vacuolization of mitochondria (arrow up), loss of cytoplasm (arrow down), and pyknotic nuclei (arrow right) (TEM, bar = 1 μm).
mitochondrial vacuolization and lipid droplet accumulation in the hepatocytes of crucian carp in vitro (Xu et al. 2009). In Xu et al. (2009), dilatation of the mitochondrial matrix, mitochondrial vacuolization, RER dilatation, and pyknotic nuclei were present, which might be caused by LPO. When hepatic ROS production exceeds the antioxidant defense capacity of the cell, increased ROS leads to lipid peroxidations and protein oxidations. These peroxidations cannot be hydrolyzed by lipases and are accumulated in hepatocytes, subsequently leading to changes in hepatocyte ultrastructure, such as cytomembrane breakage, mitochondrial vacuolization, dilatation of the RER, and nuclei pyknotion (Xu et al. 2009). Mataqueiro et al. (2009) studied that trichlorfon could cause fish (pacu Piaractus mesopotamicus) hepatocyte fusion and loss of normal cellular shape. With trichlorfon concentration increase, there were also necrotic hepatic cells with pyknotic nuclei and decreased cytoplasmatic affinity for eosin, and the liver changes were more severe with the passage of time. The same lesions were described in the liver of curimbátia Prochilodus lineatus (Rodrigues et al. 2001) exposed to trichlorfon, 0.2 µL/L and in Callychthidae (peppered corydoras Corydoras paleatus) (Fanta et al. 2003) exposed to methyl parathion (0.58 µL/L). The histological alterations in crucian carp livers suggest that the fish may face a metabolic crisis caused by tissue damage. Hepatocellular ultrastructure damage could disturb cellular function and also be associated with apo B100 expression level, which could result in a reduction in apo B100 content. Clinical studies indirectly support the fact that oxidant stress plays a substantial role in regulation of the output of apo B100 from hepatocytes (Pan et al. 2004). Karami-Mohajeri and Abdollahi (2011) reviewed the effect of pesticides, including organochlorine and carbamate compounds, on metabolic disorders and the underlying mechanism. Results indicated that organophosphorus impairs the enzymatic pathways involved in metabolism of carbohydrates, fats, and protein within cytoplasm, mitochondria, and prooximes. Organochlorines mostly affect lipid metabolism in the adipose tissues and change glucose pathway in other cells. As a shared mechanism, all organophosphorus, organochlorine, and carbamate compounds induce cellular oxidative stress via affecting mitochondrial function and therefore disrupt the neuronal and hormonal status of the body. However, the precise biological action and molecular mechanism of organophosphorus in fish lipid metabolism are still unclear and need to be elucidated in further studies.

CONCLUSION

Trichlorfon influences hepatic pathways of metabolism and transportation and the ultrastructure of hepatocytes in crucian carp, which results in lipid accumulation in the liver. The dosage of trichlorfon used to eradicate ectoparasites varies from 0.1 to 1.0 mg/L in ponds, and the findings of this study show that lipid metabolism disorders can occur in crucian carp as a result of long-term exposure to low concentrations of trichlorfon in residual water.

ACKNOWLEDGMENTS

The study was supported by the Earmarked Fund for Modern Agro-industry Technology Research System of China (CARS-46-20).

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