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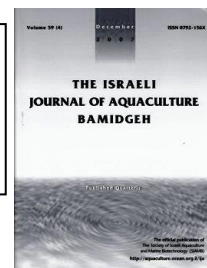**The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)**

Effects of Acute Copper Exposure on Physiological and Cytological Responses in Liver of GIFT Tilapia (*Oreochromis niloticus*)

Deng-Hang Yu[1,2], Jia-Zhi Chang[1], Gui-Fang Dong[1,2], Jun Liu[1,2]*

[1] *Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, Wuhan, 430023*

[2] *Freshwater Aquaculture Collaborative Innovation Center in Hubei Province, Wuhan, 430070*

Abstract

This study was designed to evaluate the physiological and cytological responses in liver of GIFT tilapia (*Oreochromis niloticus*) to acute copper exposure. Fish were exposed to three Cu treatments at the following concentrations: 0 (control), 1.96 mg/L and 3.92 mg/L, and liver samples were collected after 96h of exposure. Observation of oil red O staining showed that acute copper exposure increased lipid accumulation in liver, which may due to changes of hepatic enzyme activities (malic enzyme, lipoprotein lipase, hepatic lipase, fatty acid synthetase, carnitine palmitoyltransferases l) and lesions of some organelles such as mitochondria and swollen rough endoplasmic reticulum of hepatocytes. This study indicated that acute copper exposure induced hepatic lipid metabolic disturbances and damaged hepatic organelle in GIFT tilapia.

Deng-Hang Yu and Jia-zhi Chang contributed equally to this work.
* Corresponding author: Tel: +862783956175, Fax: +862783956175,
e-mail: junliu0601@163.com

Introduction

Water pollution has become an important factor that impacts negatively the development of aquaculture (Liu et al., 2010). Polluted water in fish ponds usually contains high concentrations of heavy metals, such as copper (Cu), which comes from industrial waste water, or utilization for pond disinfection (Liu and Yang 2009). Copper is an essential trace element and is of benefit to fish for some physiological functions (Watanabe et al., 1997), however high concentrations of copper usually result in toxic effects on aquatic animals (Boyd and Massaut 1999; Liu et al., 2010).

Histological investigation is an efficient tool to evaluate the effects of toxicants on target organs of fish (Capkin et al., 2009). The liver in fish plays an important role in lipid metabolism, including both synthesis and degradation of fatty acids. It is also a sensitive organ reflecting toxic effects of copper exposure in fish. Many studies have shown that copper exposure can induce a variety of histological lesions such as nucleus pyknosis, cytoplasmic dissolution, as well as cell necrosis in the liver. Copper exposure disturbs lipid metabolism by affecting hepatic enzyme activity (Figueiredo-Fernandes et al., 2007; Liu et al., 2010; Chen et al., 2013). Thus, the liver may be an appropriate organ for evaluating toxic effects of copper in fish.

Genetically improved farmed tilapia (GIFT), is popular due to its rapid growth, tasty flesh, and high adaptability (Li et al., 2010). However, information about the effects of copper exposure, especially the toxic effects on ultrastructure and lipid metabolism in the liver of this species is limited.

The objective of this study was to investigate histological and ultrastructural changes as well as several key enzymatic activities in the liver of GIFT tilapia after acute copper exposure.

Materials and Methods

Two experiments examining copper exposure were conducted. One was conducted with acute copper toxicity in GIFT tilapia, to determine the median lethal concentration (LC_{50}) after 96h, another was conducted to evaluate effects of acute copper exposure on histology, ultrastructure, and enzymatic activities related to lipid metabolism in the liver of these fish. For both experiments, copper was added as CuSO4. Stock solutions were prepared by mixing CuSO4 with distilled water and different test doses were prepared.

In experiment 1, prior to copper exposure, uniform sized GIFT tilapia (16.8 ± 0.6 g,) were collected from a tilapia breeding base (Yingshan, Hubei, China) and acclimated to experimental water conditions for 48 h in fiberglass tanks containing 50 L water. In our preliminary trials, fish were exposed to 10 different concentrations of copper (0.4, 0.6, 0.9, 1.35, 2.03, 3.04, 4.56, 6.83, 10.25 and 15.38, mg/L). During the preliminary trials fish were not fed during the acclimation and testing period. Fish mortality induced by the various concentrations was recorded. Based on the results, a series of concentrations were selected for the final acute toxicity trial (0.9, 1.35, 2.03, 3.04, 4.56 and 6.83 mg/L). 100% mortality occurred in the highest concentration and no mortality occurred in the lowest concentration. The control group with no copper added, ran simultaneously for all concentrations with 10 fish each for 96 hours. During the 96 h experiment, water was aerated continuously and each test solution was renewed daily. Fish mortality was monitored at logarithmic time intervals (24, 48, 72 and 96h) of exposure.

Experiment 2 which ran for 96 h, was designed according to Ahmed et al. (2013) and Zheng et al. (2013) with some modifications. The LC_{50} for fish exposed to three copper treatments was 0 (control), 1.96, and 3.92 mg/L, respectively. All treatments were carried out in triplicate, with 15 fish per replicate. During the experimental period, water was aerated continuously, and each test solution was renewed daily to maintain the concentration of the toxicant. During the experimental period, water temperature was maintained at 28°C, dissolved oxygen was 6.48 ± 0.3 mg/L, and pH was 7.2.

At the end of experiment 2, fish from each tank were randomly selected and dissected in ice to retrieve the liver. For enzymatic analysis, these were removed with sterile forceps and stored at -80ºC for subsequent analysis. For ultrastructural observation, livers were diced into 1mm^3 pieces, fixed in 2.5% glutaraldehyde solution, and prepared for transmission electron microscopic (TEM) analysis. For histological observation, the liver samples were sliced into 3 mm thick slabs, fixed in 10% neutral buffered formalin, and prepared for histological analysis. For hepatic lipid observation, they were frozen in cold anhydrous isopropanol (-85 °C) and maintained at -80°C until oil red O staining.

For hepatic enzyme analysis, liver samples were homogenized in ice-cold (0.65%) physiological saline using a tissue homogenizer. The homogenates were centrifuged for 15 min at -4°C. Supernatant was used to determine the activities of malic enzyme (ME), isocitrate dehydrogenase (ICDH), lipoportein lipase (LPL), hepatic lipase (HL), and fatty acid synthetase (FAS). ME activity was determined according to the method described by Wise and Ball (1964). ICDH activity was determined according to the method described by Bernt and Bergmeyer (1974). LPL and HL activities were determined according to the method described by Ballart et al. (2003) and Burgaya et al., (1989). FAS activity was measured according to the method of Chang et al. (1967) as modified by Chakrabarty and Leveille (1969). Carnitine palmitoyltransferases l (CPT-l) activity was analyzed using Elisa CPT-l assay kit for fish (No. ml036411, Mlbio biotechnology Ltd., Shanghai, China). All enzyme activities were expressed as mU per mg of soluble protein. Soluble protein content of liver homogenates was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Ultrastructural changes were determined according to the method described by (Dong et al., 2012) and examined under a FEI Tecnai (G2 F20 S-TWIN, Eindhoven, Netherlands) TEM. Liver samples were fixed for 48h in 10% neutral buffered formalin. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin. 6 µm thick sections were cut and stained with hematoxylin and eosin, and then prepared for light microscopy. Histological changes induced by treatments were photographed using photomicroscope (Olympus BX41, Japan). Frozen liver was cut on a cryostat microtome, and 8µm liver sections were stained with oil red O staining and prepared for light microscopy, according to Spisni et al. (1998).

Results are presented as mean ± SD. Data were subjected to one-way ANOVA and Duncan's multiple range tests. Difference was considered significant at $P < 0.05$. All statistical analyses were performed using the SPSS 16.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA).

Results

The data in Fig. 1 shows the average percentages of the cumulative mortality at different concentrations of copper after 96 h of exposure. 20% mortality occurred in 0.9 mg/L of copper whereas 30, 50, 70, 80 and 100% mortality were observed in 1.35, 2.03, 3.04, 4.56 and 6.83 mg/L respectively. The 96 h LC_{50} value of copper was calculated as 1.96 mg/L (Fig. 2).
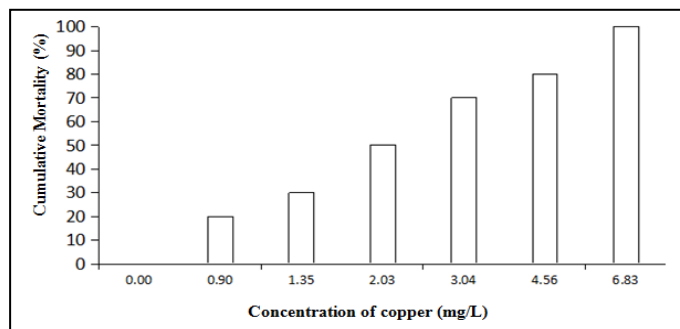

Fig.1. Cumulative mortality (%) of GIFT tilapia at different concentrations of copper after 96 h exposure time.
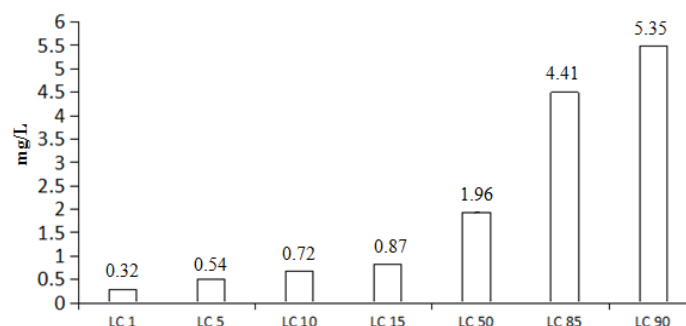

Fig.2. The LC_{50} value of copper in GIFT tilapia after 96 h exposure was 1.96 mg/L as determined by probit analysis (95% confidence limit).

Effects of copper exposure on several hepatic enzyme activities are shown in Fig. 3. After 96h exposure, FAS and CPT-l activities increased with increasing copper concentration and were significantly higher than in the control group (P<0.05). In contrast, ME and LPL activities significantly decreased with increasing copper concentration (P<0.05). ICDH and HL activities were not significantly different between the experimental groups after 96h copper exposure, although HL had an obviously decreasing trend (P>0.05).
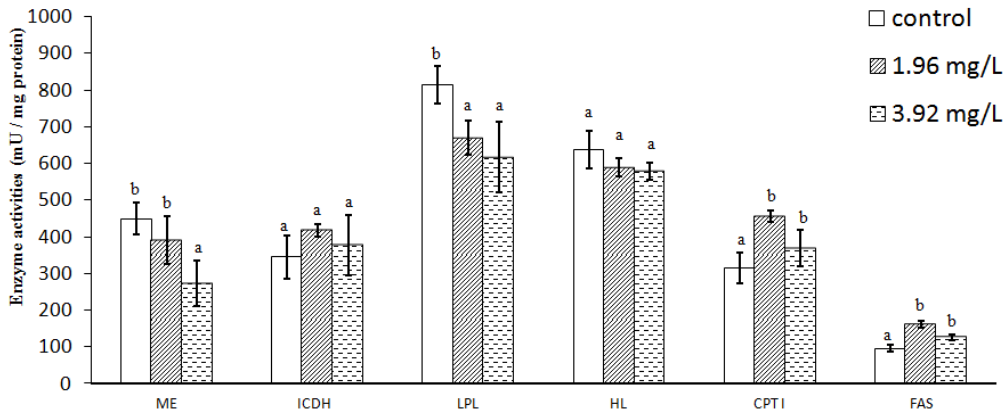

Fig.3. Effect of waterborne copper exposure on several hepatic enzymes activities of GIFT tilapia after 96h exposure. Values are expressed as mean±SD (n=3). Different letters indicated significant differences between the treatment and control groups.

Compared to the control group (Fig. 4A), oil red O staining indicated extensive lipid droplets in livers of fish exposed 1.96mg/L (Fig. 4B) and 3.92 mg/L (Fig. 4C) after 96 h. The area of lipid droplets increased in relation to increased copper concentrations.
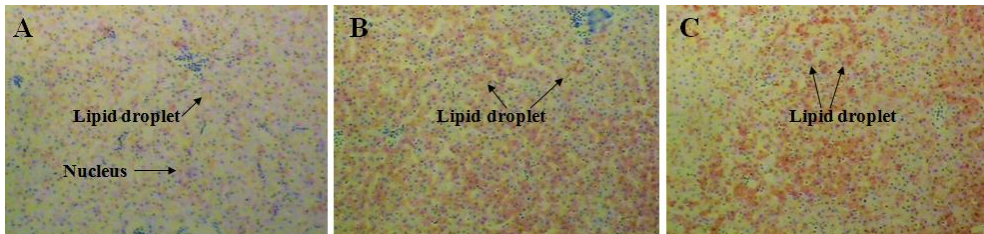
Fig.4. Light micrographs comparing intracellular lipid deposition in liver of GIFT tilapia at different treatments of copper for 96h. Control (A), 1.96 mg/L (B), and 3.92 mg/L (C). Lipid was red-colored and nucleus-blue colored after staining with oil red O. The depth of color of the red stain and the size of the lipid droplets were positively correlated with lipid content.

The hepatic parenchyma of fish exposed to waterborne copper showed hepatocellular necrosis and increased vacuolation (Fig. 5B, C), compared to the control group (Fig. 5A). In liver sections derived from the control fish, normal nucleus, intact mitochondria, and clear stacks of rough endoplasmic reticulum were observed (Fig. 6A). In the group of 1.96 mg/L copper concentration (Fig. 6B), vacuolated mitochondria (VM) and swollen rough endoplasmic reticulum were observed. When copper concentration increased to 3.92 mg/L (Fig. 6C), vacuolated mitochondria increased and lipid droplets (LDS) were observed.
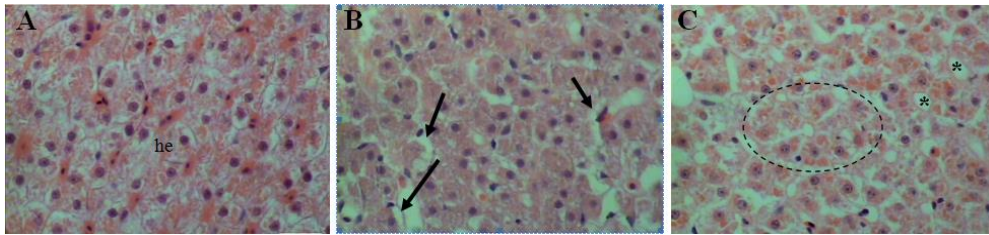
Fig.5. Liver histology of GIFT tilapia in control and copper exposed groups. (A) (× 400): Control group shows normal hepatocytes (he); (B) (× 400): liver of fish exposed to 1.96 mg/L copper, shows necrosis areas (black arrows). (C) (× 400): liver of fish exposed to 3.92 mg/L copper, shows a prominent necrosis area (circle) and vacuolation: vacuoles (*) clearly visible as white unstained areas within the hepatic cells caused by lipid accumulation.
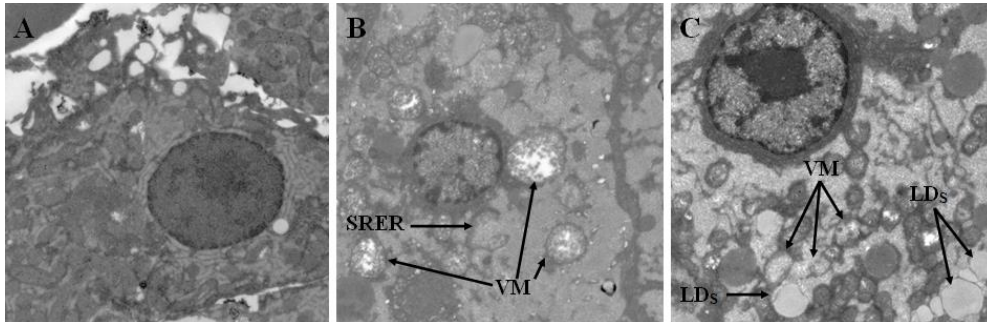
Fig.6. Hepatic ultra-structural changes in GIFT tilapia at different treatments of copper for 96 h (2500×). (A) Regular nucleus and sub-cellular organelles of control fish; (B) at 1.96 mg/L exposure: presence of vacuolated mitochondria (VM) and swollen rough endoplasmic reticulum (SRER); (C) at 3.92 mg/L exposure: presence of extensive lipid droplets (LDS) and abundant VM.

Discussion

In the present study, after 96h, LC_{50} of waterborne copper for GIFT tilapia was 1.96 mg/L, a little higher than results in other tilapia species which were reported to be 1.7 mg/L (de Vera and Pocsidio 1998), and 1.5 mg/L (Lam et al., 1998). This was obviously higher than in freshwater carp which was 0.53 mg/L (Shariff et al., 2001). The difference in copper toxicity might be due to the difference in sensitivity to copper which depends on the homeostatic regulation of copper (uptake, storage and excretion) in different species (Depledge and Rainbow 1990). The results found in this study indicated that GIFT tilapia was a more copper-tolerant species.

The enzymatic activities related to lipid metabolism in the liver were affected by copper exposure in this study. Copper exposure decreased LPL and HL activities, which were two key enzymes that participated in chylomicron and lipoprotein metabolism in the liver (Fielding and Frayn 1998; Mead et al., 2002; Santamarina-Fojo et al., 1998). This indicated that copper concentration was related to the derangement of lipid metabolism and led to lipid accumulation. Similar results were found in a study on *Synechogobius hasta* after copper exposure (Liu et al., 2010). In the present study, FAS activity, which played a key role on de novo fatty acid synthesis (Cowey and Walton 1989), significantly increased and further promoted lipid accumulation in the livers of copper-exposed fish. ME was related in the catalase production of NADPH, which is the sole hydrogen provider in hepatic fatty acid biosynthesis (Wang et al., 2005), and decreased significantly with increasing copper concentration. We speculated that decreased ME activity may be a physiological response to reduce the lipogenic rate and consequently against lipid accumulation in the liver. ME activity decreased but not significantly when there was low concentration copper exposure on *Synechogobius hasta* for 15 days (Chen et al. 2013), however, when the exposure period extended to 30 days, ME activity significantly increased. This suggests that the effect of copper exposure alone on ME activity not only depended on the copper concentration but also on the exposure period. CPT-l is a rate-limiting enzyme in fatty acids β-oxidation and the key regulatory factor of long-chain fatty acid oxidation (Morash et al., 2008). This increased significantly and was different than results reported in the study by Chen et al. (2013). This difference may be due to

the copper concentration that could damage the organelles differently. In another study, low copper concentrations (7.5% and 15% LC_{50}) were selected however the way in which these concentrations could damage the organelle were not examined (Chen et al. 2013). In the present study, observation of hepatic cell ultrastructure by TEM indicated that high copper exposure around twice the LC_{50} level, induced lesions of mitochondrion. Therefore, CPT-l activity in fish increased to enhance transferability of lipids into mitochondrion. In our results, we found that acute copper exposure affects hepatic lipid metabolic enzyme activities, and comprehensively induces lipid accumulation in the liver. This phenomenon was further supported by results of oil red O staining in the liver; the results were similar to those reported with other fish species (Liu et al., 2010; Chen et al., 2013).

Studies concerning ultrastructural changes in the liver of fish after copper exposure, are limited. In the present study, the ultrastructure of the liver in copper-exposed fish showed various changes; mitochondrial vacuolation was observed in fish exposed to 1.96 copper. Mitochondria vacuolation, caused by hydrogen peroxide-induced inhibitors, disrupts the metabolic rate of hepatocytes due to the insufficient supply of energy (Cheville 1994). Further exposure to higher copper concentrations up to 3.92 mg/L resulted in accumulative and irreversible damage to the mitochondria and the disruption of ATP synthesis (Cheville 1994). Some other changes observed in copper-exposed fish, such as swollen rough endoplasmic reticulum, indicated that hepatocytes activated the self-defense mechanism, whereas endoplasmic reticulum dilatation indicated that the liver was in the process of degeneration (Braunbeck 1998). Similarly, there are many studies indicating that waterborne pollutants could cause considerable ultrastructural alterations in the liver of other fish species (Li et al., 2004; Li et al., 2007; Liu et al., 2011).

For many cells, cellular energy is stored in the form of triacylglycerols with lipid droplets that serve as energy storehouses (Farese and Walther 2009). In the present study, the lipid droplets in the hepatocyte of copper-exposed fish also increased. On some occasions, excessive lipid accumulation may exceed the cell capacity and result in dysfunction (Farese and Walther 2009). The lipid droplets observed in the 3.92 mg/L copper group in the present study could attribute to the decline in protein synthesis accompanying cellular lesion, which blocked the utilization of lipids for lipid, protein conjugation (Cheville 1994).

In conclusion, this study demonstrated that acute exposure to copper led to ultrastructural lesions and derangement of lipid metabolism in liver of GIFT tilapia, by affecting the hepatic enzyme activities and injuring hepatic organelles.

Acknowledgments

References

Ahmed M., Habibullah-Al-Mamun M., Parvin E., Akter MS., Khan MS., 2013. Arsenic induced toxicity and histopathological changes in gill and liver tissue of freshwater fish, tilapia (*Oreochromis mossambicus*). *Exp Toxicol Pathol.* 65, 903-294.

Ballart X., Siches M., Peinado-Onsurbe J., Lopez-Tejero D., Llobera M., Ramirez I., Robert MQ., 2003. Isoproterenol increases active lipoportein lipase in adipocyte medium and in rat plasma. *Biochimie* 85, 971-982.

Bernt E., Bergmeyer HU., 1974. Isocitrate dehydrogenase. In: Bergmeyer HU (ed) *Methods of enzymatic analysis,* vol 2., Academic New York, NY, pp. 624-627.

Boyd CE., Massaut L., 1999. Risks associated with the use of chemicals in pond aquaculture. *Aquacult Eng.* 20, 113-132.

Bradford MM., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72, 248-254.

Braunbeck T., 1998. Cytological alterations in fish hepatocytes following in vivo and in vitro sublethal exposure to xenobiotics-structural biomarkers of environmental contamination. In: Fish Ecotoxicology (Braunbeck, T., Hinton, D.E. and Streit, B. eds), pp, 61-140.

Burgaya F., Peinado J., Llobera M., Ramirez I., 1989. Hepatic endothelial lipase activity in neonatal rat liver. *Bioscience Rep*. 9, 559-564.

Capkin E., Birincioglu S., Altinok I., 2009. Histopathological changes in rainbow trout (*Oncorhynchus mykiss*) after exposure to sublethal composite nitrogen fertilizers. *Ecotoxicol Environ Saf.* 72, 1999-2004.

Chen QL., Luo Z., Liu X., Song YF., Liu CX., Zheng JL., Zhao YH., 2013. Effects of waterborne chronic copper exposure on hepatic lipid metabolism and metal-element composition in *Synechogobius hasta*. *Arch Environ Contam Toxicol*. 64, 301-315.

Chakrabarty K., Leveille GA., 1969. Acetyl CoA carboxylase and fattyacid synthetase activities in liver and adipose tissue of meal-fedrats. *Exp Biol Med.* 131, 1051-1054.

Chang HC., Seidman I., Teebor G., Lane MD., 1967. Liver acetyl CoA carboxylase and fatty acid synthetase: relative activities in the normal state and in hereditary obesity. *Biochem Biophys Res Commun*. 28, 682-686.

Cheville NF., 1994. Ultrastructural Pathology: An Introduction to Interpretation, 1st edn, pp, 67-68.

Cowey CB., Walton MJ., 1989. Intermediary metabolism. In: Halver JE (ed) *Fish Nutr.*, 1st edn. Academic, New York, pp, 259-329.

Depledge MH., Rainbow PS., 1990 Models of regulation and accumulation of trace metals in marine invertebrates. *Comp Biochem Physiol* C. 97, 1-7.

de Vera MP., Pocsidio GN., 1998. Potential protective effect of calcium carbonate as limiting agent against copper toxicity in the African tilapia *Oreochromis niloticus*. *Sci Total Environ*. 214, 193-202.

Dong GF., Huang F., Zhu XM., Zhang L., Mei MX., Hu QW., Liu HY., 2012. Nutri-physiological and cytological responses of juvenile channel catfish (*Ictalurus punctatus*) to dietary oxidized fish oil. *Aquacult Nutr.* 18, 673-684.

Farese RV Jr., Walther TC., 2009 Lipid droplets finally get a little R-E-S-P-E-C-T. *Cell* 139, 855-860.

Fielding BA., Frayn KN., 1998. Lipoprotein lipase and the disposition of dietary fatty acids. *Brit J Nutr.* 80, 495-502.

Figueiredo-Fernandes A., Ferreira-Cardoso JV., Garcia-Santos S., Monteiro SM., Carrola J., Matos P., Fontaínhas-Fernandes A., 2007. Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. *Pesq Vet Bras*. 27(3), 103-109.

Lam KC., Ko PW., Wong JKY., Chan KM., 1998. Metal toxicity and metallothionein gene expression studies in common carp and tilapia. *Mar Environ Res.* 46, 563-566.

Li L., Xie P., Li S., Qiu T., Guo L., 2007. Sequential ultrastructural and biochemical changes induced in vivo by the hepatotoxic microcystins in liver of the phytoplanktivorous silver carp *Hypophthalmichthys molitrix*. *Comp Biochem Physiol C.* 146, 357-367.

Li SF., Tang SJ., Cai WQ., 2010. RAPD-SCAR markers for genetically improved NEW GIFT Nile tilapia (*Oreochromis niloticus* L.) and their application in strain identification. *Zool Res.* 31, 147-153.

Li XY., Chung IK., Kim JI., Lee JA., 2004. Subchronic oral toxicity of microcystin in common carp (*Cyprinus carpio* L.) exposed to Microcystis under laboratory conditions. *Toxicon* 44, 821-827.

Liu, J., Yang, G., 2009. Changes in Copper Content of Allogynogenetic Silver Crucian Carp after Application of Copper Sulfate to Fishponds. *Isr. J. Aquacult.-Bamidgeh, AquacultureHub*, 61(4),351-355.

Liu XJ., Luo Z., Xiong BX., Liu X., Zhao YH., Hu GF., Lv GJ., 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. *Ecotoxicol Environ Saf.* 73, 1286-1291.

Liu XJ., Luo Z., Li CH., Xiong BX., Zhao YH., Li XD., 2011. Antioxidant responses, hepatic intermediary metabolism, histology and ultrastructure in *Synechogobius hasta* exposed to waterborne cadmium. *Ecotoxicol Environ Saf.* 74, 1156-1163.

Mead JR., Irvine SA., Ramji DP., 2002. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med*. 80, 753-769.

Morash AJ., Kajimura M., McClelland GB., 2008. Intertissue regulation of carnitine palmitoyltransferase l (CPT-l): mitochondrial membrane properties and gene expression in rainbow trout (*Oncorhynchus mykiss*). *Biochim Biophys Acta*. 1778, 1382-1389.

Santamarina-Fojo S., Haudenschild C., Amar M., 1998. The role of hepatic lipase in lipoprotein metabolism and atherosclerosis. *Curr Opin Lipidol.* 9, 211-219.

Shariff M., Jayawardena PAHL., Yusoff FM., Subashinghe R., 2001. Immunological parameters of Javanese carp *Puntius gonionotus* (Bleeker) exposed to copper and challenged with Aeromonas hydrophila. *Fish Shellfish Immunol*. 11, 281-291.

Spisni E., Tugnoli M., Ponticelli A., Mordenti T., Tomasi V., 1998. Hepatic steatosis in artificially fed marine teleosts. *J Fish Dis*. 21,177-184.

Wang JT., Liu YJ., Tian LX., Mai KS., Du ZY., Wang Y., Yang HJ., 2005. Effect of dietary lipid level on growth performance, lipid deposition, hepatic lipogenesis in juvenile cobia (*Rachycentron canadum*). *Aquaculture* 249, 439-447.

Watanabe T., Kiron V., Satoh S., 1997. Trace minerals in fish nutrition. *Aquaculture* 151, 185-207.

Wise EM., Ball EG., 1964. Malic enzyme and lipogenesis. Proc Natl Acad Sci. 52, 1255-1263.

Zheng JL., Luo Z., Liu CX., Chen QL., Tan XY., Zhu QL., Gong Y., 2013. Differential effects of acute and chronic zinc (Zn) exposure on hepatic lipid deposition and metabolism in yellow catfish *Pelteobagrus fulvidraco*. *Aquat Toxicol.* 132-133, 173-181.