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Comparison of Histopathological Alterations Due to Sublethal CCl_4 on Rosy Barb (*Puntius conchonius*) and Amphioxus (*Branchiostoma belcheri*) with Implications of Liver Ontogeny

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ABSTRACT This study was undertaken to examine the histopathological effects of CCl_4 on rosy barbs and amphioxus with an aim to compare the homology between rosy barb liver and amphioxus digestive caecum. It was found that the 96 h LC_{50} values were 23.9 ± 4 mg/L and 18.9 ± 2 mg/L for rosy barbs and amphioxus, respectively. Histological examinations showed that exposure to sublethal CCl_4 caused damage to the liver, kidney, and gill in rosy barb, and to the digestive caecum and gill in amphioxus. It is clear that both rosy barb liver and amphioxus digestive caecum were the prominent target organs of CCl_4, suggesting that the digestive caecum in amphioxus is homologous to the liver in rosy barb at least in respect to toxic damages of CCl_4.

KEYWORDS Amphioxus; Carbon tetrachloride; Hepatic diverticulum; Liver, Rosy barb; Toxicity

INTRODUCTION

Liver is unique to the subphylum Vertebrata and varies little among the classes. It is the largest organ of the body, occupies a strategic position between the intestinal tract and the rest of the body, and plays a crucial role in maintaining metabolic homeostasis. Its functions include the processing of dietary amino acids, carbohydrates, lipids, and vitamins; phagocytosis of particulate materials in the portal circulation; synthesis of serum proteins; biotransformation of circulating metabolites; and detoxification and excretion of endogenous waste products and pollutant xenobiotics into the bile (Crawford 1999).

Various substances are known to cause liver damage, and one of them is carbon tetrachloride (CCl_4), which is a well-known hepatotoxin (Cassillas and Ames 1986; Kotsanis and Metcalfe 1991; Thrall et al. 2000). Within the body, CCl_4 breaks down to highly toxic trichloromethyl (CCl_3) and trichloromethyl peroxyl (CCl_3O_2) free radicals by cytochrome P450 enzyme and causes damage to hepatocytes (Abraham et al. 1999; Ohta et al. 2000). Short- and long-term exposure to CCl_4 also causes damage to the skin, brain, and blood, and in some cases results in death. Besides mammals (Sundari et al. 1997; Abraham et al. 1999; Ogeturk et al. 2005), CCl_4-induced damages have been documented in non-mammalian vertebrates such as bird (Fernandez et al. 1984) and teleosts including rainbow trout (Kotsanis and Metcalfe 1991) and tilapia (Chen et al. 2004).

It has been found that CCl_4 administration results in centrolobular necrosis of mammalian liver (Rouiller 1964). Research showed that necrotic hepatocytes were seen around central zones of each lobule, resulting in a mosaic of alternations (Smuckler and Arcasoy 1969). A study of the pattern of necrosis in trout liver following the injection of CCl_4 was found to be focal. In comparison to mammalian liver, necrotic hepatocytes were

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scattered and no regular, repeating pattern of alternation was seen in trout liver (Gingerich et al. 1978; Statham et al. 1978).

Besides the histopathological studies, responses to CCl_4 in the kidney and liver of juvenile rainbow trout at a genetic level was investigated by Krasnov et al. (2005). Furthermore, Lam and Gong (2006) recently showed that zebrafish liver tumour possess molecular similarities to human liver cancer, making a pathway for cancerogenous study using fish as model animals.

The rosy barb, *Puntius conchonius*, a member of the family Cyprinidae has a short life-span and produces great numbers of big and transparent eggs that are fertilized externally, and has become an emerging model fish for biological and biotechnological research (Amanze and Iyengar 1990; Kirankumar et al. 2003; Bhattacharya et al. 2005). In fact, it has been widely used in ecotoxicological study in recent years (Gill et al. 1990; Kirankumar and Pandian 2004; Xu et al. 2005). However, no information is available concerning the toxicity of CCl_4 to rosy barb at present. Similarly, little is known about its toxicity to amphioxus, *Branchiostoma belcheri*, an organism with close resemblance to the ancestor of vertebrates (Ruppert 1997; Holland et al. 2004). Amphioxus has a pouch-like structure called digestive caecum, which protrudes forward as an out-pocketing of the digestive tube and extends along the right side of the posterior part of the pharynx. The digestive caecum in amphioxus is considered to be the precursor of vertebrate liver (Welsch 1975; Müller 1884; Ruppert 1997). Unexpectedly, the comparative study linking the digestive caecum in amphioxus and the liver in vertebrates remains scarce (Liang et al. 2005). The aim of this study was thus to examine the acute toxicity of CCL_4 to rosy barb and amphioxus with special implications for the origin of vertebrate liver.

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride used in the experiments was purchased from Guangcheng Chemicals Ltd (Tianjin, China). All other chemicals were analytical reagents.

Animals and Treatments

Adult rosy barbs P. conchonius (body weights 2.1–2.85 g/fish) purchased from a local fish dealer were maintained in dechlorinated water at 26 ± 1°C. They were fed on live bloodworms and fish flakes (Tetramin, Germany) twice a day, and acclimatized for 2 weeks before experiments.

Healthy amphioxus *B. belcheri* with average body length of about 4 cm were collected from the sea near Shazikou, Qingdao. They were kept in sea water with sands at room temperature, and fed daily with unicellular algae. The water was aerated, and changed once a day. Amphioxus were acclimated for 1 week before experiments.

Pilot experiments were performed on both rosy barbs and amphioxus to determine the appropriate concentration of CCl_4 to carry out the experiments. On the basis of these pilot experiments, for median lethal concentration (LC_{50}) determination a total of 10 rosy barbs per group were exposed to different concentrations (0, 10, 20, 30, 40, and 50 mg/L) of CCL_4 in dechlorinated water in 20 liter glass tanks. Similarly, 10 amphioxus per group were exposed to 0, 12, 16, 20, 24 and 28 mg/L CCL_4 in sea water in 1 liter glass beakers with sands at the bottom. CCl_4 was always freshly dissolved in appropriate volume of absolute alcohol as a stock solution, and added immediately into the test solutions (dechlorinated water or sea water). Soon after addition of CCL_4, the glass tanks and beakers were sealed with double layers of cling plastic wrap to prevent volatile loss of CCl_4 (Le Blanc 1980). Note the volumes of ethanol were all equal in both treatment and control groups. Every 24 h the dead animals were removed and the surviving ones recorded. Meanwhile, test solutions were renewed to maintain the concentrations of both CCl_4 and dissolved O_2. The experiments were repeated twice, and the LC_{50} concentrations at 96 h were calculated using trimmed Spearman-Karber (Hamilton et al. 1977) estimation.

For histological examination, three sub-lethal concentrations (5, 7.5, and 10 mg/L) of CCl_4 were used for both rosy barb and amphioxus. They were both treated as above, and, 96 h after treatment, rosy barbs from each test group were sacrificed and their liver, kidney, gill, skin, muscle, testis, and ovary were dissected out and fixed in Bouin's fixative for 48 h at room temperature. Similarly, amphioxus were each severed into three to four pieces, and fixed in Bouin's fixative for 48 h. The untreated (control) fish and amphioxus were also fixed in Bouin's fixative at the same time.

Histology

All fixed samples were washed with running tap water overnight. Among them, the gills were then decalcified with decalcifying agent (5% HNO_3 and 70% alcohol) for 48 h. After dehydration with graded alcohol, all samples were embedded in paraffin and sectioned at 6 μm. The sections were double stained in hematoxylin and eosin, mounted in neutral balsam, and observed under an Olympus BX51 (Japan) microscope.

RESULTS

The LC_{50} values at 96 h for rosy barb and amphioxus were on average 23.9 ± 4 mg/L and 18.9 ± 2 mg/L, respectively. They are relatively close to the LC_{50} value (27 mg/L) at 96 h for bluegill (Buccafusco et al. 1981), the only valid acute toxicity value for fresh water fish.

Histopathological Effects on Liver, Kidney, and Gill of Rosy Barb

The livers of control rosy barb were composed of hepatocytes (parenchymal cells) arranged in typical tubular architecture, sinusoids, and blood vessels filled with numerous blood cells. The hepatocytes were morphologically polygonal, and had conspicuous nuclei with densely stained nucleoli (Fig. 1A and 1B) . In contrast, the livers of fish treated with different sublethal concentrations of CCl_4 exhibited classic histological lesions including focal necrosis of hepatocytes and mononuclear lymphocyte infiltration in a dose-dependent manner. For example, most hepatocytes of the livers from fish exposed to 5 and 7.5 mg/L CCl_4 remained morphologically and structurally normal, but the number of mononuclear lymphocytes infiltrated in sinusoids was significantly increased (Fig. 1C and 1E), and the focal necrosis of hepatocytes was observed occasionally which

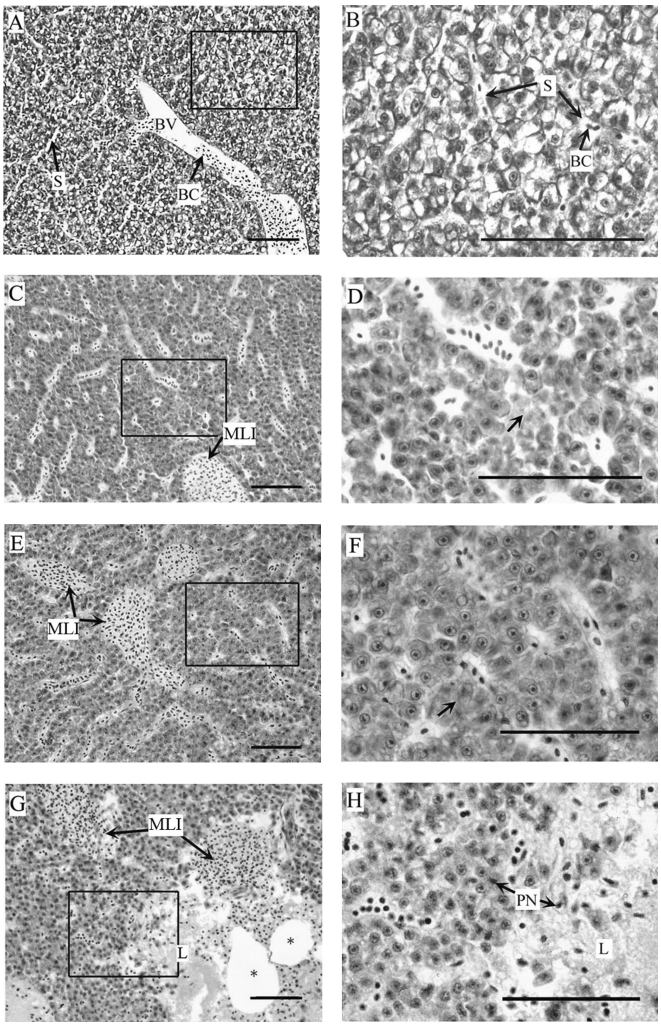

FIGURE 1 **Light micrographs of transverse section of the liver rosy barb fishes exposed to different concentrations of CCl_4 for 96 h. (A) Low magnification of controls showing hepatocytes, sinusoids and blood vessel and blood cells. Scale bar = 100 μm. (B) High magnification of the rectangle in A showing normal structure of hepatocytes. Scale bar = 50 μm. (C) and (E) Low magnification of the liver of fish exposed to 5 and 7.5 mg/L CCl_4 showing dose dependent increase in mononuclear infiltrations. Scale bar = 100 μm. (D) and (F) High magnification of the rectangle in C and E respectively showing focal necrosis (arrow) of hepatocytes. Scale bar = 50 μm. (G) Low magnification of 10 mg/L exposure showing severe mononuclear infiltrations, fatty degeneration (asterisks) and lysed areas. Scale bar = 100 μm. (H) High magnification of 10 mg/L exposure showing nuclear pyknosis, hepatocytes lacking cell boundaries and lysed areas. Scale bar = 50 μm. Abbreviations: BC, Blood Cells; BV, Blood Vessels; L, Lysis; MLI, Mononuclear Lymphocyte Infiltrations; PN, Pycnotic Nuclei; S, Sinusoids.**

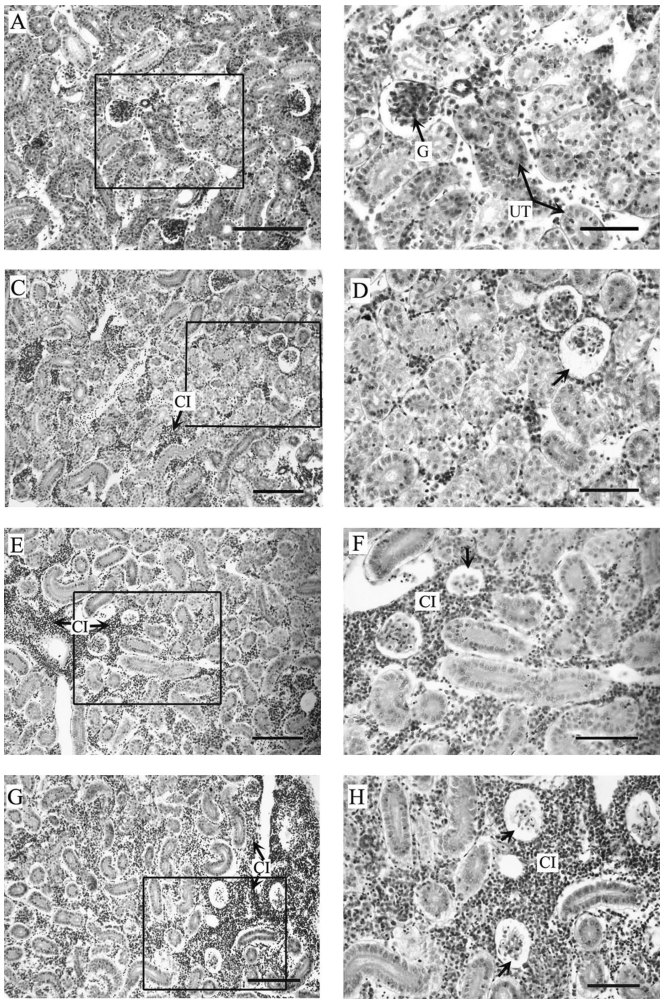

FIGURE 2 **Light micrographs of transverse section of the kidney of rosy barb fishes exposed to different concentrations of CCl_4 for 96 h. (A) Low magnification of controls showing normal appearance of kidney. Scale bar = 100 μm. (B) High magnification of the rectangle in A. showing normal structure of glomerulus and uriniferous tubules. Scale bar = 50 μm. (C), (E) and (G) Low magnification of the kidney of fish exposed to 5, 7.5 and 10 mg/L CCl_4 showing dose dependant increase in inflammatory cell infiltrations. Scale bar = 100 μm. (D), (F) and (H) High magnification of the rectangles in C, E and G showing dialated Bowman's capsule (arrow) and cell infiltrations. Scale bar = 50 μm. Abbreviations: CI, Cell Infiltrations; GL, Glomerulus; UT, Uriniferous Tubules.**

were scattered randomly (Fig. 1D and 1F). In the livers of fish exposed to 10 mg/L CCl_4, however, severe mononuclear cell infiltration throughout the liver indicative of hemorrhage was found, the cell boundaries between hepatocytes were lost, and the nuclear pyknosis and cytolysis resulting in hepatocyte death in extensive areas occurred (Fig. 1G and 1H). In addition, fatty degenerations also appeared in the livers of fish exposed to 10 mg/L CCl_4 (Fig. 1G). Nevertheless, no sign of fibrosis was seen in rosy barb livers due to the continuous exposure of CCl_4 within the studied time frame.

Trunk kidneys of control fish showed normal structural and architectural integrity of glomeruli in Bowman's capsules and uriniferous tubules (Fig. 2A and 2B). Exposure to CCl_4 caused marked morphological damages to the glomeruli and Bowman's capsules, but the effect of CCl_4 on the uriniferous tubules was limited. The kidneys of fish exposed to 5 mg/L CCl_4 had connective tissues with increased masses of inflammatory cell infiltration (Fig. 2C), and glomeruli with shrunken appearance and dilated Bowman's capsules (Fig. 2D). When the fish were exposed to 7.5 and 10 mg/L CCl_4, the kidney damages caused were histologically similar. The connective tissues had increased masses of inflammatory cell infiltration (Fig. 2E and 2G), the

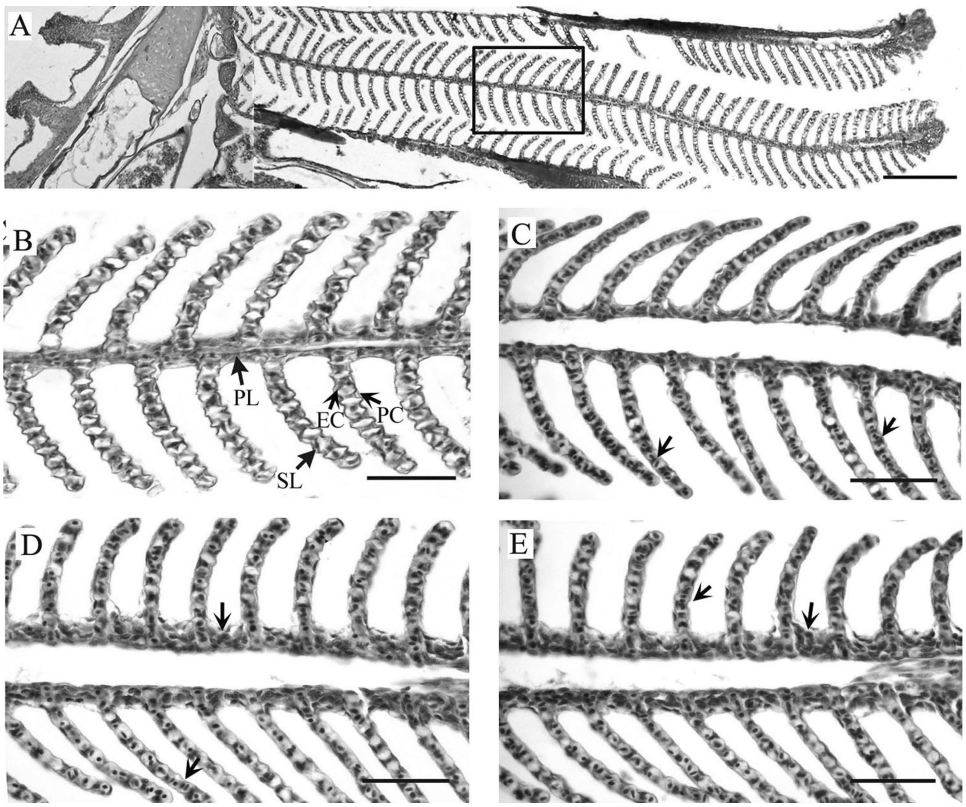

FIGURE 3 **Light micrographs of transverse section of the gills of rosy barb fishes exposed to different concentrations of CCl_4 for 96 h. (A) Low magnification of gill filament of controls showing normal appearance of primary and secondary lamellae with epithelial cells supported by pillar cells. Scale bar = 100 μm. (B) High magnification of control gills in the rectangle in A. Scale bar = 50 μm. (C), (D) and (E) High magnification of gills of fish exposed to 5, 7.5 and 10 mg/L showing multiple nuclei forming syncytia in the primary and secondary lamellae (arrows). Scale bar = 50 μm. Abbreviations: PL, Primary Lamellae; SL, Secondary Lamellae; EC, Epithelial Cells; PC, Pillar Cells.**

glomeruli and Bowman's capsules were severely degenerated, and the remains of glomeruli had a foamy appearance and seemed to be floating in Bowman's spaces (Fig. 2F and 2H).

The gills of control fish exhibited uniform arrangement of primary and secondary lamellae with unvarying inter-lamellar space (Fig. 3A). The primary lamellae comprised about two layers of cells, whereas the secondary lamellae were composed of a single layer of epithelial cells supported by pillar cells (Fig. 3B). When rosy barbs were exposed to sublethal concentrations of CCl_4, their gill gross histo-architectures were less impaired, but some epithelial cells of the primary and secondary lamellae had two-to-three nuclei, forming a syncytium (Fig. 3C, 3D, and 3E), which was not seen in control fish.

In addition to the liver, kidney, and gill, other tissues of rosy barb including skin, muscle, ovary, and testis appeared not affected by exposure to sublethal concentrations of CCl_4 (data not shown).

Histopathological Effects on Digestive Caecum and Gill of Amphioxus

Histological examination revealed that treatment with 5 mg/L CCl_4 had little effect on the structure and integrity of all tissues and organs, which were all similar to those observed in control amphioxus (Fig. 4A, 4B, and 4C). In contrast, the digestive caecum of amphioxus exposed to 7.5 mg/L CCl_4 showed some vacuolation in its columnar epithelium, and the apical cilia of columnar epithelial cells became disintegrated (Fig. 4E). When amphioxus was exposed to 10 mg/L CCl_4, most columnar epithelial cells lost their apical cilia, and large vacuoles appeared in the epithelium, resulting in destruction of the columnar cells (Fig. 4H). Moreover, the epithelium covering gill bars was dissolved (Fig. 4I). All other tissues such as nerve cord, notochord, gonads, endostyle, and muscle were not affected (Fig. 4D, 4F, and 4G).

DISCUSSION

This study demonstrates that exposure to CCl_4 caused histopathological damage to the liver, kidney, and gill of rosy barb and to the digestive caecum and gill of amphioxus. These appear to be the first reports on CCl_4 toxicity in both rosy barb and amphioxus. It is of particular interest to note that the digestive caecum of amphioxus as well as the liver of rosy barb is the prominent target organ of CCl_4, suggesting that the digestive caecum of amphioxus is homologous to the liver of rosy barb, at least in respect to toxic damages of CCl_4. This provides evidence of physiological functionality supporting the

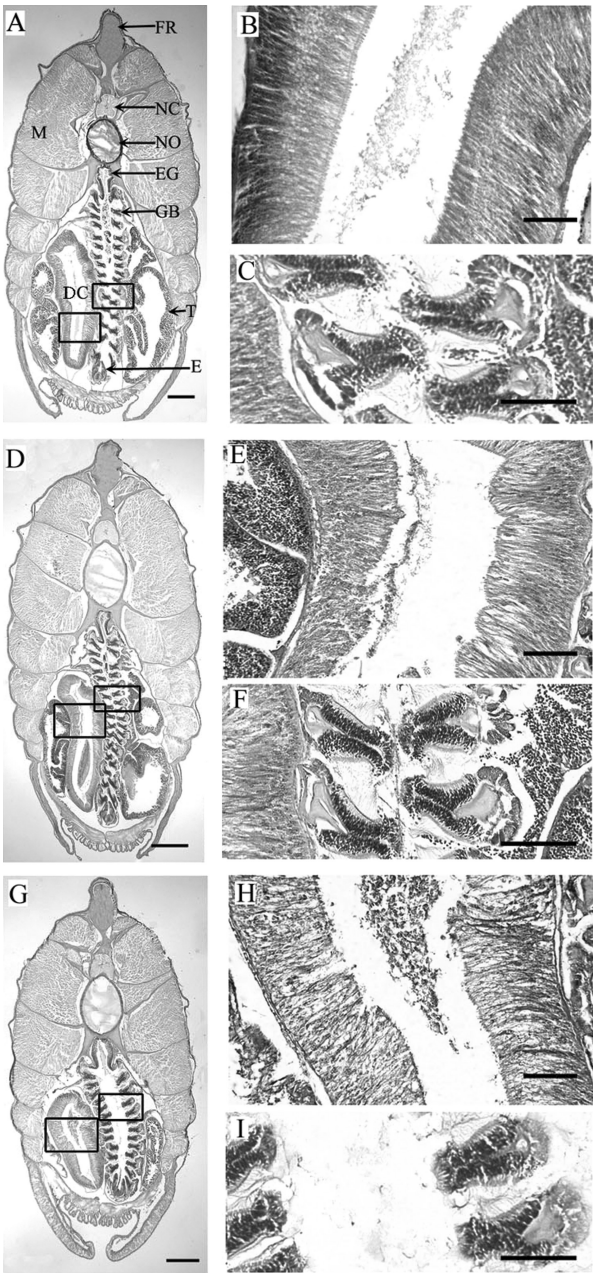

FIGURE 4 **Light micrographs of transverse section of amphioxus exposed to different concentrations of CCl_4 for 96 h. (A), (D) and (G) Low magnification of transverse section of control, 7.5 and 10 mg/L CCl_4 exposure respectively. Scale bar = 200 μm. (B), (E) and (H) High magnification of digestive caecum in the rectangle in A, D and G, respectively. There was an increase in large vacuoles and loss of apical cilia of the columner epithelial cells at high treatment concentrations. Scale bar = 50 μm. (C), (F) and (I) High magnification of gills in the rectangle in A, D and G. Dissolved epithelium of the gill bars at 10 mg/L CCl_4 exposure is noticed. Scale bar = 50 μm. Abbreviations: DC, Digestive Caecum; E, Endostyle; EG, Epithelial Groove; FR, Fin Ray; GB, Gill Bar; NC, Neural Cord; NO, Notochord; M, Muscle; T, Testis.**

hypothesis that the vertebrate liver evolved from the digestive caecum of an amphioxus-like ancestor during early chordate evolution (Müller 1844; Liang et al. 2005).

The mechanism of CCl_4 hepatoxicity has been well documented in mammalian species (Treiner-Moslen 2002). However, in adverse to centrilobular necrosis following CCl_4 administration in rats, livers of rosy barbs exposed to CCl_4 showed randomly scattered focal necrosis. This was in accordance with the observations in trout liver (Gingerich et al. 1978; Statham et al. 1978). Hampton et al. (1985) showed tubular arrangement of hepatocytes with no lobules in trout liver in contrast to the centrolobular arrangement of mammalian liver. This difference in pattern arrangement of hepatocytes could count for the observed variation in the distribution of lesions. Besides, rosy barbs treated with high concentrations of CCl_4 showed mononuclear lymphocyte infiltration in the liver. On the other hand, in rats treated with CCl_4, liver inflammation was fibrosis-induced due to accumulation of mast cells, myofibroblasts, and nerve terminal complexes (Akiyoshi and Terada 1998; Knittel et al. 1999; Cassiman et al. 2002; Kinnman et al. 2003).

The mechanism of CCl_4 on lipid peroxidation involving NADPH-cytochrome P-450 system was suggested by Slater (1966). It is believed that CCl_4 is broken down by cytochrome P450 enzyme to free radical $CCl_3^{\bullet}$. Although the intermediate $CCl_3^{\bullet}$ reacts very slowly with biomolecules, it rapidly reacts with O_2 to form highly reactive radical $CCl_3O_2^{\bullet}$. $CCl_3O_2^{\bullet}$ reacts with polyunsaturated fatty acids to initiate lipid peroxidation in liver, and results in liver injury (Slater 1982). However, it has been studied in rodents that regeneration of liver cells in cases of liver injury occur with the help of rapidly growing epithelial cells with distinct oval nucleus (Farber 1956; Sell 1990). Hematopoietic stem cells have been shown to transdifferentiate into cells of the hepatocytic lineage in both rodents and humans (Petersen et al. 1999; Oh et al. 2002; Jang et al. 2004). However, these findings have been challenged by studies showing that cell fusion rather than transdifferentiation of hematopoietic stem cells is involved in regeneration (Vassilopoulos and Russell 2003).

Recently, the mechanism of CCl_4 nephrotoxicity has also been suggested to be the same as that of the liver (Ogeturk et al. 2005), since the mammalian kidney has an affinity for CCl_4 (Abraham et al. 1999) and contains cytochrome P450 in the cortex (Rush et al. 1984; Ronis et al. 1998). We think that CCl_4 causes damages to the liver and kidney of rosy barb via the same mechanism as that of mammalian species, though the current knowledge on cytochrome P450 activity in fish liver and kidney seems limited (Buhler and Rasmusson 1968; Stegeman 1981). However, the pathogenesis of CCl_4-induced gill injury has not been clearly clarified. It has been shown that CCl_4 is a mitogenic stimulator in rainbow trout liver (Kotsanis and Metcalfe 1991). We found that CCl_4 induces syncytial formation in the gills of rosy barb. This appears to be the first such data, and the reason for this remains unclear at present. We have also found that CCl_4 causes dissolution of the epithelium covering gill bars of amphioxus. This may be due to the lipid peroxidation via free radicals CCl_3 and CCl_3O_2, which then induces destruction of cell membrane integrity (Recknagel et al. 1977).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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