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Serum Metabolic Enzyme Activities and Hepatocyte Ultrastructure of Common Carp after Gallium Exposure

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Jen-Lee Yang and Hon-Cheng Chen (2003) Serum metabolic enzyme activities and hepatocyte ultrastructure of common carp after gallium exposure. *Zoological Studies* **42**(3): 455-461. Gallium (Ga) is one of the intermetallic elements increasingly being used in making high-speed semiconductors such as gallium arsenide. The purposes of this study were to investigate the effects of gallium on serum enzyme activities and on the ultrastructure of the liver in common carp (*Cyprinus carpio*). Common carp were exposed to 3 different sublethal levels of gallium (2.0, 4.0, and 8.0 mg/l) in laboratory toxicity tests. During a 28-d testing period, serum metabolic enzyme activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) was analyzed every 14 d. An increase of enzyme activity in serum was observed, particularly at the 2 highest exposure concentrations. Electron microscopy investigations revealed ultrastructural alterations in hepatocytes which were correlated with exposure concentrations and exposure time. Cytopathological effects included nuclei with irregular outlines and heterochromatin, fragmentation and vesiculation of endoplasmic reticula, and disruption of mitochondria. Moreover, proliferation of lysosomes with electron-dense bodies and lipid inclusions were also found in the cytoplasm of hepatocytes. Our results indicate that changes in metabolic enzyme activities in serum occurred; this fact was confirmed by ultrastructural observations during the exposure period. This study also emphasizes the importance of in vivo approaches to the assessment of relative compound effects and their potential hazards in aquatic animals.
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III-V compound semiconductors, such as GaAs and InGaAs, are important materials in the manufacture of optoelectronic devices and integrated circuits in the semiconductor industry (Robinson 1983, Fowler et al. 1993, Bustamante et al. 1997). Manufacturing processes devoted to the fabrication of GaAs-based semiconductor devices generate large volumes of wastes that contain the toxic metal arsenic as well as gallium. For example, aqueous waste streams can contain from 200 to 400 mg/l of each dissolved metal in the wet polishing process of gallium arsenide (Sturgill et al. 2000). However, gallium arsenide is not a listed hazardous waste under regulations in Taiwan, but is listed as hazardous in California, USA (Sturgill et al. 1999).

The use of gallium compounds in semiconductor manufacturing was accompanied by increasing amount of toxic materials released as potential toxic wastes, which are harmful to health and the environment (Chelton et al. 1991, Sturgill et al. 2000). Gallium can interfere with calcium uptake; the element is a potent inhibitor of protein synthesis and the heme pathway enzyme, aminolevulinic acid dehydratase (Hoyes et al. 1992). Gallium also appears to inhibit DNA synthesis by action on ribonucleotide reductase (Riaz et al. 1995). Previous reports indicated that gallium compounds might cause bone marrow depression, testicular toxicity, and hemorrhagic nephritis in mammals (Webb et al. 1987, Aoki et al. 1990, Omura et al. 1996).

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However, accidental industrial spills might lead to high concentrations of toxic materials in the aquatic environment as well as effects on freshwater ecosystems with acute and chronic toxicity. Fish are particularly sensitive to water-borne environmental contamination, and are recognized as a useful model for indicating water quality (Mathis and Kevern 1975). Pollutants may significantly damage certain physiological and biochemical processes when they enter the organs of fishes (Murty 1986, Teh et al. 1997). One aquatic animal, tilapia (*Oreochromis mossambicus*), showed retardation in growth under a sublethal gallium exposure (Lin and Hwang 1998).

The use of a biochemical approach has been advocated to provide an early warning of potentially damaging changes in stressed fish. In toxicological studies of acute exposure, changes in concentrations and enzyme activities often directly reflect cell damage in specific organs (Casillas et al. 1983). Elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were presumably due to damage to the liver, but other organs may also have been damaged, like the kidney and gills (Hochachka and Mommsen 1995, Bernet et al. 2001). Further, alkaline phosphatase (ALP) is composed of several isoenzymes that are present in practically all tissues of the body, especially in cell membranes. These enzymes catalyze the hydrolysis of monophosphate esters and have wide substrate specificity (Duncan and Prasse 1986).

The liver is an important organ involved in metabolic processes and in detoxification of xenobiotics. In some situations, materials may accumulate in the liver to toxic levels and cause pathological alterations (Meyers and Hendricks 1984, Ferguson 1989, Braunbeck et al. 1990). There are many studies of liver ultrastructural alterations induced by heavy metals in aquatic environments (Koyama et al. 1979, Khangarot 1992). The type of liver injury is often dependent upon not only the particular agent and its mechanism of action but also on the length of exposure (Jacobson-Kram and Keller 2001). The prolonged hepatic ultrastructural effects of gallium need to be better understood.

Because the common carp is an important cultured fish species in fishponds near semiconductor manufacturing districts in Taiwan, it is a suitable model species to study the toxicity of semiconductor-related metals. Therefore, the purposes of this study were to investigate the effects of sublethal gallium concentrations on biochemical and ultrastructural alterations of the carp, so that this evidence could then be used to determine the possible adverse effects of gallium.

MATERIALS AND METHODS

Animal maintenance and chemicals

Common carp (*Cyprinus carpio*) were obtained from the Chupei Branch of the Taiwan Fisheries Research Institute, Hsinchu. Fish were transported to the glass aquarium in our laboratory which was equipped with a water-cycling device, and dechlorinated tap water (pH 7.4-7.8; dissolved oxygen concentration 7.3-8.1 mg/l; hardness 38-45 mg $CaCO_3$/l, ammonia < 0.5 mg/l, and nitrite 0.05-0.1 mg L^{-1}/l) was used. Fish were acclimated for 14 d and fed with aquarium fish mixture every 2 d. The temperature was maintained at 25.0 ± 0.5°C, and the photoperiod was set at 8 h of light and 16 h of dark during the entire experiment. Carp (12 wk old, 2.3 ± 0.19 g in body weight) were used for serum enzyme activity analysis and cytopathological examinations in the initial experiments.

Gallium sulfate (purity 99.999%) was purchased from Alfa Aesar (Ward Hill, MA, USA). A stock solution was prepared in deionized water (1000 mg/l Ga in 0.1% nitric acid).

Experimental protocol

Common carp for toxicity tests were randomly placed in 100-L plastic tanks. Every tank contained 15 fish which were exposed to the following concentrations: 2.0, 4.0, and 8.0 mg/l Ga test solutions and a control, respectively. Sublethal levels of gallium were equivalent to approximately 10%, 20%, and 40% of the 96-h LC_{50} value (19.78 mg/l) according to the static renewal method for acute toxicity testing (Buikema et al. 1982) in our laboratory. Six fish per exposure concentration were anesthetized with MS-222 (Sigma Chemical, St. Louis, MO, USA) and sacrificed at 0600 h after 14 d and 28 d of exposure. Blood samples were taken from each fish by puncture of the caudal vessel. Blood was allowed to coagulate at room temperature for 2 h. Serum was obtained by centrifugation of an amount of blood at 1500 xg (10 min, 4°C) and was prepared for enzyme activity analyses. Livers were removed for electron microscopy studies.

Enzyme assay

Enzyme activities were measured using a Johnson & Johnson (New York, NY, USA) Ektachem 250 biochemical analyzer. Assays were run in triplicate. Aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) activities were measured according to the method of Bergmeyer et al. (1985), and alkaline phosphatase (ALP, EC 3.1.3.1) activities were determined by the method of McComb and Bowers (1972). Test kits from Johnson & Johnson were used for determinations.

Transmission electron microscopy (TEM)

For electron microscopic examination, small pieces of carp livers from control and treated specimens were fixed for 2 h in cold cacodylate-buffered 4.0% glutaraldehyde at pH 7.2 (in a 7.5% sucrose solution, 4°C), rinsed twice with 0.1 cacodylate buffer, and postfixed with 2% osmium tetraoxide in 0.2 M cacodylate buffer (1:1). Samples were dehydrated in a graded series of alcohol and embedded in Spurr's medium. Semithin samples (0.5 μ) were stained with toluidine blue. Ultrathin sections (50-70 nm) were contrasted using lead citrate and uranyl acetate, and examined through a transmission electron microscope (JEM-1200EX, JEOL, Tokyo, Japan).

Statistical analysis

Values of the enzyme assay (at the same exposure times) were analyzed statistically by analysis of variance using SAS statistical software (SAS 1988). Duncan's multiple range test was used to evaluate the mean difference among individual groups at the 0.05 significance level.

RESULTS

Serum enzyme activity

Results of enzyme activity analysis are presented in table 1. No significant changes occurred in the activities of the 3 enzymes (ALT, AST, and ALP) under 2.0 mg/l Ga after 14 d of exposure. Both ALT and ALP, but not AST, activities at an exposure of 2.0 mg/l Ga for 28 d exhibited higher values than those of the control group. In treatment with 4.0 mg/l Ga, there was no significant difference in serum AST activities compared with the control group. On the other hand, ALT and ALP activities in serum of treated carp were significantly higher than those of the control group after 14 and 28 d at the same level of gallium administration. Statistically significant increases in all metabolic enzyme activities were recorded at the highest gallium concentrations after 14 and 28 d of exposure. Values recorded for serum activity of the above enzymes were about 2-3 times higher than those of the control group. The present study shows that gallium-induced alterations in serum metabolic enzyme activities in intoxicated carp.

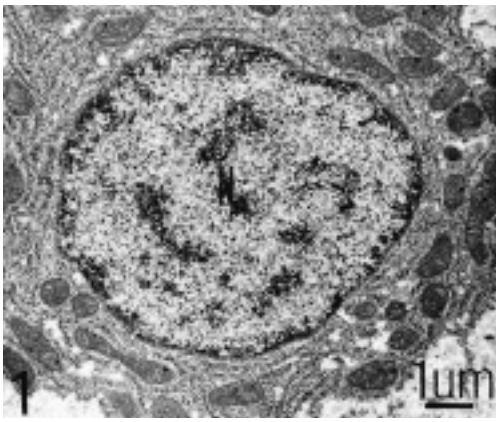

Fig. 1. Untreated carp hepatocytes showing a clear, round nucleus with little heterochromatin. Small stacks of non-fenestrated parallel cisternae of the rough endoplasmic reticula and a large amount of mitochondria surround the cell nucleus. N, nucleus.

Table 1. Serum enzyme activities of common carp exposed to gallium

	ALT (U/l)		AST (U/l)		ALP (U/l)	
	14 d	28 d	14 d	28 d	14 d	28 d
Control	32.33 ± 5.21^{a}	33.33 ± 4.13^{a}	36.23 ± 3.84^{a}	37.70 ± 11.09^{a}	18.67 ± 4.97^{a}	19.83 ± 4.02^{a}
2.0 mg/l Ga	31.31 ± 2.88^{a}	42.90 ± 2.57^{b}	38.54 ± 12.98^{a}	42.24 ± 11.97^{a}	20.00 ± 2.10^{ab}	27.93 ± 4.39^{b}
4.0 mg/l Ga	41.75 ± 3.84^{b}	54.39 ± 17.97^{c}	44.30 ± 14.92^{a}	49.66 ± 9.60^{a}	26.00 ± 2.76^{b}	36.66 ± 5.76^{c}
8.0 mg/l Ga	66.83 ± 4.41^{c}	108.79 ± 16.13^{d}	56.99 ± 10.20^{b}	65.17 ± 12.60^{b}	39.00 ± 4.41^{c}	64.88 ± 12.04^{d}

All values are given as the mean ± SD; $n = 6$.
Values in the same column with different superscripts significantly differ at $p < 0.05$.

Hepatocyte ultrastructural changes

The ultrastructure of untreated carp hepatocytes is shown in figure 1. The centrally located nucleus (5.0-6.5 μ in diameter) normally exhibited little heterochromatin. Non-fenestrated parallel cisternae of the rough endoplasmic reticulum (RER) were arranged around the nucleus. Mitochondria with an ovoid or rod shape were located prominently near the nucleus. A small number of peroxisomes were scattered in the cytoplasm. Myelinated bodies and lipid inclusions were not found in untreated hepatocytes.

In the hepatocytes of carp exposed to 2.0 mg/l Ga for 14 d, nuclei showed a spherical shape with little scattered heterochromatin, and a nuclear envelope whose integrity was similar to that of the control liver. Cisternae of variable length of the RER formed parallel stacks, and many peroxisomes of various sizes (0.1-0.9 μ in diameter) were observed in the cell matrix (Fig. 2A). Hepatic ultrastructural alterations of fish exposed to 4.0 mg/l Ga for 14 d included degranulation of the cisternae of the RER, and the proliferation and vesicular transformation of the smooth endoplasmic reticulum (SER) (Fig. 2B). As to alterations when exposed to 8.0 mg/l Ga for 14 d, mitochondrial cisternae displayed lysis and destruction of membranes. Fragmentation of the RER was also frequently exhibited in the cytoplasm (Fig. 2C).

After 28 d of exposure, carp hepatocytes displayed nuclear alterations in the groups treated with 2.0 and 4.0 mg/l Ga. Nuclei appeared to have slightly irregular outlines and an expansion of electron-dense heterochromatin fields on the margin of the inner envelope. In hepatocytes of carp exposed to 2.0 mg/l Ga, non-fenestrated parallel cisternae of the RER appeared near the nucleus. Lysosomes with myelin-like membrane whorls, multivesicular bodies, and peripheral glycogen fields could be found in the cytoplasm (Fig. 3A). Hepatocytes of fish exposed to 4.0 mg/l Ga displayed additional secondary lysosomes scattered in the cytoplasm; their length and width were both determined to be about 2.0 μ. The effects also included cluster formation by mitochondria, and lipid inclusions (0.7-2.0 μ in diameter) showed dramatic accumulation (Fig. 3B). Alterations in the group exposed to 8.0 mg/l Ga were stronger than those of the other exposure groups. Nuclei (2.5-3.0 μ in diameter) showed significant shrinkage and deformation. Dilation of nuclear pores and large expansion of heterochromatin fields were observed on the nuclear margin. Further, many swollen mitochondria were located near the nucleus (Fig. 3C). Both outer and inner membranes of the mitochondria were severely damaged; the RER was broken into small fragments, and vari-

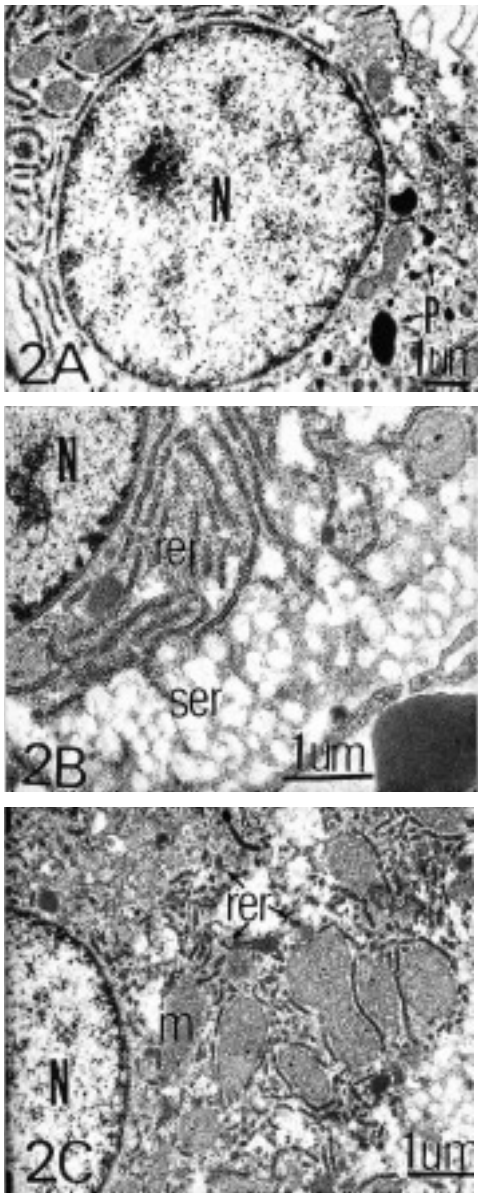

Fig. 2. Alterations of carp hepatocytes after 14 d. In the 2.0 mg/l Ga group (A), the nucleus still has a spherical shape. Parallel cisternae of the rough endoplasmic reticulum forms stacks and many peroxisomes of various sizes can be observed in the cell matrix. In the 4.0 mg/l Ga group (B), degranulation of the cisternae of the rough endoplasmic reticulum can be observed, while vesiculation of the smooth endoplasmic reticulum is elevated. In the 8.0 mg/l Ga group (C), mitochondrial membranes are severely damaged, and the rough endoplasmic reticula are broken into small fragments. N, nucleus; p, peroxisome; rer, rough endoplasmic reticula; ser, smooth endoplasmic reticula; m, mitochondria.

able sizes of lipid inclusions were also observed (Fig. 3D). Ultrastructural alterations of hepatocytes were observed in most of the treated fish, and these lesions appeared with increased severity at higher gallium concentrations.

DISCUSSION

Recently, there has been considerable research on the physiological, biochemical, and molecular aspects of xenobiotic absorption, distribution, biotransformation, and excretion in fish. Although there has been much more extensive biochemical toxicological research in mammals than in fish, it is clear that there is considerable overlap in many of the basic aspects of these processes, a fact that is not surprising since many other biochemical similarities exist among vertebrate species (Duncan and Prasse 1986, Hochachka and Mommsen 1995). Cell injury of certain organs leads to the release of tissue-specific enzymes into the bloodstream (Heath 1987, Burtis and Ashwood 1996). The increased transaminase (AST and ALT) activity in fish exposed to gallium may reveal possible leakage of enzymes across damaged plasma membranes and/or the increased synthesis of enzymes by the liver. Meanwhile, elevation of serum ALP also correlates with exposure levels and exposure time of carp. Increased serum activities of ALP have been explained by pathological processes such as liver impairment, kidney dysfunction, and bone disease. Although its precise biochemical functions which act in the organism are not known (Bogin et al. 1994, Atroshi et al. 2000), gallium treatment increases serum AST, ALT, and ALP activities of fishes reflecting a situation of tissue damage and

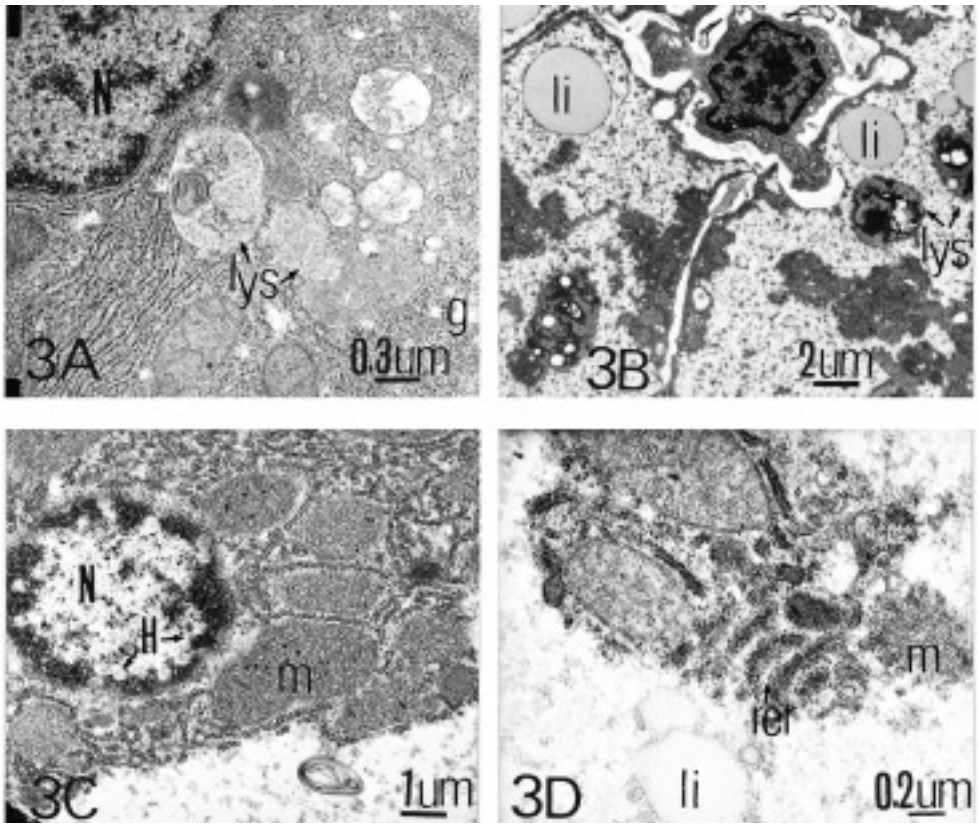

Fig. 3. Alterations of carp hepatocytes after 28 d. In the 2.0 mg/l Ga group (A), the nucleus displays an irregular outline, and condensed heterochromatin is located on the margin of the nuclear envelope. The cytoplasm contains lysosomes with myelin-like membrane whorls, multivesicular bodies, and peripheral glycogen fields. In the 4.0 mg/l Ga group (B), the amount of secondary lysosomes and lipid inclusions is elevated. Cluster formation of mitochondria is pronounced in the cytoplasm. In the 8.0 mg/l Ga group (C), the nucleus displays significant deformation, heterochromatin condensation and swelling of mitochondria. Furthermore, some mitochondria and rough endoplasmic reticula are severely damaged. Variable sizes of lipid inclusions can also be observed (D). N, nucleus; lys, lysosome; g, glycogen; li, lipid inclusion; H, heterochromatin; m, mitrochondria; rer, rough endoplasmic reticula.

stress. This biochemical evidence agrees with observations of damage to organelles within hepatocytes in the present work.

Due to the high sensitivity of the hepatocytic ultrastructure, a higher gallium concentration in the aquatic environment resulted in more-extensive degenerative hepatocyte responses. Pronounced changes in hepatic nuclei, such as heterochromatin condensation and marginalization, as well as an irregular nuclear envelope, were seen in treated fish. Braunbeck (1994) interpreted such changes of the heterochromatin as suggesting progressive inactivation of the nuclear components; cell nuclei are regarded as a major intoxication site. The severity of nuclear lesions increased with increased exposed concentration; deformation of the nuclear envelope was exhibited in the group exposed to 8.0 mg/l Ga after only 28 d.

In the present study, sloughing of mitochondria and changes in cristae were frequently seen. A large amount of enzymes is involved in phospholipid metabolism and fatty acid synthesis in mitochondria (Constantinides 1984). In particular, the inner mitochondrial membrane contains the respiratory chain of enzymes which oxidizes substrates to form ATP (Bozzola and Russell 1992). Damage to mitochondria may be a common response of fish hepatocytes to heavy metal stressors (Bowler and Duncan 1970). Because gallium resembles ferric iron with respect to its charge and atomic radius, it therefore appears to be capable of interacting with iron-binding proteins. The metal gallium (transferrin-gallium) inhibits iron uptake by cells and decreases cellular ferritin content (Chitambar et al. 1991, Riaz et al. 1995). A possible site of action may include the mitochondrial respiratory chain, which is dependent on the function of a number of iron-containing enzymes (Jacobson et al. 1993).

Previous reports have demonstrated the detachment of ribosomes as well as dilation and fragmentation of the RER in fish hepatocytes following exposure to copper and cadmium. There are general responses that occur in fish after treatment with certain drugs or chemicals (Khangarot 1992, Biagianti-Risbourg et al. 1996). SER is involved in lipid metabolism and metabolism of toxic substances as well as the breakdown of glycogen. Extensive proliferation and vesiculation of the SER suggest active detoxification processes in the hepatocytes of mammals and fish (Braunbeck et al. 1989, Bozzola and Russell 1992, Wu et al. 1999).

Lysosomes with electron-dense bodies were also found in our investigations; these changes might be associated with the digestion of damaged cytoplasmic debris, which is a frequent phenomenon of liver injury. Biagianti-Risbourg (1996) indicated that moderate increases in the number of autophagosomes might be viewed as a defense mechanism that allows the segregation and elimination of altered parts of the cytoplasm. Increases in secondary lysosomes and autophagosomes in hepatocytes can be accompanied by a significant increase in cell proteolysis and lipolysis with many liver injuries induced by such processes as chemical intoxication, shock, prolonged fasting, and aging (Phillip et al. 1987).

The major findings of this study are that gallium is a toxic substance in carp, with severe hepatic cytopathological alterations as well as elevated enzyme activities in serum of fish exposed to various concentrations. Because of the widespread, large-volume, high-frequency use of gallium in semiconductor manufacturing, we must be aware of its toxicity in aquatic environments. This awareness must include knowledge of its effect on fishes, more specifically of its acute toxicity, cytopathology, histopathology, and influence on biochemical parameters, growth, reproduction, etc.

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