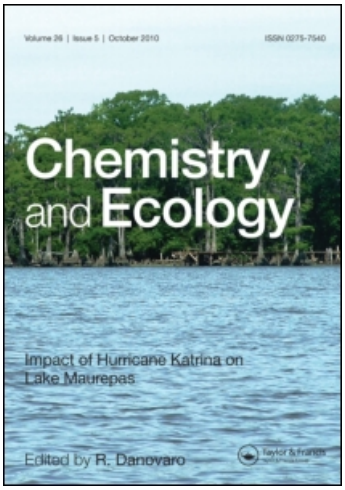

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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To cite this Article Hwang, Deng-Fwu , Lin, Jui-Fen and Jeng, Sen-Shyong(1996) 'Comparative Toxicity of Copper and Zinc to Isolated Eel Hepatocytes', Chemistry and Ecology, 12: 1, 109 — 114
To link to this Article: DOI: 10.1080/02757549608035351
URL: http://dx.doi.org/10.1080/02757549608035351

Chemistry and Ecology, 1996, Vol. 12, pp. 109–114
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COMPARATIVE TOXICITY OF COPPER AND ZINC TO ISOLATED EEL HEPATOCYTES

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(Received 24 August 1995; Revised 29 November 1995)

Attempts were made to elucidate lethal responses of hepatocytes of Japanese eel (*Anguilla japonica*) exposed to Krebs-HEPES buffer at different concentrations of Cu^{++} and Zn^{++}. It was found that the 2-hr LC_{50} value of Cu^{++} and Zn^{++} on eel hepatocytes was 490 and 1576 μM for Cu^{++} and Zn^{++} in single solution, and 1734 and 5200 μM in mixed solution. The toxic effects of Cu^{++} and Zn^{++} on eel hepatocytes were characteristic of antagonistic interaction. After 2-hr exposure, the amount of accumulated metal on the hepatocytes and the mortality of hepatocytes were increased with increasing metal concentration in Krebs-HEPES buffer. The accumulated amount of both metals was significantly decreased when the eel hepatocytes were exposed to mixed metals than to single metal.

KEY WORDS: Copper, zinc, Japanese eel hepatocytes, toxicity, LC_{50}.

INTRODUCTION

Recently, the use of isolated fish hepatocytes as a model system for general toxicity studies has been developed and reviewed (Baksi and Frazier, 1990). Denizeau and Marion (1990) investigated the effects of cadmium, mercury and copper, either in single or in various binary combination, on isolated rainbow trout (*Onchorynchus mykiss*) hepatocytes. They found that cadmium and mercury in combination produced a synergistic interaction while other combinations did not demonstrate interaction. The induction of metallothionein-like proteins by cadmium in isolated striped bass (*Morone saxatilis*) hepatocytes was investigated (Baksi and Frazier, 1988). Ballatori *et al.* (1988) reported the effects of various mercurials and cadmium chloride on plasma membrane permeability of isolated skate (*Raja erinacea*) hepatocytes, and found that plasma membrane was a sensitive target for metal induced toxicity. Rainbow trout hepatocytes were sensitive to aflatoxin B_1 and acetaminophen as assessed by lactate dehydrogenase leakage (Kocal *et al.*, 1988).

In West Taiwan, copper and zinc are often reported as pollutants in aquaculture area. Although there have many reports on the toxicity of copper and zinc on fish (Taylor *et al.*, 1985; Elsa, 1991), toxic effects of both pollutants on fish hepatocytes are not reported so far. Because Japanese eel (*Anguilla japonica*) is an important species for aquaculture in western Taiwan, the study of acute toxic effects of copper and zinc on the eel hepatocytes was undertaken to evaluate concentration-response relationship and to know the mode of interaction between copper and zinc.

MATERIALS AND METHODS

Animals

Japanese cultured eels, *Anguilla japonica*, weighing from 350–450 g, were purchased from a local aquaculturer in Ilan Prefecture. They were kept in freshwater aquaria and fasted for about three days before use.

Chemicals

Tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MD, USA). Copper chloride, zinc sulphate and other reagents were from E. Merck & Co. (Darmstadt, Germany).

Hepatocyte Isolation

The eels were anaethesized with 0.3% ethylene glycol monophenol ether. The liver was isolated and perfused as described previously (Hwang *et al.*, 1994). Briefly, the eel livers were continuously perfused with Hanks buffer, Hanks EGTA buffer, Hanks enzyme buffer, and Krebs-HEPES buffer, and then suspended with Krebs-HEPES buffer. The viability of suspended hepatocytes was determined by trypan blue dye exclusion.

Test for Toxicity of Copper and Zinc

The suspended hepatocytes were diluted with Krebs-HEPES buffer to a cell concentration of 2×10^7 cells/ml into tubes. The diluted hepatocytes solution in each tube was separately exposed to different copper (as $CuCl_2$ form) concentrations [control (Cu^{++}-free), 50, 100, 200, 400 and 600 μM Cu^{++}], zinc (as $ZnSO_4$ form) concentrations [control (Zn^{++}-free), 50, 100, 500, 1000 and 2000 μM Zn^{++}], and Cu^{++}-Zn^{++} mixed concentrations [control (Cu^{++}-Zn^{++}-free, 200 μM Cu^{++}-600 μM Zn^{++}, 400 μM Cu^{++}-1200 μM Zn^{++}, 800 μM Cu^{++}-2400 μM Zn^{++}, 1200 μm Cu^{++}-3600 μM Zn^{++}, 1600 μM CU^{++}-4800 μM Zn^{++}, and 2000 μM Cu^{++}-6000 μM Zn^{++}]. After 2-hr exposure, the solution in the tube was pipetted vigorously to mix and then number of the hepatocytes of the suspension obtained was counted by a blood counter. The experiment for each test was determined in triplicate. The data on hepatocyte viability were collected following methods described by Buikema *et al.* (1982), Trevors and Lusty (1985), and Roy (1988) to provide 2-hr median lethal concentration (LC_{50}) values and the associated 95% confidence intervals (CI). The above pairing ratio of Zn^{++} to Cu^{++} was three; this ratio was obtained from 2-hr LC_{50} value of Zn^{++} to that of Cu^{++}. On the other hand, 1 ml of hepatocyte solution from each test tube was removed and centrifuged at 500 rpm for 5 min. The precipitated hepatocytes were determined for metal content by using a flame atomic absorption spectrophotometer (Hitachi Z-8100, Hitachi Co., Japan) (Apha, 1985).

RESULTS

When the eel hepatocytes were exposed to either Zn^{++} or Cu^{++} in single solution for 2 hr, the relationships between mortality and concentration of both metals are shown in Figures 1 and 2. There are two positive linear relationships for $Y = 13.28 + 0.075\ X$ ($r = 0.99$) and $Y = 19.87 + 0.019\ X$ ($r = 0.98$) for Cu^{++} and Zn^{++}, respectively. The eel hepatocytes demonstrated more sensitivity to Cu^{++} than to Zn^{++}. The 2-hr LC_{50} values and their 95% confidence limits for Cu^{++} and Zn^{++} on eel hepatocytes were 490 μM and 227–753 μM for Cu^{++}, and 1576 μM and 904–2248 μM for Zn^{++}. This indicated that 2-hr LC_{50} value of Zn^{++} was about three fold that of Cu^{++} for eel hepatocytes.

The relationships between mortality and mixed concentration of Zn^{++} and Cu^{++} for eel hepatocytes exposed for 2 hr are shown in Figure 3. There are also two positive linear relationships for $Y = 15.15 + 0.0067\ X$ and $Y = 15.15 + 0.02\ X$ ($r = 0.98$) for Zn^{++} and Cu^{++}, respectively. The respective LC_{50} value of Zn^{++} and Cu^{++} was 5200 μM and 1734 μM. Their 95% confidence limits for Zn^{++} and Cu^{++} on eel hepatocytes

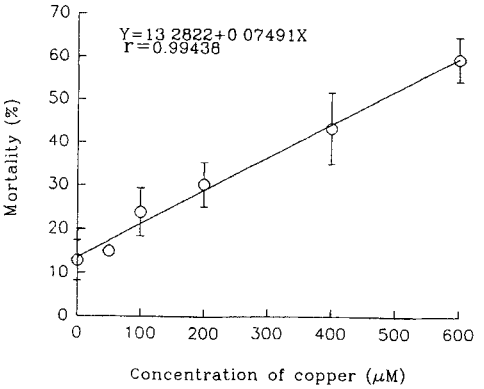

Figure 1 Relationships between mortality and concentration of copper on eel hepatocytes exposed for 2 hr.

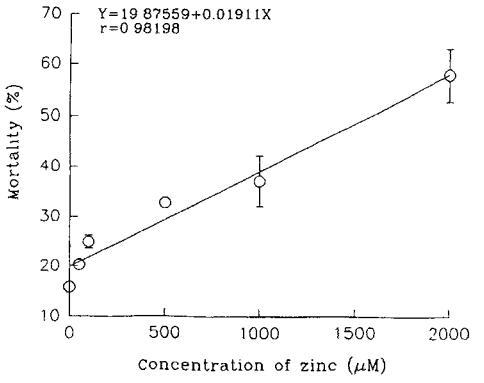

Figure 2 Relationships between mortality and concentration of zinc on eel hepatocytes exposed for 2 hr.

were 3617–6784 and 1206–2263 μM, respectively. The 2-hr LC_{50} values of Zn^{++} and Cu^{++}, when eel hepatocytes were exposed to Zn^{++}-Cu^{++} mixed solution, were about 3.5 fold those when the hepatocytes were separately exposed to Zn^{++} or Cu^{++} solution. This indicate the toxic effects of Zn^{++} and Cu^{++} on eel hepatocytes are antagonistic interaction.

When the eel hepatocytes were exposed to Zn^{++} and Cu^{++} in single solution, accumulated amounts of Zn^{++} and Cu^{++} on the eel hepatocytes are shown in Figure 4. It was found that amounts of accumulated of both metals were increased with increasing the concentration of exposed metal, especially when the concentration of Cu^{++} and Zn^{++} in the buffer was more than 200 μM and 500 μM, respectively. The relationships between accumulated and exposed concentrations of metals were as follows: $Y = 0.40 - 0.0023\,X + 3.63 \times 10^{-6}\,X^2 (r = 0.99)$ for Cu^{++} and

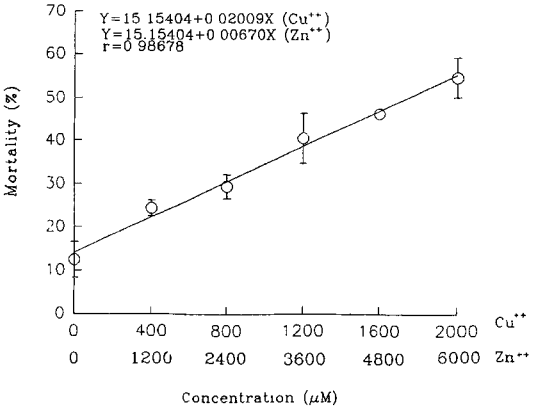

Figure 3 Relationships between mortality and concentration of copper and zinc on eel hepatocytes exposed for 2 hr.

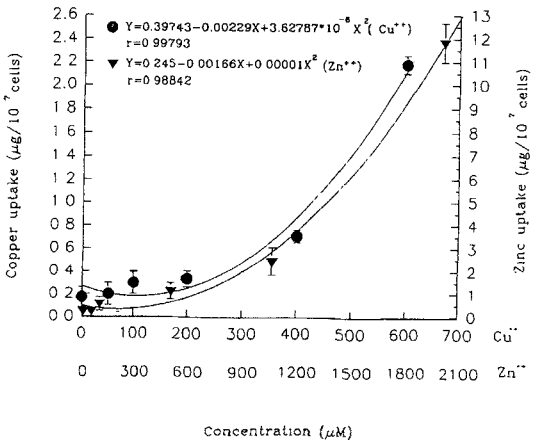

Figure 4 Copper and zinc uptake in eel hepatocytes incubated in Krebs-HEPES buffer (pH 7.5) with different concentration of single metal for 2 hr.

$Y = 0.24 - 0.0017\ X + 0.00001\ X^2$ ($r = 0.99$) for Zn^{++}, respectively. The pattern of relationships between accumulated and exposed concentrations of both metals in the eel hepatocytes were rather similar to each other. It means that both metals might have the same passway to enter into eel hepatocytes.

When the eel hepatocytes were exposed to $Cu^{++} - Zn^{++}$ mixed solutions, the accumulated amounts of Cu^{++} and Zn^{++} on the eel hepatocytes are shown in Figure 5. It was also found that amounts of both accumulated metals were increased with increasing the concentration of exposed metal. The amounts accumulated of both metals in eel hepatocytes were less in mixed solution than in single solution. The relationships between accumulated and exposed concentrations of metals were as follows: $Y = 0.43 - 0.00018\ X + 2.20 \times 10^{-6}\ X^2$ ($r = 0.99$) for Cu^{++} and $Y = 1.24 - 0.0003\ X + 1.047 \times 10^{-6} X^2$ ($r = 0.99$) for Zn^{++} respectively.

DISCUSSION

The responses of Japanese eel hepatocytes exposed to lethal level of Zn^{++} and Cu^{++} solutions were demonstrated in this study. It was found that the respective 2-hr LC_{50} value of Cu^{++} and Zn^{++} on eel hepatocytes was 490 and 1576 μM for Cu^{++} and Zn^{++} in single solution, and 1734 and 5200 μM in mixed solution. The toxic effect of Cu^{++} on Japanese eel hepatocytes was higher than that of Zn^{++}. This phenomenon was the same as that on other fish (Thompson *et al.*, 1980, 1980; Taylor *et al.*, 1985; Cusimano and Brakke, 1986). Moreover, the toxic effects of Cu^{++} and Zn^{++} on Japanese eel hepatocytes were characteristic of antagonistic interaction. However, the toxic effects of Cu^{++} and Zn^{++} on fish were synergistic and/or additive interactions (Lloyd, 1961; Sprague and Ramsay, 1965; Lewis, 1978; Thompson *et al.*, 1980; Elsa, 1991; Alabaster *et al.*, 1994).

No matter how Japanese eel hepatocytes were exposed in Cu^{++} and/or Zn^{++} solutions, the amount of accumulated metal on the hepatocytes and the mortality of

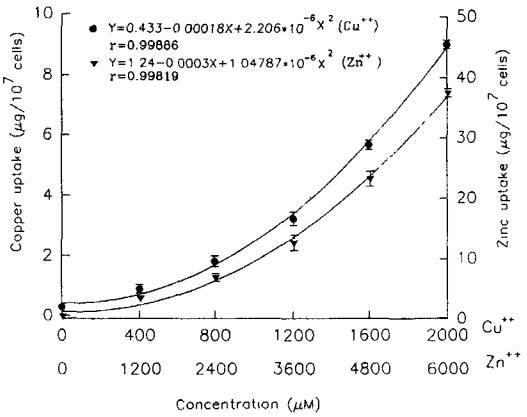

Figure 5 Copper and zinc uptake in eel hepatocytes incubated in Krebs-HEPES buffer (pH 7.5) with different concentrations of mixed metals for 2 hr.

hepatocytes were increased with increasing the concentration of exposed metal. It means that the death of eel hepatocytes might be caused by increasing accumulated amounts of Cu^{++} and Zn^{++} on the hepatocytes. The amounts of both accumulated metals were significantly decreased when the Japanese eel hepatocytes were exposed to mixed solution than to single solution. This phenomenon might elucidate why the toxic effects of Cu^{++} and Zn^{++} on the Japanese eel hepatocytes were antagonistic interaction. The mechanism of antagonistic interaction for the toxic effects of Cu^{++} and Zn^{++} on the eel hepatocytes is now being studied.

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