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In Vitro Toxicity of Copper, Cadmium, and Chromium to Isolated Hepatocytes from Carp, *Cyprinus carpio* L.

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Heavy metals are, due to increased activity of industry and agriculture, increasingly entering aquatic ecosystems and have accumulated in the tissues of aquatic vertebrates. The liver, playing a crucial role in a variety of metabolic pathways and detoxification processes, is a target organ for accumulation of metals in fish. Among these metals copper (Cu), cadmium (Cd) and hexavalent chromium (Cr(VI)) are of particular importance due to their toxic effects in fish, including hepatotocixity and cancerogenicity. Thus, although Cu is an essential trace element, it has been found to be acutely toxic for hepatocytes from both rat (Pourahmad and O'Brien 2000) and trout (Manzl et al. 2003) when surpassing physiological levels. In comparison, Cd, which has no known functions in fish metabolism, was not acutely toxic in trout hepatocytes (Manzl et al. 2003). However, after 6 hr of exposure it induced DNA strand breaks in these cells and thereby proved to be toxic (Risso-de Faverney et al. 2001). And Cr(VI), which can cross the cell membrane by an anion transporter and is reduced to Cr(III) by intracellular reductants, may also exert toxic effects in fish hepatocytes, as recently demonstrated in a study on goldfish hepatocytes (Nawaz and Krumschnabel 2004).

Although deleterious effects of metal-exposure to isolated hepatocytes have thus been demonstrated in several studies, the mechanisms underlying their toxicity are still only partly understood. One known consequence of metal-exposure is an enhanced formation of radicals, including reactive oxygen species (ROS). ROS production was induced after exposure to Cu in trout hepatocytes (Manzl et al. 2004a), and to Cr(VI) in goldfish hepatocytes (Nawaz and Krumschnabel 2004), and all the three metals enhanced lipid peroxidation in rat hepatocytes (Pourahmad et al. 2003). In some cases metal-exposure may disrupt cellular Ca^{2+} homeostasis, leading to impairment of mitochondrial function and finally to cell death (Nicotera et al. 1992). As a further mechanism of toxicity metals may inactivate cellular enzymes by interacting with their sulfhydryl groups. For example, Cu-induced inhibition of tubulin polymerisation by modifying the sulfhydryl group of the cytoskeletal element has been demonstrated (Arai et al. 1975). Since the cytoskeleton plays an important role in many cellular functions, alterations of any of the three major protein filaments, i.e. microfilaments (actin), microtubules, and intermediate filaments, can cause adverse effects to the cells.

The aim of the present study was the investigation and comparative evaluation of toxic effects of three metals on isolated hepatocytes from the common carp.

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MATERIALS AND METHODS

The acute cytotoxicity of Cu, Cd and Cr(VI) was evaluated and several parameters known to be involved in metal-induced cell death were investigated, including formation of ROS, lipid peroxidation, and intracellular free Ca^{2+} (Ca^{2+}i). Furthermore, toxic effects exerted on cellular integrity were investigated by visualisation of two major proteins of the cytoskeleton, actin filaments and microtubules.

Bovine serum albumin (BSA), fetal calf serum (FCS), and collagenase (Type VIII) were purchased from Sigma. Alexa fluor phalloidin 488, dichlorofluorescein diacetate (DCF-DA), and Fura 2-AM were from Molecular Probes. Mouse anti ß-tubulin monoclonal antibody was obtained from Chemicon and TRITC-labelled anti-mouse antibody was from Dako. CytoTox-ONE™ assay was purchased from Promega. All other chemicals were obtained from local suppliers. Cu, Cd and Cr(VI) were from Merck (Germany) and were applied as $CuCl_2 \cdot 2H_2O$, $CdCl_2$ and $K_2Cr_2O_7$ from stock solution prepared in distilled water.

Common carp (*Cyprinus carpio*) weighing 50-150 g were obtained from a local commercial supplier. Fish were maintained in 200-L aquaria with running water at 20°C and were fed with a mixture of trout pellets (EWOS Aquaculture International) and carp flakes (JBL Pond Coloron) *ad libitum*. Hepatocytes were isolated as previously described (Krumschnabel et al. 1994). After isolation cells were suspended in standard saline including 1% BSA and were incubated for recovery for 1 hr in a shaking water bath thermostated to 19°C. The standard saline contained (in mM): 10 HEPES, 135.2 NaCl, 3.8 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 10 $NaHCO_3$, and 1.3 $CaCl_2$, pH titrated to 7.6. Initial cell viability was determined using the Trypan blue exclusion method and was > 90%.

For experiments on single cells hepatocytes were suspended at a concentration of 2×10^6/ml in sterile, modified Leibovitz L15 medium (L15 medium plus 10 mM HEPES, 5 mM $NaHCO_3$, 50 µg/ml gentamycin, and 100 µg/ml kanamycin, pH 7.6). Cells were plated onto poly-L-lysine coated coverslips and were left to attach in an incubator at 19°C and 0.5% CO_2 overnight. Before using the primary cultures in experiment, cells were washed several times with fresh standard saline to remove non-adherent cells and debris.

The release of lactate dehydrogenase (LDH) from cells with a damaged membrane into the culture medium was determined by using the CytoTox-ONE™ assay and served as a measure of cell viability. Measurements were performed according to manufacturer's instructions using 100 µl aliquots of cell suspensions adjusted to a cell density of 1×10^6/ml.

Measurement of metal-induced alterations of Ca^{2+}i in single cells was monitored by an inverted epifluorescence microscope (Axiovert 100, Zeiss) using the intracellular calcium indicator Fura 2-AM as previously described (Krumschnabel et al. 2001). Briefly, primary cultures (2×10^6/ml) were loaded with Fura 2-AM (3 µM) in standard saline for 1 h, then washed twice to remove surplus fluorescent

dye, mounted in a measuring chamber and were covered with 1 ml of standard saline. The chamber was fixed on the stage of the microscope and fluorescence images were taken through a 40x ultraviolet objective every 60 s with excitation wavelengths set to 340 nm and 380 nm and emission detected > 510 nm. After a stable signal was obtained 500 µl of saline covering the cells were exchanged for an equal volume of saline containing 10 µM of either Cu, Cd or Cr(VI) and the measurement was continued for 1 hr. Since a calibration process with a Ca^{2+}-ionophore could not be performed due to relatively weak attachment of the cells, results of these experiments were expressed as the 340/380 nm ratio.

The production of ROS in isolated carp hepatocytes was determined using DCF-DA as previously described for trout hepatocytes (Manzl et al. 2004a). DCF-DA can cross cell membrane and inside the cells the diacetate is removed by esterases. In the presence of intracellular ROS DCF forms the fluorescent product 2',7'dichlorofluoroscein, which can be detected by a fluorescence microplate-reader. Briefly, 100 µl aliquots of hepatocytes suspension (5×10^6/ml) were transferred into the wells of a 96 well plate and mixed with 150 µl of standard saline containing Tris-HCl instead of HEPES and including DCF-DA (3.6 µM). The cells were then exposed to 10 µM of either Cu, Cd or Cr(VI) while cells treated with an equal volume of distilled water served as controls. Measurements of fluorescence intensity were taken after 0, 0.5, 1, 2, 3 and 6 h, with excitation and emission wavelengths set to 485 and 538 nm, respectively. Between measurements sample plates were placed in an incubator with temperature set to 19°C and 0.5% CO_2.

Lipid peroxidation was determined by measurement of thiobarbituric acid reactive substances (TBARS). Hepatocytes were diluted to a concentration of 1×10^7/ml and were incubated under control conditions or with Cu, Cd or Cr(VI) at final concentrations of 10 µM in a shaking water bath at 19°C. After 0, 3 and 6 hr 300 µl of the cell suspensions were transferred into a reaction tube, were mixed with an equal volume of 10% ice cold trichloroacetic acid (TCA) containing 2% w/v butylated hydroxyl toluene (BHT). Samples were placed on ice for 2 min and centrifuged at 6000×g for 30 s. Finally, 500 µl of the supernatant were mixed with an equal volume of 0.62% thiobarbituric acid (TBA) and the samples were boiled at 95°C for 30 min. Absorbance of TBARS was then measured using a Hitachi F-2000 spectrophotometer at 532 nm.

To analyze metal-induced alterations of the actin filamentous network in carp hepatocytes, cells were diluted to a concentration of $2x10^6$/ml in modified L15 medium and were exposed to 10 µM Cu, Cd or Cr(VI) for 24 hr. After metal-exposure hepatocytes were washed 2-3 times with phosphate buffered saline (PBS, pH 7.6) and were fixed in 4% paraformaldehyde at room temperature for 1 h. After fixation cells were washed twice with PBS for 5 min each and then permeabilized with PBS containing 0.1% Triton X-100 (PBS-T) for 15 min. Subsequently the cells were incubated with Alexa fluor phalloidin 488 (1:150 in PBS) for 30 min at room temperature in the dark. Cells were again washed twice with PBS for 15 min and finally the coverslip, to which the cells were attached, was placed, in inverted position, on an object slide treated with a drop of an

antifade mounting medium (Vectashield, Vector Laboratories Burlingarne, CA). Stained hepatocytes were visualized using a laser scanning microscope, LSM 510 (Zeiss), with excitation wavelength set to 488 nm (Argon laser) and emission being monitored through a band-pass filter (505-550 nm). Images for 3D-reconstruction of the hepatocytes were created from serial optical slices (1 µm) acquired by the use of LSM 510 software version 3.2.

To visualize changes caused by metal-exposure of a second cytoskeleton element we stained β-tubulin of carp hepatocytes. Primary cultures were carefully washed with standard saline and were incubated and fixed as described above for actin. Cells were permeabilized by treatment with PBS-T for 30 min and were treated with a PBS blocking solution containing 0.1% Triton X-100, 1% BSA and 10% FCS for 1 hr. Subsequently the cells were incubated overnight in a humid dark chamber at 4°C with a mouse anti β-tubulin monoclonal antibody (1:200 in blocking solution) followed by three washings in PBS-T. Hepatocytes were then incubated with a secondary TRITC-labelled anti mouse antibody (1:150 in blocking solution) for 1 hr at room temperature, washed with PBS and the coverslips were fixed on object slides treated with mounting medium. Images of β-tubulin were taken with the laser scanning microscope using a Helium/Neon laser with excitation at 543 nm and detection through a long pass filter > 585 nm.

Data are presented as means ± SE of the number n independent preparations or individual cells from at least 3 preparations. Statistical differences were calculated using ANOVA followed by appropriate post-tests. A p-value < 0.05 was considered as significant.

RESULTS AND DISCUSSION

After one hour of metal exposure cell viability was not affected (not shown), but after 3 hr of incubation a significant decrease of cell viability from 87 ± 3% in controls ($n = 5$ for each treatment) to 64 ± 9% in Cu-treated hepatocytes was observed. Exposure to Cd and Cr(VI) over the same period had no effect on cell viability, which amounted to 87 ± 4% and 86 ± 6%, respectively. This clearly indicates that, acutely, exposure to Cu is most toxic for carp hepatocytes, resulting in a significant decrease of cell viability, whereas Cd and Cr(VI) exerted marked toxic effects as well (see below), but did not kill the cells in the short term. These results are in line with a previous study on trout hepatocytes where, in contrast to Cu, Cd has been found to be non-toxic even in a 10-fold higher concentration during short-term exposure to the metal (Manzl et al. 2003). Similarly, although Cr(VI) was found to be acutely toxic in goldfish hepatocytes, a concentration of as much as 250 µM was required to cause cell killing and no effect was noted at 10 µM as used in the present study (Nawaz and Krumschnabel 2004).

The impact of metal exposure on $Ca^{2+}i$ of carp hepatocytes is depicted in Fig. 1. As can be seen, a strong and steady increase of $Ca^{2+}i$ was elicited by the presence of Cu during 1 hr of exposure. This increase was not immediate but occurred after a delay of approximately 15 min. Exposure to 10 µM Cd or Cr(VI) caused a more sudden increase, being significant after 5 min of incubation. However, in both

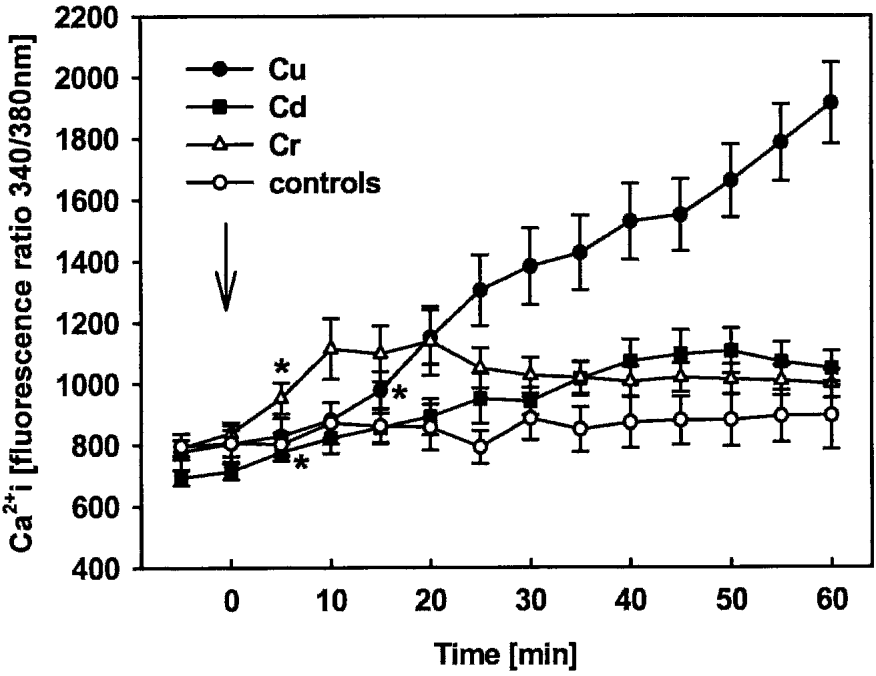


Figure 1. Cytosolic free calcium ($Ca^{2+}i$) of carp hepatocytes during incubation with 10 µM Cu, Cd or Cr(VI). Metals were added at time zero (arrow). Data are means ± SE of 42-50 individual cells from 4-6 independent cultures obtained from 4-6 independent preparations. * denotes first point where $p < 0.05$ compared to time zero. $Ca^{2+}i$ was not significantly altered in controls.

cases the relative increase was much less pronounced as compared to Cu and a $Ca^{2+}i$ peak was observed after 10 and 50 min for Cr(VI) and Cd, respectively. In controls $Ca^{2+}i$ was more or less constant during the entire experimental period. In comparison, in previous studies we found that neither Cd nor Cr(VI) caused an elevation of $Ca^{2+}i$ in hepatocytes from trout (Manzl et al. 2003) and goldfish (Nawaz and Krumschnabel 2004), respectively, indicating that carp hepatocytes respond more sensitive with regard to maintenance of Ca^{2+}-homeostasis. The more pronounced increase of $Ca^{2+}i$ induced by Cu resembled that seen in trout hepatocytes (Manzl et al. 2003). Since only Cu reduced cell viability within the experimental period studied, this suggests that a certain $Ca^{2+}i$ threshold must be surpassed to cause cell death, which is in line with previous observations (Krumschnabel et al. 1999). Similarly, although Cd was cytotoxic in a grass carp cell line, a much higher relative increase of $Ca^{2+}i$ was associated with this effect (Xiang and Shao 2003).

Intracellular ROS formation was assessed by measurement of the DCF-oxidation. Hepatocytes exposed to 10 µM Cu showed an increase in cellular ROS which was

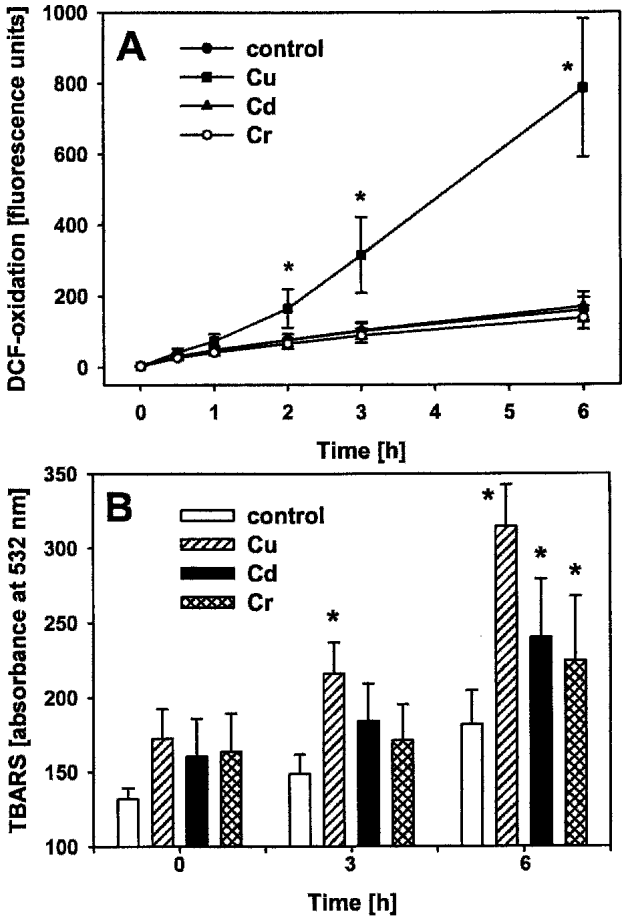
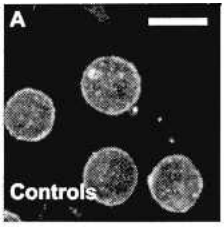
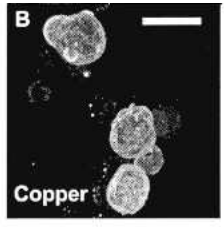
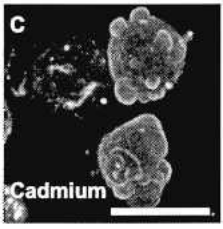


Figure 2. A. Production of reactive oxygen species and **B.** lipid peroxidation in hepatocytes isolated from the common carp incubated in standard saline (control) or in the presence of 10 μM Cu, Cd or Cr(VI). Data are means ± SE of 5-6 independent preparations. * <0.05 compared to controls.







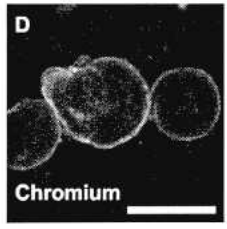
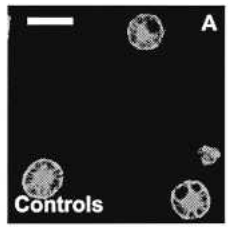
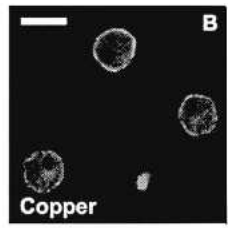
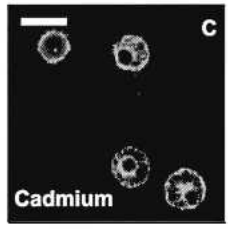


Figure 3. 3D reconstruction of actin filamentous network in carp hepatocytes monitored after 24 hr of incubation under control conditions or in the presence of 10 μM of the metal indicated. Bars denote 20 μm.







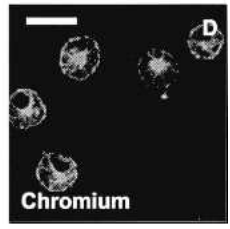


Figure 4. Tubular meshwork of primary cultures of carp hepatocytes presented as an optical slice through the equatorial plane of the cells. Cells were incubated for 24 hr under control conditions or in the presence of 10 μM of the metal indicated. Bars denote 20 μm.

significant after 3 and 6 hr of incubation (Fig. 2A). In contrast, exposure to either Cd or Cr(VI) caused no significant increase of ROS formation over 6 hr of measurement compared to the basal level observed in controls. Measurement of TBARS, reflecting the level of lipid peroxidation, indicated that, compared to controls, the addition of Cu elevated this parameter 1.5- to 1.7-fold after 3 and 6 h, respectively (Fig. 2B). Different from the absence of an effect on ROS formation, a significant elevation of TBARS could also be observed after 6 hr of exposure to Cd or Cr(VI). The relatively strong effect of Cu on ROS formation and lipid peroxidation is consistent with previous observations on hepatocytes from trout (Manzl et al. 2004a) and rat (Pourahmad et al. 2003), underlining the huge redox-reactivity of this metal. Similarly, the lack of enhanced ROS production with Cd despite elevated lipid peroxidation is in agreement with earlier findings (Muller 1986; Ochi et al. 1987; Watjen and Beyersmann 2004). This corroborates the notion that Cd is not directly involved in redox cycling, but rather exerts its toxic action via interference with membrane stability and cellular antioxidative protection mechanisms (Stohs et al. 2001). In contrast, the increase of TBARS formation induced by Cr(VI) in the absence of a measurable enhancement of ROS is at variance with the well known redox-cycling potential of this metal (Stohs et al. 2001). However, numerous studies have shown that rather high concentrations of Cr(VI), up to 1 mM (Pourahmad et al. 2003), are required to induce short term toxic effects (Nawaz and Krumschnabel 2004). The present results may thus indicate that, at low concentrations, lipid peroxidation is a more sensitive indicator for Cr(VI) toxicity as compared to other parameters.

Toxic effects on cytoskeleton elements caused by metal-exposure were investigated in hepatocytes stained to visualize actin filaments or microtubules. Acutely, we found no significant alterations of the cytoskeleton (not shown), for which reason we investigated this parameter in hepatocytes exposed to the metals for an extended period. The vast majority of the Cu-treated cells had completely lost cell integrity after 24 hr of Cu-incubation, rendering staining and visualization of the actin filamentous network impossible in these cells. However, in those Cu-treated cells which survived an incubation of 24 h, actin filaments, which formed a smooth cortical sheath in control cells, appeared disassembled (Fig. 3A+B). Furthermore, the phalloidin derivative protruded into numerous cell surface blebs which had formed. A similar loss of overall cell viability was seen in cells treated with Cd or Cr(VI), and in the former case surviving cells were characterized by even more pronounced surface blebbing as compared to Cu-treated cells (Fig. 3C). In hepatocytes incubated with Cr(VI) overall staining for actin was very weak and it appeared that the actin filamentous network in these cells was most strongly disrupted by metal-exposure (Fig. 3D). Compared to the devastating effect of the metals on actin filaments, the impact on microtubules was less dramatic (Fig. 4). However, in many hepatocytes an overall decrease of microtubular structures was noted, being most pronounced in Cu-treated cells. Disruption of the actin filamentous network as a response to Cu, Cd and Cr(VI)-exposure has been reported by several studies (Gunaratnam and Grant 2002; Manzl et al. 2004b). These results can be explained by the fact that these metals may interact with actin filament integrity by directly binding to cytoskeletal elements or to proteins involved in actin filament polymerization, or via disruption of Ca^{2+} homeostasis disturbing the function of these proteins (Wang and Templeton 1996). Alternatively, metal-induced ROS formation may damage filaments, e.g. by interacting with actin thiol groups (Kuo et al. 1997). Metal effects on microtubules have received much less attention so far, and at least the present results seem to indicate that they are indeed less sensitive than actin filaments. Nevertheless, a reduction of the microtubular meshwork could be observed, with the possible mechanisms underlying this effect presumably resembling those outlined for actin. For example, polymerization of tubulin was found to be reduced in the presence of 50 μM Cu in cell free extracts of bovine brain (Liliom et al. 1999), and in cortical neurons microtubules were even more sensitive to oxidative stress than actin filaments (Allani et al. 2004).

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