Physiological and oxidative stress biomarkers in Oreochromis mossambicus exposed to hexavalent chromium

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INTRODUCTION

Chromium (Cr) is a naturally occurring heavy metal found commonly in the environment in two valence states: trivalent Cr (III) and hexavalent Cr (VI). It is widely used in numerous industrial processes and as a result, is a contaminant of many environmental systems (Cohen et al., 1993). Exposure to acidic environments and ROS can act synergistically to cause extensive DNA damage leading to apoptosis (programmed cell death) (Antonova et al., 2009). Antioxidant enzyme systems are a well-developed regulatory mechanism protecting against oxidative stress. Under normal physiological states, ROS are rapidly eliminated by antioxidant enzymes, including superoxide dismutase (SOD) and Catalase (Yu, 1994; Abele and Puntarulo, 2004; Mohankumar and Ramasamy, 2006). The SOD catalyses the dismutation of two superoxide radicals to hydrogen peroxide (H₂O₂), whereas CAT degrade H₂O₂ (Holmlblad and Soderhall, 1999; Mohankumar and Ramasamy, 2006). The effects of Chromium exposure on the enzyme activity of SOD and Catalase (CAT) were investigated in blood cells of Oreochromis mossambicus. The results showed that pollution stress induced down regulation of SOD and CAT. This provides the first evidence that Chromium induced oxidative stress may cause DNA damage, and cooperatively activate the expression of checkpoint-related proteins and antioxidant enzymes, through the activation of a p53-mediated signal transduction pathway.

MATERIALS AND METHODS

Experimental Design

Groups of six fishes each were treated with four different potassium dichromate dose levels. Potassium dichromate was diluted with water (as required) and exposed animals at the doses of 2.5, 5.0, 7.5, and 10.0 mg/kg BW, one dose per 24 hrs given for 7 days. We selected toxic induced via water because it is the most commonly used method that is simple and also, for many agents such as chromium compounds, it will tend to maximize chemical exposure to the target organs. Each fish received a total of five doses at 24 hrs intervals. At the end of the exposure, after overnight starvation, the liver and both gills were removed under anesthesia. The organs were washed thoroughly in ice-cold physiological saline and weighed. The biological material not used immediately was stored frozen at -80°C until further analysis.

Animal and blood cells collection

The blood samples (approximately 1mL per individual) were collected using a syringe from the tail muscle. The samples from each Tilapia were immediately centrifuged at 800xg at 4°C for 10 min to collect the blood cells. Three replicates were examined at each sampling time.

KEYWORDS:
Chromium, SOD, catalase, DNA damage and Oreochromis mossambicus

ABSTRACT

Fish constitute an excellent model to understand the mechanistic aspects of metal toxicity vis-à-vis oxidative stress in aquatic ecosystems. Hexavalent chromium (Cr (VI)), due to its redox potential can induce oxidative stress (OS) in fish and impair their health. In the present investigation, Potassium dichromate was exposed to Oreochromis mossambicus for 7 days with the doses of 2.5, 5.0, 7.5, and 10.0 mg/kg body weight per day. Oxidative stress including the level of reactive oxygen species (ROS), the extent of lipid peroxidation (TBARS) and the activity of antioxidant enzymes (SOD, CAT) in both liver and gill was determined. DNA damage in blood cells was determined by single cell gel electrophoresis (comet assay). The results indicated that administration of Cr (VI) had caused a significant increase of ROS level in both liver and gill after 7 days of exposure, accompanied with a dose-dependent increase in superoxide dismutase and catalase activities. The malondialdehyde content in liver and gill was elevated as compared with the control animals. Dose- and time-dependent effects were observed on DNA damage after 24, 48, 72, and 96 hrs post treatment. Taking together the findings of this study are helpful in organ-specific risk assessment of Cr (VI)-induced oxidative stress biomarkers and genotoxicity in fish.

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**Lipid peroxidation**

Plasma levels of malonaldehyde, a marker of lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) by fluorescence methodology (Yagi, 1976). Briefly, plasma (200 l) was treated with 8.1% SDS and 20% acetic acid to solubilize protein and precipitate it and then heated with Thiobarbituric acid for 1 h at 95°C. The supernatant was then extracted with butanol: pyridine (15: 1) to yield a fluorescent product, which was detected by excitation at 535 nm and emission at 535 nm. Absolute malonaldehyde levels were calculated using the regression parameters obtained using various concentrations (0.25–5.0 nM) of the standard. 1, 1’, 3, 3’,-tetramethoxypropane. Inter- and intra-assay coefficients of variation of the above method were < 5 and 10%, respectively. (Ganesan et al, 2011)

**ROS measurement**

Intracellular Reactive Oxygen Species (ROS) generation was measured using DCF-DA dye method. Once the cells were collected according to the experimental conditions, cells were incubated with 15µM DCF-DA for 45 minutes, briefly centrifuged down to remove the dye and resuspended in HEPES buffer and change in fluorescence was measured in a Spectrofluorimeter set at 485nm excitation and 530nm emission. Cells were challenged with PMA to induct ROS generation.

**Comet assay**

DNA strand breaks and FPG-sensitive sites were detected in blood cells by single cell gel electrophoresis, the comet assay (Singh, McCoy, Tice, & Schneider, 1988). Clear microscope slides were pre-coated with 1% normal melting agarose. For each slide, 100 µl of cell suspension (approximately 10,000 cells) was mixed with 200 µl of 0.5% low melting point agarose, spotted as first layer onto the pre-coated slide and covered with a coverslip. After agarose solidification the cover slip was gently removed; a second layer of 200 µl of normal melting agarose (NMA) was added over the first layer, covered with a coverslip and allowed to solidify. Cover slips were removed and slides were placed in chilled lysis buffer (2.5MNaCl, 100mM EDTA, 10mM Tris–HCl; pH 10, 1% Triton X-100 and 10% DMSO added just before use) at 4°C for 1 h. After lysis, the slides were placed on the platform in an electrophoresis tank that contains the pre-chilled (4°C for at least 1 hr) electrophoresis solution (300mM NaOH, 1mM EDTA, pH 13). The buffer should just barely cover the slides and was incubated for 30 min at 4°C before beginning electrophoresis. The electrophoresis was subsequently conducted at 25V constant voltage and 300mA for 30 min. Then slides were removed from electrophoresis apparatus and washed with three changes of neutralization buffer in staining jar for 5 min each at 4°C. Each slide was stained with 75 µl of ethidium bromide (20µg/ml) and covered with a cover slip. The slides were examined under a fluorescent microscope and analyzed within 3–4 hrs. Slides were scored using an image analysis system (Comet Imager 1.2.13) attached to a fluorescent microscope (Carl-Zeiss, Germany) equipped with appropriate filter. The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to software for analysis. The final magnification was 400×. Analysis of mean % DNA in the tail, one of the reliable indicators of DNA damage was done using image analysis software. Images from 50 cells (25 from each duplicate slide) were analyzed. To show the reproducibility of the method, DNA damage was measured in blood cells from Tilapia sp collected at five different sites of Chrompet Lake. For this, blood samples were taken twice from the same subject on different sites; the respective samples were used for the comet assay and checked for and significance (p < 0.05).

**Antioxidant enzyme activity**

After sacrificing the fishes, fresh blood samples were collected in monovettes with EDTA and transported on ice. Liver were dissected, stored in ice-cold 1X PBS (Phosphate Buffer Saline) and transported on ice. Blood samples were centrifuged for 15minutes at 4000rpm and collected plasma fractions were divided for each analysis separately. Then, blood cells were hemolized with distilled water and DNA-se (1 mg/ml), and cells were frozen over night at -80°C. Next day the hemolise fractions were centrifuged again and hemolise fractions were divided for each analyses. The specimens of hemolise were frozen and stored at -80°C until use. Superoxide dismutase (SOD; EC 1.15.1.1) activities were measured by the ferricytochrome C method using xantine/xantine oxidase as a source of superoxide radicals. Enzyme activity of the present analysis can be reported in units of SOD per milligram of Hb or protein. One unit of activity is defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate (McCord and Fridovich, 1969). Catalase (CAT; EC 1.11.1.6) activities was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm according to Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH=7) and 10.6 mM H2O2 freshly added.

**Statistical analysis**

Comparison between groups were performed using one-way ANOVA with p<0.05 as the criterion for significance. All analysis was done using windows based SPSS statistical package (version 12.0, Chicago, IL).

**RESULTS**

**TBARS in Liver and Gills**

Oxidative stress as determined by lipid peroxidation (TBARS). Potassium dichromate exposure significantly (P<0.05) increased the concentration of MDA in both liver (13.4± 0.25 –15.53± 1.29 nmol/g liver) and gills (14.6± 0.74 – 15.9± 0.89 nmol/g gills) when compared with the control (11.12 - liver; 11.3 - gills). Results are illustrated in Figure 1. The increase in MDA concentration in both liver and gills was found to be dose-dependent, indicating a gradual increase with increasing dose of potassium dichromate. However, the liver exhibited more oxidative stress than the gills.

![Figure 1 Effect of potassium dichromate on detection of TBARS in the liver and gills of Oreochromis mossambicus. Each experiment was done in triplicate. Values are expressed as means ± SD. (*) P<0.05 compared to control.](image-url)
ROS detection in Liver and Gills

The administration of Cr (VI) to fish significantly enhanced the ROS level at four tested doses as compared with the control animals and increases were dose-dependent. However, the level of ROS in Liver was lower compared with gills at all four dose levels. Figure 2 (135.67 ± 2.45 to 670.23 ± 4.87; P<0.001) represents the results of ROS detection.

DNA damage in tilapia blood cells

The level of DNA damage in tilapia RBC cells was shown as the tail moment, tail length and as the percentage of migrated DNA. An endpoint “tail moment” is defined as the product of the tail length and the fraction of DNA in the tail. The results are shown in Fig. 3. ([B] Tail Length 2.01±0.2 - 7.67±0.12 & [C] % DNA damage 4.01±1.2-17.67±0.98) DNA damage in blood cells demonstrated that there was a significant difference (P < 0.05) between the fish from Chromium exposed and the control, in which there was approximately double the number of cells with DNA damage, indicating genotoxicity in the environment.

Figure 2 Effect of potassium dichromate on detection of ROS in the liver and gills of Oreochromis mossambicus. Each experiment was done in triplicate. Values are expressed as means ± SD. (#) P<0.001 compared to control.

Enzyme activity of SOD in Liver and Gills

The effect of chromium exposure on the enzyme activity of SOD in tilapia Liver and Gills is shown in Fig. 4. Potassium dichromate exposure significantly (P<0.05) increased the concentration of enzyme activity in both liver (3.1± 0.75 – 5.5± 0.37 U/mg) and gills (2.4± 0.14 – 3.8± 1.01 U/mg) when compared with the control (1.33 - liver; 1.9 - gills). All are statistically significantly compared with the control groups.

Enzyme activity of CAT in Liver and Gills

The effect of chromium exposure on the enzyme activity of CAT in tilapia Liver and Gills is shown in Fig. 5. The results are significantly (P<0.05) increased the concentration of enzyme activity in both liver (0.6± 0.03 – 2.1± 0.6 U/mg) and gills (1.3± 0.64 – 2.6± 0.92 U/mg) when compared with the control (0.1 - liver; 0.3 - gills).
CONCLUSION

The current study demonstrates that the Cr (VI) induced oxidative stress biomarkers effects in Oreochromis mossambicus goldfish liver and gills at all the tested concentrations (for 24h to 96h) exposure. As a result of oxidative stress, the antioxidant enzyme levels were significantly modulated in both organs in a concentration-dependent manner. Cr (VI) induced significant DNA damage in Oreochromis mossambicus liver and gills cells leading to believe that the elevated levels of antioxidants were inadequate to combat the high level of ROS generated.

References

Mohankumar, K., and Ramasamy, P. 2006. Whist spot syndrome virus infection decreases the activity of antioxidant enzymes in Fenneropenaeus indicus, Virus Res. 15, 69 -75.

DISCUSSION

Several studies showed that the heavy metal related loss of a number of functions is associated with an accrual of oxidative damage in the tissues mediating those functions. With antioxidant systems become deregulated, just like so many other cellular components, and so oxidative damage occurs. Therefore, the production of ROS is not merely a cause of havoc but rather a complex and critical system whose disruption in disease and aging leads to oxidative damage (de Magalhaes and Church, 2006). The increased oxidative stress, DNA damage and antioxidant enzyme activity are thought to contribute to the aging process by oxidative modification of different macromolecules, such as lipids, proteins, and genomic DNA. In conclusion, our study presents a possible pathway how toxicity affects Oreochromis mossambicus exposed to Chromium, Values are expressed as means ± SD. (*) P<0.05 compared to control.

Figure 5 Enzyme activity of CAT in Liver and Gills of Oreochromis mossambicus exposed to Chromium. Values are expressed as means ± SD. (*) P<0.05 compared to control.

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