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# Application of fish cell lines for evaluating the chromium induced cytotoxicity, genotoxicity and oxidative stress

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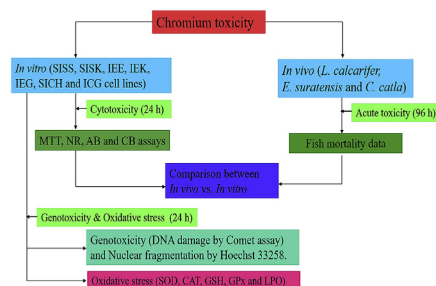
## HIGHLIGHTS

- To assess the chromium toxicity by *in vitro* and *in vivo* assays.
- *In vitro* assay using seven fish cell lines.
- *In vivo* assay using three fish species of different environments.
- Cytotoxicity measured by MTT, NR, AB and CB assays.
- Compared the *in vitro* and *in vivo* assays to determine chromium toxicity.

## GRAPHICAL ABSTRACT

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## ABSTRACT

In the present study, we hypothesize that cytotoxicity, genotoxicity and oxidative stress play a key role in chromium induced toxicity in SISS, SISK, IEE, IEK, IEG, SICH and ICG cell lines after 24 h exposure. Three fish species namely *Lates calcarifer, Etroplus suratensis* and *Catla catla* were exposed to the concentrations of 0, 10, 20, 30, 40 and 50 mg/L of chromium for 96 h under static conditions for conducting acute toxicity tests. $LC_{50}$ was then calculated. The percentage cell survival was assessed by multiple endpoints such as MTT, NR, AB and CB assays in the seven fish cell lines exposed to different concentrations of chromium and $EC_{50}$ values of all the four endpoints were calculated. High significances were noted in the correlations between each *in vitro* cytotoxicity assays and *in vivo* mortality data. Cell shrinkage, cell detachment, vacuolations and cell swelling at the highest concentration of chromium (50 mg/L) were seen on microscopic examination of cell morphology. Comet assay and Hoechst staining were carried out to assess DNA damage and nuclear fragmentation in the seven fish lines exposed to chromium. The results of antioxidant parameters obtained indicate a significant reduction in the level of catalase, superoxide dismutase, glutathione S-transferase and Glutathione peroxidase, and increased level of lipid peroxidation in all the cell lines exposed to chromium. These results confirm that fish cell lines could be used as an alternative to whole fish for cytotoxicity, genotoxicity and oxidative stress assessment in chromium toxicity studies.

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## 1. Introduction

A serious environmental problem facing the modern world is heavy metal pollution of water. At global level heavy metal pollution is increasing in the environment due to increase in number of

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industries (Chidambaram et al., 2009). Industrial effluents are discharged into the sewage canals, rivers and irrigation water, causing major pollution and health hazards (Baddesha and Rao, 1986). Lambert et al. (2000) reported the presence of heavy metals such as arsenic, cadmium, chromium, copper, lead, nickel, and zinc in the waste water and their risk to human health and environment. Usually toxic heavy metals are mostly absorbed and get accumulated in various plant parts as free metals which may adversely affect the plant growth and metabolism (Barman and Lal, 1994). Among heavy metals, chromium plays a major role in polluting our aquatic environment. In nature, chromium occurs predominately in two valances Cr (III) and Cr (VI). Hexavalent chromium [Cr (VI)] predominates over the Cr (III) form in natural waters. Hexavalent chromium [Cr (VI)] particulates enter the aquatic medium through effluents discharged from leather tanning, textiles, chrome electroplating, metal finishing, dyeing and printing industries and several other industries.

The Cr (VI) penetrates biological membranes easily and causes cellular damage by oxidative stress (Irwin et al., 1997; Begum et al., 2006), its unselective exposure may pose a serious threat to aquatic communities including fish. Toxic effects of Cr (VI) on enzymological/biochemical (Al-Akel and Shamsi, 1996; Vutukuru et al., 2007; Oner et al., 2008), hematological (Gautam and Gupta, 1989; Al-Akel and Shamsi, 1996), immunological (Prabakaran et al., 2007) parameters, endocrine toxicity (Mishra and Mohanty, 2009) and genotoxicity (Chen et al., 2011) have been reported in many teleost fishes.

The toxicity tests on fish have involved the use of lethality as the endpoint to assess environmental risk. On the other hand, whole animal bioassay is expensive and requires huge quantity of toxicant. The exposure time required in the case of fish cell lines for cytotoxicity test is only 24 h, whereas in the case of bioassay (*in vivo*) it is 96 h. This reduces the cost of labor, lab facilities, test time and allows a decision on toxicity to be taken more quickly. Nevertheless, toxicity testing with fish is an essential part of environmental risk assessment procedures (Castaño et al., 2003). For all these considerations, the development and use of *in vitro* assays that could measure early stages of toxicity in vertebrates represent an approach that could be very useful to monitoring environmental risk assessment (Walker, 1999). Over the last four decades, cell and tissue culture methods have been refined and have now become an essential tool in environmental research. There are a lot of ethical, scientific and economical reasons that support the development of *in vitro* methods for use in ecotoxicology (Castano and Gomez-Lechon, 2005; Bols et al., 2005; Schirmer, 2006; Fent, 2007; Taju et al., 2012, 2013, 2014). The use of fish cell lines as *in vitro* model for environmental toxicology has been studied and positively assessed mainly with regards to cytotoxicity (Babich and Borenfreund, 1991; Castaño et al., 2003; Fent, 2001). Multiple endpoints, including the measurements of cell death (apoptosis), cell viability, cellular morphology, cell metabolism, cell attachment/detachment, cell membrane permeability, proliferation, growth kinetics, genotoxicity and oxidative stress can be examined by making use of cytotoxicity assessments (Maracine and Segner, 1998; Li and Zhang, 2002; Shuilleabhain et al., 2004; Taju et al., 2014).

*Lates calcarifer* (Marine), *Etroplus suratensis* (Brackishwater) and *Catla catla* (freshwater) are excellent food fishes with good market demand in India, Malaysia, Bangladesh and Pakistan. In the present study they were selected as representatives of their respective aquatic environments to study their suitability for *in vivo* acute toxicity test to evaluate the potential risk of chromium (Cr). Attempts were previously made to study an *in vivo* acute toxicity in sea bass, pearl spot and catla using various toxicants (Chezhian et al., 2010; Azmat, 2011, 2012; Bhat et al., 2012; Taju et al., 2012, 2013). The seven fish cell lines namely SISK and SISS cell lines derived from *L. calcarifer* (Sahul Hameed et al., 2006; Parameswaran et al., 2006), SICH and ICG cell lines derived from *C. catla* (Ishaq Ahmed et al., 2009b; Taju et al., 2014), and IEE, IEK and IEG cell lines derived from *E. suratensis* (Sarath Babu et al., 2012) were used as an *in vitro* assay to evaluate the cytotoxicity, genotoxicity and oxidative stress after exposing them to chromium. In addition, the results of cytotoxicity were compared with the results of *in vivo* bioassay test using fish.

## 2. Material and methods

### 2.1. Chemicals and reagents

Tissue culture media and chemicals were obtained from GIBCO (Invitrogen Corporation, USA). Potassium dichromate ($K_2Cr_2O_7$), EDTA, Trichloroacetic acid, DTNB [5,5-dithio-bis-(2-nitrobenzoic acid)], Thiobarbituric acid, Hydrogen peroxide, Nitro blue tetrazolium (NBT), Riboflavin, Hydroxylamine–HCl, Triton X-100, Ethidium bromide, Methanol, Acetic acid, Sodium chloride, Sodium hydroxide and Coomassie Blue were purchased from SRL chemicals, India.

### 2.2. Collection of experimental animals

*Lates calcarifer* and *Etroplus suratensis* were collected from Central Institute of Brackishwater Aquaculture (CIBA), Chennai. *Catla catla* was obtained from a local fish farmer in Walajapet, Vellore - District, Tamil Nadu, India. The experimental fishes were 2–3 g in body weight. Specimens were transported live in oxygen bags or buckets to the laboratory. They were acclimatized and maintained for 20–30 days in a salinity range of 5–10 ppt for *E. suratensis*, 20–25 ppt for *L. calcarifer* and in freshwater in the case of *C. catla* under an ambient photoperiod in the laboratory for 10 days prior to experiments at room temperature (23–28 °C). Commercial pellet feed was given twice a day. Then they were starved for 24 h before and during the experiments.

### 2.3. *In vivo* fish acute toxicity test

Acute toxicity tests were conducted by exposing *E. suratensis*, *L. calcarifer* and *C. catla* (N = 10 per aquarium) for 96 h to chromium under static conditions (OECD 203, 1992). Five different concentrations of chromium i.e., 10, 20, 30, 40 and 50 mg/L were diluted with seawater (5 ppt) for *E. suratensis* and *L. calcarifer* and freshwater for *C. catla* while control groups kept in sea water and freshwater alone were tested to determine the $LC_{50}$ (concentration at which 50% of the fish population dies). Based on the body weight of fishes (1 g fish/L), aquarium tanks with a working volume of 30 L were used. Dead fishes were counted and removed immediately every day. All the experiments were conducted in triplicates. The standard guideline was used to determine the acute toxicity and mortality in fish (OECD 203, 1992).

### 2.4. Fish cell lines

A total of seven cell lines established from different organs of *L. calcarifer* (SISS-seabass spleen, SISK-kidney), *E. suratensis* (IEE – Etroplus eye, IEG - gill, IEK - kidney) and *C. catla* (SICH – Catla heart, ICG - gill) were tested for their sensitivity to chromium. These fish cell lines were propagated at 28 °C in Leibovitz's L-15 medium (pH 7.0–7.4) with 2 mM L-glutamine, 10% foetal bovine serum (FBS), penicillin 100 IU/mL and streptomycin 100 μg/mL. The osmolarity ranged from 300 to 360 mOsm $kg^{-1}$. These cells were sub-cultured every 2–3 days using standard procedure. For *in vitro* cytotoxicity tests cells at exponential growth phase were harvested and used.

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