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Developmental toxicity, uptake and distribution of sodium chromate assayed by frog embryo teratogenesis assay-*Xenopus* (FETAX)

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ABSTRACT

The embryotoxicity and teratogenicity of Cr(VI) on the survival and morphology of the anuran *Xenopus laevis* have been assessed by frog embryo teratogenesis assay-*Xenopus* (FETAX). The lethal median (LC_{50}) and teratogenic median (TC_{50}) concentration values of Cr(VI) were 890 μM and 260 μM, respectively. The calculated teratogenic index (TI) value was 3.42, suggesting that hexavalent chromium has a teratogenic potential. Malformations of embryos included lifting of the body, coiling of the tail and body oedema. Furthermore, the chromium salt caused significant growth retardation at 25 μM exposure concentrations. The use of radiolabelled $^{51}Cr(VI)$ allowed the determination of the time course uptake of Cr in *Xenopus* exposed to concentrations ranging from 0.025 to 500 μM. The evaluation of its distribution into the body (head–abdomen–tail) was evaluated at different exposure times. Chromium is taken up at 24 h by *Xenopus* embryos for all concentrations tested. At 48 h post fertilization (stage of larva) the amount of Cr accumulated by the two-day-old larva ranged from 0.42 to 580 pg mg^{-1} wet weight at 0.025 and 500 μM respectively. These amounts were lower than those at 24 h (2.77 to 11016 pg mg^{-1} wet weight embryo) reaching values of the same order of magnitude at 120 h (five-days-old larva). Since at 48 h *Xenopus* development leads to a swimming embryo, the observed uptake at 24 h could be the result of the binding of Cr to jelly coat compounds surrounding the embryo body as confirmed by gel filtration experiments on ^{51}Cr-jelly coat. The interaction of Cr with jelly coat is in agreement with the role of jelly coat in protecting the embryo against pathogen and chemical toxins to ensure fertilization. This work further supports the hypothesis that Cr contamination of surface waters could contribute to explain the reported worldwide depletion of frog population.

1. Introduction

A worldwide decline in amphibian populations has been described in the last few years (Williams, 2007). Several causes have been suggested such as natural fluctuations, parasites, habitat fragmentation, climatic changes, acid rain, parasites, UV exposure due to ozone layer depletion, oil and chemical pollution, including trace metals (Lefcort et al., 1998; Mendelson et al., 2004). In fact, trace metals are well known for their toxicity in living organisms also at low levels of exposure (De Forest et al., 2007). In this context, chromium is one of the most widely distributed trace metals in the earth's crust although its presence in the environment has been mainly related to anthropogenic sources (Goyer, 1996). In particular, hexavalent chromium compounds are well known mutagens in bacterial and mammalian assays and are established carcinogens in humans, being classified within Group 1A by IARC (i.e., there is sufficient epidemiological evidences for the chemicals to be considered carcinogens) (WHO, 1988). Moreover, hexavalent, but not trivalent, chromium compounds are teratogens in mice, resulting in a high incidence of embryo deaths and skeletal abnormalities (Trivedi et al., 1989). In natural waters, chromium occurs mainly as Cr(III) and Cr(VI) (Comber and Gardner, 2003) which greatly differ in their chemistry and toxicology (Sivakumar and Subbhuraam, 2005). Since Cr(VI) is more toxic than Cr(III), information regarding its potential embryotoxic and teratogenic effects in amphibians is of particular interest in understanding if it could be a cause of the extinction of amphibian populations.

Well established protocols and the ease use of *Xenopus* in the laboratory has made FETAX an appropriate bioassay for the assessment of many questions concerning amphibian toxicology (Hoke and Ankley,

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2005). By its three endpoints (embryotoxicity, teratogenicity and growth retardation), FETAX can detect chemicals that affect embryonic development (Richards and Cole, 2006). Moreover, inter-laboratory validation studies have shown that the FETAX assay appears to satisfy the requirements of low cost, reliability and reproducibility, representing one of the most extensively used toxicity screening test systems for alternative developmental toxicity testing (Brown et al., 1995). However, few papers have reported on the application of FETAX to metal toxicology including $NiCl_2$, $CdCl_2$ and $CoCl_2$ (Plowman et al., 1994), $CuCl_2$ and $ZnCl_2$ (Luo et al., 2006), $HgCl_2$ and CH_3HgCl (Prati et al., 2002), $NaAsO_2$, Na_2HAsO_4 and dimehylarsinic acid (Gornati et al., 2002) as well as $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$ (Monetti et al., 2003).

The aims of the present study were: 1) the determination of embryotoxicity, teratogenicity and growth retardation of Na_2CrO_4 in *Xenopus laevis* by FETAX, in order to establish concentration–effect relationship; 2) the study of the time course of metabolic patterns of Cr (uptake, repartition among body sections and binding with body biocomponents) at different developmental stages of *Xenopus* by means of radiotracer in combination with bioanalytical techniques.

2. Experimental procedures

2.1. Chemicals, solutions and materials

$Na_2CrO_4 \cdot 4H_2O$ (hereafter named Cr(VI)) was purchased from Alfa Aesar; formaldehyde, 3-aminobenzoic ethyl ester acid (MS 222), cacodilic acid and sucrose from Sigma Aldrich, Cologno Monzese, Italy; human chorionic gonadotropin (Profasi) from Serono, Latina, Italy. All other reagents were supplied by Sigma Aldrich, Cologno Monzese, Italy. Neutron Activation Analysis was performed by irradiating 120 h old *Xenopus* embryos. The elemental impurities of Cr(VI) salt were determined by Neutron Activation Analysis and Inductively Coupled Plasma Mass Spectroscopy (Sabbioni et al., 1993). The composition and the preparation of FETAX and DeBoer-Tris solutions were previously described (Monetti et al., 2003). Sephacryl S-200 resin was supplied by Pharmacia; gel filtration molecular standard proteins kit (molecular weights 6500–66,000) from FLUKA, Cologno Monzese, Italy. Centricon (YM-1) Centrifugal Filter Devices (nominal molecular weight cut off 100 kDa) were supplied by Amicon, Millipore.

2.2. Radiochemicals and radioactive counting

$Na_2^{51}CrO_4$ radiotracer in aqueous saline ($T_{1/2} = 27.7$ d, specific radioactivity 16.7 MBq $mgCr^{-1}$, radiochemical purity>99%, radioactive concentration 185 MBq mL^{-1}) was purchased by Perkin Elmer Life Sciences, Massachusetts, USA. The $Na_2^{51}CrO_4$ was used to prepare ^{51}Cr-labelled solutions (hereafter named ^{51}Cr(VI)) at concentrations ranging from 0.025 to 500 μM by mixing appropriate amounts of cold Cr(VI) with aliquots of $Na_2^{51}CrO_4$ in FETAX. Solutions were equilibrated overnight at room temperature and checked by Minoia's method (Minoia et al., 1983) for the chemical form. More than 99% of the ^{51}Cr remained Cr(VI), being extracted in the organic phase.

^{51}Cr radioactivity was measured by Wallac 1480 3 in. automatic γ-counting system (Perkin Elmer, Massachusetts, USA) using the characteristic line of 320 keV photon emission. ^{51}Cr radioactivity was interpreted in term of Cr concentration by comparison with ^{51}Cr standards.

2.3. In vitro fertilization

Mature *X. laevis* were purchased from a local supplier (Rettili, Varese, Italy) and maintained in aquariums (Tecniplast, Varese, Italy). FETAX was performed as previously reported (Monetti et al., 2003). Sexually mature females were injected with 700–800 IU of human chorionic gonadotropin in the evening. The day after, females deposited eggs in 90 mm polystyrene Petri dishes. Eggs were immediately inseminated with sperm suspension and after 1 min, 30 mL of FETAX solution were added to each Petri dish. *Xenopus* sperm suspension was obtained by mincing the testes in 1–2 mL of cold DBT solution. The osmolarity of DBT is higher than FETAX solution therefore preventing the spermatozoa from initiating flagellar movement (Bernardini et al., 1998). Successful insemination was detected when after a few minutes the eggs were orientated with the dark side (animal pole) up. Unfertilized eggs and abnormal embryos were removed. Viable embryos were placed in Petri dishes (25 embryos per dish) and maintained at 23 °C until the end of the assay.

2.4. Embryo treatment and data collection analysis

The bioassays were carried out as previously described (Bernardini et al., 1996). A total number of 2000 embryos were exposed to different concentrations. All embryos of the same assay came from the same female. Briefly, the assay consisted of a series of Petri dishes with 25 embryos per dish containing eight increasing concentrations (from 0.025 to 2 mM, 5 dishes per each concentration) of Cr(VI) in 10 mL FETAX solution (ASTM, 1998). The control was a set of 5 dishes per each concentration containing FETAX solution alone. The experiments were carried out in duplicate.

Embryos were kept at 23 °C and maintained until the end of the test (120 h post fertilization). The medium was replaced and dead embryos, if any, were removed daily. At the end of the test, embryos were evaluated for mortality (absence of heartbeat). Surviving embryos were anaesthetised with tricaine methanesulfonate (MS 222, 100 mg mL^{-1}) and scored under a stereomicroscope to detect malformations. The embryos scored as normal were fixed in 4% formaldehyde and head–tail length measured.

Lethality and malformation data were analysed by using a PriProbit program (Sakuma, 1998) and median lethal (LC_{50}) and teratogenic (TC_{50}) concentrations were obtained. The Teratogenic Index (TI) was calculated as the ratio between LC_{50} and TC_{50}. The head–tail length data of normal embryos were analysed by ANOVA for unbalanced mixed effects, with females as the random factor, to check for growth retardation (Prati et al., 2002).

2.5. Chromium uptake

Xenopus embryos at blastula stage were divided in seven groups (one group for each Cr concentration tested (six) and one as control, 25 embryos/dish). The embryos were placed in 60 mm Petri dishes and incubated at 23 °C in a final volume of 10 mL of FETAX medium containing the following concentrations: 0 (control), 0.025, 0.1, 1, 25, 100 and 500 μM of ^{51}Cr(VI). After 24 h the ^{51}Cr-containing FETAX medium was removed by gentle aspiration. Then, the embryos were washed 3 times with 6 mL of cold FETAX medium and immediately counted for the ^{51}Cr content to measure the ^{51}Cr content. The ^{51}Cr-containing embryos were re-incubated again for other 24 h (stage of 2-days-old larva) with 10 mL of new FETAX solution containing increasing concentrations of ^{51}Cr(VI) as described above. Then, the ^{51}Cr-containing FETAX medium was removed by gentle aspiration and washed 3 times with 6 mL of FETAX solution and immediately counted for the ^{51}Cr content. Then the ^{51}Cr-containing larvae were incubated for other 96 h with 10 mL of new FETAX solution containing the increasing concentrations of ^{51}Cr(VI). After the removal of the FETAX medium and 3 washings with 6 mL of cold FETAX solution the five-days-old larvae (hereafter named 5 d-old ^{51}Cr-larvae) were counted for the ^{51}Cr content.

2.6. Release of chromium

The release experiments concerned the use of 5 d-old ^{51}Cr-larvae originated from embryos exposed to 1 μM of ^{51}Cr(VI) (see above