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Cytotoxic and genotoxic potential of Cr(VI), Cr(III)-nitrate and Cr(III)-EDTA complex in human hepatoma (HepG2) cells

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HIGHLIGHTS

- Cr(VI) increased the formation of DNA strand breaks and micronuclei in HepG2 cells.
- Cr(III)-nitrate increased the formation of DNA strand breaks in HepG2 cells.
- Cr(III)-EDTA did not induce the formation of DNA strand breaks in HepG2 cells.
- Cr(III)-nitrate and Cr(III)-EDTA had no effect on genomic stability of HepG2 cells.

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ABSTRACT

Chromium (Cr) and ethylenediaminetetraacetate (EDTA) are common environmental pollutants and can be present in high concentrations in surface waters at the same time. Therefore, chelation of Cr with EDTA can occur and thereby stable Cr(III)-EDTA complex is formed. Since there are no literature data on Cr(III)-EDTA toxicity, the aim of our work was to evaluate and compare Cr(III)-EDTA cytotoxic and genotoxic activity with those of Cr(VI) and Cr(III)-nitrate in human hepatoma (HepG2) cell line. First the effect of Cr(VI), Cr(III)-nitrate and Cr(III)-EDTA on cell viability was studied in the concentration range from 0.04 μg mL^{-1} to 25 μg mL^{-1} after 24 h exposure. Further the influence of non-cytotoxic concentrations of Cr(VI), Cr(III)-nitrate and Cr(III)-EDTA on DNA damage and genomic stability was determined with the comet assay and cytokinesis block micronucleus cytome assay, respectively. Cell viability was decreased only by Cr(VI) at concentrations above 1.0 μg mL^{-1}. Cr(VI) at ≥0.2 μg mL^{-1} and Cr(III) at ≥1.0 μg mL^{-1} induced DNA damage, while after Cr(III)-EDTA exposure no formation DNA strand breaks was determined. Statistically significant formation of micronuclei was induced only by Cr(VI) at ≥0.2 μg mL^{-1}, while no influence on the frequency of nuclear buds nor nucleoplasmic bridges was observed at any exposure. This study provides the first evidence that Cr(III)-EDTA did not induce DNA damage and had no influence on the genomic stability of HepG2 cells.

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1. Introduction

Chromium (Cr) is a common environmental contaminant due to its wide use in steel and electroplating industry, in leather tanning and in pigment manufacture (International Agency for Research on Cancer, 1990). The toxicity and essentiality of Cr depend on its oxidation state. Cr(III) is the most common species found in the environment and is considered to be an essential micronutrient, whereas Cr(VI) is known to be highly toxic (Langård and Costa, 2007) and is reported as the major metal pollutant creating an environmental and human health hazard. Cr(VI) can enter cells via nonspecific phosphate/sulphate anionic transporters (O'Brien, 2003) due to the similarity of tetrahedral chromate (CrO_4^{2-}) and sulphate (SO_4^{2-}) anions (Nies, 1999). Once inside the cell Cr(VI) is quickly reduced, causing cellular damage in several ways. The reduction of Cr(VI) via unstable and reactive Cr(V) generates reactive oxygen species that cause oxidative damage to lipids, proteins and DNA (Beyersmann and Hartwig, 2008). Additionally, reactive

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Cr(V) and Cr(IV) can bind with DNA inducing DNA damage. And lastly, the formed Cr(III) binds to cellular macromolecules including DNA, creating stable Cr(III)-DNA adducts, DNA-Cr-DNA crosslinks, protein-Cr-DNA crosslinks (Zhitkovich, 2011) as well as single (Ueno et al., 2001) and double DNA strand breaks (Ha et al., 2004) that lead to mutations and chromosomal breaks (Zhitkovich, 2011). The genotoxicity of Cr(VI) is well reported and has been demonstrated *in vitro* (for review see Nickens et al. (2010)), in plants (Rodriguez et al., 2011), in test animals *in vivo* (Ahmed et al., 2013; Bigorgne et al., 2010; Dana Devi et al., 2001; Kumar et al., 2013; Manerikar et al., 2008) and in occupationally exposed workers [for review see Benova et al. (2002), Li et al. (2014) and Proctor et al. (2014)]. Cr(VI) induced damage can lead to dysfunctional DNA replication and transcription, dysregulated DNA repair mechanisms, aberrant cell cycle checkpoints, microsatellite and genomic instability, epigenetic modifications, which all play an important role in Cr(VI) induced carcinogenesis (Nickens et al., 2010). There is sufficient evidence in experimental animals as well as in humans for the carcinogenicity of Cr(VI) compounds; therefore, International Agency for Research on Cancer (IARC) classified Cr(VI) compounds as carcinogenic to humans (Group 1) (International Agency for Research on Cancer, 1990).

On the other hand, Cr(III) compounds are considered to be far less toxic than Cr(VI) due to poor ability of Cr(III) ions to penetrate cell membranes. In aqueous solutions at pH below 4, Cr(III) exists as hexa-aqua positively charged complex $[Cr(H_2O)_6]^{3+}$ with octahedral structure and cannot enter cells via anion channels. It is assumed that cells take up Cr(III) compounds with passive diffusion or even pynocytosis or phagocytosis (Eastmond et al., 2008).

For many decades it has been believed that Cr(III) compounds have no harmful effects, but in recent years many studies have been published on genotoxicity of Cr(III) salts and Cr(III) compounds *in vitro* and *in vivo* (Eastmond et al., 2008). Cr(III) has been shown to induce DNA damage (Rudolf et al., 2008), sister chromatid exchanges (Cohen et al., 1993), centromere positive and negative micronuclei (Seoane and Dulout, 2001), oxidative DNA damage and Cr-DNA adducts (Levina and Lay, 2008). In tannery workers exposed to Cr(III) in the form of basic Cr(III)-sulphate, DNA damage was reported and correlated with Cr levels in the blood (Zhang et al., 2008). Blasiak and Kowalik (2000) have reported that DNA fragmentation in lymphocytes induced by Cr(III)-chloride was even greater than by Cr(VI). In contrast, several studies reported that no DNA damage could be detected *in vitro* by Cr(III)-chloride and Cr(III) complexes with histidinate and picolinate under physiological conditions (Andersson et al., 2007; Hininger et al., 2007; Itoh and Shimada, 1996) and even protective properties of these molecules against oxidative stress in human keratinocytes (HaCaT cells) have been suggested (Hininger et al., 2007). It was demonstrated that the rate with which Cr(III) passes the cell membrane, the concentration and the type of complexing ligand such as picolinate, histidinate, EDTA and salen, play a crucial role in Cr(III) toxicity. In general it appears that very high Cr(III) concentrations and long exposure times result in elevated intracellular concentrations and related Cr(III) genotoxic effects. The IARC has classified Cr(III) under Group 3, not classifiable as to its carcinogenicity to humans (International Agency for Research on Cancer, 1990).

Ethylenediaminetetraacetate (EDTA) is a common chelating agent used in textile and paper industry, as a preservative and stabiliser in food industry, in the production of pesticides, washing powders and personal care products such as shampoos, soaps, creams and cleansers to improve the product stability (United States Environmental Protection Agency, 2004; World Health Organization, 1998). Moreover EDTA is extensively used in household products such as detergents and personal care products as well as in the pulp, paper and textile industry. Additionally, it is poorly biodegradable in the environment and is therefore one of the most common contaminants in European surface waters (Oviedo and Rodriguez, 2003). Another use of EDTA is in soil remediation where soil washing with EDTA is performed to remove contaminants such as Pb and Cd (Voglar and Lestan, 2013). Balaska et al. (2012) described the removal of chromium (III) ions from aqueous solutions by using EDTA as chelating agent for Cr(III) complexation. EDTA is also used in plant bioremediation to increase Cr bioavailability and bioaccumulation (Aydin and Coskun, 2013; Chen and Cutright, 2001), since Cr(III)-EDTA can efficiently pass the plant root system. Milačič and Štupar (1994) demonstrated that Cr(III)-EDTA is effectively taken up by plants, without changing its speciation. However, due to its extensive use, EDTA has become a persistent environmental contaminant and has been determined at high concentrations in many surface and drinking waters (Nörtemann, 1999). EDTA's ecotoxicology is mainly associated with the enhanced bioavailability and mobility of heavy metals and its toxicological effects vary greatly between different test organisms used and metals evaluated. EDTA forms complexes with major ions (Ca, Mg) as well as with trace metals (Cu, Zn, Fe, Cd, Pb and Cr). This could potentially lead to formation of Cr(III)-EDTA complexes in surface waters.

Available literature data on human and environmental hazards of Cr(III)-EDTA are scarce. In general, metal complexes with EDTA (Zn(II)-EDTA, Cu(II)-EDTA, Fe(III)-EDTA) have appreciatively higher toxicity in comparison to free EDTA (Sorvari and Silanpaa, 1996). The opposite is true for free metals (Hg, Cr(II) and Cr(IV)), where EDTA chelation has no effect (Guilhermino et al., 1997) or even reduces the toxicity of the metal (Cd, Cu, Cr(VI), Cr(III) and Zn) (Lermanda et al., 2009; Sorvari and Silanpaa, 1996; Urrutia et al., 2008).

As there are many indications pointing out that EDTA is a promising scavenger for chromium by forming a stable complex and since the data on Cr(III)-EDTA genotoxicity are lacking, the aim of our study was to evaluate the cytotoxicity and genotoxicity of Cr(III)-EDTA and to compare its genotoxic activity with Cr(VI) and Cr(III)-nitrate. Genotoxicity was studied with the comet assay and cytokinesis-block micronucleus (CBMN) cytome assay on human hepatoma cell model (HepG2 cells).

2. Materials and methods

2.1. Chemicals

Cr(III) was purchased in the form of $Cr(NO_3)_3 \times 9H_2O$ (Fluka Analytical, St. Louis, USA) and Cr(VI) as Na_2CrO_4 (Riedel de Haën, Hanover, Germany). Cr(III)-EDTA complex was prepared by dissolving the same amount of moles of Na_2EDTA (Carlo Erba, Milan, Italy) and $Cr(NO_3)_3 \times 9H_2O$ to achieve an equimolar Cr(III)-EDTA complex. The mixture was left overnight at 70 °C to form the Cr(III)-EDTA complex. Intensive violet colour was indicative of complex formation. An equimolar complex of Cr(III)-EDTA was prepared to eliminate the possibility of any negative effect of the surplus of EDTA.

William's medium E, cytochalasin B (Cyt-B), acridine orange (AO), benzo(*a*)pyrene (BaP, CAS-No. 50-32-8, >96% purity) and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (St. Louis, USA), etoposide (ET) was from Santa Cruz Biotechnology (Dallas, USA). Penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine, phosphate buffered saline (PBS) were obtain from PAA Laboratories (Dartmouth, USA). Triton X-100 was from Fisher Sciences (New Jersey, USA). Normal melting point agarose (NMP) and low melting point agarose (LMP) were from Invitrogen (Paisley, Scotland). Trypsin was from BD-Difco (Le Pont-De-Claix Cedex, France). All other chemical reagents were of the purest grade

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