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ANTIOXIDANTS ENHANCE CHROMIUM (VI) MEDIATED TOXICITY IN *NEUROSPORA CRASSA*

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ABSTRACT

Chromium can be found in the environment in two main valence states: hexavalent Cr (VI) and trivalent Cr (III). Reduction activation of carcinogenic Cr (VI) is required for the induction of DNA damage and mutations. There is a controversy over antioxidants' role in Cr (VI) toxicity and cell death in mammalian systems. In the present study we compared the effect of antioxidants and insulin on Cr (VI) induced genotoxicity in wall-less mutant of *Neurospora crassa* (slime), which contains insulin receptor on its cell surface as a model system. Analysis by comet assay indicated Potassium dichromate ($K_2Cr_2O_7$) induced DNA damage in slime cells, as increase in comet tail moment and the effect was dose dependent. Simultaneous addition of N-acetyl cysteine (NAC) & Sodium ascorbate (ASC) increased the extent of DNA damage whereas insulin decreased the extent of DNA damage induced by Cr (VI). The simultaneous exposure of antioxidants and insulin in presence of Cr (VI) showed enhanced cytotoxicity as observed by MTT assay. The results obtained suggest that N-acetyl cysteine and Sodium ascorbate reduce Cr (VI) to Cr (III) enhancing the levels of reactive oxygen species. Absence of protective role of insulin in presence of antioxidants indicate that a differential route may be adopted by the cells in response to metallic stress.

KEY WORDS: Chromium (VI), Insulin, Antioxidants, *Neurospora crassa*, Reactive oxygen species.

INTRODUCTION

Chromium is a prominent heavy metal found in the environment. Industries such as mining, electroplating, painting, tanning, and textiles contribute to chromium contamination. Chromium is known to exist in different valency forms as Cr (VI/V/IV/III) ((Von Burg *et al*., 1993). Of all forms, hexavalent chromium [Cr (VI)] is considered as highly toxic (De Flora, *et al*., 1989) and is associated with chronic bronchitis, skin irritations, liver problems, renal failure, lung cancer, contact dermatitis with prolonged exposure (Mohanty *et al*., 2023). The toxicity of Cr (VI) containing compounds is due to the generation of reactive intermediates upon its reduction. (Stearns *et al.,* 1997; Arslan *et al*., 1987; De Flora *et al*., 1990). Cr (VI) is readily absorbed while Cr (III) is poorly absorbed. All the ingested Cr (VI) is converted to Cr (III) before it enters the bloodstream. Cr (VI) passes through anion transporters present in the cell membranes and metabolically reduced to Cr (III) (Pourahmad *et al*., 2001).

Cr (VI) does not interact with complex molecules like proteins, lipids and nucleic acids. However, once internalized the intermediates Cr (V) and Cr (III) are able to form covalent interactions with macromolecules. Cr (III) is a vital nutrient required by the human body to promote insulin action, facilitating the metabolism of fats, sugars and proteins (Shrivastava, *et al*., 2002). Cr (III) mimics the insulin receptor, thereby inducing the amplification of cellular insulin receptors and stimulates insulin receptor kinases, enhancing the insulin sensitivity (Anderson, 2000). An uptake reduction model of Cr (VI) toxicity (Connett, *et al.,* 1983; O'Brien *et al.,* 2003)

stated that Cr (VI) reduction to lower oxidation states Cr(V/IV/III) by multiple reducing agents includes Glutathione reductase (GR), Glutathione and ascorbic acid (Zhitkovich *et al.,* 2003; Shi, *et al.,* 1989; Codd *et al.,* 2003).

The cellular defense system can decrease the Cr (VI) mediated toxicity in the cells. The antioxidant system in the cells include both enzymatic (glutathione peroxidase, superoxide dismutase, catalase) and non-enzymatic (Ascorbic acid, α-tocopherol) antioxidants. Once the defense system of the cell is overpowered, redox balance is disrupted leading to oxidative stress (Mates, 2000). N-acetylcysteine (NAC) and thiol-containing antioxidants are used to mitigate oxidative stress injury (Cotgreave, 1996). Despite this, the precise mechanisms by which antioxidants rescue cells from the harmful effects of Cr (VI) remain unclear. Sodium Ascorbate (ASC) is known to shield the cells from oxidative stress with the aid of glutathione-transferase and quinone reductase (Munday *et al.,* 2004). The antioxidants NAC, Vitamin E and sodium ascorbate increase insulin sensitivity in patients with insulin resistance. Insulin promotes the active cellular uptake of ASC (Cunningham, 1998).

In addition to its genotoxic properties, Chromium is known to have certain beneficial effects. Besides its carcinogenic, teratogenic and mutagenic effects, it is interesting to note that Cr (III) acts as insulin mimetic agent (Mckenzie *et al.,* 1988). The toxicity of Cr (VI) is dependent on insulin availability and this may become a serious issue in diabetes due to insulin deficiency (Gaddameedi, *et al.,* 2011).

The current study explores the antioxidants' role in Cr (VI) mediated toxicity in the presence of insulin in *Neurospora crassa* (slime), known to have insulin receptor. The wall-less *N. crassa* mutant (slime) was used for the study as the fungal cell wall interferes with the metal transport by binding to the metal (Gaddameedi, *et al.,* 2011). The response of the slime (wall-less mutant *N. crassa*, FGSC 4761) against chromium stress on exposure of insulin and antioxidants was explored.

MATERIALS AND METHODS

Organism

The wall-less mutant of *Neurospora crassa* [FGSC 4761] (Vogel, 1956) was obtained from the Fungal Genetic Stock Centre (Department of Microbiology, University of Kansas Medical Centre, Kansas City, KS). The cells were grown for 72 h at 28 °C in Vogel's supplemented medium (VSM) (Fawell *et al*., 1988) with 2% (W/V) sucrose as the carbon source.

Cell viability and the atomic absorption spectrophotometry

Cell viability of *N.crassa*cells was checked by trypan blue staining before treatment with Cr (VI) in the form of $K_2Cr_2O_7$ (100, 500 µmol) and / orinsulin, N-acetyl cysteine and Sodium ascorbate (Vitamin C). All the treatments were carried out by dissolving $K_2Cr_2O_7$, N-acetyl cysteine and Sodium ascorbate in Vogel's medium. In case of controls, only Vogel's medium was used. After the treatments, the cells were washed twice with PBS (Phosphate buffered saline) and then pelleted. The pellet was acid digested and further atomic absorption spectrometry was performed.

MTT Assay

To determine the cell viability, the colorimetric MTT metabolic activity assay was used. *N.crassa* cells (1×10^4 cells/well) were cultured in a 96-well plate at 28 °C and exposed to Cr (VI) concentrations in the range of 100 - 500 µmol and 0.4 IU, 4IU of insulin, 50 µM of NAC and ASC for 3 h. After removing the medium from each well, 200 µl of MTT solution (8 mg/ ml prepared in Vogel's medium) was added. After incubation for another 2h, the resultant formazan crystals were dissolved in dimethyl sulfoxide (200 µl) and the absorbance was measured in a microplate reader (Bio-RAD 680, USA) at 450 nm with a reference wavelength of 620 nm. All experiments were performed in duplicates, and the relative cell viability was plotted relative to the untreated cells.

Comet Assay

Chemical treatment

A stock solution of $K_2Cr_2O_7$ was prepared using phosphate - buffered saline (PBS, pH 7.2). A Slime cells (2×10^6 cells/ml) were taken and inoculated with different concentrations of Cr (VI) (100, 500 µmol) or insulin 0.4 units. To examine DNA damage, thecells were incubated for a period of 3 h at 28 °C. Each experiment included treatment with hydrogen peroxide (20 µM) as positive control. In experiments with ASC and NAC (GSH precursor) treatments, cell wereincubated with Cr (VI) which was preceded by the addition of ASC or NAC (1mM) to the cell suspension to give a final concentration of 50 µM.

RESULTS

Cell viability

The results of cell viability after incubation with Cr (VI) and / or insulin are presented in Fig. 1. A dose dependent reduction in cell viability was observed in Cr (VI) treatments, whereas simultaneous incubation with insulin enhanced cell viability. Insulin reversed the cytotoxic effect of chromium by increasing the cell viability of 100 and 500 µmol Cr treated cells. The uptake of Cr in the presence of insulin and antioxidants is presented in Fig. 2.

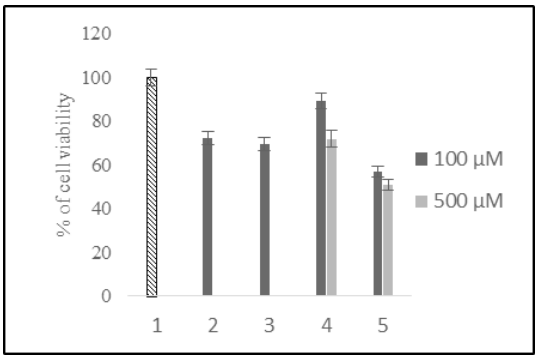

Fig. 1. Graph showing changes in cell viability in *N.crassa* cells upon treatment with Cr (VI).
N. crassa cells were treated with Cr (VI) in the presence or absence of insulin. Cell viability was determined by trypan blue exclusion method. Treatments: 1) control, 2)100 µM Cr (VI), 3) 500 µM Cr (VI), 4) 0.4 IU insulin, 5) 4 IU insulin.

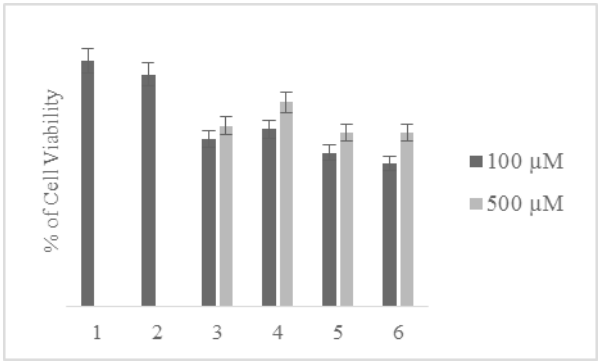

Fig. 2. Graph showing precentage of cell viability of *N. crassa* cells treated with Cr (VI), insulin and antioxidants
N.crassa cells were treated with Cr (VI) in the presence or absence of insulin and antioxidants. Cell viability was determined by trypan blue exclusion method.

Effect of antioxidants in the presence of insulin

Antioxidants act as free radical scavengers and reduce reactive oxygen species levels, thereby restoring cell viability. The antioxidants ASC and NAC in presence of Cr (VI) interestingly increased the total reactive oxygen species (ROS) levels and decreased cell viability. The control cells treated with vitamin C or N-acetyl cysteine but not Cr (VI) had no effect on cell viability whereas the cells exposed to Cr (VI) upon subsequent treatment with vitamin C or N-acetyl cysteine specifically showed decreased cell viability. Presence of insulin therefore seemed to have reversed the effect of antioxidants, resulting in increased the cell viability.

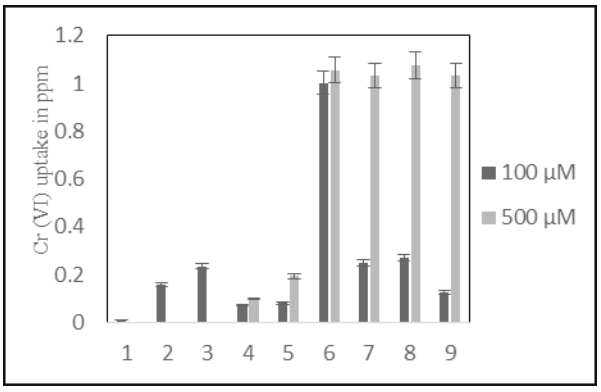

Fig. 3. Measurement of Cr (VI) uptake in presence of insulin and antioxidants.
N. crassa cells were treated with Cr (VI) in the presence or absence of insulin and antioxidants. Cr (VI) uptake was measured by atomic absorption spectrometry. Graph represents Cr (VI) in ppm. Treatments: 1) control; 2) 100 µM Cr (VI); 3) 500 µM Cr (VI); 4) 0.4 IU insulin; 5) 4 IU insulin; 6) 0.4 IU insulin + 50 µM ASC; 7) 4 IU insulin + 50µM ASC; 8) 0.4 IU insulin+ 50 µM NAC; 9) 4 IU insulin + 50 µM NAC.

Vitamin C or N-acetyl cysteine or insulin treatment

The mean comet tail moments for the slime cells treated with Cr (VI)/insulin at indicated concentrations and subsequent treatment with ASC and NAC or insulin, are displayed in Fig. 4. It can be seen that both the antioxidants increased the mean comet tail moment of the cells exposed to Cr (VI) at 100 and 500 µm, whereas, insulin reduced the tail moment of slime cells.

DISCUSSION

In the present study, Cr(VI) substantially increased comet tail lengths in slime cells signifying its genotoxicity. This effect is due to the reduction of Cr (VI) to Cr (V)/(IV)/(III) and generates reactive oxygen species (ROS). ROS cause oxidative damage

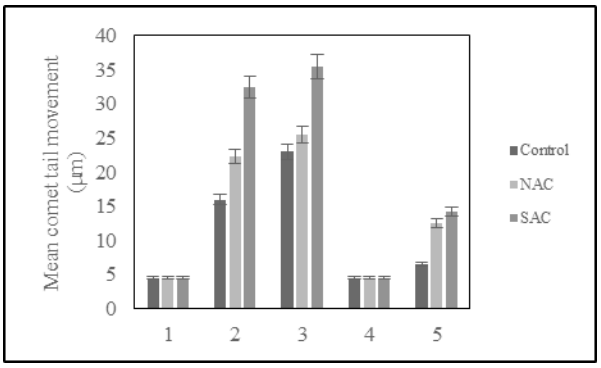

Fig. 4. Changes in mean comet tail moment of Cr (VI) treated *N.crassa* cells in response to insulin and antioxidants

N. crassa cellstreated with Cr (VI) in presence and absence of insulin and or antioxidants were processed to perform comet assay. Mean comet tail moment of slime cells exposed for 1 h at 28 C to chromium/insulin in the presence and absence of vitamin C and *N*-acetyl cysteine was measured. The number of cells observed in each treatment was 100. The results are mean ± SD of two independent experiments.

to cellular components including DNA (Bal *et al.,* 2002). It has been evident that Cr (III) can directly form DNA – DNA & DNA- protein crosslinks (Voitkun *et al.,* 1999). Moreover, the reactive intermediates Cr(V), Cr(IV) also contribute to the formation of DNA lesions. The concentrations of the chemicals used were below 50% inhibitory concentrations as judged by trypan blue exclusion staining. The occupational exposure limit for Cr (VI) is stated as 0.05 mg/m^3 but previous reports suggested that the concentration of Cr (VI) in chromate production workers is much higher than the above (Gao *et al.,* 1994).

The observed increase in comet tails moment is resultant of DNA strand breaks, formation of DNA adducts and formation of apurinic/apyrimidinic sites, which can eventually transform into strand breaks and result in comet lengths. This observation is in accordance with the findings of (Ceryak *et al.,* 2004; O'Brien *et al.,* 2003) Cr(VI) mediated genotoxicity. It has been reported that the genotoxic effects of Cr(VI) may be associated to the intermediates formed during the reduction of Cr (VI), with reactive intermediates such as Cr (V) and Cr (IV) (Wang, *et al.,* 1997).

NAC and Sodium ascorbate (Vit C) acts as antioxidants that combat free radical damage. In the present study increased ROS levels were observed in the presence of antioxidants indicating genotoxic role of NAC and Vitamin C in the presence of Cr (VI). Previous reports suggest that the increase of ROS levels is due to increased conversion of Cr (VI) to Cr(III) (Quievryn, *et al.,* 2003). These results are surprisingly against the results obtained by Blasik *et al.,* 2000, where they demonstrated the protective role of Vitamin C in Cr(VI) mediated toxicity. Therefore, it can be concluded that vitamin C and N-acetyl cysteine could play a dual role in toxification and detoxification of hexavalent chromium and theirmode of action seems to depend on the extracellular and intra-cellular levels. Cr(III) is known to play a role in insulin receptor activation and there by affectfat and sugar metabolism. In this study insulin was observed to reduce Cr(VI) induced genotoxicity in slime cells but no protective role of insulin in the presence of the antioxidants was observed. The surprising results produced in the presence of insulin may be due to its free radical scavenging activity.

CONCLUSION

N.crassa cells on exposure to Cr (VI) showed decreased cell viability which was restored insulin. This suggests that insulin has a protective role in Cr (VI) induced genotoxicity. Elevated levels of ROS in the presence of antioxidants such as Vitamin C and N-acetyl cysteine indicate that Cr (VI) is reduced in their presence leading to elevated ROS levels leading to cell death. Cell death due to reduction of Cr (VI) by antioxidants could not be reversed by addition of the free radical scavenging insulin. This may have important implications in conditions of insulin deficiency such as diabetes where exposure to Cr (VI) may have enhanced toxicity due to lower and in such conditions, antioxidants may contribute to Cr (VI) toxicity. These findings suggest a potential interaction between insulin signalling and chromium-mediated ROS production and cell damage.

Conflict of Interest - None

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