RESPONSE OF ANTIOXIDANT DEFENSE SYSTEM TO CHROMIUM (VI) - INDUCED OXIDATIVE STRESS IN EMBRYONARY FIBROBLASTS

MIHAELA ROCSANA LUCA, RALUCA CIUBĂR**, CRISTINA IANCU CIOFRÂNGEANU**, VALENTINA MITRAN**, ANIŞOARA CÎMPEAN**, DANA IORDĂCHESCU***

*Bios Diagnostic, Bucharest, Romania
**Department of Biochemistry and Molecular Biology, University of Bucharest, 93, Splaiul Independenței, Bucharest 6, Romania

Abstract. Evaluation of biological effects of chromium (VI) is very important taking into account its toxicity seen at people with organ prostheses manufactured from chrome alloys and at workers in galvanic chromium plating and the beneficial effects of chromium on serum glucose and lipid and insulin resistance in diabetic patients. We have evaluated the effects of sodium chromate on the viability and oxidative status of human embryonary fibroblasts (HEF). Our results show that hexavalent chromium has not cytotoxic effects upon HEFs at 5 – 50 μM concentration. Addition of 100 – 200 μM Cr (VI) in the culture medium caused decreases in cell viability in a time and dose-dependent manner and an appreciable increase in intracellular ROS production. GSH depletion in cells treated with the highest doses tested may be explained by its scavanging role as well as by Cr (VI) reduction by GSH to Cr (V). Catalase (CAT) and glutathione peroxidase (GPx) activities were increased after 3-h and decreased after 24-h exposure to higher doses of chromate. The activity of Cu,Zn-superoxide dismutase (SOD) was inhibited only as a result of cell incubation for 24 h with 200 μM Na_2CrO_4 while MnSOD activity was activated by 100 μM Cr (VI) and inhibited after 24-h exposure to 200 μM chromate.

Key words: chromium (VI), fibroblasts, reactive oxygen species, TBARS, glutathione, glutathione peroxidase, catalase, superoxide dismutase.

INTRODUCTION

The widespread use of chromium in industrial applications such as chemical production of pigments, refractory brick production, tanning, metallurgy, electroplating, and combustion of fuels has lead to human occupational exposure and to its increased introduction into the environment [5].

Chromium (Cr) is present in the environment in two main oxidation states: Cr (III) and Cr (VI) that have a different toxicity. Since Cr (III) cannot usually cross cell membranes its toxicity is considered to be relatively low [11], whereas Cr (VI) is transported through the sulfate ion transporter as chromate (CrO_4^{2-}) [7], and therefore accumulates in cells. Hexavalent Cr [Cr (VI)] is the primary toxic form to which organisms are exposed. Once inside the cell, Cr (VI) is rapidly reduced by

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different intracellular components (such as glutathione, ascorbate, tocopherols, NAD(P)H, etc.) into several reactive intermediate species, and finally to stable Cr (III). During this reduction process, these reactive intermediates, which may include Cr (V), Cr (IV), and reactive oxygen species (ROS), bind both protein and DNA, and cause many types of oxidative lesions [3, 15, 22, 23].

Earlier studies have shown that in cellular systems, Cr (VI) is reduced by certain flavoenzymes such as glutathione reductase to generate Cr (V) [17–19]. During this process, molecular oxygen is reduced to $O_2^{\bullet-}$, which generates H_2O_2 via dismutation [19]. The resultant Cr (V) reacts with H_2O_2 to generate HO• radical via a Fenton-like reaction. Thus, during the one-electron reduction of Cr (VI), a whole spectrum of ROS is generated.

$$\text{Cr (III)} + O_2^{\bullet-} \rightarrow \text{Cr (II)} + O_2 \quad (1)$$

$$\text{Cr (II)} + H_2O_2 \rightarrow \text{Cr (III)} + HO^{\bullet} + HO^{-} \quad (2)$$

$$\text{Cr (VI)} + O_2^{\bullet-} \rightarrow \text{Cr (V)} + O_2 \quad (3)$$

$$\text{Cr (V)} + H_2O_2 \rightarrow \text{Cr (VI)} + HO^{\bullet} + HO^{-} \quad (4)$$

It is known that ROS play an important role in carcinogenesis induced by a variety of carcinogens. The net effect of the response is determined by a balance between intracellular oxidants and antioxidants. Generation of hydroxyl radical by chromate might play a significant role in the mechanism of Cr (VI) cytotoxicity [12, 13] and is responsible for tissue damaging effects. A variety of DNA lesions are formed during the reduction of Cr (VI) to Cr (III), including DNA strand breaks, alkali-labile sites, DNA-protein and DNA-DNA crosslinks, and oxidative DNA damage, such as 8-oxo-deoxyguanosine [8, 14]. Cr (VI) has been recognized as a highly toxic elemental species on the basis of experimental and epidemiological evidence, and has been classified as a class I human carcinogen by the International Agency for Research on Cancer (IARC). The mechanistic cytotoxicity of chromium (VI) is not completely understood, however, a large number of studies suggested that chromium (VI) induces oxidative stress, DNA damage, apoptotic cell death and altered gene expression [6, 21].

In the present communication, we examined the effect of treatment with sodium chromate on oxidative status in a relevant human cell culture model, human embryonary fibroblasts (HEF).

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

HEFs were obtained in our laboratory by the explants culture method from embryonary pieces. Cells were seeded at a density of 2×10^5 cells per well in a six-well plate and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal

bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were studied between passages 5 and 7.

After the culture reached 60 – 70% confluence, the monolayers were rinsed with PBS and incubated at 37 ºC with DMEM with 0.5% FBS containing various concentrations of Na_2CrO_4 (5 – 200 μM) for the indicated time periods.

The cells which reached confluence were harvested by scraping with a rubber policeman, washed with PBS and suspended in 50 mM potassium phosphate buffer pH 7.4, containing 0.1% Triton X-100. Subsequently, HEFs were sonicated on ice (for 1 min at intervals of 15 sec) and centrifuged at 4 ºC, 10,000 × g, for 10 min to remove cellular debris. Supernatants were then used for enzyme assays.

CYTOTOXICITY ASSAY

The MTT test measures the cell activity, proliferation rate and cell viability. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenases, to the corresponding blue formazan. HEF were treated in 24-well plates and incubated with 0.2 mg/ml MTT in DMEM without phenol red for 4 h. After this time the MTT solution was removed and the formazan released from the cells with 0.5 ml DMSO. Absorbance of the supernatant was measured at 550 nm. Absorbance values that are lower than the control cells indicate a reduction in the cell activity. Conversely a higher absorbance indicates an increase in cell activity/ proliferation.

DETECTION OF ROS AND OXIDATIVE LESIONS

Detection of intracellular ROS in intact cells treated with chromate was performed by using chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). This nonpolar and nonfluorescent compound passively diffuses into cells, where it is converted to the membrane-impermeant polar derivative CM-H_2DCF by esterases when it is taken up by the cell. CM-H_2DCF is nonfluorescent but it is rapidly oxidized to the highly fluorescent CM-DCF by intracellular ROS, mainly H_2O_2 [24]. HEFs were plated in 12-well plates at a density of 2×10^4 cells/well and allowed to adhere for 24 h in DMEM with 10% FBS. After 20 h of conditioning in DMEM supplemented with 0.5% FBS, cells were rinsed with PBS and incubated with 5 μM CM-H_2DCFDA in 0.5 ml PBS for 1 h at 37 ºC. Cells were then rinsed twice with PBS to remove the excess dye and were exposed to sodium chromate for various periods. The intracellular ROS levels were measured by using a fluorescence plate reader (TECAN) at an excitation wavelength of 485 nm and emission wavelength of 530 nm. Thus, values of the fluorescence in the cell cultures are constantly rising in this assay due to the accumulation of ROS. Values are given as arbitrary units of fluorescence relative to control.

Lipid peroxidation was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) by the method of Esterbauer and Cheeseman [9]. After Cr (VI) treatment, the supernatant was collected and 900 µl were kept frozen (–20 ºC) after addition of 90 µl butylated hydroxytoluene (BHT) 2% (vol/vol in ethanol) until the TBARS assay. The TBA reagent was prepared by dissolving 0.67% TBA (wt/vol) in 50% acetic acid and adding 0.01% (wt/vol) BHT. To 0.5 ml of supernatant, 0.5 ml of TBA reagent was added and heated in a boiling water bath for 15 min. After cooling on ice, the fluorescence intensities of samples were finally measured at excitation and emission wavelengths of 535 nm and 553 nm, respectively. For quantification a standard curve was prepared by using a 100 mM 1,1,3,3-tetraethoxypropane stock solution diluted in ethanol, and normalized to the cell protein content. Data were expressed as pmol TBARS/ µg of protein.

ANTIOXIDANT ENZYMES AND GSH ASSAYS

Determination of catalase (CAT) activity was performed at room temperature in a 1-ml mixture containing clear cell lysate, 100 mM of phosphate buffer (pH 7.0) and 10 mM of H_2O_2 as described by Aebi [1]. The decomposition of H_2O_2 was followed directly by a decrease in absorbance at 240 nm. Enzyme activity was expressed in µmol of H_2O_2 decrease/min/mg protein.

Total SOD, Mn-SOD, and Cu,Zn-SOD were determined by using the pyrogallol assay following the procedure described by Marklund and Marklund [16], based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, spectrophotometrically read at 420 nm. Briefly, 50 µl of the sample was added with 1,870 µl of 50 mM Tris/1 mM diethylenetriaminepentaacetic acid/20% cacodylic acid buffer (pH 8.3) and with 80 µl of 10 mM pyrogallol to induce an absorbance change of 0.02 in the absence of SOD. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions was defined as one SOD unit. The specific Cu/Zn SOD inhibition by KCN (60 µl of 54 mM KCN added to 300 µl of lysate) allows the Mn SOD determination in the same conditions. Each sample was assayed twice, and results were expressed as SOD units and normalized to the cell protein content.

Glutathione peroxidase (GPx) activity of the cell supernatants was measured by assessing oxidation of GSH with t-butyl hydroperoxide according to the method of Flohe and Gunzler [10]. The rate of GSSG formation was measured by means of GR, and oxidation of NADPH was followed at 340 nm. Both sample and reference tubes contained 0.05 M phosphate buffer, pH 7.2, 4.3 mM EDTA, 0.28 mM NADPH, 0.5 U of GR, 4 mM glutathione, 0.18 mM t-butyl hydroperoxide and the appropriate amount of HEF lysates. One mol of NADPH is oxidized for each mol t-butyl hydroperoxide reduced. One unit of GPx was defined as 1 µM substrate converted per minute.

Protein concentration was determined by the Bradford method [4].

Total glutathione (GSH + GSSG) was measured in 96-well plates using a TECAN microplate reader according to the procedure described in the Glutathione

Assay Kit (Sigma). The assay is based on the enzymatic recycling method, using glutathione reductase and 5',5'-dithiobis-2-nitrobenzoic acid (DTNB). Briefly, after the treatment, the fibroblasts (5×10^5 cells) were washed twice with phosphate-buffered saline (PBS) and the cells were recovered by centrifugation at 200 × g, for 10 minutes at 4°C. The resulting cell pellet was lysed by adding 80 µl HCl 10 mM and freeze-thawing. The lysate was deproteinized by addition of 20 µl 5-sulfosalicylic acid 5%. Precipitated proteins were removed by centrifugation at 8000 × g, for 10 minutes and the supernatant was submitted immediately to the analysis according to the manufacturer's protocol. GSSG was quantified after derivatization of reduced glutathione with 2-vinylpyridine (VP). Briefly, 100 µl of the protein-free supernatant was mixed with 6 µl triethanolamine and 2 mg 2VP and incubated for 30 min at room temperature. All the samples and standards (GSH and GSSG) were treated the same way and the extinction at 412 nm was followed over 5 min in 30 sec intervals. The intracellular levels of glutathione were calculated based on cellular protein concentration and data are reported as percent of control. Reduced glutathione in the native samples could then be obtained by the subtraction of GSSG content from total glutathione (GSH+GSSG) content.

RESULTS AND DISCUSSION

Initially, a range-finding study was conducted to determine the Cr (VI) exposure condition that resulted in cytotoxic effects. In these studies, HEFs were exposed to Cr (VI) at concentrations ranging from 5 to 200 µM for 3, 24 and 48 h and assayed immediately for cytotoxicity using MTT test (Fig. 1).

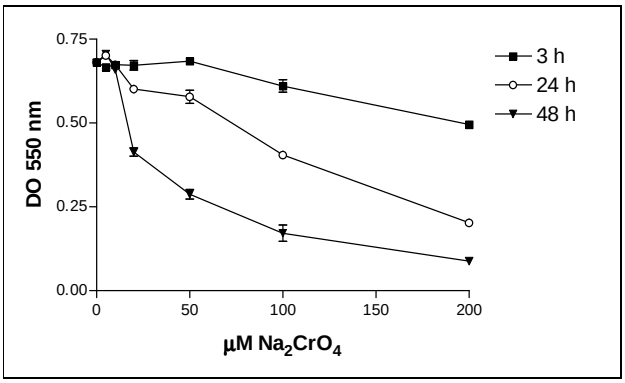


Fig. 1. The effect of sodium chromate on cell viability (MTT test). Cells were treated with different concentrations of Cr (VI) for 3, 24 and 48 h. Data are expressed as mean ± SD of two separate experiments performed in triplicate.

Chromium (VI) was not cytotoxic at 5–50 µM concentration when the cells were incubated for 3 hours. However, marked decreases on the viability of fibroblasts were observed at concentrations of 100 and 200 µM, following 24 and

48 hours of exposure, when it is noticed a time and dose-dependent cytotoxicity. Therefore, after 48-h exposure, cell survival decreased with 74.7% and 86.9% as a result of treatment with 100 μM and 200 μM Cr (VI), respectively. After 48 h of cells incubation with Cr (VI), a decreased viability was observed also at lower concentrations with 39.2% (20 μM) and 57.6% (50 μM).

Oxidative stress plays a role in the mechanisms of toxicity of a number of compounds, whether by production of free radicals or by depletion of cellular antioxidant capacity. CM-H_2DCFDA is widely used to measure ROS generation in cells following 3-h exposure to different concentrations of Cr (VI) (Fig. 2A) and during 24 h of incubation with 0 – 100 and 200 μM chromate (Fig. 2B).

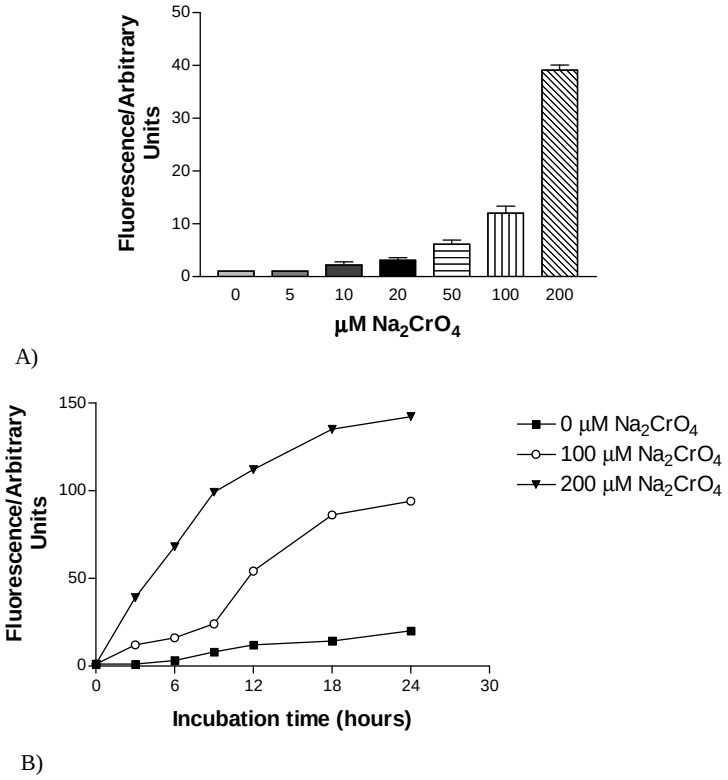
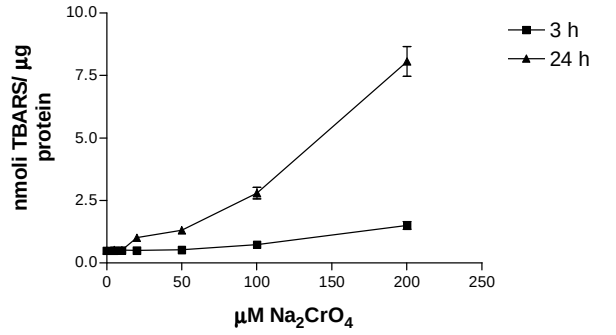
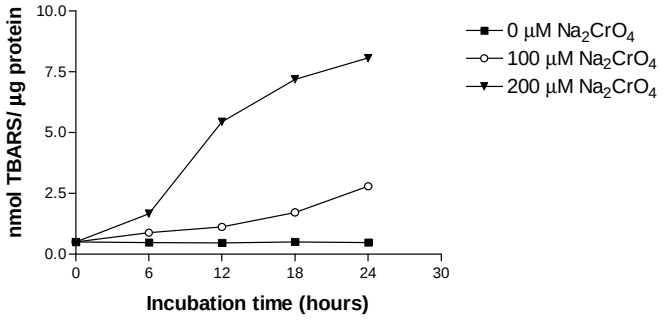


Fig. 2. Effect of sodium chromate on intracellular ROS production. Cells were incubated with: A) various chromate concentrations for 3 h (data are expressed as mean ± SD of three separate experiments performed in triplicate); B) 0 – 100 – 200 μM Cr (VI) for several incubation periods (duplicate readings did not vary by more than 5%; each experiment was performed 3 times with similar results; representative experiment is shown).

As seen in Fig. 2, addition of Cr (VI) in the culture medium resulted in a dose- and time-dependent increase of intracellular ROS production. After 3 h of treatment the level of ROS increased significantly only for the chromate doses of 100 and 200 μM, 12- and 39-fold, respectively.



A



B

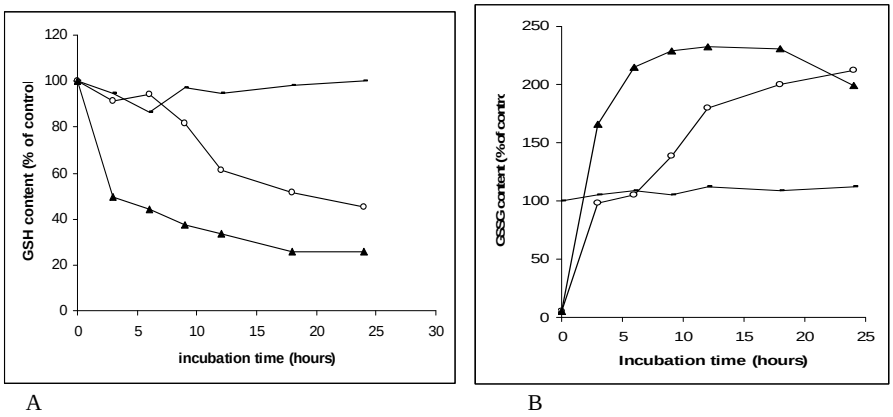
Fig. 3. Increase in TBARS released in the supernatant of HEFs exposed to sodium chromate as function of concentration and time. A. Dose dependent response after 3 and 24 h of incubation. B. Time course of TBARS generation by cells; Duplicate readings did not vary by more than 5%; each experiment was performed 3 times with similar results; representative experiment is shown.

Monitoring oxidative stress in cells can be done indirectly at best, by assaying products of oxidative damage or by investigating the potential of a cell to withstand further oxidation. In this study, we will therefore deal with both methods. Lipid peroxidation is a well-known example of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures. Lipid hydroperoxides in the presence or absence of catalytic metal ions give rise to a large variety of products, including short and long chain aldehydes – which can be used to assess the degree of LPO in a system. One of the most frequently used biomarkers providing an indication of the overall lipid peroxidation level is the concentration of malondialdehyde (MDA), one of several byproducts of lipid peroxidation processes. We have quantified MDA by the thiobarbituric acid (TBA) test. TBA-reactive substances (TBARS) formed in the HEFs treated with different concentrations of Cr (VI) for 3 or 24 h, at 37 ºC, are shown in Fig. 3.

Our results show an increase in extracellular TBARS in a dose- and time-manner as a result of Cr (VI) treatment. Thus, after 3-h exposure of cells to Cr (VI) the TBARS level increased significantly only at doses of 100 and 200 µM, by 51%

and 206%, respectively. The level of oxidative peroxidation was low in the first 6-h, but abruptly increased upon prolonged incubation: about 11-fold (12 h) and 16-fold (after 24 h treatment) over the basal level, when the HEF cells were treated with 200 μM Cr (VI). Our study clearly demonstrates that 100–200 μM Cr (VI) induces an oxidative stress as assessed by measurement of extracellular TBARS levels.

In order to better understand the mechanism of Cr (VI) cytotoxicity, we examined the changes in the levels of some components of the antioxidant defense system, GSH/GSSG and antioxidant enzyme activities.



A B

Fig. 4. Effect of sodium chromate on intracellular reduced (GSH) and oxidized (GSSG) glutathione levels. Fibroblasts were exposed during 24 h to 100 μM [○] and 200 μM Na_2CrO_4 [▲]. At the indicated time points, GSH (A) and GSSG (B) concentrations were determined in control and treated cell lysates. Data are expressed as% control and represent the mean of three separate experiments.

The analysis of intracellular glutathione levels showed that, under normal growth conditions, control cells exhibited the GSH and GSSG levels of 11.25 ± 1.05 mmol/g protein and 5.50 ± 0.85 mmol/g protein, respectively. The relatively constant GSH level in control culture during 24-h of incubation at 37 ºC shows that the cells were grown in a well-balanced redox medium, as shown in Fig. 4. The GSH concentration decreases slowly only in the first 9 h of treatment (by 18.6%), thereafter the oxidative process emphasizes that after 24h its content declines by 54.9%, for 100 μM Cr (VI) dose. After a 3-h exposure, GSH level in cells treated with 200 μM chromate suddenly decreases by 50.44%, then the GSH oxidizing goes on but with a lower rate, thus after 24-h it was decreased by 74.3%. These data are correlated with the evolution of GSSG level in the three experimental variants. Besides the antioxidant role of glutathione in cell defense against the deleterious effects of ROS which is well-documented, GSH depletion in cells treated with chromate may be explained by interaction of Cr (VI) with GSH leading to the formation of two Cr (V) complexes and glutathione thiyl radical [2]. Once formed Cr (V) can react via Fenton reaction with H_2O_2 forming the hydroxyl radical capable of causing DNA damage [20].

It is widely known that the cell response to an oxidative injury of a xenobiotic compound is dependent on the activities of antioxidant enzymes in cultured normal HEFs. Among them, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) play a central role. SOD is a critical enzyme responsible for the elimination of superoxide radicals and is considered to be a key anti-oxidant in aerobic cells. There are two forms of intracellular SOD in mammalian cells: Cu,Zn-SOD, localized in the cytoplasm and the nucleus; and Mn-SOD, which is found in the mitochondrial matrix.

To assess the potential role of antioxidant enzymes in sodium chromate toxicity, CAT, GPx, Cu,Zn-SOD (SOD 1) and Mn-SOD (SOD2) were analyzed in control HEFs and cells exposed to Cr (VI) for 3 and 24 h.

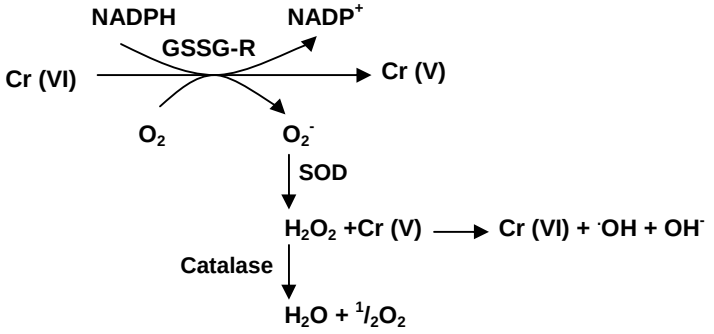


Fig. 5. Intracellular reduction of Cr (VI) with glutathione (GSH), Fenton-type reaction with hydrogen peroxide, formation of hydroxyl radical and involvement of antioxidant enzymes CAT, GPx and SOD.

The reactions catalysed by CAT and GPx are extremely important in the cell because they prevent the accumulation of hydrogen peroxide, a strong oxidizing agent which tends to disrupt the delicate balance of cell biochemistry.

$$2\ H_2O_2 \xrightarrow{\text{CAT}} 2\ H_2O + O_2 \tag{5}$$

$$H_2O_2 + 2\ GSH \xrightarrow{\text{GPx}} G\text{-}S\text{-}S\text{-}G + 2\ H_2O \tag{6}$$

The purpose of this study was to elucidate the differential contribution of CAT and GPx to H_2O_2 scavenging in cultured human embryonary fibroblasts. Table 1 shows the activation of the GPx and CAT after 3-h treatment to a greater extent at 100 µM chromate, by 110% for GPx and 88% for CAT activity. Prolongation of treatment until 24 h leads to inhibition of both enzymatic activities, more prominent at the maximum dose, by 62% for GPx and 91.2% for CAT activity. Although both GPx and CAT decompose H_2O_2, their contributions vary depending on the amount and the site of H_2O_2 production. Higher sensibility of the GPx activity to Cr (VI) treatment may be due to the changes in the intracellular

GSH level that is an enzyme substrate. These results with human embryonary fibroblasts indicate that catalase acts as a primary defence against oxidative stress from exogenous or endogenous H_2O_2 at low concentrations. In contrast, GPx helps protect the cell from damage during exposure to high concentrations of H_2O_2.

Table 1

The effect of sodium chromate on some antioxidant enzyme activities in human embryonary fibroblasts*

Chromate treatment	GPx activity	CAT activity	Cu/Zn SOD activity	Mn SOD
	μmol/min/mg	μmol/min/mg	mUnits/mg	mU/mg
Control	11.06 ± 2.02	10.05 ± 1.82	4.8 ± 0.62	1.24 ± 0.22
100 μM – 3 h	24.04 ± 2.80	18.9 ± 1.78	5.2 ± 1.02	1.81 ± 0,35
100 μM – 24 h	5.06 ± 1.01	5.35 ± 0.72	5.6 ±1.10	4.10 ± 0.65
200 μM – 3 h	19.0 ± 2.38	15.2 ± 2.15	4.7 ± 0.84	1.68 ± 0,30
200 μM – 24 h	0.98 ± 0.18	3.88 ± 0.26	1.4 ± 0.18	0.78 ± 0,15

*Data are expressed as mean ± SD of triplicate determinations.

SOD is is a critical enzyme responsible for the elimination of superoxide radicals

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad (7)$$

and is considered to be a key anti-oxidant in aerobic cells. Accumulation of ROS results in cellular oxidative stress and, if not corrected, can lead to the damage of important biomolecules such as membrane lipids, proteins and DNA.

In HEF cells treated for 3 h with 100 and 200 μM chromate and for 24 h with 100 μM Cr (VI), our data show that cytosolic CuZn-SOD is resistant. On the other hand, the activity of cytosolic SOD was inhibited by 70.8% as a result of cell incubation for 24 h with 200 μM Na_2CrO_4.

An absolutely different response was seen in the case of MnSOD activity. Thus, the cell treatment with 100 μM Cr (VI) caused the activation of mitochondrial SOD to a greater extent as a result of 24-h treatment (by 230.6%), while the result of 24-h exposure to 200 μM chromate was the inhibition by 37% of MnSOD activity. These results may be explained in the context of the primary role of Mn-SOD in protection of cells and mitochodria from free radical damage due to ROS and they are in accord with enhanced levels of ROS noticed for this highest dose. Since SOD is the key enzyme in the first metabolic step of superoxide elimination, inhibition of the enzyme activity may cause severe accumulation of $O_2^{\bullet -}$ in cells and lead to cell death. Prolonged accumulation of high levels of free radicals in cells may cause irreversible cellular injury and ultimately result in cell death.

The results obtained from the present study support the following conclusions: (a) Hexavalent chromium Cr (VI) has not cytotoxic effects upon HEF cell at 5 – 50 μM concentration; (b) Addition of 100 and 200 μM chromate in the culture medium caused decreases in cell viability in a time and dose-dependent

manner; (c) Cytotoxicity caused by Cr (VI) in higher doses was due to increased intracellular ROS production and arisen oxidative lesions; (d) GSH depletion in cells treated with 100 – 200 μM chromate may be explained by its scavenging role as well as by interaction of Cr (VI) with GSH leading to reduction to Cr (V); (e) Compared to baseline activity, in treated cells (with 100 and 200 μM chromate), there were significant changes in activity of CAT, GPx, Cu,Zn-SOD and Mn-SOD, although the magnitude of the modification varied among the enzymes. The CAT and GPx activities were increased after 3-h and decreased after 24-h exposure to higher doses of chromate. The activity of Cu,Zn- SOD was inhibited only as a result of cell incubation for 24 h with 200 μM Na_2CrO_4 while MnSOD activity was activated by 100 μM Cr (VI) and inhibited by 24-h exposure to 200 μM chromate.

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