

Uranium induces oxidative stress in lung epithelial cells

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Abstract Uranium compounds are widely used in the nuclear fuel cycle, antitank weapons, tank armor, and also as a pigment to color ceramics and glass. Effective management of waste uranium compounds is necessary to prevent exposure to avoid adverse health effects on the population. Health risks associated with uranium exposure includes kidney disease and respiratory disorders. In addition, several published results have shown uranium or depleted uranium causes DNA damage, mutagenicity, cancer and neurological defects. In the current study, uranium toxicity was evaluated in rat lung epithelial cells. The study shows uranium induces significant oxidative stress in rat lung epithelial cells followed by concomitant decrease in the antioxidant potential of the cells. Treatment with uranium to rat lung epithelial cells also decreased cell proliferation after 72 h in culture. The decrease in cell proliferation was attributed to loss of total glutathione and superoxide dismutase in the presence of uranium. Thus the results indicate the ineffectiveness of antioxidant system's response to the oxidative stress induced by uranium in the cells.

Keywords Uranium · Glutathione · Reactive oxygen species · Superoxide dismutase · Cell proliferation · Lipid hydroperoxide

Introduction

Uranium, a heavy metal, is found widespread in nature, always in combination with other elements. Uranium has sixteen radioactive isotopes. Uranium isotopes, like other radioactive materials, emit ionizing radiation that is strong enough to damage or destroy living cells. Natural uranium emits gamma radiation and alpha particles. However, alpha radiation rarely appears without being accompanied by other emitters and because they penetrate a very short distance, these particles concentrate on a few micrometers of tissue and are therefore very harmful.

Exposure to uranium often occurs during mining, processing, and from mine tailings (Yazzie et al. 2003). Therefore, effective management of waste uranium compounds is necessary to prevent exposure and adverse health effects cause a growing concern. It has been documented that inhalation is the most common route of uranium exposure. But uranium can also be absorbed through the skin or wounds, ingested with food or drink, and injected into the system intravenously (Sanchez et al. 2001). In recent years, it has been noticed that depleted uranium (DU) in munitions is also another route of exposure to the heavy metal through contact with the skin. In addition, it has been found that people subjected to heavy amounts of stress are more susceptible to uranium toxicity (Albina et al. 2003). Previous studies have shown cancer mortality in men living in counties of Colorado where uranium tailings were used as construction fills (Yazzie et al. 2003). It is further demonstrated that uranium can be correlated with their mutagenicity in a dose and time dependent manner (Stearns et al. 2005).

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As mentioned earlier, the route of exposure to uranium is largely through inhalation and therefore it makes the lung tissue as one of its target organs. DU of size $<5\ \mu\text{m}$ can lodge deep into the lung alveoli, thus producing toxicological impact at the site of contact (Monleau et al. 2006). Uranium toxic studies in lung reported the toxic response to the metal. In macrophage cell line increased secretion of TNF α in association with JNK and P38 activation has been reported (Gazin et al. 2004). The impact of cancer risk upon uranium exposure is worked out in epidemiological studies. These studies have shown the risk of lung cancer in miners formerly exposed to uranium during mining (Bruske-Hohlfeld et al. 2006). The occurrence of a genomic instability in lymphocytes of uranium miners, especially those who developed cancer was observed by studying the cytogenetic endpoint: marker micronuclei (Mn) (Kryscio et al. 2001). Further in vitro analysis with uranium acetate have shown significant increase in DNA single strand breaks in presence of ascorbic acid (Yazzie et al. 2003). Treatment of DU to J774 macrophage cells resulted in the apoptotic death of these cells as confirmed by both DNA ladder and annexin V staining (Kalinich et al. 2002). A mechanistic approach to study the effect of uranium on genomic DNA in CHO EM9 cells has suggested DNA strand breaks or cross links as UA-induced mutagenic lesions. These unique mutagenic lesion induced mutation spectrum elicited by exposure to uranium suggested that uranium generates mutations in ways that are different from spontaneous and that are mediated by free radical as well as through radiological mechanisms (Coryell and Stearns 2006). In addition to the mutagenic potential of uranium it is also observed to alter the proteome in human lung cells (Malard et al. 2005). The proteomic analysis showed cytokeratin and peroxiredoxin 1, of which the later is involved in protection against oxidative injury.

Taken together with the studies on uranium toxicity it reveals to be an environmental health hazard and requires further insights into the mechanism of its action. Towards such an attempt we have investigated the effect of UA on rat lung epithelial cells and to study the response due to induction of oxidative stress. Further, the role of antioxidant on the oxidative stress induced due to UA exposure was also addressed.

Materials and methods

Rat lung epithelial cell line (LE, RL 65, ATCC; CRL-10354) from ATTC, was grown at 37°C in an atmosphere

of 5% CO_2 , in complete growth medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS).

Uranium solutions were prepared as described by Prat et al. (2005). In brief, all experiments were performed using 0.1 M stock solutions of Uranyl (VI) acetate (Amresco, Catalog number 541093, Ohio) pH 4, in water. For cell treatments, 100 mM uranyl bicarbonate was prepared using appropriate dilutions of the stock solution in aqueous 0.1 M bicarbonate solutions and filtered through $0.22\ \mu\text{m}$ filter.

The oxidative stress generated in the cells were measured by a real time assay as described by Sarkar et al. (2006). To study the induction of oxidative stress in LE cells; 1×10^4 cells/well were seeded in 96 well plate and grown overnight under standard culture conditions. The cells were then treated with $10\ \mu\text{M}$ of dichlorofluorescein [5-(and-6)-carboxy-2, 7'-dichloro-dihydroxyfluorescein diacetate, H_2DCFDA , (C-400, Molecular Probes, Eugene, OR)] for 3 h in Hank's balanced salt solution (HBSS) in incubator. Following 3 h of incubation, cells were washed with phosphate buffered saline (PBS) and uranium at 0, 0.5 and 1.0 mM concentration was added for incubation. For studying the effect of antioxidants in LE cell: cells were co-incubated with antioxidants in presence or absence of uranium. Cells were incubated in an incubator for 3 h as detailed in the figure legend and fluorescence was measured at excitation wavelength of 485 nm and emission at 527 nm (Thermo Lab Systems, Franklin, MA). The pre-incubation of the cells with antioxidants did not influence the oxidative stress. Therefore, we resorted to study the effect of antioxidants by co-incubation with uranium in our studies.

Uranium induced lipidperoxidation (LPO) was determined by detection of thiobarbituric acid-reactive malondialdehyde (MDA), an end product of the peroxidation reaction of polyunsaturated fatty acids and related esters using a kit from Cayman; Catalog number 705002 (Wise et al. 2005). LE cells were suspended in chloroform methanol mixture and the hydroperoxides were extracted and used for estimation of LPO according to the protocol described in the kit. The chromogen formed was detected at a wavelength of 500 nm in a spectrophotometer.

The cytotoxicity assay was essentially performed as described by Manna et al. (2005). In brief, LE cells were seeded at 5×10^3 cells/well in a 96 well plate and grown overnight. After 18 h in serum-free medium, cells were treated with different concentrations of uranium and grown for 72 h. At the end of the incubation, cells were additionally treated with 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide]

(MTT; Sigma, St. Louis) MTT for 3 h. The cells were then washed with chilled PBS and formazon formed was extracted in 150 μ L of acidic methanol and the absorbance was read at 570 nm.

The concentration of GSH present in cells were measured by glutathione assay kit as per the instructions provided by the manufacturer and as described by Sharma et al. (2006). 4×10^5 cells were seeded/well in a six well plate and grown overnight. After 24 h, the cells were starved with serum free media for 24 h. Cells were then treated additionally with uranium for 24 h before assaying for GSH. GSH assay was performed in cell lysate using kit from Sigma, St. Louis MO (Catalog number CS0260). In brief, the cells were scraped and homogenized in PBS and deproteinized with 5% 5-sulfosalicylic acid and centrifuged to remove protein precipitate. The supernatant was treated with 5, 5-dithiobis (2-nitrobenzoic acid; DTNB). GSH reduced DTNB to TNB and oxidized to GSSG. Oxidized GSSG present in cells react with added NADPH to give GSH, which later also reacts with DTNB to give TNB. The total TNB formed is measured by absorption at 412 nm in a spectrophotometer.

Western blot was performed as described by Manna et al. (2005). To prepare cell extracts, cells were washed three times with PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 140 mM Sodium chloride, 10% glycerol, 1% Nonidet P-40, 100 mM Sodium fluoride, 200 mM Sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin for 15 min on ice. The lysates were centrifuged for 15 min at 4°C and used for immunoblotting. The proteins at concentration of 75 μ g was boiled with sample buffer for 3 min at 95°C in a water bath. The proteins were resolved in 12.5% SDS-PAGE and transferred to nylon membrane. The membranes were blocked in 5% skimmed milk incubated with primary antibodies specific to superoxide dismutase-2 (Santa Cruz, SC-18503). The blots were incubated with anti-rabbit horse raddish-peroxidase coupled antibody and blots were incubated with ECL reagent (Amersham, NJ) and exposed to X-Ray film.

Statistical analysis

For significant changes, the results were analyzed by Student's *t* test.

Results

LE cells were treated with 0.25, 0.5 and 1 mM uranium for 3 h and significant induction of reactive oxy-

gen species (ROS) was observed at 0.5 and 1 mM concentrations (Fig. 1a). The increase in ROS by uranium was dose dependent and therefore we performed time kinetics to study the increase in ROS as a function of time (Fig. 1b). The increase in ROS was significant as early as 30 min for 0.5 and 1 mM uranium and remained increasing till 60 min. However, at later time intervals the increase in ROS kinetics

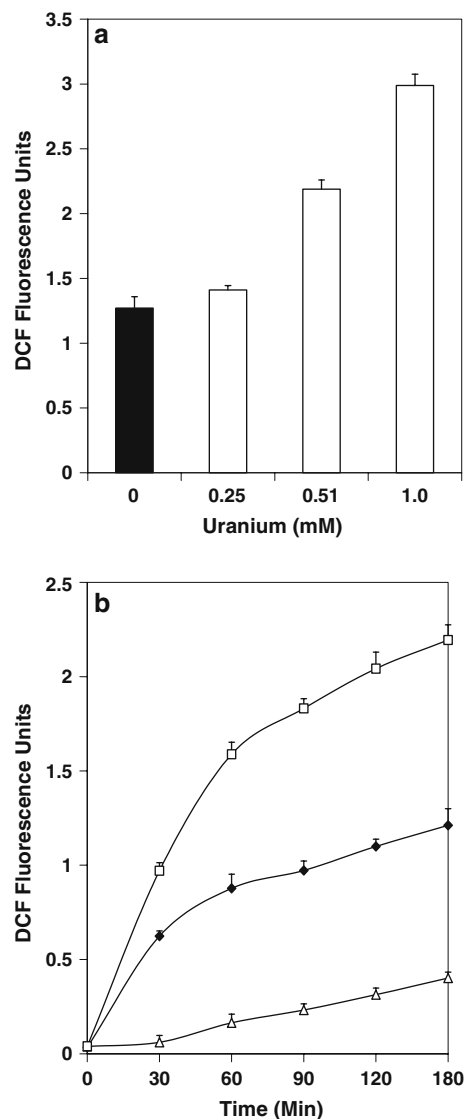


Fig. 1 Uranium induces ROS in LE Cells. **a** 1×10^4 cells/well were seeded in a 96 well plate and incubated for 18–24 h. Cells were then washed with phosphate buffered saline (PBS) followed by incubation in CO_2 incubator with 10 μ M DCF for 3 h in HBSS. The cells were then washed with PBS and incubated with different concentrations of uranium in HBSS. Fluorescence was read at the end of 3 h. **b** Treatment and culture of the cells were similar described as above. The change in DCF fluorescence was measured after each time interval as shown. Values are mean \pm SD of eight wells and are a representative from three experiments performed independently. In absence of uranium (*open triangle*); in presence of 0.5 mM (*filled diamond*) and 1.0 mM (*open square*) uranium

observed was less as compared to 30 min. DU by inhalation resulted in increase of inflammatory cytokine expression and production of hydroperoxides in lung tissue as a consequence of the inflammatory processes and oxidative stress (Monleau et al. 2006). Therefore, uranium is known to induce oxidative stress and in similar fashion the present results are in line with the earlier findings.

As shown in (Fig. 1a, b) exposure of LE cells to uranium results in increased ROS which implicates that such an event could induce lipid peroxidation. Therefore we investigated the lipid peroxidation levels in LE cells exposed to uranium for 24 h. The levels of MDA reflect the measure of lipid peroxidation. The lipid peroxidation was found to be significantly increased at 0.5 and 1 mM of uranium compared to control (Fig. 2). This suggests that elevated levels of lipid peroxides may cause cell death in uranium treated cells.

Oxidative assault can lead to inhibition of cell proliferation or even cell death (Filomeni and Ciriolo 2006; Domej et al. 2006). To further analyze the extent of damage by uranium, LE cells were assessed for cell proliferation using MTT assay (Fig. 3). The results of MTT assay showed a dose dependent decrease in cell viability after 72 h of culture in uranium. A significant decrease in cell viability was observed from 0.5 to

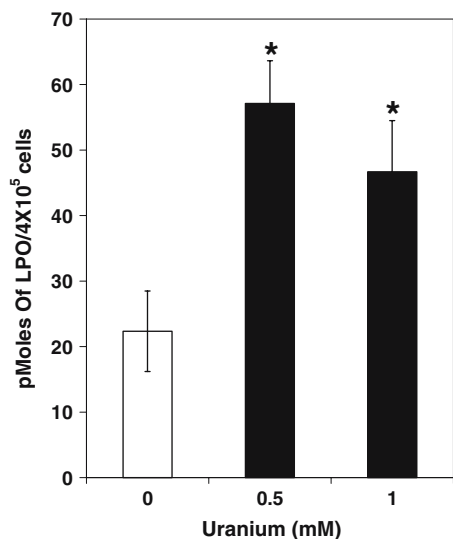


Fig. 2 Uranium increases lipidperoxidation in LE cells. 4×10^5 cells were seeded in a six well plate in DMEM containing 10% FBS and grown under standard cell culture condition for 18–24 h. Following overnight incubation, cells were starved in serum free medium for 24 h. Cells were then treated with different concentrations of uranium and allowed to grow for 24 h. Lipidperoxidation was then measured in the chloroform–methanol extract as described in [Materials and methods](#) section. Values are mean \pm SD of three treatments and done experiments independently. * $P < 0.01$ significance as estimated by Student's *t* Test

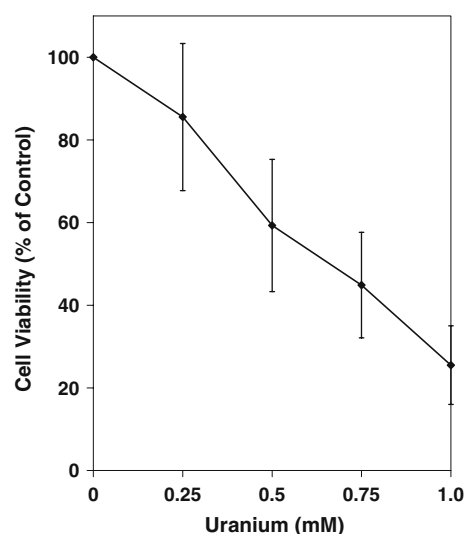


Fig. 3 Uranium decreases cell viability in LE cells. 5×10^3 cells/well were seeded in a 96 well plate in DMEM containing 10% FBS and grown under standard cell culture condition for 18 h. Following overnight incubation, cells were starved in serum free medium for 24 h. Cells were then treated with different concentrations of uranium and allowed to grow for 72 h. The MTT assay was then performed as described in [Materials and methods](#). The mean absorbance at 570 nm is represented as percent of control and is mean \pm SD of eight wells. The values are a representative from three experiments performed independently

1 mM, suggesting the possibility of significant damage induced by the oxidative stress in presence of uranium. Increase in oxidative stress could result from failure of the antioxidant system that constantly counteracts the formation of oxidative species in the cells. Heavy metals like Pb, Cd, Hg and Cr are capable of inducing oxidative stress both in vivo and in vitro (Dick et al. 2003). The cause of the oxidative stress due to these metals pertains to failure in antioxidant status in the system. Therefore, to look for a similar cause with uranium we estimated the levels of glutathione (GSH) in uranium treated cells and compared to control cells.

Cells treated with various concentrations of uranium for 24 h were subjected to GSH analysis according to the procedure described in [Materials and methods](#) section. As expected the amount of GSH was significantly decreased in cells treated with uranium (Fig. 4). GSH is one of the major antioxidant proteins present in the cells to counteract oxidative assaults. Cellular redox potential is largely determined by glutathione (GSH), which accounts for more than 90% of cellular non-protein thiols (Meister 1994). Therefore, the decrease in GSH might be a factor responsible leading to increase in oxidative stress by uranium in LE cells.

In addition to GSH, the antioxidant enzymes comprising of Superoxide dismutase-1, Superoxide dismutase-2, glutathione peroxidase and catalase are also

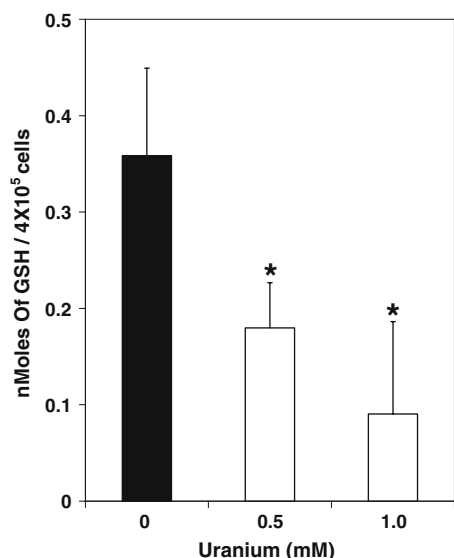


Fig. 4 Uranium depletes GSH levels in LE Cells. 4×10^5 cells/well were seeded in a six well plate in DMEM containing 10% FBS and grown under standard cell culture condition for 18 h. Following overnight incubation, cells were starved in serum free medium for 24 h. Cells were then treated with different concentrations of uranium and allowed to grow for 24 h. The cells were scraped and homogenized in PBS and assayed as described in [Materials and methods](#). The total 2-Nitro-5-ThioBenzoic acid (TNB) formed was measured in a spectrophotometer at 412 nm and is proportional to the concentration of GSH. The results are expressed as nm of GSH present/ 4×10^5 cells. Values are mean \pm SD of three treatments and are a representative from three experiments performed independently. * $P < 0.01$ significance as estimated by Student's *t* Test

effective in scavenging free radicals (Ahlemeyer et al. 2001; Rahman et al. 2006). Therefore, to study the effect of uranium on SOD-1 and SOD-2 enzymes, we did immunoblot analysis in the cell lysates treated with uranium for 24 h. The immunoblot analysis as showed in Fig. 5 suggests the loss of SOD-2 expression by approximately 50% at a dose of 1 mM when compared to control cell lysate. However, under similar treatments, the same lysate SOD-1 did not show any change in expression. The beta-actin used in the same immunoblot analysis was used for equal loading of proteins. The loss of SOD-2 expression by uranium indicates that the cells were unable to counteract the induced oxidative stress.

The above results suggest that the increase in ROS by uranium in LE cells were possibly due to the failure of the antioxidant mechanisms to counteract the rising oxidative species in the cells. Therefore, supplementation of antioxidants could preferably counteract the oxidative stress induced by uranium. Towards such an effort we have cultured the cells in presence of uranium and various antioxidants and have measured the ROS after 3 h of incubation. Figure 6 represents one of such

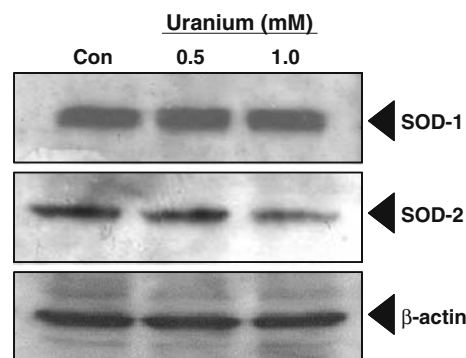


Fig. 5 Uranium decreases the SOD-2 expression in LE cells. Cells were treated with different concentrations of uranium as described in Fig. 4. The cell lysate was resolved in 12.5% SDS-PAGE and immunoblot analysis was performed as described in the [Materials and methods](#) section. Beta-actin immunoblot analysis was performed in the same cell lysate for equal protein loading. The figure is a representative of three independent experiments

experiments and shows the counteractive profile of various antioxidants on uranium induced oxidative stress in LE cells. Oxidative stress induced by uranium at concentrations of 0.5 and 1 mM were decreased in the presence of GSH by ratios of approximately 1.6. NAC, another effective antioxidant also decreased the oxidative stress induced by uranium by ratios of 2 and 2.5 at concentrations of 0.5 and 1 mM uranium respectively. Vitamin E was also effective in counteracting the oxidative stress by uranium at both the concentrations

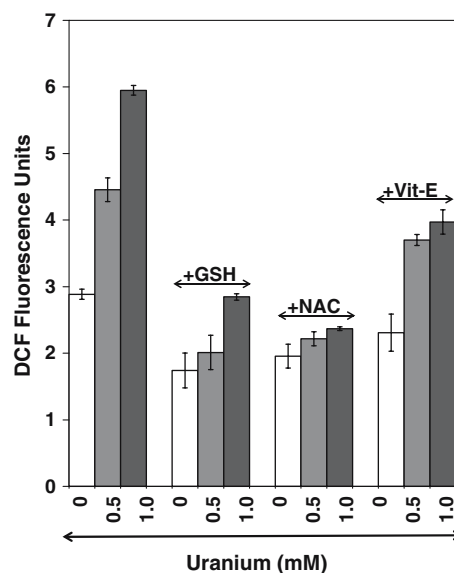


Fig. 6 Antioxidants counteract uranium induced ROS in LE cells. Cells were incubated as previously described in Fig. 1. Cells were grown for 3 h in presence or absence of uranium and antioxidants and changes in fluorescence were measured. Values are mean \pm SD of eight wells and are a representative from three experiments performed independently

with ratios of 1.5 and 2.5, respectively. The results of this experiment suggest that oxidative stress was indeed induced by uranium in LE cells. The antioxidants used were all effective to quench the oxidative radicals to molecular species and thus allowing a decrease in DCF fluorescence. The counteraction of antioxidants has been shown in various cell culture models, wherein oxidative stress was generated by heavy metals (Valko et al. 2006; Hussain et al. 1977). Uranium in the present study also shows a similar response to the antioxidant treatments revealing that a mechanism of oxidative stress induction may be through the pathways already worked out for heavy metals. However, due to lack of studies delineating the uranium signaling it is too early to speculate the mechanism similar to heavy metals and thus needs more extensive studies.

Taken together all these findings, it is reasonable to state that uranium induces oxidative stress in LE cells. The increase in the oxidative stress by uranium in LE cells can be attributed to loss of effectiveness of the antioxidant systems in the cells.

Discussion

Metals can cause oxidative damage by free radical mechanism (Fenton type chemistry) or through direct interactions. Uranium has been earlier shown to produce DNA damaging hydroxyl radical in presence of ascorbate (Yazzie et al. 2003). There is also growing number of reports documenting the possibility of genotoxicity by uranium possibly through a free radical mechanism. The vulnerability of the lung to uranium rises from the inhalation exposure route often encountered during occupational exposure and very few studies have reported on the toxic mechanism of uranium in lung. Our results have shown induction of oxidative stress by uranium in LE cells at 0.5 and 1 mM concentrations and the response correlated to dose and time. The induction of oxidative stress by heavy metals are known and in addition uranium is also a radioisotope. The early induction of ROS by uranium in LE cells suggest the possibility of the involvement of early response proteins being affected. The increase in ROS leading to cell death was reviewed extensively and therefore in the present study uranium followed a similar outcome leading to loss of cell proliferation as revealed by MTT assay. The increased ROS induce LPO and that may lead to cell membrane damage and subsequent cell death. Further, the toxic cell death by uranium might also be associated with the activation of apoptotic pathways induced by oxidative stress. Oxida-

tive stress generated in cells is known to activate apoptotic pathways leading to cell death (Yao et al. 2006). This area needs to be explored to get insights into the mechanism of cell death by uranium. The increase in ROS due to over production of oxidative radicals generated the oxidative stress by uranium. It is possible that uranium may catalyze oxidative reactions as it has been reported to behave in a similar fashion in *in vitro* with DNA in presence of ascorbic acid. The other explanation is the loss of antioxidant defense capacity of the cells to counteract the rising oxygen radicals in the cells. The decrease in GSH by uranium in LE cells clearly indicates a correlation between loss of antioxidants and increase in oxidative stress. In addition to loss of GSH, decrease in the expression of SOD-2 by uranium also may be a reason for increase in ROS. SOD-2 is localized in the mitochondria and is involved in the production of oxygen free radicals in the mitochondria. SOD-2 decrease in presence of 1 mM uranium may be due to transcriptional inactivation or due to proteolysis degradation. Loss of SOD-2 by proteolysis degradation has been observed in presence of strau-sosporine in primary cultures of astrocytes (Ahlemeyer et al. 2001). Uranium in the presence of rotenone, an electron transport chain inhibitor, failed to induce ROS, suggests that probably free radicals were not generated in the mitochondria (data not shown). However, the influence of ROS generated in the cytosol could be sufficient enough to affect the mitochondria. The oxidative stress generated due to loss of antioxidants can be restored by supplementation of antioxidants (Yadav et al. 1994; Hussain et al. 1977). In the present study, we supplemented antioxidants and shown the protection against oxidative stress generated by Uranium. The effect of antioxidants suggest the generation of oxidative stress by uranium and the protection reveals the excessive generation of oxidative species leading to loss of antioxidant response and cell death.

In summary uranium induce oxidative stress in LE cells and loss of antioxidant response. The increase in oxidative stress by uranium may be one of the reasons that lead to DNA mutation as shown earlier by others.

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