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***In vitro* Evaluation of Uranium Induced Immunotoxicity in Chicken Lymphocytes**

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Abstract: In this present investigation *in vitro* Uranyl Nitrate (UN) induced immunotoxicity in chicken spleens lymphocytes was evaluated. At dose levels 15×10^{-4} mL⁻¹ UN induced maximum DNA toxicity within 90 min. It was observed shrunken cells, migration of chromatin, karyorehxis, budding and phagocytic apoptotic bodies were observed after by transmission electron microscopy analysis (TEM). Abortive DNA detected by agarose gel electrophoresis and damaged lymphoid cells was observed by immunoperoxidase assay. Hence, it was concluded that low concentration uranium can produce immunotoxicity.

Key words: Uranium, immunotoxicity, lymphocytes, apoptosis

INTRODUCTION

Uranium is a heavy metal and present at 2 ppm (parts per million) concentration in the earth's crust. Elevated concentration of naturally occurring radioactive material, including ²³⁸U, ²³²Th and their progeny found in underground deposits (Raaretnam and Spitz, 2000). This element is largely limited to use as a nuclear fuel. Depleted Uranium (DU) has been used as a substitute for the fissionable enriched uranium component of atomic weapons. While uranium is a radioactive element, it also has chemical toxicity and this may possibly present a hazard unrelated to radioactivity. Moreover, enhancement of uranium phytoaccumulation takes place from contaminated soils (Shahandeh and Hossner, 2002). Risk calculations and biokinetic modeling and solubility data of uranium showed that there is risks of uranium toxicity from soil and ground water (Elless *et al.*, 1997).

Recently, oral administration of uranyl nitrate in calves was found to significantly suppress serum antibody titer, mitogen induced T-cell blastogenesis and Delayed Type Hypersensitivity (DTH) reaction (Sharma *et al.*, 2001). Heavy metals may cause irreparable damage to DNA leading to deregulated apoptosis (Thompson, 1995). However, no work has been done to investigate DNA toxicity induced by uranium in small concentration and there is no establish data about the toxicity uranium at small concentration. Thus study was therefore undertaken to *in vitro* investigate the effect of UN at minimum concentration in DNA toxicity of spleen lymphocytes in chicken.

MATERIALS AND METHODS

UN was purchased from Loba Chemicals from Loba, USA and immunoperoxidase kits from Sigma, USA. All other chemical reagents were of analytical grade purchased from different sources.

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Isolation and Treatment of Lymphocytes

Spleens of healthy poultry birds were collected under aseptic conditions from slaughter house of Panthnagar, Uttaranchal, India with permission of intuitional animal ethical permission. Splenocytes were separated as methods described by Kelin *et al.* (2006). Separate spleens from the experimental chicken were collected aseptically in ice cold PBS. Briefly, spleens were then placed on a sterile, autoclavable nylon mesh pre-wetted with PBS. Capsule of the organ were removed using a pair of forceps and needles. The organ were disintegrated in to many pieces and minced in RPMI - 1640 media with HEPES buffer and 10% fetal calf serum (Sigma, St Louis, USA) with plunger of a 10 mL glass syringe. Suspension was filtered through sterile muslin cloth and the cells were washed thrice with media. The lymphocyte count was adjusted as 10^7 cells mL^{-1} in media by trypan blue (0.5%) dye exclusion test. Finally 144 mL suspension of spleens lymphocytes was prepared which was divided into 72 equal parts of 2 mL each in sterile vials.

Groups and Dose Selection

Initially UN dissolved in RPMI-1640 media and prepared initially 1.5 ppm concentration. Final dilutions were made in RPMI-1640 media at following concentration and divide in the following groups:

Group I

Served as control group where only 2 mL spleens lymphocytes and 1 mL RPMI-1640 media used.

Group II

UN 1 mL at concentration 15×10^{-4} mg mL^{-1} and 2 mL spleens lymphocytes were used.

Group III

UN 1 mL at concentration 15×10^{-6} mg mL^{-1} and 2 mL spleens lymphocytes were used.

Group IV

UN 1 mL at concentration 15×10^{-9} mg mL^{-1} and 2 mL spleens lymphocytes were used.

After that all lymphocytes were incubated at 37°C in 5% carbon dioxide incubator for 30, 60 and 90 min, respectively.

Assessment of Immunotoxicity

Apoptotic cells were isolated by apoptotic cell separation kit (Sigma, St. Louis, USA) and followed as per manufacturer instruction and immunoperoxidase assay was followed as methods described by Chauhan (1988) and number of apoptotic lymphocytes cells/100 lymphocytes were studied. DNA was extracted from spleen lymphocytes as method described by Herrmann *et al.* (1994). Gel electrophoresis of DNA (10 μg /well) was performed at 100 mV for 1 h on 1% agarose gel containing 0.004% ethidium bromide in 10 mM tris (hydroxymethyl) aminomethane (Tris) -1 mM EDTA (pH 8.0). The loading buffer consisted of 0.25% xylene cynole FF, 0.25% bromophenol blue, 40% sucrose and 1 mM ethidium bromide. The gels were visualized and photographed (Patel *et al.*, 1994). Transmission Electron Microscopy (TEM) was performed as per the methods described by Malorni *et al.* (1998). Scanning electron microscopy was performed as per the method described by Chauhan (2003).

Statistical Analysis

Measured values were expressed as the mean \pm standard error of mean. Kruskal-Wallis test and Mann-Whitnly test were used to compare the means of each group using the SPSS for Window release 10.0 packages. A p-value 0.05 was considered statistically significant.

RESULTS

Immunoperoxidase Assay

Immunoperoxidase study showed that increased apoptotic spleen lymphocytes in presence of UN (Table 1, Fig. 2). When compared with control group (Group I, Fig. 1) it was evident that there was significant increase ($p < 0.05$) in the content of apoptotic lymphocytes in all UN treated groups (Group II, III and IV) at 30, 60 and 90 min, respectively. At 90 min, the apoptotic lymphocytes content were found peak level when compared with 30 and 60 min UN treated groups.

Apoptotic lymphocytes increased significantly ($p < 0.05$) in group II when compare with other groups.

Transmission Electron Microscopy

UN treated lymphocytes at all dilution and all exposure times irrespectively found to be shrunken and denser nucleus as compared to untreated lymphocytes (Fig. 3). Group II lymphocytes at 90 min

Table 1: Effect of UN in apoptotic lymphocytes cells counts/100 lymphocytes at UN treated different groups and at different time intervals

Dose	30 min	60 min	90 min
Group I	1±0.17	3±0.23	3±0.21
Group II	38±1.23*	44±1.37*	50±1.81*
Group III	30±1.19*	37±1.56*	42±0.53*
Group IV	24±0.49*	26±1.16*	26±1.37*

*Significant difference ($p \leq 0.05$)

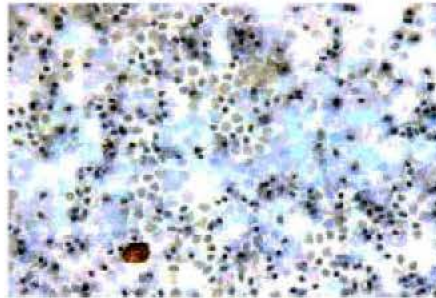


Fig. 1: Photomicrograph of control group lymphocytes stained with immunoperoxidase technique (x400)

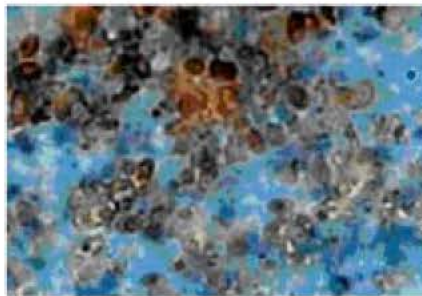


Fig. 2: Photomicrograph of Group II chicken lymphocytes stained (Brown) with immunoperoxidase technique showing apoptosis induced by UN ($15 \times 10^{-4} \text{ mg mL}^{-1}$) at 90 min (x400)

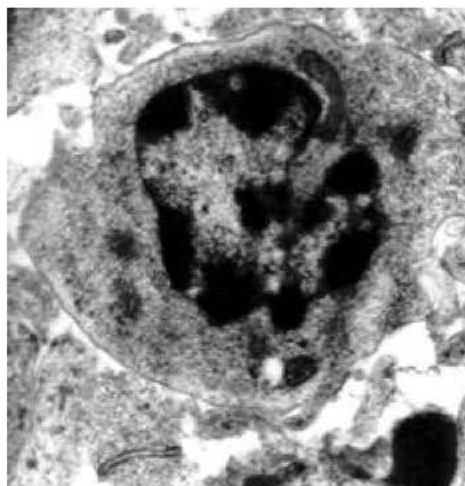


Fig. 3: Transmission electron microphotograph of normal lymphocytes at 90 min (x15000). Showing prominent nucleus

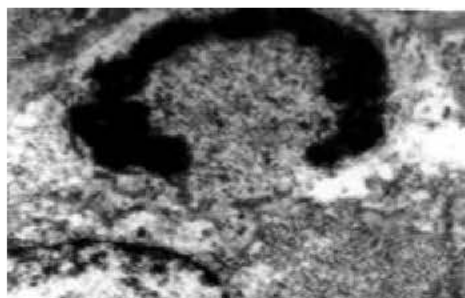


Fig. 4: Transmission electron microphotograph of Group II at 90 min (x15000). Showing migration of chromatin material on nuclear membrane

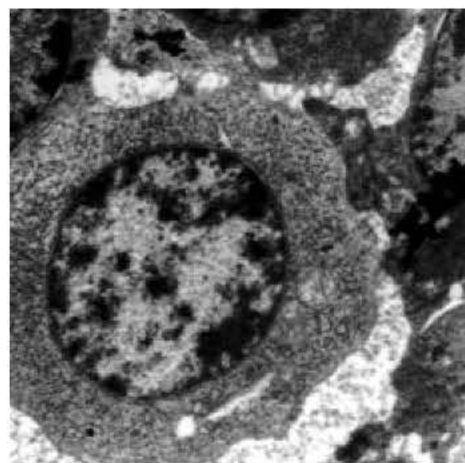


Fig. 5: Transmission electron microphotograph of control group (x15000). Showing well distinguished cell membrane

was observed pyknotic and karyorehxis chromatin and looks like rarified, bud shaped nucleus (Fig. 4) when compare others UN treated groups. These bud shaped processes were seen to break off from the cell, giving rise to apoptotic bodies (budding).

Scanning Electron Microscopy

Ultra structural alterations such as formation of apoptotic bodies and budding were evident on surface of cells treated with UN for 60 min and above, at all the dilutions (Fig. 5) when compared control group (Group I). Maximum apoptotic body formation was found in Group II (Fig. 6) when compared with others UN treated lymphocytes groups.

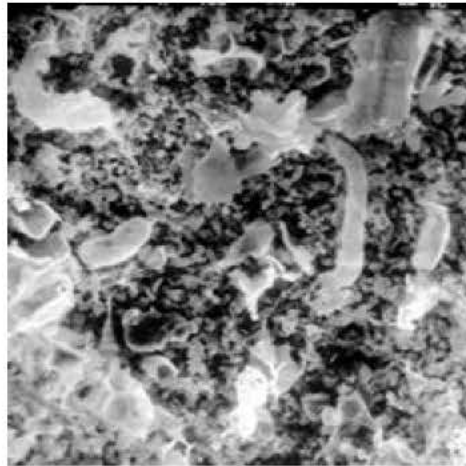


Fig. 6: Transmission electron microphotograph of Group II at 90 min (x15000). Showing budding of apoptotic bodies

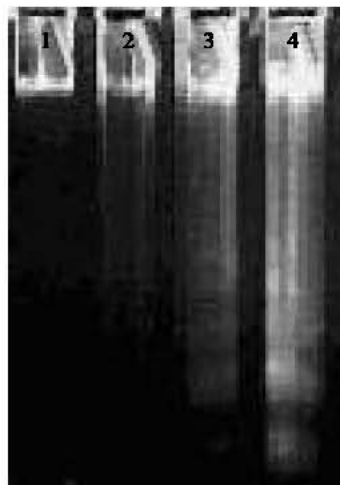


Fig. 7: Agarose gel electrophoresis of DNA isolated from lymphocytes. Lane 1: Normal DNA, Lane 2: 30 min UN treated DNA, Lane 3: 60 min UN treated DNA and Lane 4: 90 min UN treated DNA

Agarose Gel Electrophoresis

DNA isolated from lymphocytes and fragmentation were observed at concentration in UN treated lymphocytes in Group II and Group III for 90 min. Group III and Group I lymphocytes after 90 min exposure showed intact band of DNA (Fig. 7).

DISCUSSION

The present study was conducted *in vitro* to investigate uranium induced apoptosis of chicken lymphocytes. It is reported that lymphocytes toxicity directly related with immunological toxicity (Klein *et al.*, 2006). Naturally occurring radioactive materials produces environmental hazard. Recent survey of wheat plant in India reported that UN²³⁸ concentration were present in between range 4.0×10^{-4} to 2.1×10^{-3} (Pullani, 2005). Lal *et al.* (1983) reported the present of U²³⁸ in cow milk. Several reported various toxic effects of Uranium. Kurtio *et al.* (2006) reported that kidney toxicity of ingested uranium from drinking water. Genotoxicity Monleau *et al.* (2006) of uranium also reported. The assessment of uranium chemical and radiological consequences depends on the physiochemical properties of these radiochemical and the environmental fate. Although uranium is the source of all these fissionable isotopes, its effects in cells toxicity has been poorly investigated. In this research paper we investigated the cell toxicity of UN at various concentration at various time. The changes were dose and time dependent as most prominent alterations were recorded at 1.5×10^{-4} concentration for 90 min exposure, while least at treatment with 1.5×10^{-9} concentration for 30 min.

Kalinch *et al.* (2002) reported uranium chloride (*in vitro*) induced apoptosis in mouse J 774 macrophages. Present study supported that UN induced apoptosis in chicken lymphocytes Durakovic (2001) reported that depleted uranium produced Balkan syndrome, a type immunosuppressant after gulf war and these studies also supported immunosuppressant effect of UN. Immunosuppressant effect of UN because uranium produced apoptotic effect on lymphocytes. A plethora of findings contributing to the knowledge of apoptosis, e.g., the role of sub-cellular structures and organelles, have been widely published (Kerr, 1994; Allen, 1997; Sunetal *et al.*, 2006). Extensive chromatin clumping, cytoplasmic condensation, membrane blebbing and nuclear fragmentation were observed in cells undergoing apoptosis. In the present study condensed chromatin material and detection of apoptotic bodies observed in early and late stages of apoptosis.

Different changes on the surface of apoptotic cells such as the expression of thrombospondin binding sites, loss of sialic acid residues and exposure of phospholipids like Phosphatidylserine (PS) were previously described (Wyllie *et al.*, 1984). In the early stages of apoptosis, a change in the composition of the cell membrane occurs and phosphatidylserine is translocated to the outer leaflet of the cell membrane, where it functions as marker for recognition by other cells. Changes in PS asymmetry, which is analyzed by measuring Annexin-V binding to the cell membrane, were detected before morphological changes associated with apoptosis occurred (Fadok *et al.*, 1992). In the present investigation some of the apoptotic cells had condensed chromatin material, reflecting early stage of apoptosis, while few of them also exhibited vesicle like structures, probably representing apoptotic bodies, featuring late stage of apoptosis.

Apoptosis caused by uranium may be due to direct action on cell membrane or by acting on cell organelles, altering signal transduction pathways or affecting the intracellular enzymes responsible for proper functioning and survival of the cell. In conclusion, the current finds indicate uranium is a dangerous immunosuppressant, immunotoxic agents and produces cell toxicity with in short time. Radiological health community required to proper management and regulation naturally occurring radioactive materials like uranium. Further studies to elucidate the molecular mechanism of action of UN on the expression of genes during apoptosis needs to be conducted.

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