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Radiation exposure from depleted uranium: The radiation bystander effect

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ABSTRACT

Depleted uranium (DU) is a radioactive heavy metal used primarily in military applications. Published data from our laboratory have demonstrated that DU exposure *in vitro* to immortalized human osteoblast cells (HOS) is both neoplastically transforming and genotoxic. *In vivo* studies have also demonstrated that DU is leukemogenic and genotoxic. DU possesses both a radiological (alpha particle) and chemical (metal) component but is generally considered a chemical biohazard. Studies have shown that alpha particle radiation does play a role in DU's toxic effects. Evidence has accumulated that non-irradiated cells in the vicinity of irradiated cells can have a response to ionization events. The purpose of this study was to determine if these "bystander effects" play a role in DU's toxic and neoplastic effects using HOS cells. We investigated the bystander responses between DU-exposed cells and non-exposed cells by co-culturing the two equal populations. Decreased cell survival and increased neoplastic transformation were observed in the non-DU exposed cells following 4 or 24 h co-culture. In contrast Ni (II)- or Cr(VI)- exposed cells were unable to alter those biological effects in non-Ni(II) or non-Cr(VI) exposed co-cultured cells. Transfer experiments using medium from the DU-exposed and non-exposed co-cultured cells was able to cause adverse biological responses in cells; these results demonstrated that a factor (s) is secreted into the co-culture medium which is involved in this DU-associated bystander effect. This novel effect of DU exposure could have implications for radiation risk and for health risk assessment associated with DU exposure.

1. Introduction

Depleted uranium (DU) is a dense heavy metal and an alpha particle emitter used in military applications. It has been used in military conflicts in Iraq, Bosnia, Kosovo and the technology has been established for future use by to multiple nations. Exposure can occur *via* wounding, ingestion, or inhalation. During the 1991 Gulf War and the recent Iraq War, several soldiers were wounded during friendly fire accidents and now have chronic internal exposure to DU; additionally the extent of DU inhalation by soldiers and civilians during previous military operations is difficult to verify.

Recent studies have investigated the potential health effects of this unique heavy metal which is also a radioactive heavy metal [Miller, 2007; McClain and Miller, 2006]. These *in vitro* investigations, reviewed in Fig. 1, have not only demonstrated the neoplastic transforming ability, the mutagenicity, and the genotoxicity of DU, but also that DU exposure can induce genomic instability in a human cell model [Miller, 2007a; McClain and Miller, 2006]. Furthermore some results demonstrated that alpha particle radiation is responsible for some of the cellular damage induced by DU [Miller et al., 2002a; Miller et al., 2007] while others suggested that chemical damage is responsible for DU-effects (Miller et al., 2002b; Stearns et al., 2005). *In vivo* studies of DU implants in rodent models, reviewed in Fig. 2, have demonstrated the carcinogenicity (Hahn et al., 2002), neurotoxicity (Pellmar et al., 1999), and leukemogenic effect (Miller, 2005; Miller et al., 2009) of chronic long-term internal exposure to embedded DU. Renal dysfunction following long-term chronic exposure has been observed as well (Zhu et al., 2009). Inhalation studies *in vivo* have also demonstrated that inhaled DU is genotoxic (Monleau et al., 2006), inhibits vitamin metabolism (Tissandie et al., 2006), accumulates in brain (Houpert et al., 2007), and adversely affects rodent behavior (Monleau et al., 2005).

DU has a low specific activity in comparison to natural uranium, and was not considered to be a significant external radiological hazard prior to recent uses. However, studies have demonstrated that DU exposure intracellularly can cause radiation effects *in vitro* (Miller et al., 2001;

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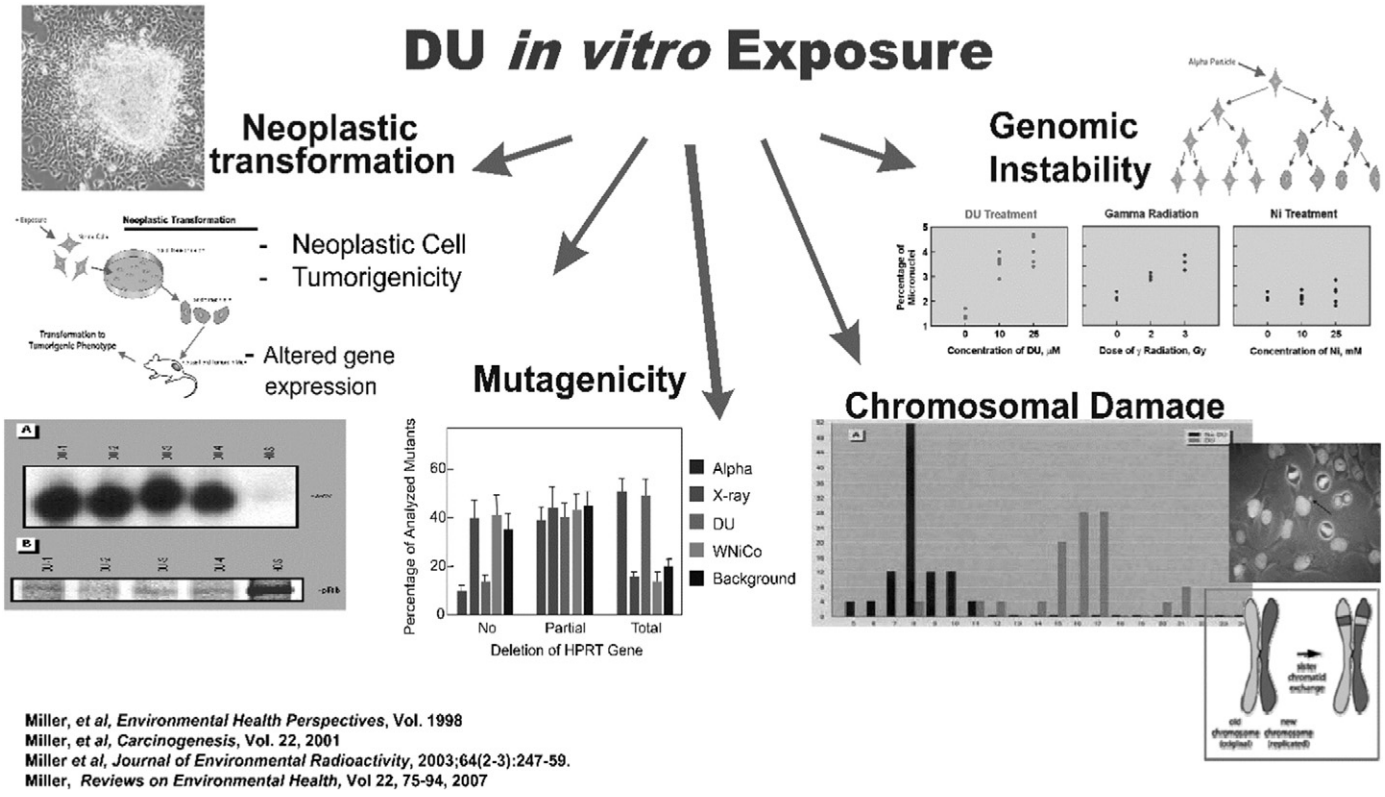

Fig. 1. Review of depleted uranium *in vitro* studies. Counterclockwise from top left: DU-induced Neoplastic Transformation; DU-induced mutagenicity; DU-induced chromosomal damage; DU-induced genomic instability *in vitro*. References for each study are listed in the figure.

Miller et al., 2007). Cytogenetic studies showed that DU exposure could induce chromosomal dicentrics which are considered a radiation-specific chromosomal damage (Miller et al., 2001). A second approach was used to further evaluate DU radiation-specific damage. The induction of mutagenic damage in V79 cells was examined using three uranyl nitrate compounds each containing a different isotope of uranium to determine if radiation plays a role in the induction of that damage. Mutagenicity assessments at the hypoxanthine (guanine) phosphoribo syltransferase *(hprt)* locus demonstrated that equal uranium concentrations with increasing specific activities could induce a specific activity-

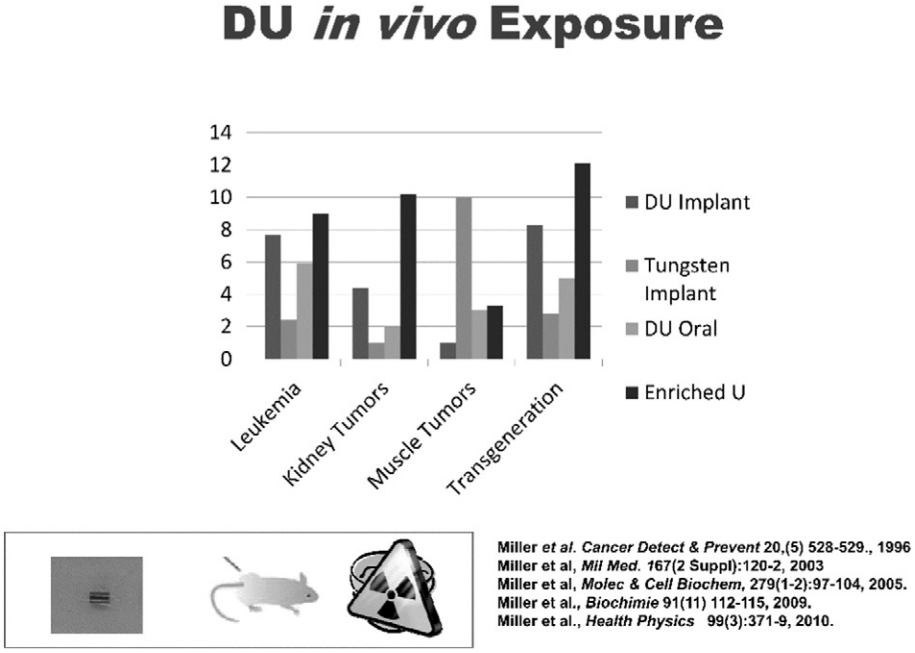

Fig. 2. Review of depleted uranium *in vivo* studies. The bar graph illustrates several studies done to evaluate DU implants in comparison to tungsten implants, DU oral administration, or Enriched 235U oral exposure in mice. Endpoints assessed included leukemia, kidney tumors, muscle tumors, and transgenerational effects. The bottom panel illustrates the implant model system. References for each study are listed in the figure.

dependent mutagenesis (Miller et al., 2007). Taken together these data support earlier results (Miller et al., 2002a, 2002b) showing that dicentric chromosomes were formed after DU treatment, that radiation can play a role in DU-induced biological effects *in vitro*. The potential contribution of radiation to DU-induced biological effects is important and could have significant implications for current risk estimates for internalized DU exposure since risk estimates are based on DU's chemical toxicity and not its radiological activity which is considered to be "low dose".

Evidence has now emerged for a biological radiation phenomenon called "the bystander effect" that may be important in determining the cellular response to low doses of radiation (Mothersill and Seymour, 2015; Morgan, 2003; Sawant et al., 2001). The bystander effect is defined as the observation of a biological response in cells that are not themselves traversed by ionizing radiation, but which can communicate with cells that are (reviewed in Morgan, 2003). Multiple studies have shown that alpha particles are more likely than gamma or X-rays to be involved in this bystander effect (reviewed in Morgan, 2003, Hei and Zhou, 2008). This would have implications for the applicability of the radiation risk models in extrapolating DU data into the low- radiation dose region as current radiation risk models do not consider adverse effects in unirradiated cells. It is necessary to determine the extent and impact of this radiation phenomenon on DU biological effects.

The present study used a novel co-culturing procedure to assess the potential radiation "bystander effect" of DU for the endpoints of clonogenic survival, neoplastic transformation, and micronuclei formation. We have further examined how this phenomenon may be mediated by an extracellular secretion into the culture medium by DU exposed cells. Alpha particles delivered *via* a broad beam were evaluated for comparison as were $NiSO_4$ (NiII) and K_2CrO_4 (CrVI) exposures.

2. Materials and methods

2.1. Heavy metals

DU as uranyl nitrate ($UO_2(NO_3)_2$) with a 234U/238U activity ratio of 0.12 and a specific activity of 0.43 pCi/g was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA). $NiSO_4$ and K_2CrO_4 were obtained from Sigma-Aldrich (St Louis, MO). Both $NiSO_4$ and K_2CrO_4 were used in this study as a comparison to DU since they are non-radioactive heavy metals that are carcinogenic and have been studied extensively (Costa et al., 2001 review).

2.2. Cell culture

Human osteoblast cells (HOS) were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with Eagle's minimum essential medium (EMEM, Sigma) containing L-Glutamine and $NaHCO_3$ supplemented with 10% fetal bovine serum (FBS, ATCC), 100 μg/ml streptomycin and 100 units/ml penicillin. Seeding about 1.0×10^6 and 2.5×10^6 cells/dish in a T75 flask (Corning) was done 24–36 h before treatment so that log-phase cells would be used in all experiments.

2.3. Cell heavy metal incubation

Concentrations of heavy metals were chosen to result in equivalent levels of cell death following cellular exposure. HOS cells were treated in complete media with DU-NO_3 (25 μM, 4 or 24 h), $NiSO_4$ (60 μM, 4 or 24 h), or K_2CrO_4 (3 μM, 4 or 24 h). After treatment with the heavy metals, cells were rinsed with PBS 3× and prepared for cell survival colony formation or neoplastic transformation assays as detailed below.

2.4. Cell Co-culture technique

Immediately after HOS cell incubation with $DUNO_3$ (25 μM, 4 or 24 h), $NiSO_4$ (60 μM, 4 or 24 h), or K_2CrO_4 (3 μM, 4 or 24 h) these HOS + (DU), HOS + (Ni), HOS + (Cr) cultures were rinsed 3× with PBS and counted. The HOS cells incubated with DU (or Ni or Cr) are called HOS-donor cells. The HOS-donor cells were then co-cultured in complete media for either 4 or 24 h with equal numbers of HOS that had never been treated with DU (or Ni or Cr). HOS cells that are co-cultured with HOS-donor cells are called HOS-recipient cells.

2.5. Colony formation assay

Cell survival and plating efficiency (PE) were measured by the standard colony-formation assay. The co-cultivated cells were harvested by trypsinization and re-suspended in *E*-MEM. The diluted cells were seeded in 60 mm dishes in order to form approximately 50–100 surviving cell colonies. After 10 days of incubation, the formed colonies were fixed with 10% methanol and stained with 1% methylene blue; the plating efficiency and surviving fraction were then calculated.

2.6. Cell survival and oncogenic transformation

Survival and mutation assay: Clonogenic survival was measured as previously described (Miller et al., 1998a, 1998b, 2001, 2002a, and b). To assess radiation-induced oncogenic transformation and clonogenic survival, approximately 300 or 100 viable cells were plated into 100 mm dishes respectively. For transformation studies, culture medium was changed at 12 day intervals during the 7-week incubation. The cells were then fixed in formalin, stained with Giemsa and transformed foci types II and III scored as described (Reznikoff et al., 1973). Cells plated for clonogenic survival were incubated for 2 weeks without medium change, stained with 2% crystal violet and colonies with 50 cells scored. Data from a minimum of three independent experiments were pooled. All data for clonogenic survival were presented as a mean with standard error. The statistical significance of differences between groups was tested by Student's *t*-test.

2.7. Uranium analysis

After cell incubation with DU, cellular uranium content was measured by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Finnigan MAT, Bremen, Germany). The methods were as previously described17. HOS cells incubated with DU and HOS cells that were co-cultured with DU-incubated cells were both measured for uranium content. The quantitation limit for the instrument was 0.002 ppb. Values are expressed as nM/cell.

2.8. Medium transfer experiments

HOS cells were treated with DU-NO_3 (25 μM, 4 or 24 h), $NiSO_4$ (60 μM, 4 or 24 h), or K_2CrO_4 (3 μM, 4 or 24 h). As detailed above, equal numbers of the heavy-metal donor cells were then incubated for 24 h with equal numbers of non-heavy-metal HOS cells. The cells were collected for further evaluation of colony formation survival and neoplastic transformation as described above. Following the 24 h co-culture, the DU co-culture media was collected and assessed for uranium content. Similarly, following the 24 h co-culture, the Ni- or Cr- co-culture medium was collected and assessed for Ni content. The respective co-culture media were filtered and used for additional medium transfer experiments. Non-heavy metal treated HOS cells in exponential growth were then incubated for 24 h with the sterile co-culture media from either DU, Ni, or Cr experiments. For comparison, non-heavy metal treated HOS cells in exponential growth were incubated for 24 h with sterile conditioned medium. Conditioned medium is medium that was obtained from control HOS cells that were incubated for 24 h in that medium

prior to its collection for the transfer experiments. The cells from the different transfer medium experiments were then processed for colony survival assay and neoplastic transformation as described above.

2.9. Alpha particle exposure

Alpha particle radiation was conducted at Columbia University, Radiological Research Accelerator Facility (RARAF). RARAF is a Biomedical Technology Resource *(P41)* Center supported by National Institute of Biomedical Imaging and Bioengineering *(NIBIB)* through grant: P41 EB002033. The track segment facility is a broad beam charged-particle irradiator. This system can uniformly irradiate confluent cell dishes rapidly with high doses of alpha particles. The Exponentially growing HOS cells were grown on mylar-rimed rings. The rings were centered onto a movable wheel to allow for irradiation. Cells were irradiated at a range of doses (1 cGy to 200 cGy) at 2.4 Gy/min. Following radiation cells were plated for colony formation or for neoplastic transformation assessment.

3. Results

To determine how much uranium was taken up by DU-treated cells and to determine whether the co-culture process resulted in uptake of uranium by the non-DU treated cells, cellular uranium content was measured using ICP-MS. Fig. 4 shows that the intracellular concentration of uranium increased dramatically over a period of 24 h in DU-treated cells. In contrast the mixed population of cells, after 4 or 24 h co-culture showed a significantly reduced amount of uranium per cell. This reduced amount is expected since only half of the co-culture population cells were previously treated with DU. To test whether the DU-treated cells, when mixed with untreated cells were able to secrete DU into the media which might then have been taken up by untreated cells, we measured the uranium content in the media at five time points – immediately post-mixing the cells populations, and at 1, 4, 12, or 24 h post co-culture initiation. The data in Fig. 4 demonstrate that there was no significant uranium in the media at the end of any of the time points tested. Therefore it appears that there was no uranium available in the co-culture mix media for the untreated recipient cells to take up.

To evaluate whether radiation bystander effects may be involved in DU effects, we compared DU to Ni(II), Cr(VI), and alpha particles. For the most effective comparison we are comparing results with the individual exposures at equitoxic doses of DU, Ni(II), Cr(VI) and alpha particles (Fig. 3). After a series of survival experiments to determine equitoxic doses (data not shown), the exposures used were DU-NO_3 (25 μM, 24 h), $NiSO_4$ (60 μM, 24 h), K_2CrO_4 (3 μM, 24 h) and alpha particles (12 cGy); each of these exposures resulted in a survival percent of 10 ± 2%. Results shown in Fig. 5 demonstrate a significant decrease in surviving fraction from control levels was observed in the co-cultured cells following 24 h co-culture with cells treated with DU (24 h: SF = 0.31 ± 0.03). Control HOS cells demonstrated a baseline surviving fraction of 0.99 ± 0.05 and regardless of the 4 or 24 h co-culture, survival of the co-cultured cells was adversely affected by co-culture with DU-treated cells. There was a significant difference in survival between the recipient cells co-cultured with DU donor cells for 4 h and recipient cells co-cultured with DU donor cells for 24 h indicating that the longer co-culture time did induce an increase in cell killing. In contrast either Ni (II) or Cr (VI) recipient cells did not show a significant change in survival level following a 4 or 24 h co-culture with Ni (II)-treated cells (4 h: SF = 0.95 ± 0.06; 24 h: SF = 0.93 ± 0.05) or Cr (VI)-treated cells ((4 h: SF = 0.96 ± 0.06; 24 h: SF = 0.92 ± 0.06). There was no significant difference in Ni (II) or Cr (VI) recipient cells dependent on time of co-culture (4 or 24 h). These data demonstrate that DU treated cells caused an adverse effect in non-DU exposed recipient cells that was not observed in cells co-cultured with other carcinogenic heavy metals *i.e.*, Ni (II)- or Cr (VI)- treated cells. However, the cells irradiated with alpha particles and then co-cultured with recipient cells demonstrated an increase in cell killing following co-culture. The co-culture phenomenon of increased cell killing was only observed in the DU, an alpha emitter, and alpha particle exposure cells.

The neoplastic transformation frequency was evaluated in heavy metal-treated cells and cells co-cultured with heavy metal-treated cells for either 4 or 24 h. A comparison to alpha particles was also done. Data demonstrating the neoplastic frequencies are shown in Fig. 6 and indicate that the neoplastic frequencies in cells co-cultured with DU-treated cells (4 or 24 h) were significantly increased in comparison to background transformation levels ($p < 0.0001$, respectively). The equitoxic concentrations of the heavy metals and alpha particles were used and DU-treated, Ni(II)-treated, and Cr(VI)-treated cells are shown for comparison and demonstrate as expected that DU, Ni(II), or Cr(VI) treatment caused significant increase in neoplastic transformation. However, as was observed for clonogenic survival, cells co-cultured with cells following equitoxic Ni(II) or Cr(VI) exposure did not show a significant difference in transformation frequency from control levels ($p < 0.0001$). In contrast, cells co-cultured with cells that had been alpha particle irradiated also demonstrated a significant increase in transformation frequency.

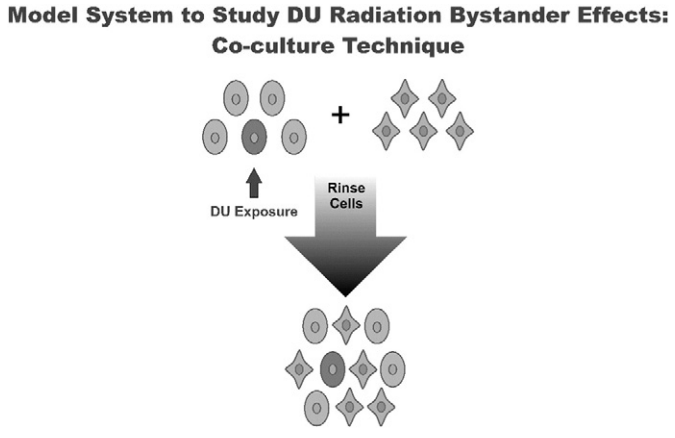

Fig. 3. Model system to study du radiation bystander effects: co-culture technique. HOS cells were exposed to DU (50 μM for 24 h). After exposure. Cells were washed and counted. Equal numbers of DU-treated HOS cells and non-DU treated cells were co-cultured for either 4 or 24 h. The co-culture which consisted of equal numbers of DU-treated and non-treated cells were processed for additional evaluation including uranium content, survival, neoplastic transformation, and micronuclei.

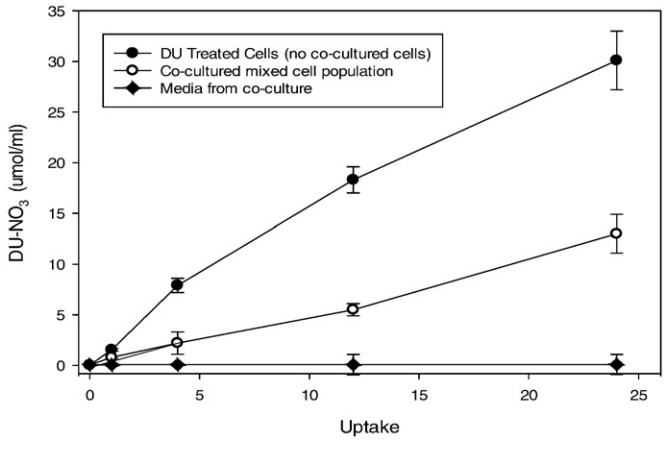

Fig. 4. Cellular uranium uptake. HOS cells were treated with DU-NH2 (25 μM) for either 0, 1, 4, 12, or 24 h (●) and processed for ICP-MS analysis of uranium content. Co-cultured cells were assessed at 4 and 24 h (○) with ICP-MS analysis. The co-culture media was collected at 1, 4, 12, and 24 h post co-culture initiation (♦) and analyzed with ICP-MS for uranium content. Data are pooled from three independent experiments (mean ± SEM).

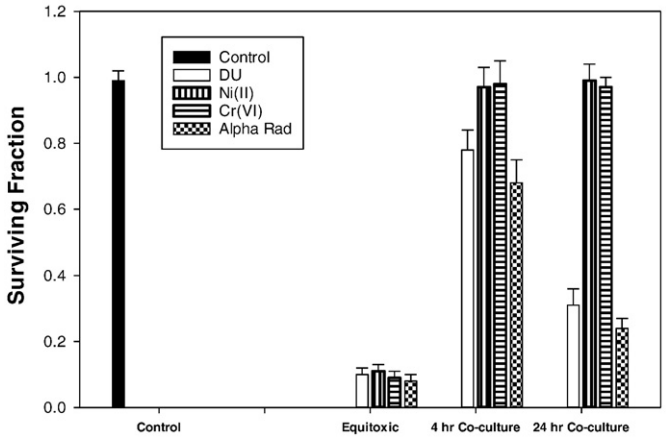

Fig. 5. Surviving fraction of bystander HOS cells. The surviving fraction of HOS cells treated with DU (25 μM) (white bar), Ni(II) (60 μM) (vertical bar), Cr(VI) (3 μM) (horizontal bar), and alpha particle (22 cGy) (checkered bar) for 24 h is shown. The surviving fraction of HOS cells co-cultured for 4 h (white bar) or 24 h (white bar) with DU-treated HOS cells is shown. The surviving fraction of HOS cells co-cultured for 4 or 24 h (vertical bar) with Ni(II)-treated HOS cells, Cr(VI) (horizontal bar), or alpha particles (checkered bar) is shown for comparison. Data are pooled from three to five independent experiments (mean ± SEM).

Radiation studies have shown that release of a factor which is directly cytotoxic or a signal transduction mechanism may be involved in the decreased survival and increased neoplastic transformation in bystander cells (Morgan, 2003; Mothersill and Seymour, 1997; Mothersill et al., 2015).

To investigate whether DU, Ni(II), Cr(VI), or alpha particle co-culture media contained some secreted factor that was associated with the decreased survival and increased transformation in the media of cells co-cultured with DU or alpha particles, transfer media experiments were done. Transfer experiments using the post-co-culture medium from either the DU, Ni(II), Cr(VI), or alpha particles co-culture experiments are shown in Fig. 7. Medium from DU donor and recipient cells co-culture was used to assess survival and neoplastic transformation following a 24 h incubation with DU co-culture media. A comparison to HOS cells incubated for 24 h in conditioned medium or incubated for 24 h with

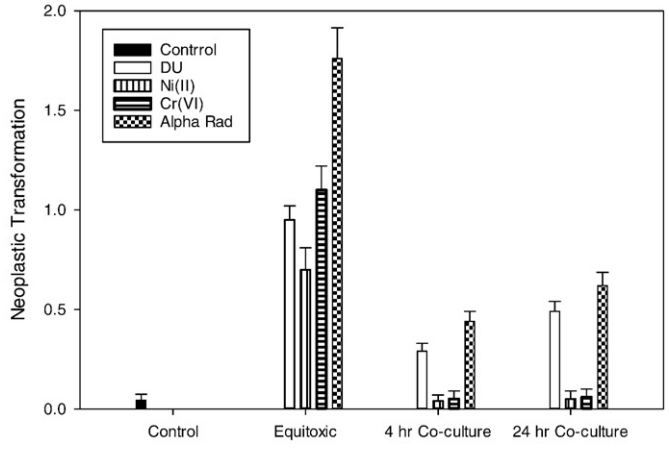

Fig. 6. Neoplastic transformation of bystander HOS cells. The neoplastic transformation frequency of HOS cells treated with DU (25 μM) (white bar), Ni(II) (60 μM) (vertical bar), Cr(VI) (3 μM) (horizontal bar), and alpha particle (22 cGy) (checkered bar) for 24 h is shown. The neoplastic transformation frequency of HOS cells co-cultured for 4 h (white bar) or 24 h (white bar) with DU-treated HOS cells is shown. The neoplastic transformation frequency of HOS cells co-cultured for 4 or 24 h (vertical bar) with Ni(II)-treated HOS cells, Cr(VI) (horizontal bar), or alpha particles (checkered bar) is shown for comparison. Data are pooled from three to five independent experiments (mean ± SEM).

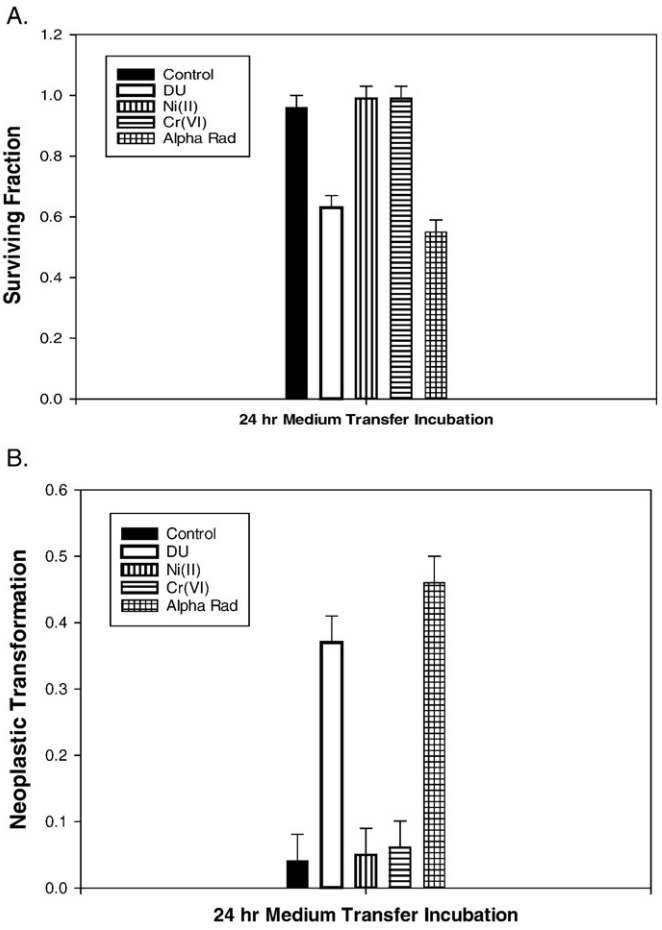

Fig. 7. Survival and neoplastic transformation frequency in HOS cells incubated in transfer medium. Media from DU, Ni(II)-, Cr(VI)- or alpha particle donor and recipient cells co-culture was used to assess survival and neoplastic transformation (DU: white bar, Ni(II): vertical bar, Cr(VI): horizontal bar, ad alpha particles: checkered bar) following a 24 h incubation with the transfer medium. Cells incubated with conditioned medium (black bar) are shown for comparison. Data are pooled from three to five independent experiments (mean ± SEM).

Ni(II), Cr(VI)-, or alpha particle radiation- transfer medium was also done. Transfer experiments using medium from the DU co-culture resulted in a decrease in survival while transfer medium from Ni(II) or Cr(VI) co-culture did not affect survival. Transfer experiments using medium from alpha particle radiation co-culture did result in a decrease in survival. Furthermore, HOS cell incubation in DU transfer medium also increased neoplastic transformation in comparison to HOS cells incubated in conditioned medium or transfer media from Ni(II) or Cr(VI) co-cultures. HOS cell incubation with medium from alpha particle radiation co-cultures also demonstrated an increase in neoplastic transformation above background rate. These data demonstrate that only DU co-cultured cell medium and alpha particle co-cultured cell medium contained an unknown factor that was able to decrease cell survival and increased neoplastic transformation. This phenomenon was not observed for the non-radioactive Ni(II) or Cr(VI).

4. Discussion

Depleted uranium is a radioactive heavy metal emitting alpha particles and although it is considered to be primarily a chemical hazard, it is none the less radioactive and studies have shown that its radioactivity is a significant contributor to its adverse biological effects (Miller et al., 2001; Miller et al., 2007). Therefore the extent of its radiation component to the development of neoplastic transformation, chromosomal damage, and other adverse responses should be addressed.

Radiation dogma has long held that the biological effects of radiation were exclusively caused by deposition of energy in the cell nucleus (Morgan, 2003). Considerable evidence has accumulated in the past 20 years demonstrating that bystander effects can occur in cells that were not themselves irradiated but were either in the vicinity of irradiated cells, hence "bystander", or were exposed to medium from irradiated cells (Belykov et al., 2001; Brenner et al., 2001; Kadhim et al., 2013, Morgan, 2003; Mothersill and Seymour, 2015). In particular, it has been shown that very low doses of alpha particles can lead to multiple types of chromosomal damage, chromosomal instability, proteomic changes, and oncogenic transformation (Brenner et al., 2001) in more cells than would have been traversed by an α particle. This was confirmed *via* Microbeam radiation studies which permit the traversal of one cell or part of a cell in a field with a charged particle beam. Results showed that effects of single cell irradiation are not limited to the exposed cell but affect other cells in the vicinity. It has also been demonstrated that medium from epithelial cells irradiated with radiation, then transferred to cultures that have not been irradiated, induced apoptotic cell death in the unirradiated cells (Mothersill and Seymour, 1997). These studies show that there is significant evidence that alpha particle exposure is associated with radiation bystander effects.

In the present study, a significant bystander effect was seen for clonogenic survival, oncogenic transformation, and micronuclei yields in non-DU-treated, bystander cells after 4 or 24 h of incubation with DU-treated cells. No effect on these endpoints by co-culture with Ni(II)- or Cr(VI)- treated cells was observed for these non-radioactive but neoplastically transforming heavy metals. As previously shown by us and others, cells treated with either DU, Ni(II), or Cr(VI) directly did exhibit significant increases in cell killing and neoplastic transformation (Costa et al., 1994; Miller et al., 1998a; Sun et al., 2011). However, only cells co-cultured with DU treated cells demonstrated increases in cell killing and neoplastic transformation, while cells co-cultured with Ni- or Cr- treated cells did not. Whatever caused the adverse biological effects was only observed in the alpha-particle emitting heavy metal, while Ni did not demonstrate the same effect, suggesting that DU can induce a bystander effect that Ni cannot. Each of the heavy metals studied are known to induce reactive oxygen species (ROS) and oxidative damage so it would appear that the bystander effect induced by DU co-culture medium is not just an oxidative response (Huang et al., 1995) since Ni(II) and Cr(VI) did not induce the bystander effect.

Significant support for the existence of a radiation bystander effects is available in the literature as mentioned above, but the mechanisms by which the bystander signal is produced and transduced have yet to be elucidated. There are two primary possibilities. It appears that multiple pathways are involved in the bystander phenomenon, and different cell types respond differently to bystander signaling. Multiple studies have identified a number of cellular signaling genes, including cyclooxygenase-2 (COX-2), TGFβ2, and the mitogen-activated protein kinase (MAPK) signaling cascade in the bystander process (Vodovotz et al., 1999; Iyer and Lehnert, 2000). Furthermore, cells deficient in mitochondrial DNA showed a significantly reduced response to bystander signaling, suggesting a functional role of mitochondria in the signaling process. Inhibitors of nitric oxide (NO) synthase (NOS) and mitochondrial calcium uptake provided evidence that NO and calcium signaling are part of the bystander signaling cascade as well (Hei and Zhou, 2008). Our laboratory is currently investigating the role that signaling pathways play in bystander phenomenon following DU exposure.

Cell-density dependence of the bystander effect has been observed and implies cell-to-cell contact in the process (Hei and Zhou, 2008). The relationship between gap junctional activity and α-particle-induced bystander mutagenicity was investigated using lindane to inhibit gap-junction-mediated intercellular communication and also by using genetically engineered cells that lack gap junctions. (Azzam et al., 1998, 2001; Lyng et al., 2002; Shao et al., 2003; Zhou et al., 2000). Although these results indicate a role of gap junctions in the bystander response, the role of gap-junctions in the observed DU effects is not well understood.

A third possibility is that the effect of alpha particle radiation on cells produces a transmissible or clastogenic factor (s) that induce cell death, stimulates the neoplastic transformation process, or acts as a chromosome breakage factor. Our data with the medium transfer experiments suggest that there was an extracellular factor that the co-culture of DU-treated and non-treated cells, and the co-culture of alpha particle irradiated cell and non-treated cells, produced since that medium was able to produce adverse biological effects in medium-transfer cultured cells. In contrast, the co-culture medium from Ni(II)- or Cr(VI)- treated and non-treated cells, when used to incubate cells, did not produce any increased adverse effects in those cells and no biologically adverse extracellular factor. At the density at which the cells were plated in this current study and the long incubation time (24 h), the vast majority of cells were in close contact at the time of co-culture. ICP-MS measurements indicated that the observed effects were not due to residual uranium in the medium. Therefore, it is possible that the DU-treated cells could transmit the bystander signal to non-DU-treated cells either through the secretion of a soluble, extracellular factor into the medium and/or through direct cell-to-cell communication via gap junctions. Gap junction studies remain to be done to address this possibility.

An alternative hypothesis should be considered and that is that the observed bystander effect and unknown secreted factor in the medium is not exclusively a radiation phenomenon. Limited studies have shown that bystander effects are not unique to ionizing radiation. Chemotherapeutic drugs such as mitomycin C (MMC) (Rugo et al., 2005), chloroethylnitrosourea (Demiden et al., 2006) and paclitaxel (Alexandre et al., 2007) were shown to induce bystander effects characterized by increased genomic instability, persistent tumor cell growth, and generation of extracellular ROS causing cell death. Adverse changes in growth characteristics and adhesion were also found in cells following bystander exposure to photosensitizers and UV radiation (Dahle et al., 2001). It was also demonstrated that the ability of two DNA-damaging chemicals, MMC and phleomycin (PHL), which have very different modes of action, could induce micronuclei in bystander cells (Asur et al., 2009; Asur et al., 2010). Thus, bystander effects appear to represent tissue or cellular responses to a range of genotoxic stresses that include chemicals and radiation. In some cases like DU, alpha radiation, and paclitaxel, generation of ROS occurs, while in others like phleomycin, it does not. Whether the bystander effect observed with DU is exclusively a radiation-effect or involves a chemical effect, is unknown at this time since some other ROS- and non-ROS- generating chemicals have shown bystander effects.

Our understanding of how the bystander effect impacts the cellular damage induced by the alpha particle emitter DU, is still in the early stages. This study does demonstrate that non-DU exposed cells are influenced by their proximity to DU exposed cells and that this phenomenon is not observed with carcinogenic non-radioactive heavy metals like Ni(II) or Cr(VI). The present finding of bystander effects induced by DU-exposed cells mediated through a secreted-extracellular factor may not only have significance as it supports the finding of radiation involvement in DU effects, but may have significant impact on the cancer risk assessment of DU exposure.

Disclaimer

This work is exclusively the opinion of the authors and is not the official position of the U.S. Government, Department of Defense, USUHS, or AFRRI.

Conflicts of interest statement

All the authors report that they do not have conflicts of interests. COIs are attached to the journal website with the manuscript.

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References

Alexandre, J., Hu, Y., Lu, W., Pelicano, H., Huang, P., 2007. Novel action of paclitaxel against cancer cells: bystander effect mediated by reactive oxygen species. Cancer Res. 67, 3512–3517.

Asur, R.S., Thomas, R.A., Tucker, J.D., 2009. Chemical induction of the bystander effect in normal human lymphoblastoid cells. Mutat. Res. 676 (1–2):11–6. http://dx.doi.org/10.1016/j.mrgentox.2009.02.012 Epub Mar 13.

Asur, R.S., Balasubramaniam, M., Marples, B., Thomas, R.A., Tucker, J.D., 2010. Bystander effects induced by chemicals and ionizing radiation: evaluation of changes in gene expression of downstream MAPK targets. Mutagenesis 25 (3), 271–279.

Azzam, E.I., de Toledo, S.M., Little, J.B., 1998. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from α-particle irradiated to non-irradiated cells. Radiat. Res. 150, 497–504.

Azzam, E.I., de Toledo, S.M., Little, J.B., 2001. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from α-particle irradiated to non-irradiated cells. PNAS 98 (2), 473–478.

Belykov, O.V., Malcolmson, A.M., Folkard, M., Prise, K.M., Michael, B.D., 2001. Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. Br. J. Cancer 84, 674–679.

Brenner, D.J., Little, J.K., Sachs, R.H., 2001. The bystander effect in radiation oncogenesis: II. A quantitative mode. Radiat. Res. 155, 402–408.

Costa, M., Salnikow, C., Cosentino, S., Klein, C.B., Huang, X., Zhuang, Z., 1994. Molecular mechanisms of nickel carcinogenesis. Environ. Health Perspect. 102 (Suppl. 3) September.

Costa, M., Sutherlandurname, J.E., Peng, W., et al., 2001. Molecular biology of nickel carcinogenesis. Mol. Cell. Biochem. 2001 (222):205. http://dx.doi.org/10.1023/A:1017978500031.

Dahle, J., Angell-Petersen, E., Steen, H.B., Moan, J., 2001. Bystander effects in cell death induced by photodynamic treatment UVA radiation and inhibitors of ATP synthesis. Photochem. Photobiol. 73 (4), 378–387 Apr.

Demiden, A., Morvan, D., Madelmont, J.C., 2006. Bystander effects are induced by CENU treatment and associated with altered protein secretory activity of treated tumor cells: a relay for chemotherapy. Int. J. Cancer 119, 992–1004.

Hahn, F.F., Guilmette, R.A., Hoover, M.D., 2002. Implanted depleted uranium fragments cause soft tissue sarcomas in the muscles of rats. Environ. Health Perspect. 110, 51–59.

Hei, T., Zhou, Z., 2008. Mechanism of radiation-induced bystander effects: a unifying model. J. Pharm. Pharmacol. 60 (8):943–950 August. http://dx.doi.org/10.1211/jpp.60.8.0001.

Houpert, P., Frelon, S., Monleau, M., Bussy, C., Chazel, V., Paquet, F., 2007. Heterogeneous accumulation of uranium in the brain of rats. Radiat. Prot. Dosim. 127, 86–89.

Huang, X., Kitahara, J., Zhitkovich, A., Dowjat, K., Costa, M., 1995. Heterochromatic proteins specifically enhance nickel-induced 8-oxo-dG formation. Carcinogenesis 16 (8), 1753–1759.

Iyer, R., Lehnert, B.E., 2000. Factors underlying the cell growth related bystander responses to alpha particles. Cancer Res. 60, 1290–1298.

Kadhim, M., Salomaa, S., Wright, E., Hildebrandt, G., Belyakov, O.V., Prise, K.M., Little, M.P., 2013. Mutat. Res. 752 (1):84–98. http://dx.doi.org/10.1016/j.mrrev.2012.12.001.

Lyng, F.M., Seymour, C.B., Mothersill, C., 2002. Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: a possible mechanism for "bystander" induced genomic instability. Radiat. Res. 157 (2), 365–370.

McClain, D., Miller, A.C., 2006. Depleted uranium biological effects. In: Miller, A.C. (Ed.), Depleted Uranium: Properties, Uses, And Health Consequences. CRC Press Taylor and Francis, Boca Raton, FL, pp. 1–26.

Miller, A.C., 2005. Leukemic transformation of hematopoietic cells in mice internally exposed to depleted uranium. Mol. Cell. Biochem. 279, 97–104.

Miller, A.C., Fuciarelli, A.F., Jackson, W.E., Ejnik, J.W., Emond, C.A., Strocko, S., Hogan, J., Page, N., Pellmar, T., 1998a. Urinary and serum mutagenicity studies with rats implanted with depleted uranium or tantalum pellets. Mutagenesis 13, 643–648.

Miller, A.C., Miller, W.F., Blakely, D., Livengood, T., Whittaker, J., Xu, J., Ejnik, W., Hamilton, M.M., Parlette, E., John, T.S., Gerstenberg, H.M., Hsu, H., 1998b. Transformation of human osteoblast cells to the tumorigenic phenotype by depleted uranium-uranyl chloride. Environ. Health Perspect. 106, 465–471.

Miller, A.C., Mog, S., McKinney, L., Luo, L., Allen, J., Xu, J., Page, N., 2001. Neoplastic transformation of human osteoblast cells to the tumorigenic phenotype by heavy-metal tungsten-alloy metals: induction of genotoxic effects. Carcinogenesis 22, 115–125.

Miller, A.C., Xu, J., Stewart, M., Brooks, K., Hodge, S., Shi, L., Page, N., McClain, D., 2002a. Observation of radiation-specific damage in human cells exposed to depleted uranium: dicentric frequency and neoplastic transformation as endpoints. Radiat. Prot. Dosim. 99, 275–278.

Miller, A.C., Xu, J., Prasanna, P.G.S., Page, N., 2002b. Potential late health effects of the heavy metals, depleted uranium and tungsten, used in armor piercing munitions: comparison of neoplastic transformation and genotoxicity using the known carcinogen nickel. Military Med. 167, 120–122.

Miller, A.C., Stewart, M., Rivas, R., Marino, S., Randers-Pehrson, G., Mitchell, S., Shi, L., 2007. Observation of radiation specific damage. Radiat. Meas. 42, 1090–1094.

Miller, A.C., Stewart, M., Rivas, R., 2009. DNA methylation during depleted uranium-induced leukemia. Biochimie 91, 1328–1330.

Monleau, M., Bussy, C., Lestaevel, P., Houpert, P., Paquet, F., Chazel, V., 2005. Bioaccumulation and behavioral effects of depleted uranium in rats exposed to repeated inhalations. Neurosci. Lett. 390, 31–36.

Monleau, M., DeMeo, M., Freion, S., Paquet, F., Donnadieu-Claraz, M., Dumenil, G., Chazel, V., 2006. Distribution and genotoxic effects after successive exposure to different uranium oxide particles inhaled by rats. Inhal. Toxicol. 18, 885–894.

Morgan, W., May, 2003. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. Radiat. Res. 159 (5), 567–580.

Mothersill, C., Seymour, C., 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. Int. J. Radiat. Biol. 71 (4).

Mothersill, C., Seymour, C., 2015. Radiation-induced non-targeted effects: some open questions. Radiat. Prot.Dosim. 166 (1–4):125–130. http://dx.doi.org/10.1093/rpd/ncv155 (Epub 2015).

Pellmar, T.C., Keyser, D.O., Emery, C., Hogan, J.B., 1999. Electrophysiological changes in hippocampal slices isolated from rats embedded with depleted uranium fragments. Neurotoxicology 20, 785–792.

Reznikoff, C.A., Bertram, J.S., Brankow, D.W., Heidelberger, C., 1973. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to post-confluence inhibition of cell division. Cancer Res. 33 (12), 3239–3249 Dec.

Rugo, R.E., Almeida, K.H., Hendricks, C.A., Jonnalagadda, V.S., Engelward, B.P., 2005. A single acute exposure to a chemotherapeutic agent induces hyper-recombination in distantly descendant cells and in their neighbors. Oncogene 24 (32), 5016–5025 Jul 28.

Sawant, S.G., Randers-Pehrson, G., Geard, C.R., Brenner, D.J., Hall, E.J., 2001. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. Radiat. Res. 155, 397–401.

Shao, C., Stewart, V., Folkard, M., Michael, B.D., Prise, K.M., 2003. Nitric oxide-mediated signaling in the bystander response of individually targeted glioma. Cell.

Stearns, D.M., Yazzie, M., Bradley, A.S., Coryell, V.H., Shelley, J.T., Ashby, A., Asplund, C.S., Lantz, R.C., 2005. Uranyl acetate induces hprt mutations and uranium-DNA adducts in Chinese hamster ovary EM9 cells. Mutagenesis 20, 417–423.

Sun, H., Clancy, H., Kluz, T., Zavadil, J., Costa, M., 2011. Comparison of gene expression profiles in chromate transformed BEAS2B cells. Plos One 6 (3), 17982.

Tissandie, E., Guegun, Y., Lobaccaro, J.M., Paquet, F., Aigueperse, J., Souidi, M., 2006. Effects of depleted uranium after short-term exposure on vitamin D metabolism in rat. Arch. Toxicol. 80, 473–480.

Vodovotz, Y., Chesler, L., Chong, H., Kim, S.J., Simpson, J.T., DeGraff, W., Cox, G.W., Roberts, A.B., Wink, D.A., Barcellos-Hoff, M.H., 1999. Regulation of transforming growth factor beta1 by nitric oxide. Cancer Res. 59 (9), 2142–2149 1999 May 1.

Zhou, H., Randers-Pehrson, G., Waldren, C.A., Vannais, D., Hall, E.J., Hei, T.K., 2000. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. 97, 2099–2104.

Zhu, G., Xiang, X., Chen, X., Wang, L., Hu, H., Wend, S., 2009. Renal dysfunction induced by long-term exposure to depleted uranium in rat. Arch. Toxicol. 83, 37–46.