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INTRODUCTION (Based on original abstract)

Recent studies of Gulf War veterans with depleted uranium (DU) embedded fragments in their soft tissues point to DU-induced effects on neurobehavioral and cognitive function (McDiarmid et al., 2000). These observations are corroborated by electrophysiological changes in hippocampal slices isolated from rats embedded with DU fragments (Pellmar et al., 1999a; Pellmar et al., 1999b). Notably, studies from the same group also suggest, for the first time, that uranium accumulates within brain tissue (Pellmar et al., 1999a). It is presently unknown how uranium is transported into the brain, and there are no pharmacological modalities to reduce its accumulation within the central nervous system (CNS). The purpose of this project is to identify the substrate specificity of uranium transport in the CNS, the working hypothesis being that the divalent metal transporter (DMT-1) which has an unusually broad substrate range that includes Fe^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} , and Pb^{2+} , is mediating uranium transport in the CNS. This project focuses on examining this hypothesis from an in vitro approach utilizing endothelial cell culture models (Technical Objective 1.0) as well as an in vivo approach to delineate the pharmacokinetics of uranium transport across the BBB in rats embedded with DU fragments (Technical Objective 2.0). The studies will test the hypothesis that a relationship exists between blood and brain uranium concentrations, determining whether rats with the highest blood uranium concentrations also accumulate the highest uranium concentrations in the CNS. Thus, the studies will facilitate risk assessment in veterans, and will determine whether those with the highest uranium blood levels are more prone to accumulate uranium in the CNS compared to veterans with low blood uranium levels.

BODY

Stated Technical Objectives for Year 3 of this proposal were:

- 1.0 To determine the *in vitro* transport of uranium across the BBB in *in vitro* endothelial cell culture models (RBE4 and bovine brain endothelial cells).
- 2.0 Study the *in vivo* transport of uranium across the BBB.

SUMMARY REVIEW:

Technical Objective 1:

Studies on Gulf War veterans with depleted uranium (DU) fragments embedded in their soft tissues have led to suggestions of possible DU-induced neurotoxicity. We investigated DU uptake into cultured rat brain endothelial cells (RBE4). Following the determination that DU readily enters RBE4 cells, cytotoxic effects were analyzed using assays for cell volume increase, heat shock protein 90 (Hsp90) expression, 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction, and lactate dehydrogenase (LDH) activity. The results of these studies show that uptake of the U₃O₈ uranyl chloride form of DU into RBE4 cells is efficient, but there are little or no resulting cytotoxic effects on these cells as detected by common biomarkers. Thus, the present experimental paradigm is rather reassuring, and provides no indication for overt cytotoxicity in endothelial cells exposed to DU.

Materials and Methods

Culture and Treatment of RBE4 Cells

The RBE4 cell line was provided as a gift from Neurotech, S.A., Evry, France. Cells of passage 20-80 were grown on collagen-coated tissue culture flasks (Becton Dickinson Labware, UK). The RBE4 cells were maintained in medium of the following composition: 1:1 Ham's F10/minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), basic fibroblast growth factor (bFGF, 1 ng/ml), and Geneticin (300 µg/ml). For the present experiments, these cells were treated with depleted uranium in the oxidized form of U₃O₈ uranyl chloride diluted in HEPES buffer to final concentrations of 10, 50, or 100 µM or HEPES buffer alone.

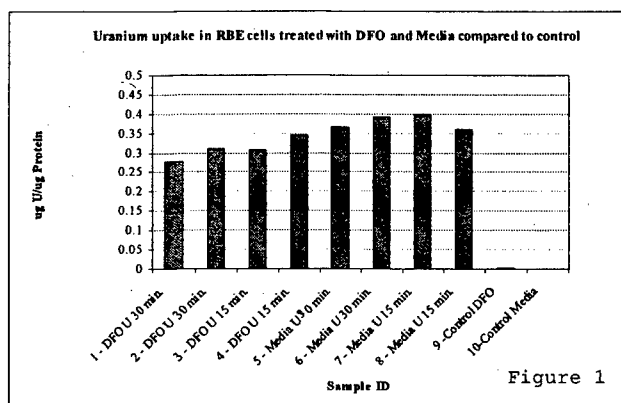
Uranium Uptake Assay Using RBE4 Monolayer and Neutron Activation Analysis

RBE4 cells were pretreated with HEPES alone or 100 µM deferoxamine (DFO) in HEPES for 30 minutes at 37°C, and then

they were treated with HEPES or U_3O_8 uranyl chloride dissolved in HEPES buffer at concentrations of $10\mu M$ or $50\mu M$ for either 15 or 30 minutes at $37^\circ C$. The cells were then washed four times with ice cold Mannitol buffer (290 mM mannitol, 10 mM TRIS-nitrate, 0.5 mM $Ca(NO_3)_2$, pH 7.4) and then lysed with 1N NaOH for 10 minutes. Aliquots of 0.75 mL were then frozen for shipment to North Carolina State University for neutron activation analysis.

Depleted uranium in HEPES buffer readily entered cultured RBE4 endothelial cells as measured by neutron activation analysis (NAA). Cultured RBE4 cells were pretreated with HEPES-only control buffer for 30 minutes. To investigate other possible

influences on cellular uptake of DU, additional samples were pretreated with $100\mu M$ DFO to chelate iron. Following removal of pretreatment media, cells were next treated with DU-containing HEPES or HEPES-only control buffer for 15 or 30 minutes. Cells were



then rinsed, collected, pelleted, and frozen for NAA. Time points of 15 and 30 minutes after replacement of normal culture media with DU-containing HEPES or HEPES-only control buffer are shown in Figure 1. All DU-treated samples showed between 0.27 and $0.4\mu g U_3O_8 / \mu g$ protein analyzed. No differences were observed between 15 minute and 30 minute exposures, and DFO showed no effect on uptake. These data demonstrate rapid entry of DU into the cells.

HSP90 induction

Western blots were performed using protein samples from RBE4 cells treated with control media or 10 or $100\mu M$ DU for 10 minutes, 30 minutes, or one hour. The cells were then washed 3x with cold phosphate-buffered saline (PBS). Subsequently, the cells were harvested with PBS/0.5 mM EDTA, and immediately centrifuged at $12,000 \times g$ for 10 min. The supernatant was removed and the remaining pellet was resuspended in WANG buffer (25 mM HEPES, pH 7.0, 250 mM sucrose, 100 μM EDTA, 1 $\mu g/ml$ leupeptin, 0.5 $\mu g/ml$ pepstatin A, 1 mM DTT, 0.2% Triton-X 100). Following

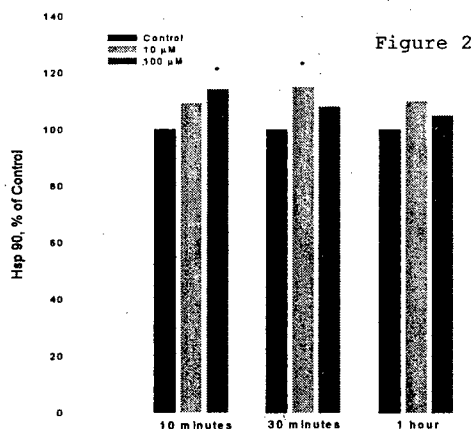
sample sonication, the protein content was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Aliquots of protein (100 µg) were mixed with 5x sample buffer (0.25 M Tris, pH 6.8, 10% SDS, 50% glycerol) and 1M DTT, and separated by denaturing SDS-PAGE using 5% stacking, and 8% resolving acrylamide gels. Proteins were electrophoretically transferred to a nitrocellulose membrane (Protran BA83, Schleicher and Schuell, Keene, NH) in 20% methanol, 0.1% SDS, 25 mM Tris, and 192 mM glycine for 3 hours at 60V. Membranes were then blocked with 5% non-fat powdered milk in TBST (Tris-buffered saline with 0.1% Tween 20, 150 mM NaCl; 20 mM Tris) for 1 hour. Hsp90 protein expression was detected with a rabbit monoclonal antibody (Alpha Diagnostic International; San Antonio, TX) diluted 1:2500 in TBST and 5% milk overnight, followed by a 1 hour incubation with an HRP-conjugated anti-rabbit secondary antibody (1:5000). Protein bands were visualized with Enhanced Chemiluminescence System (New England Nuclear; Boston, MA) followed by exposure to x-ray film. Films were digitized and band density was determined using the TINA v2.09e program (Raytest USA, Inc., Wilmington, NC).

Western blot techniques were used to assess the ability of U₃O₈ to alter Hsp90 expression in RBE4 cells. As shown in Figure 2, U₃O₈ caused a small but significant (p<0.05) increase in Hsp90 levels at 10 and 30 minutes (100 and 10 µM, respectively) compared to control treated

RBE4 cells, but this effect was not apparent after 1 hour (note y-axis represents % change over control, which is standardized to 100%). Accordingly, we conclude that any effect of U₃O₈ is fast and short lasting, and the consequences should be investigated for further detail in future studies.

LDH toxicity assay

RBE4 cells in 96-well culture plates were treated with 10 or 50 µM U₃O₈ in HEPES buffer or HEPES alone at 37°C for 3 hr. or 6 hr. The treatment media was removed and the CytoTox96 Non-Radioactive Cytotoxicity assay for LDH activity (Promega) was employed to assess differences



between LDH content of the samples. Briefly, the cells were lysed and combined with LDH substrate according to the manufacturer's recommendations. The assay for total LDH utilizes colorimetric measurement for conversion of a tetrazolium salt (INT) into a red formazan product at 490 nm in a spectrophotometric 96-well plate reader. Toxic metals effectively remove some of the cellular LDH by inducing events of toxicity, including loss of cell membrane integrity. Therefore, total LDH activity is effectively reduced in those samples treated with toxic metals. Treated samples are reported as percentage of control viability.

MTT toxicity assay

The CellTiter96 Aqueous One Solution Cell Proliferation assay for MTT reduction to formazan (Promega) was also used to assess cells treated with 10 or 50 μM U_3O_8 in HEPES buffer or HEPES alone at 37°C for 3 hr. or 6 hr. MTT reagent was then added to the cells and further incubated at 37°C before colorimetric measurement for substrate conversion at 490 nm in a spectrophotometric 96-well plate reader, according to manufacturer's recommendations. Toxicity results in decreased ability to reduce MTT to formazan. Treated samples are reported as percentage of control viability.

The assays for LDH activity and MTT activity are widely utilized as indicators of cytotoxicity. Both demonstrated that 10 μM or 50 μM U_3O_8 causes little or no toxicity to the RBE4 cells (Figure 3). Additionally, caspase assays showed no induction of caspases 8 or 9 following U_3O_8 exposure (data not shown).

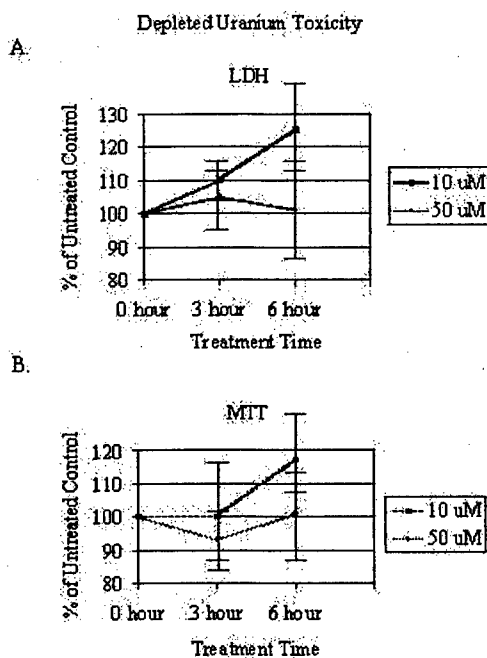


Figure 3

**Technical Objective 2:
Materials and Methods**

Chemicals: Depleted uranium (DU) pellets (1 mm diameter, part # AOT PN 900397) were obtained from Aerojet Metals (Jonesboro, TN). Tantalum (Ta) pellets (diameter x 2 mm, stock # 77611, lot # A16N11) were specified by Alpha Aesar (Ward Hill, MA).

Animals: The Wake Forest University School of Animal Care and Use Committee approved all of the procedures conducted in this study. Adult male Dawley rats (Harlan, ~250 g at study initiation) were housed two per cage in an approved laboratory under 12:12 light:dark cycle with access to food *ad libitum*. Rats were randomly divided into three treatment cohorts. Each cohort was further divided into five groups: Non-surgical control (NS Control), 0 DU pellets/20 Ta pellets (Sham); 4 DU pellets/16 Ta pellets (Low); 10 DU pellets/10 Ta pellets (Medium); 16 DU pellets/0 Ta pellets (High). To assess general health during the study, the animals were weighed weekly and observed for signs of morbidity.

Surgical procedure: Animals were anesthetized with xylazine and 80 mg/kg ketamine prior to surgery. The gastrocnemius was shaved with an electric razor and the surgical area was cleaned with betadine. Rats were draped with sterile surgical drape for the procedure, and the following technique was followed. Pellets were implanted. A 1 cm initial incision (approximately 3 cm long) was made parallel to the muscle. An 18-gauge needle was used to produce a guide-hole perpendicular to the muscle fiber for each pellet. Pellets were implanted to a depth of approximately 1 cm. Following the implantation, the incision was closed with suture and antibiotic ointment was applied prior to the animal regaining consciousness. Animals were rehydrated with 2 mL sterile isotonic saline injected subcutaneously, and then kept in a warm insulated box until fully conscious (about 45 minutes post-surgery). Animals were returned to their cages and monitored daily for a week for infection and pain, and then weekly thereafter until sacrifice.

Tissue collection: At the conclusion of the study, rats were anesthetized with 12 mg/kg xylazine and 80 mg/kg ketamine. Following anesthetization, animals were perfused with 200 mL phosphate buffered saline (PBS).

7.4), through the left ventricle with drainage through the right atrium until effluent was clear. This perfusion ensured that brain tissue was relatively free from blood contamination. Brains were removed and immediately dissected into six regions: cerebellum (CB), pons/medulla [grouped together as brain stem (BS)], midbrain (MB), striatum (STR), hippocampus (HP) and cortex (CX). Following initial weighing, sections were quick frozen on dry ice. Samples were lyophilized prior to analysis.

Inductively-coupled plasma mass spectrometry (ICP-MS): Concentrated nitric acid (HNO₃) (Suprapur, Merck) was added to the lyophilized samples in the following amounts: 1 mL HNO₃ for 0.05 - 0.159 g tissue; 2 mL HNO₃ for 0.16 - 0.259 g tissue; 3 mL HNO₃ to 0.26 - 0.359 g tissue; or 6 mL HNO₃ for 0.66 - 0.96 g tissue. Samples were allowed to sit at room temperature for 24 hours prior to a 1-hr digestion in a block heater at 110 °C. Samples were further digested in a microwave oven (Multiwave 3000, Anton Paar) for the following specifications: Ramp 8 min from 0 - 130 °C, then holding at 130 °C for 3-6 min, depending of sample mass. Finally, samples were diluted to 0.6 M HNO₃ with 18.2 MΩ water.

All brain regions were analyzed for DU content by ICP-MS using a Thermo (Finnigan) model Element 2 instrument (Bremen, Germany), as previously published (Erikson et al. 2004) except that RF-power was now set at 1300 W. Briefly, the sample is introduced using a CETAC ASX 510 autosampler (Omaha, USA) with a peristaltic pump (pump speed 1 mL/min). The instrument is equipped with a concentric Meinhard nebulizer connected to a Scott spray chamber, and a quartz burner with a guard electrode. The nebulizer argon gas flow rate is adjusted daily to give a stable signal with maximum intensity for the nuclide ¹¹⁵In and ²³⁸U. The instrument is calibrated using 0.6 M HNO₃ solutions of multi-element standards at appropriate concentrations. After each sample 0.1 M HNO₃ was flushed through the sample introduction system to reduce memory effects. To check for possible drift in the instrument, a standard solution with known elemental concentrations was analyzed for every 10 samples. In addition, blank samples (0.6 M HNO₃) were analyzed for approximately every 10 samples. Limit of detection (LOD) was 0.4 ng ²³⁸U/ L corresponding to 0.06 - 0.12 ng ²³⁸U / g wet mass of tissue. All digested samples were above the LOD.

Statistical analysis: One-way analysis of variance (ANOVA), with Tukey's posttest for multiple comparisons, was used to determine whether differences in DU doses affected weight

gain during the course of the experiment at each time point. Two-way ANOVA comparing treatment groups at either 3- or 6 months with each specific brain region was performed. Further three-way ANOVAs, with Bonferonni's posttest for multiple comparisons, examined the potential interaction among specific treatment groups in each region at either 3 or 6 months. For each analysis, the overall p-value for the ANOVA is given, as well as the statistical significance of any reported results for discrete regions or treatment groups. Differences among group means was accepted as significant if $p \leq 0.05$. Data is presented as mean \pm SEM, with $n = 5-7$ animals for each group in each cohort.

Results

Treatment with DU does not result in overt toxicity. One week post-surgery, visual inspections suggested that animals had fully recovered and were as ambulatory as non-surgical controls (NS controls). While there was no significant difference in body mass by the end of the respective studies among groups for either the 3- or 6-month cohorts, DU animals consumed greater quantities of food as determined by how quickly rodent chow was replaced. Water consumption did not seem to differ from NS controls for either cohort.

In the 3-month cohort, sham rats were initially statistically significantly smaller than the other groups (figure 1A). At week 1, one-way ANOVA indicated that the group means were highly statistically significantly different ($p < 0.0001$). Specifically, NS control ($p < 0.001$), low and high DU dose rats were different from shams ($p < 0.05$ for both). Additionally, low, medium and high groups were different compared to NS Controls ($p < 0.05$ for all). At week two, the difference among means was still significant ($p < 0.0005$): NS control ($p < 0.001$) and high ($p < 0.05$) were significantly different compared to sham, while low and medium were different compared to NS control ($p < 0.05$ for both). By weeks 4 and 5, only NS control ($p < 0.01$ and $p < 0.05$, respectively) differed compared to sham. By week 6, the body masses of the groups were indistinguishable.

Figure 1B indicates that, while the mean body mass was not statistically significantly different after week 5, the percent gain (initial body mass compared to subsequent weeks) differed among groups. One-way ANOVA indicated differences among groups for weeks 6-12 (see figure 1B). Thus, although variations in body mass among groups were

apparent in the early time points, differences in percent mass increase among groups did not appear until week 6. From week 6 until the conclusion of the 3 month experiment at week 12, the mean percent increase in body mass for the NS controls was significantly lower compared to shams ($p < 0.05$ for weeks 6-8, $p < 0.01$ for weeks 9-12). Additionally, by week 9 and continuing until week 12, the percent mass gain in the low group was statistically decreased compared to the shams ($p < 0.05$ for all weeks).

Figure 1C depicts the actual average body mass of the 6-month group. As for the 3-month cohort, early differences in body masses compared to sham were observed. At week 1, one-way ANOVA indicated ($p < 0.005$) that both medium ($p < 0.05$) and high ($p < 0.01$) groups were lighter than sham rats. One-way ANOVA indicated that only animals in the high group remained lighter for weeks 2-5 ($p < 0.05$ for each week), and at week 9 ($p < 0.01$). However, by the conclusion of the experiment at week 22, the differences in body mass among the groups was indistinguishable. When percent gain relative to initial body mass was calculated (figure 1D), there was no statistically significant differences among groups until the last time point at week 22. Here the medium group was significantly different from sham animals ($p < 0.05$).

Regional brain mass was not different among groups. Two-way ANOVA of the data for the mass (g) of

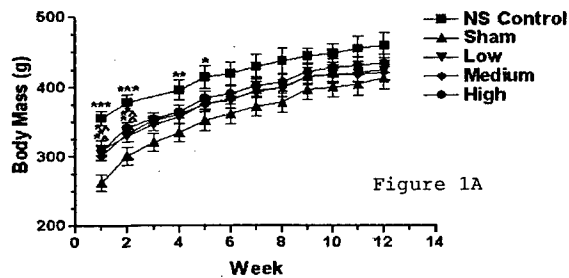


Figure 1A

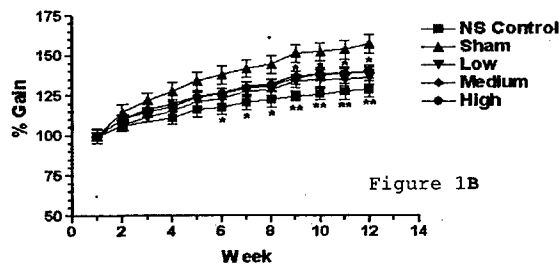


Figure 1B

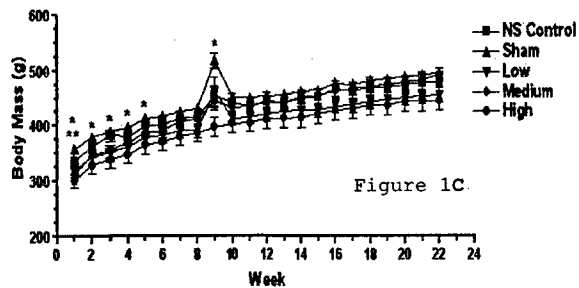


Figure 1C

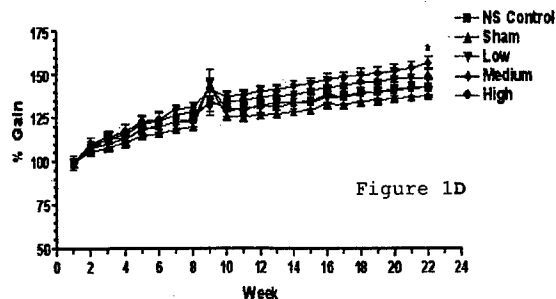
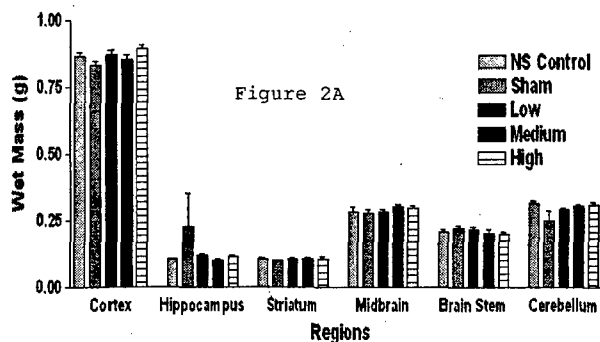
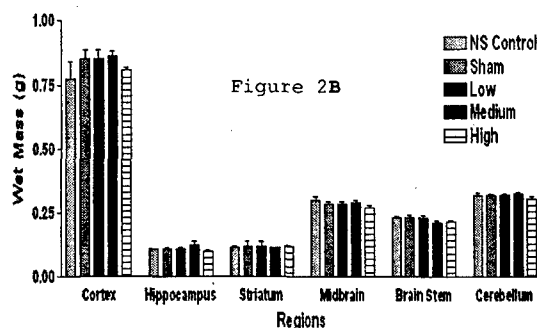


Figure 1D

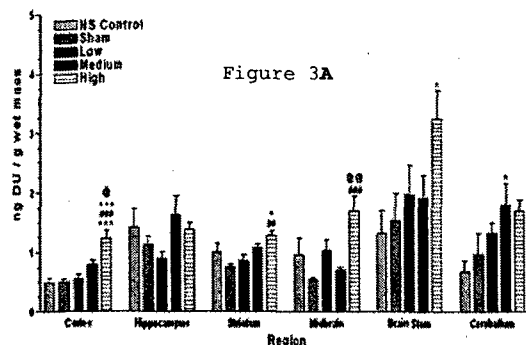
different brain regions from rats in the 3-month cohort (figure 2A) indicated that the source of variation among the brain regions was due to actual differences in regional sizes ($p < 0.0001$). Univariate analysis within brain regions indicated that there were no significant differences in wet brain mass among any of the treatment groups.



The results were similar for the 6-months cohort (figure 2B). Although two-way ANOVA indicated differences among group means was due solely to the differences in regional wet masses ($p < 0.0001$), one-way ANOVA indicated no differences in mass of specific regions from animals in the respective treatment groups. As the body masses of the groups did not differ significantly by the end of either study, brain mass was not normalized to body mass.

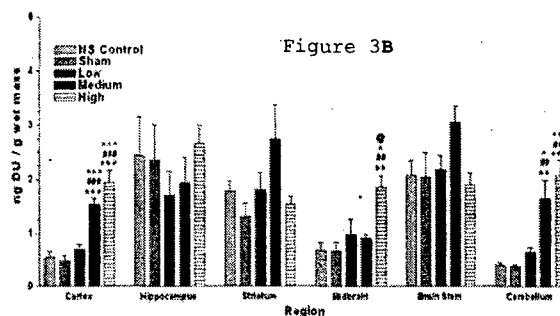


Regional brain DU deposition. By 3 months, a dose-dependent pattern of DU accumulation was apparent in cortex, striatum, brain stem and cerebellum (figure 3A). Two-way ANOVA indicated that both treatment and regional differences accounted for the variation observed in the data ($p < 0.0001$ for both). DU accumulation was significantly increased (one-way ANOVA within brain regions) from animals in the sham group in the cortex, midbrain ($p < 0.001$ for both) and striatum ($p < 0.01$), and different from NS controls in the brain stem ($p < 0.05$) for the high groups. The DU accumulation in the cerebellum was greater than NS controls only in the medium group ($p < 0.05$).



By 6 months (figure 3B), two-way ANOVA indicated variation due not only to treatment and regional ($p < 0.0001$ for both) differences, but also to an interaction between the

two ($p < 0.01$). Univariate analysis within cortex and cerebellum revealed that both medium ($p < 0.001$ and $p < 0.01$, respectively) and high groups ($p < 0.001$ and $p < 0.001$, respectively) had significantly greater levels of DU compared to sham animals. The accumulation of DU was no longer statistically different from sham animals in the striatum or brain stem, although the midbrain from the high-dose group still had statistically significantly more DU than shams ($p < 0.01$).



Regional changes in DU accumulation over time. Further analysis using three-way ANOVA for treatment, region and time indicated an overall interaction ($p < 0.02$). When the individual analysis for separate comparisons was examined, statistically significant differences in DU accumulation over time were observed in the midbrains of sham animals ($p < 0.05$), and the cortex ($p < 0.05$), hippocampus ($p < 0.01$), midbrain and brain stem ($p < 0.05$ for both) of animals in the low treatment group. In general, however, the difference in the amount of DU accumulation in specific regions does not change significantly from 3 to 6 months. This suggests that by 3 months, DU deposition had either reached a maximum, protective mechanisms to prevent further metal accumulation have been activated, or that transport of the metal across the blood-brain barrier reached a steady-state equilibrium with DU uptake equaling clearance.

Key Research Accomplishments

- U_3O_8 does not appear to cause overt cytotoxicity in RBE4 cells.
- U_3O_8 increased Hsp90 protein expression in RBE4 cells, but this effect did not appear to be concentration-dependent or time-dependent.
- U_3O_8 did not affect 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction, and lactate dehydrogenase (LDH) activity in RBE4 cells, suggesting little or no resulting cytotoxic effects on these cells as detected by common biomarkers.
- In vivo studies have been completed and all cohorts have been treated and tissues collected for U analysis. Analyses of regional U levels has been completed this year.
- After 3 months post-implantation, DU significantly accumulated in all brain regions except the hippocampus in animals receiving the highest dose of DU ($p < 0.05$).
- By 6 months, however, significant accumulation was measured only in cortex, midbrain and cerebellum ($p < 0.01$).
- Our data suggest that DU implanted in peripheral tissues can preferentially accumulate in specific brain regions.
- Finally, our data confirm the hypothesis that DU can cross the BBB and deposit in the brain parenchyma.

Reportable Outcome

Dobson A, Erikson KM, Lack K, Aschner M. Depleted uranium is not toxic to rat brain endothelial (RBE4) cells. Biol Trace Element Res 2005; in press.

Fitsanakis VA, Erikson KM, Garcia SJ, Evje L, Syversen T, Aschner M. Brain accumulation of depleted uranium (DU) in rats following 3- or 6-month treatment with implanted DU pellets. Toxicology 2005; Submitted July 2005.

Jiang GC-T, Aschner M. Neurotoxicity of depleted uranium - reasons for increased concern. Biol Trace Element Res 2005; in press. A copy of this review is included in the Appendix.

Conclusions

In Vitro

Studies on Gulf War veterans with depleted uranium (DU) fragments embedded in their soft tissues have led to suggestions of possible DU-induced neurotoxicity. We investigated DU uptake into cultured rat brain endothelial cells (RBE4). Following the determination that DU readily enters RBE4 cells, cytotoxic effects were analyzed using assays for cell volume increase, heat shock protein 90 (Hsp90) expression, 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction, and lactate dehydrogenase (LDH) activity. The results of these studies show that uptake of the U_3O_8 uranyl chloride form of DU into RBE4 cells is efficient, but there are little or no resulting cytotoxic effects on these cells as detected by common biomarkers. Thus, the present experimental paradigm is rather reassuring, and provides no indication for overt cytotoxicity in endothelial cells exposed to DU.

In Vivo

Depleted uranium (DU) is used to reinforce armor shielding and increase penetrability of military munitions. Although the data is conflicting, DU has been invoked as a potential etiological factor in Gulf War Syndrome. We examined regional brain DU accumulation following surgical implantation of metal pellets in male Sprague-Dawley rats for 3 or 6 months. Prior to surgery, rats were randomly divided into five groups: Non-surgical control (NS Control); 0 DU pellets/20 tantalum (Ta) pellets (Sham); 4 DU pellets/16 Ta pellets (Low); 10 DU pellets/ 10 Ta pellets (Medium); 20 DU pellets/0 Ta pellets (High). Rats were weighed weekly as a measure of general health, with no statistically significant differences observed among groups in either cohort. At the conclusion of the respective studies, animals were perfused with phosphate buffered saline, pH 7.4, to prevent contamination of brain tissue with DU from blood. Brains were removed and dissected into six regions: cerebellum, brain stem (pons and medulla), midbrain, hippocampus, striatum and cortex. Uranium content was measured in digested samples as its ^{238}U -isotope by

high resolution inductively coupled plasma mass spectrometry (ICP-MS). After 3 months post-implantation, DU significantly accumulated in all brain regions except the hippocampus in animals receiving the highest dose of DU ($p < 0.05$). By 6 months, however, significant accumulation was measured only in cortex, midbrain and cerebellum ($p < 0.01$). Our data suggest that DU implanted in peripheral tissues can preferentially accumulate in specific brain regions.

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

APPENDIX

NEUROTOXICITY OF DEPLETED URANIUM - REASONS FOR INCREASED CONCERN

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Abstract

Depleted uranium (DU) is a by-product of the enrichment process of uranium for its more radioactive isotopes to be used in nuclear energy. Because DU is pyrophoric and a dense metal with unique features when combined in alloys, it is used by the military in armor and ammunitions. There has been significant public concern regarding the use of DU by such armed forces, and it has been hypothesized to play a role in Gulf War Syndrome. In light of experimental evidence from cell cultures, rats, and humans, there is justification for such concern. However, there is limited data on the neurotoxicity of DU. This review reports on uranium uses and its published health effects, with a major focus on *in vitro* and *in vivo* studies that escalate concerns that exposure to DU may be associated with neurotoxic health sequelae.

Key Words:

Uranium, Depleted Uranium, Neurotoxicity, Gulf War Syndrome, Review

Introduction

Uranium (U) is a naturally occurring heavy metal that is both radioactive and ubiquitous (1). Small amounts of uranium are found in rock, soil, air, water and food. It is estimated that total annual intake of uranium by human adults approximates 460 μg by ingestion of food and water, and 0.6 μg by inhalation (2-5). Natural uranium is composed of three isotopes ^{234}U , ^{235}U , and ^{238}U in the proportions shown in Figure 1.

Depleted uranium (DU) is a by-product of the enrichment of naturally occurring uranium for its most radioactive isotope ^{235}U (Figure 1). During enrichment, for every one kilogram of uranium enriched to 3% ^{235}U , about 5 kg of DU (as a fluoride, UF_6) is produced (6). It is estimated that 700,000 tons of UF_6 are stored in the USA (7). Each year the mass of accumulated DU increases by 30,000 tons (6). DU has 40% less radioactivity than natural uranium, though it may contain trace levels of plutonium, neptunium, americium, technetium, and ^{236}U (8). These additional trace elements increase the radioactivity by 1% but are insignificant with respect to chemical and radiological toxicity (9). The extremely dense and pyrophoric properties of DU make it an excellent metallic substrate for radiation shielding, counterbalances, and in armor and ammunition. It is well documented that DU can be internalized as shrapnel or inhaled from battlefield exposure, but it remains to be determined whether it has a role in the etiology of Gulf War Syndrome. This review reports on uranium uses and its published health effects, with a major focus on *in vitro* and *in vivo* studies that escalate the concerns that exposure to DU may be associated with neurotoxic health sequelae.

Civilian uses of DU

The technical civilian uses of DU are marginal compared to the military applications (6). Commercially, DU is used frequently as ballasts in yachts and counterbalances in commercial jets (8, 10), shielding in radiation therapy, and containers for transportation of radioactive materials. Considering the various exposure routes, typical civilian exposure to uranium from food, water, and air, are considered minimal under normal circumstances (11). Typical exposure levels are well below daily tolerable intake levels for soluble uranium (0.5 $\mu\text{g}/\text{kg}$ body weight), insoluble uranium (5 $\mu\text{g}/\text{kg}$ body weight), and inhaled uranium

(1 $\mu\text{g}/\text{m}^3$ in the respirable fraction), as set forth by the World Health Organization and other US regulatory agencies (9). However, in war-ravaged regions, uranium is present in greater levels and presents a potentially greater exposure danger (6).

Military uses of DU

The use of DU by the military in armor and ammunition takes advantage of its unique metallic properties. Uranium is the heaviest naturally occurring element, and it is extremely dense (approximately 1.7 times the density of lead), such that uranium rods are resistant to deformation (6).

Furthermore, uranium alloys containing 2% molybdenum or 0.75% tungsten sharpen themselves on impact with a hard target, which allows for greater penetration of the uranium-based projectiles compared to traditional tungsten-based alloys. Moreover, DU is pyrophoric - fine particles burn rapidly at relatively low temperatures (150-175°C), and above 600°C particles will spontaneously burn violently with air, releasing heat and uranium oxide aerosols (1, 6).

It is estimated that during the 1991 Gulf War, 300 tons of DU were used in the aircraft rounds and tank-fired shells in Kuwait and southern Iraq over an area of 20,000 km^3 (6). Table 1 illustrates the number of DU ammunitions used during the 1991 Gulf War. Tests on DU penetrators indicate that upon hitting armored targets, 17-28% (up to 70% according to other reports) of a projectile's mass is converted into DU aerosols (1, 6, 12). Of these aerosols, 83% are S-type (S for slow dissolution) oxides, while 17% are M-type (M for medium dissolution) oxides, and the respirable fraction (diameter < 10 μm) may be 50% of the total mass of the aerosol. Accordingly, of the 50 tons of tank-fired and 25 tons of aircraft-fired DU munitions, 10 tons of uranium oxides would have been released in the air in the form of respirable uranium doses. US Department of Defense (DOD) studies have evaluated the distribution of aerosolized DU after destruction of an armored vehicle. Inside a tank, just after an explosion caused by a DU penetrator, the concentration of uranium could reach hundreds or thousands mg/m^3 . Because of the high density of DU, the aerosolized particles should fall within 10 meters of a burning tank. US DOD estimates that DU intake of a person in the vicinity of a tank hit by a single 120 mm projectile is 0.1 mg U, and the maximum intake in traversing a cloud of smoke plume that is 200 meters in length is 0.8 μg U (1, 6, 12). Thus, the US DOD concludes

that the risks of uranium inhalation are primarily greatest for crews of damaged tanks and rescue teams. The use of DU munitions in the Gulf War, and other battlefields, has undoubtedly exposed both civilian and military units to copious amounts of DU dust, vapors, and aerosols, but exposure data for these individuals is unknown.

Exposure pathways and body retention of DU

The three traditional exposure pathways are inhalation, ingestion and dermal contact. In non-military situations, the main routes of uranium uptake are by inhalation and ingestion. Recently, internalization of DU fragments resulting from embedding of projectile fragments (shrapnel) has increased due to the military use of DU in ammunition, and must now be considered as a potentially significant route of exposure for DU (Figure 2). Follow-up studies of a small group of 1991 Gulf War veterans with embedded DU shrapnel seek to determine if there are health effects associated with internalized DU (13-17).

Inhalation

This is the most likely route of intake of DU. Normally, natural uranium is inhaled in very small amounts. Measurements on people in New York City show that about 1 μg of uranium is inhaled each year by each person (1, 2). Of the natural uranium inhaled, 75% is exhaled and 25% is retained in the lungs. Of that 25%, bronchial clearance removes 80%, while 15% ends up in lymph nodes and 5% enters the blood, such that less than 1% of the originally inhaled uranium will actually end up in the kidneys (1, 20).

The risk of uranium inhalation increases during or following the use of DU munitions. This is because the impact of the ammunition will cause DU to become aerosolized, forming oxides and small particles that become suspended in the air by the wind, or settle into the environment for later resuspension. In Kuwait, the estimated mean annual concentration of suspended matter in ambient air is 200 $\mu\text{g}/\text{dm}^3$, one of the highest concentrations in the world (6). The particle size of the aerosolized DU, and its correlated physical and chemical properties (e.g., solubility), have a significant impact on how far into the lungs the particles will penetrate, and if they will be effectively cleared by mucociliary clearance or result in deposition in the respiratory tract. Particles less than 10 μm in diameter will have a greater tendency to accumulate in the bronchioles and alveoli, and it is these respirable

particles that present a potential health hazard from uranium inhalation (1, 9, 19).

Ingestion

This route of entry becomes important if food and drinking water are contaminated by DU. Additionally, ingestion of soil by children is considered a potentially important exposure pathway. The daily intake of uranium in food is estimated to be between 1 and 2 $\mu\text{g}/\text{day}$ (1). Bone ash data indicates significant baseline uranium differences across countries (11), but there is no clear indication as yet that this has led to any adverse health consequences (1). Yet, contamination of food and drinking water is a major concern in battlefield arenas, such as Iraq, Kuwait, the Balkans and Afghanistan. Numerous studies have demonstrated increased uranium levels in the soil of these conflict sites years later after the actual battles are over (20, 21, 22, 23). Other studies have attempted to link higher malignancy rates and genotoxic effects in these regions (24, 25, 26), with varying degrees of correlation.

The solubility of the uranium compound to which exposure has occurred is an important consideration in determining adsorption and distribution, particularly in the case of ingestion, as toxicity is related to uptake efficiency of the GI tract. Uranium oxides (U_3O_8 and UO_2) are relatively insoluble (Types M and S) while uranium trioxide (UO_3) is more soluble (between Type M and F; F for fast dissolution). The more insoluble forms are less likely to enter the bloodstream and cause toxicity. Unlike other heavy metals, uranium is not efficiently absorbed through the intestinal lumen (20). Only 0.2-2.0% of uranium in food and water is absorbed by GI tract (9). Of the amount that is absorbed, 67% will be filtered out by the kidneys and excreted in the urine within the first 24 hours.

Dermal contact and embedded fragments

Typically, dermal contact is a relatively unimportant route of exposure since DU does not pass through the skin into the blood unless there are open wounds or embedded fragments (1, 9). If not removed, the DU-containing shrapnel is a permanent exposure source within the body, and the DU will easily enter the systemic circulation. As the use of DU munitions is relatively recent, there is little published literature on exposure to embedded fragments. The DU Follow-Up Program at the Baltimore Veterans Administration Medical Center has been surveying a small population of approximately 230 soldiers since the

1991 Gulf War. Reports indicate that the embedded fragments are not inert, and thus a permanent source of DU exposure, resulting in elevated urinary uranium levels compared to control patients (14, 15, 16, 17, 18, 29). To date, data indicate there may be some physiological manifestations related to the chemical or radiological effects of DU. For example, there are perturbations in biochemical and neuropsychological endpoints correlated with elevated urinary uranium levels, whose clinical significance remains unclear (1, 14, 15, 16, 17, 18).

Health effects of uranium exposure

Interestingly, the first studies of natural uranium in the mid-19th Century led scientists to believe that uranium had homeopathic properties. Specifically, it was thought that uranium would be an effective treatment for diabetes and albuminuria, after promising results in animals and humans dosed with uranium (30). It was only in the early 1900s, when publications with a plethora of animal studies clearly demonstrated therapeutic failures, that the misuse of uranium in humans was halted (30).

Kidney disease

The renal toxicity associated with uranium have been known for two centuries, as the kidneys work to eliminate internalized uranium (31). Numerous studies confirm this and a recent study actually demonstrated a concentration-dependent effect of uranium on renal cells *in vitro* (32, 33). Nephrotoxicity is a chemically related risk associated with uranium exposure and has been documented in animal studies at high exposure levels (1). It should be noted that any observed toxicity results from acute exposure. To date, there is no evidence that DU has any long-term effects on renal function. There is no medical evidence that shows that long-term renal impairments will develop if no acute effects are seen (1). Indeed, there is evidence in animals and humans of repair of damaged tubular epithelial tissue (1, 31).

Bone cancer

Bones are the secondary target organ of uranium toxicity, and the potential for increased risk of bone cancer has been explored (1). There is a time-dependent correlation between implanted uranium and both oncogene expression (34) and genomic instability (35, 36), suggesting that DU has mutagenic effects. *In vitro* studies with uranyl chloride (soluble DU, UO₂Cl₂) demonstrated a ten-fold increase in

neoplastic transformation of human osteoblasts, compared to a seven-fold increase by the known carcinogen nickel sulfate (35). The insoluble DU (DU-UO₂) also transformed the human osteoblasts to a neoplastic phenotype, resulting in a twenty-five fold increase in neoplasias, compared to a ten-fold increase with nickel (35). This data is in agreement with reports that there is an increased carcinogenic risk in lungs of Gulf War I veterans (37, 38). There are numerous other studies which demonstrate the deleterious effects of DU on viability, micronuclei, chromosomal instability and sister chromatid exchanges by human bronchial, bone marrow, and Chinese hamster ovary cells (39, 40, 41, 42; 43, 44, 45, 46, 47, 48).

To mimic Gulf War veterans' injuries and assess the chronic effects of internalized DU, recent studies have utilized rats implanted with DU. The carcinogenicity of DU fragments has been evaluated by surgically implanted DU pellets in the muscles of rats (49). The authors concluded that DU fragments of sufficient size cause localized proliferative reactions and soft tissue sarcomas, and the carcinogenicity is related to the size of the fragments. Urine and serum mutagenicity studies with rats implanted with DU or control rats implanted with tantalum pellets demonstrated enhanced mutagenic activity in *Salmonella* TA98 strain and Ames II mixed strain (TA7001-7006) in a dose-dependent manner with excreted urinary concentration (34). It should be noted that it has yet to be determined whether these effects are a result of the chemical or radiological effects of DU. The risk of osteosarcoma is clearly related to the amount of radiation exposure as shown in *in vitro* studies of bone cells and in animal models (35, 26, 43, 44, 45). However, researchers have postulated that the amount of radiation emitted by DU is insufficient to raise a significant risk in humans, especially in adults whose bones are not growing rapidly.

Lung cancer

As the lungs are the primary portal of inhaled uranium, there are numerous studies which examined the health effects of inhaled uranium. As shown in the 1940s, and previously mentioned, the most soluble uranium compounds are the most toxic (50, 51). In the 1970s, Leach and colleagues examined the long-term effects of DU inhalation of 1 μ m particles of uranium dioxide (UO₂) in monkeys, dogs and rats (52, 53). These researchers found that the lungs and tracheobronchial lymph nodes were the major sites of uranium accumulation of these large particles and relatively insoluble form of uranium, and there were

fibrotic changes in the lung tissue suggestive of radiation injury. Insoluble particles deposited in the lungs have a long residual time and result in increased risk for cancer (7, 54). Early animal studies did not reveal significant animal mortality from inhalation unless very soluble forms of uranium were used. Typical health consequences in the uranium inhalation studies included the development of pneumonia and chemically irritated passages (50, 51, 55, 56), which could be considered early signs of lung cancer. As previously stated, there is data that indicate an increased carcinogenic risk in lungs of GWI veterans (37, 38), and numerous studies that demonstrate the deleterious effects of DU on viability, micronuclei, chromosomal instability and sister chromatid exchanges in cells (39-48). The debate continues as to whether these particles are the causative agents for lung cancer as it is often difficult to pinpoint specific cancerous agents using epidemiological studies (57).

Reproductive effects

Lin *et al.* (47) demonstrated the cytotoxicity of DU *in vitro* in Chinese hamster ovary cells by examining cell viability, cell cycle kinetics, micronuclei formation, chromosomal aberrations and sister-chromatid exchanges. Domingo (58) reviews the reproductive effects of DU as demonstrated most recently in animal models. Uranium is a developmental toxicant when given orally or subcutaneously, resulting in decreased fertility, embryo/fetal toxicity including teratogenicity, and reduced growth of offspring, following uranium exposure at different gestation periods (58, 59). Studies that address male reproductive effects in miners, uranium processors and Gulf War veterans, show that these individuals have uranium in their semen but do not otherwise show any detrimental reproductive effects (1, 16, 29, 58, 59, 60).

Growing evidence that DU may affect the central nervous system (CNS)

Since the focus of DU studies has been on the major sites of deposition (kidneys, bones, lungs), there have been a limited number of studies which examine the effects of DU on the CNS. However, recent studies, both in rats and humans, suggest that DU may cause subtle changes in CNS function without any corresponding nephrotoxicity (14, 15, 16, 17, 18, 29, 61-64). This growing body of evidence suggests that DU may be a frank neurotoxicant, and has led to public concerns regarding the use of DU in armor and

ammunitions by the military. One of the earliest studies evaluating the specific effects of DU on the CNS focused on the presynaptic action in phrenic nerve preparations from mice. It demonstrated that uranyl nitrate facilitated the release of acetylcholine from the nerve terminals to potentiate muscle contraction (65).

Recent studies indicate that uranium crosses the blood-brain barrier and will accumulate in the brain (61, 62, 66-69; Aschner et al., unpublished results). Lemercier and colleagues (69) demonstrated transfer of uranium across the blood-brain barrier in a study in which researchers performed *in situ* rat brain perfusions followed by inductively-coupled plasma-mass spectrometry (ICP-MS). They found that a significant amount of uranium accumulated in the brain after a 2 minute perfusion (69). This group estimated the quantity of uranium in the blood vessels and/or nervous parenchyma to be 32 ng/g (69). Studies in rats embedded with DU and/or tantalum pellets (0, 4, 10, or 20) for 1 day, 6, 12, and 18 months, confirmed previous biodistribution data that demonstrated kidneys and bone as the primary target organs, but also identified other sites in the lymphatic, respiratory, reproductive and CNS (61). Kinetic phosphorimetry, which was used to measure uranium concentrations throughout the body, indicated that high-dose (20 pellets) DU implantation resulted in significantly increased uranium levels in the skull after only 1 day (61). Tantalum control rats were measured to contain approximately 1.41 ng U/g skull tissue while high-dose rats had approximately 12.5 ng U/g skull tissue (61). By 6 months, there was a significant difference in the amount of uranium in brain samples from high-dose rats (approximately 5000 ng U/g tissue) compared to tantalum control samples (approximately 50 ng U/g tissue) (61). As previously demonstrated with other heavy metals, there were significant differences in brain region distribution of uranium after 18 months of implantation. Statistically significant increase in uranium levels could be seen in a number of discrete brain regions after high-dose DU treatments, such as in cerebellum, midbrain, frontal and motor cortexes (61).

Differences were also seen in electrophysiological studies in hippocampal slices of rats implanted with DU (62). The hippocampus was selected because of its known role in learning, memory consolidation, and spatial orientation functions (personal communication with Dr. T.C. Pellmar). The 6-month and 12-month high-dose groups exhibited decreased neuronal excitability compared to

controls, as measured by excitatory postsynaptic potential/spike (E/S) coupling. Rats in the 18-month DU-treatment groups did not show significant changes in neuronal excitability, but this was surmised to have been the result of aging of the rats, which may have obscured the consequences of metal exposure (62). There were no significant differences in hippocampal weights of DU treated animals compared to controls (personal communication with Dr. T.C. Pellmar). In all of the rat DU implantation studies, kidney and urine levels of uranium indicated high levels, but with no indications of nephrotoxicity.

Researchers at Duke University have investigated the effects of uranyl acetate on sensorimotor behavior, generation of nitric oxide (NO), and the central cholinergic system of male Sprague-Dawley rats (70). Intramuscular injection of 0.1 and 1.0 mg/kg for 7 days, followed by a 30 day observational period, resulted in sensorimotor deficits in rat behavior, differential levels of NO (increased levels in 0.1 mg/kg dosed animals, decreased levels in 1.0 mg/kg dosed animals in cortex and midbrain, but not in brainstem or cerebellum), and increased acetylcholinesterase activity in the cortex of animals dosed with 1 mg/kg. These results show that multiple exposures to low-doses of uranyl acetate (similar to the exposures of Gulf War veterans) cause prolonged neurobehavioral deficits in rats after the initial exposure has ceased (70).

Most recently, Briner and Murray have demonstrated short-term and long-term differences in brain lipid oxidation and open-field behavioral differences in rats exposed to DU (63). After two weeks of DU exposure, brain lipid oxidation, as measured by the thiobarbituric assay, was increased and correlated with increases in line crossing and rearing behavior. However, though the open-field behavior differences remained after 6 months of DU exposure, brain lipid oxidation could no longer be clearly correlated with the behavioral changes. The researchers also demonstrated that male rats appeared to be more sensitive to the behavioral effects of DU compared to female rats (63). The results of these studies support previous studies which indicate that DU is a toxicant capable of crossing the blood-brain barrier and producing prolonged behavioral changes. Indeed, the gender differences seen by Briner and Murray may further concern the public's fear of DU use by the military, especially considering the US armed forces deployment to Kosovo,

Bosnia and the Persian Gulf in 2000 were approximately 91% males (71). Today, the US military is still predominantly male - roughly 84% with deployed troops being 87% male.

In humans, several studies have followed Gulf War veterans that were subjected to friendly fire and embedded with shrapnel from DU projectiles (14-18, 29, 72). These fragments are a permanent exposure source inside the body and these veterans, compared to controls without DU shrapnel, demonstrated consistently higher urine levels of uranium even after ten years of exposure (18). Urine uranium concentrations for the 2001 cohort ranged from 0.001 $\mu\text{g/g}$ creatinine to 78.125 $\mu\text{g/g}$ creatinine, where all participants with embedded shrapnel had over 0.1 $\mu\text{g/g}$ creatinine (18). Such values are of concern, considering the upper dietary limit of uranium is 0.365 $\mu\text{g/L}$ (18, 73). Interestingly, these Gulf War veterans do not show evidence of kidney damage or dysfunction, but there is an indication that increased uranium exposure may be marginally correlated with decreased neurocognitive performance, as measured by paper and pencil, and automated tests (1, 13-16, 18, 29). Further studies were proposed to examine the potential relationship between DU exposure and cognition (13-16, 29). Extensive reviews of the uranium literature (6, 8, 37, 57, 74) and publications by RAND (1), US DOD (75, 76), WHO (9), and the Royal Society (77-79), suggest that further studies are warranted, as there is a significant gap in the current understanding as to how DU affects the CNS.

Based on the limited number of DU-CNS studies published to date, there is no specific mechanism that emerges to explain the results indicating decreased excitability in rat hippocampi exposed to chronic DU (62), prolonged sensorimotor deficits and open-field behavior (63), and facilitated release of acetylcholine from nerve terminals (65). As there has been a lack of focused cellular studies, there is a significant gap in understanding the cellular mechanisms underlying uranium toxicity in cells of the CNS. An appealing hypothesis for the specific mechanism by which DU leads to neurotoxicity is that DU results in oxidative stress, which could potentially lead to cell death, perhaps by apoptosis. It has been demonstrated that uranyl compounds have high affinity for phosphate, carboxyl, and hydroxyl groups, and easily combine with proteins and nucleotides to form stable complexes (80). Furthermore, DU leads to oxidative DNA damage by catalyzing hydrogen peroxide and ascorbate reactions (81), as uranium with ascorbate in the presence

of hydrogen peroxide leads to single strand breaks in plasmid DNA *in vitro* (82). Evidence for induction of oxidative stress and reactive oxygen species (ROS) by DU has been shown by increases in NO in rat brains (69), brain lipid oxidation (63), transcriptomic, and proteomic changes in kidney cells (83, 84). Moreover, some studies indicate that heat shock proteins may be involved in the cellular response to DU exposure and acquired resistance to uranium rechallenge (85-87). These data strongly suggests the possibility that uranium may result in the formation of reactive oxygen species, leading to cell death via apoptotic mechanisms. Support for an apoptotic mechanism comes from studies by Kalinich and colleagues, whom demonstrated significant apoptotic events in mouse macrophage cells treated with 100 μ M DU (88).

Summary

The extremely dense and pyrophoric properties of DU make it an excellent metallic substrate for radiation shielding, and for armor and ammunition by the military. The use of DU in these materials is expected to grow, as is the potential black market to use DU as a weapon for bioterrorism. It is well documented that DU can be internalized as shrapnel or inhaled from battlefield exposure, but the controversy still remains as to whether it may be implicated in Gulf War Syndrome. Previous studies with DU have focused on the effects of DU on the kidneys and bones, as these are the major sites of accumulation. Recent animal data suggesting brain accumulation of DU, electrophysiological changes in hippocampal slices, and lowered neurocognitive results in Gulf War veterans, reveal that there is a considerable gap in the current understanding of how DU affects the CNS.

Like most heavy metals, DU possesses high chemical affinity for proteins and other biological molecules (1). Few studies have focused directly on the neurobiological effects of DU, although recent studies suggest these interactions should not be overlooked. Based on preliminary work of this laboratory and others, we are attempting to further understand the underlying molecular changes associated with the neurological sequelae of exposure to DU. Data derived from cell culture and animal models, in addition to the invaluable data from Gulf War veterans, will provide novel insight into the potential mechanism(s) of cellular alterations after DU exposure. These will direct future efforts to understand the acute

and chronic physiological effects of this metal, and facilitate the potential development of pharmacological interventions. In particular, while there is data that implies accumulation of uranium in the brain, a number of important questions remain. For example, what are the resulting functional changes associated with increased brain uranium accumulation, and what are the neurobiological and cellular pathways that change in response to such an insult? Clearly, there is a need for more focused studies by the research community to evaluate the relationship of DU and the CNS. Only with a better understanding of the neurotoxic mechanisms of DU can we alleviate the concerns of the public regarding this "metal of dishonor" (89).

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Figure 1 - Uranium forms and isotope percentages

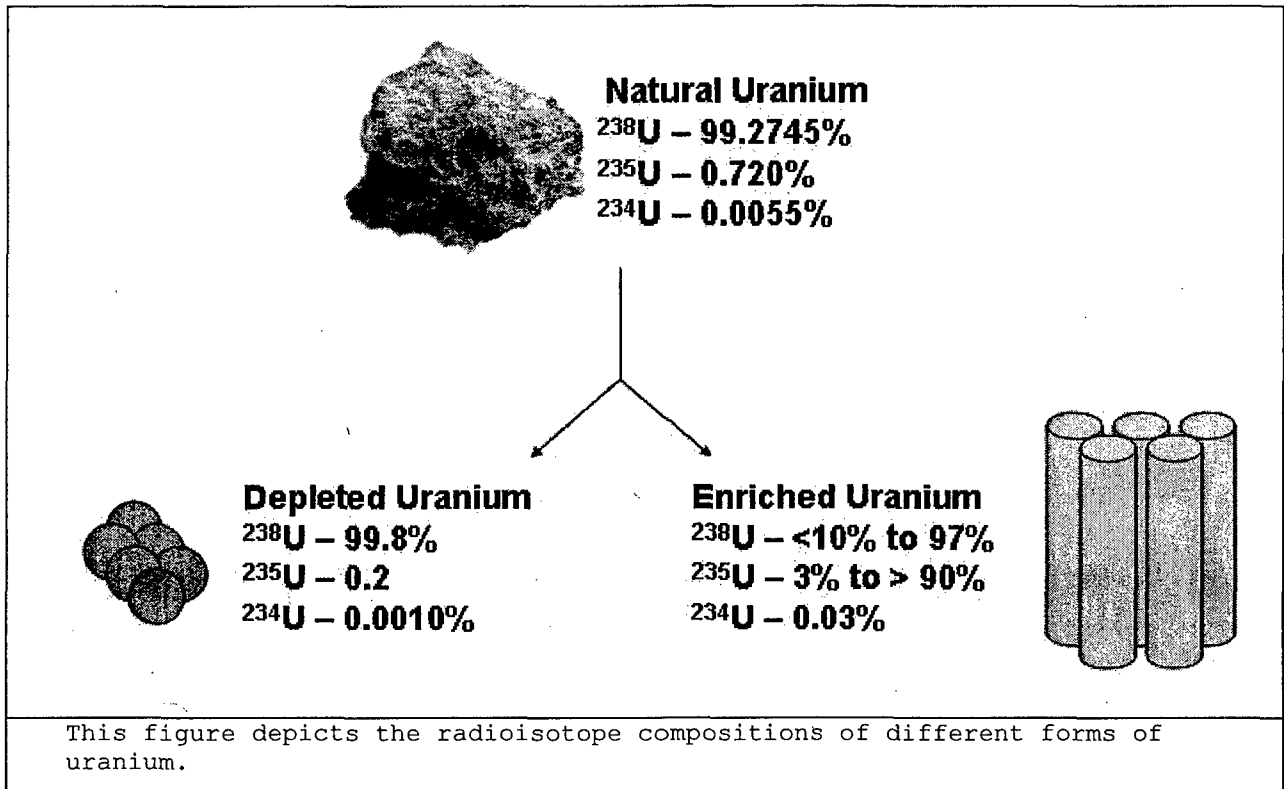


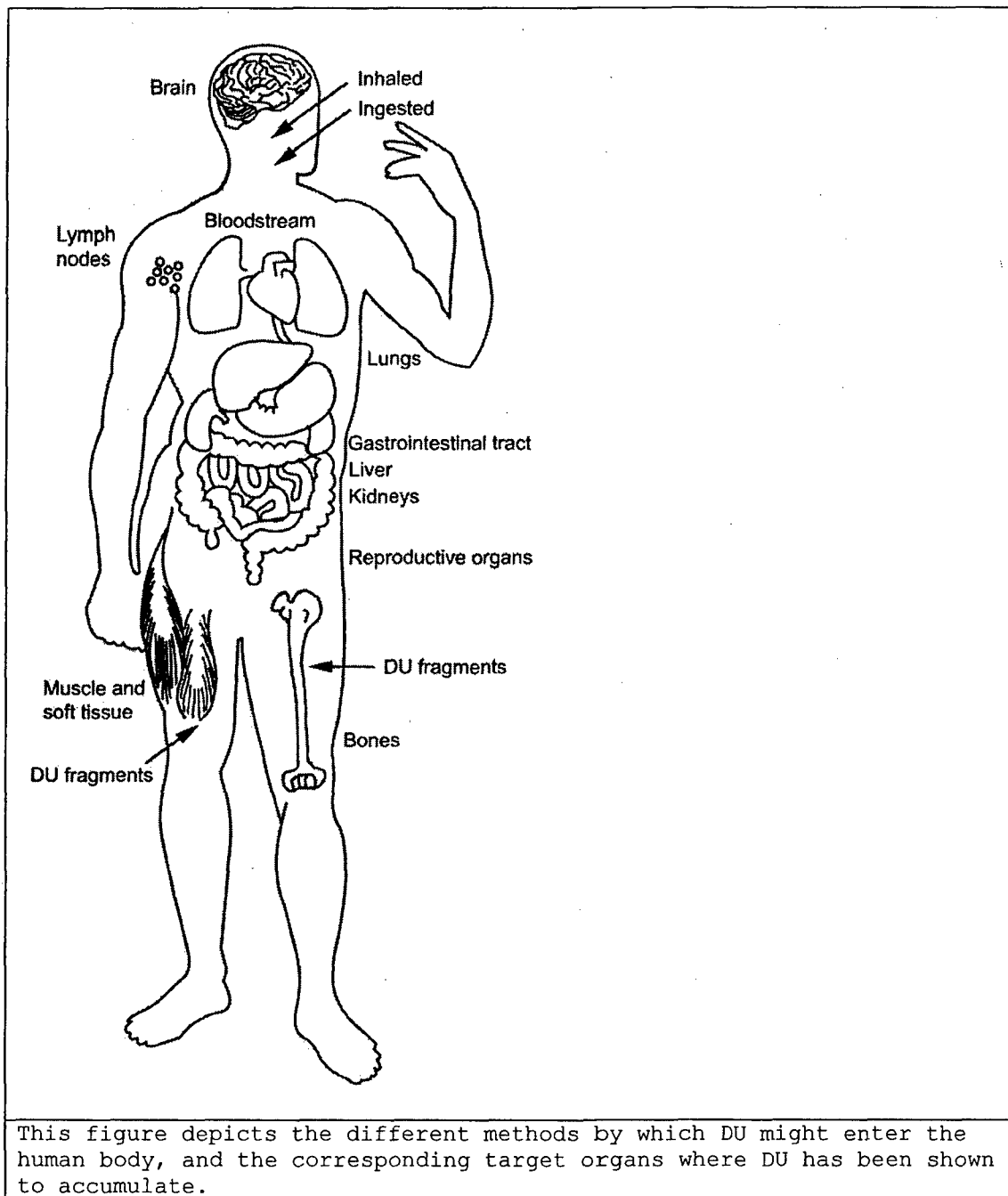
Table 1 - DU Ammunition Used in the 1991 Gulf War

Branch	Weapon System	Ammo Size	Quantity of DU Rounds	Weight of DU (kg)
US Army	M1 tank	105mm	504	1,930
	M1A1 tank	120mm	9,048	37,293
US Air Force	A-10 jet	30mm	782,514	236,319
	A-16 jet	30mm	1,000	302
US Marine Corps	AV-8b Harrier	25mm	67,436	9,981
	M60A3, M1A1	105, 120mm	Unknown	Unknown
US Navy	Phalanx gun	20mm	Unknown	Unknown
	Tomahawk cruise missiles	variable	Unknown (4-288?)	Unknown
UK Army	Challenger tank	120mm	88	405
Totals			Tanks - 9,640 Jets - 850,950	Tanks - 39,631 Jets - 246,602

Table compiled from data from 6 and 89.

**Total -
286,233**

Figure 2 - Routes of Depleted Uranium Entry



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