Depleted Uranium Is Not Toxic to Rat Brain Endothelial (RBE4) Cells

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Abstract:
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.
Index Entries: Depleted uranium (DU); heavey metal toxicity; blood–brain barrier; endothelium.

Article:
INTRODUCTION
Depleted uranium (DU) is a component of military munitions and is therefore the subject of important toxicity studies. Specifically, the possibility of DU neurotoxicity is under investigation. DU is a dense heavy metal used without reserve in many military applications. Chemically similar to natural uranium, but depleted of much of the radioactivity of the ^{235}U and ^{234}U isotopes, DU is a low-specific-activity metal that has several advantages for use as weapons material. Neurotoxicity could potentially arise from the chemical or radioactive properties of DU, and the level of neurotoxicity is as yet undetermined *(1–3).*

Gulf War veterans with DU fragments embedded in their soft tissues were studied and the results suggested that there might be DU-associated effects on behavior and cognition *(1–4).* Rats embedded with DU fragments accumulated uranium in a range of tissues, with early levels highest in the kidney and a gradual increase in bone accumulation. Brain tissues were found to have far lower levels, with the hippocampus showing high levels among the brain regions following physiologically relevant exposures and cerebellum accumulating the highest levels upon extremely high exposure levels *(4,5).* This group also reports electrophysiological changes in the hippocampus *(5).* There have been several recent summary reports concerning follow-ups on the Gulf War veterans, and although urine excretion levels remain high in some subjects, no nephrotoxicity has been found *(1–3).* However, hypoxanthine phosphoribosyl transferase (HPRT) gene mutation rates are elevated in subjects with high urinary uranium excretion *(1).* There is also the possibility of exposure resulting from inhaled DU dust from munitions impact *(6).*

For a blood-borne contaminant to cause neurotoxicity, it must first cross the blood–brain barrier (BBB). This barrier protects the central nervous system (CNS) from toxicants in the blood, and its ability to protect against metal neurotoxicity was reviewed by Zheng et al. *(7).* The capillaries of the brain are lined with endothelial cells acting as the first line of defense in the BBB. The high degree of tightness of the junctions that link the endothelial cells virtually prevents any paracellular passage from occurring in physiological conditions. In addition, several transport proteins can increase the brain-to-blood efflux of various compounds (e.g., P-glycoproteins or multidrug resistance proteins [MDR] of the ATP-binding cassette [ABC]) *(7).* The molecular

details of uranium transport across the BBB are completely unknown, but Lemercier et al. have investigated the process through an *in situ* unilateral brain perfusion technique *(8).* They report significantly higher uranium in the parenchyma of the exposed hemisphere, indicating that DU does cross the BBB. We have studied RBE4 rat brain endothelial cells for transport of other metals *(9),* and the divalent metal transporter (DMT- 1) is a likely putative candidate for the transport of DU into endothelial cells and across the BBB. This transporter 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+}, and it is plausible that it mediates uranium transport into the CNS *(10,11).* If DU does enter endothelial cells, the toxicity it causes to these cells is also completely unknown.

The rat brain endothelial cell line (RBE4) was derived from rat brain microvascular endothelial cells immortalized with the plasmid pE1A-neo, containing the E1A region of adenovirus 2 and a neomycin-resistance gene *(12,13).* RBE4 cells preserve the endothelial phenotype, show differentiation characteristics of brain endothelium in the presence of glial factors, and express P-glycoprotein *(14,15).* We report in this study the uptake of DU into RBE4 cells and three biochemical indicators of cell toxicity following the DU exposures. These include induction of a heat shock protein (Hsp90), a stress response indicator known to increase with many heavy metal exposures, lactate dehydrogenase (LDH) release, which demonstrates loss of cell membrane integrity, and 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) conversion to formazan, which is primarily an indicator of normal mitochondrial function.

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 U_3O_8 uranyl chloride diluted in HEPES buffer to final concentrations of 10, 50, or 100 µ*M* or HEPES buffer alone.

Uranium Uptake Assay Using RBE Monolayer and Neutron Activation Analysis

RBE4 cells were pretreated with HEPES alone or 100 µ*M* deferoxamine (DFO) in HEPES for 30 min at 37°C, and then they were treated with HEPES or U_3O_8 uranyl chloride dissolved in HEPES buffer at concentrations of 10 or 50 µ*M* for either 15 or 30 min at 37°C. The cells were washed four times with ice-cold mannitol buffer (290 m*M* mannitol, 10 m*M* Trisnitrate, 0.5 m*M* $Ca(NO_3)_2$, pH 7.4) and then lysed with 1 *N* NaOH for 10 min. Aliquots of 0.75 mL were then frozen for shipment to North Carolina State University for neutron activation analysis *(16).*

HSP90 Induction

Western blots were performed using protein samples from RBE4 cells treated with control media or 10 or 100 µ*M* DU for 10 min, 30 min, or 1 h. The cells were then washed three times with cold phosphate-buffered saline (PBS). Subsequently, the cells were harvested with PBS/0.5 m*M* EDTA and immediately centrifuged at 12,000*g* for 10 min. The supernatant was removed and the remaining pellet was resuspended in WANG buffer (25 m*M* HEPES, pH 7.0, 250 m*M* sucrose, 100 µ*M* EDTA, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A, 1 m*M* dithiothreitol [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-HCl, pH 6.8, 10% sodium dodecyl sulfate [SDS], 50% glycerol) and 1 *M* DTT and separated by denaturing SDS-PAGE (Polyacrylamide gel electrophoresis 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 m*M* Tris-HCl, and 192 m*M* glycine for 3 h at 60 V. Membranes were then blocked with 5% nonfat powdered milk in TBST (Tris-buffered saline with 0.1% Tween-20, 150 m*M* NaCl; 20 m*M* Tris-HCl) for 1 h. 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-h incubation with an horseradish perocidase (HRP)-conjugated anti-rabbit secondary antibody (1 : 5000). Protein bands were visualized with the 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).

Volume Measurements in RBE4 Cells Treated with DU

The RBE4 cell volume was determined by modification of the electrical impedance method *(17).* Cell monolayers grown on a No. 1.5 cover slip were placed in a channel that joins two chambers. The total height of the channel was approx 250 µm. Each chamber contained a silver wire electrode (5 cm in length) that was soldered at the distal end to a copper-insulated wire for connection through a large resistance (1 MΩ), to a lock-in amplifier that supplied a 500-Hz, 5-V signal to the system. There was a continuous flow of solution through the channel (1 mL/min). As the volume of the cell monolayer increased, the volume of the solution within the channel and above the cells available for current flow decreased proportionately, resulting in an increase in the measured resistance in the channel above the cells. Because $V=IR$ (where V is voltage, I is current, and R is resistance) and I was kept constant, changes in V are directly proportional to changes in R.

The monolayer of RBE4 cells was bathed in a HEPES-buffered solution consisting of the following: 122 m*M* NaCl, 3.3 m*M* KCl, 0.4 m*M* $MgSO_4$, 1.3 m*M* CaCl2, 1.2 m*M* KH_2PO_4, 10 m*M* D(+) glucose, 25 m*M* HEPES. All buffers were adjusted to pH 7.4 by addition of 10 *N* NaOH. The osmolarity of the isotonic solution was approx 300 mOsmol as measured by a freezing-point osmometer (Advanced Instruments, Inc., Needham Heights, MA). The protocol for all experiments involved a 15-min exposure to isotonic buffer, followed by a 60-min exposure to isotonic buffer containing various concentrations of U_3O_8 (0–100 µ*M*).

LDH Toxicity Assay

The RBE4 cells in 96-well culture plates were treated with 10 or 50 µ*M* U_3O_8 in HEPES buffer or HEPES alone at 37°C for 3 or 6 h. The treatment media was removed and the CytoTox96 Non-Radioactive Cytotoxicity assay for lactate dehydrogenase (LDH) activity (Promega) was employed to assess differences between LDH content of the samples. The cells were lysed and combined with the 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 sample 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 µ*M* U_3O_8 in HEPES buffer or HEPES alone at 37°C for 3 or 6 h. 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 the manufacturer's recommendations. Toxicity results in decreased ability to reduce MTT to formazan. Treated samples are reported as percentage of control sample viability.

RESULTS

Heavy metals can be toxic to mammalian cells in culture at relatively low doses. This study focuses on concentrations of DU in the potential physiological range, according to previous reports *(1–3).* Concentrations of 10 µ*M*, 50 µ*M*, and, in some cases, 100 µ*M* U_3O_8 were used for RBE4 treatments in the following studies.

Uptake of DU into RBE4 cells

Depleted uranium in HEPES buffer readily entered cultured RBE4 endothelial cells as measured by neutron activation analysis (NAA) *(16).* Cultured RBE4 cells were pretreated with HEPES-only control buffer for 30 min. To investigate other possible influences on cellular uptake of DU, additional samples were pretreated with 100 µ*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 min. Cells were then rinsed, collected, pelleted, and frozen for NAA. Time-points of 15 and 30 min after replacement of normal culture media with DU-containing HEPES or HEPES-only control buffer are shown in Fig. 1. All DU-treated samples showed between 0.27 and 0.4 µg U_3O_8/µg protein analyzed. No differences were observed between 15-min and 30-min exposures, and DFO showed no effect on uptake. These data demonstrate rapid entry of DU into the cells.

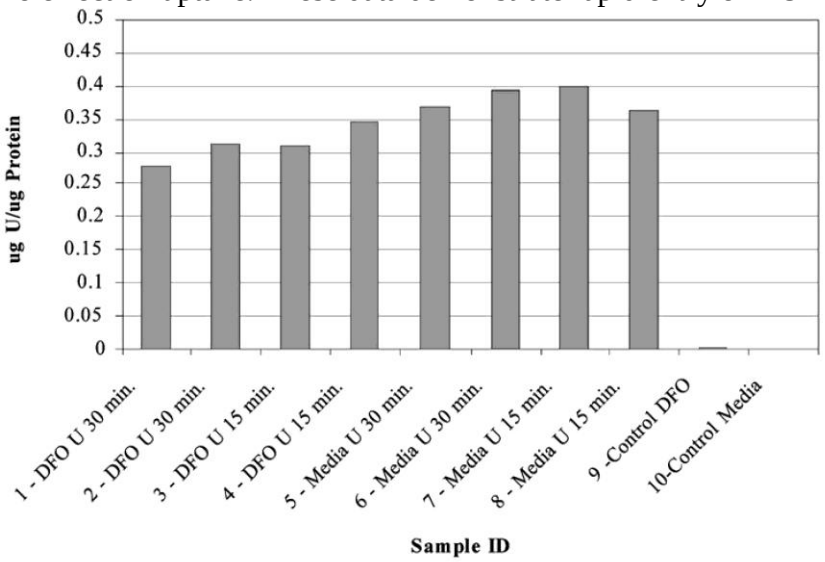

Fig. 1. Uptake of U_3O_8 depleted uranium. Cultured RBE4 cells were pretreated with HEPES only or HEPES containing DFO, an iron chelator, for 30 min. They were next treated with DU-containing HEPES or HEPES-only control buffer for 15 or 30 min. Treatment media was then removed and the cells were rinsed, collected, pelleted, and frozen for measurement of DU content by NAA.

Effects of DU on Hsp90 Expression in RBE4 Cells

Western blot techniques were used to assess the ability of U_3O_8 to alter Hsp90 expression in RBE4 cells. As shown in Fig. 2, U_3O_8 caused a small but significant ($p < 0.05$) increase in Hsp90 levels at 10 and 30 min (100 and 10 µ*M*, respectively) compared to control treated RBE4 cells, but this effect was not apparent after 1 h (note *y*-axis represents percent change over control, which is standardized to 100%). Accordingly, we conclude that any effect of U_3O_8 is fast and short-lasting, and the consequences should be investigated for further detail in future studies.

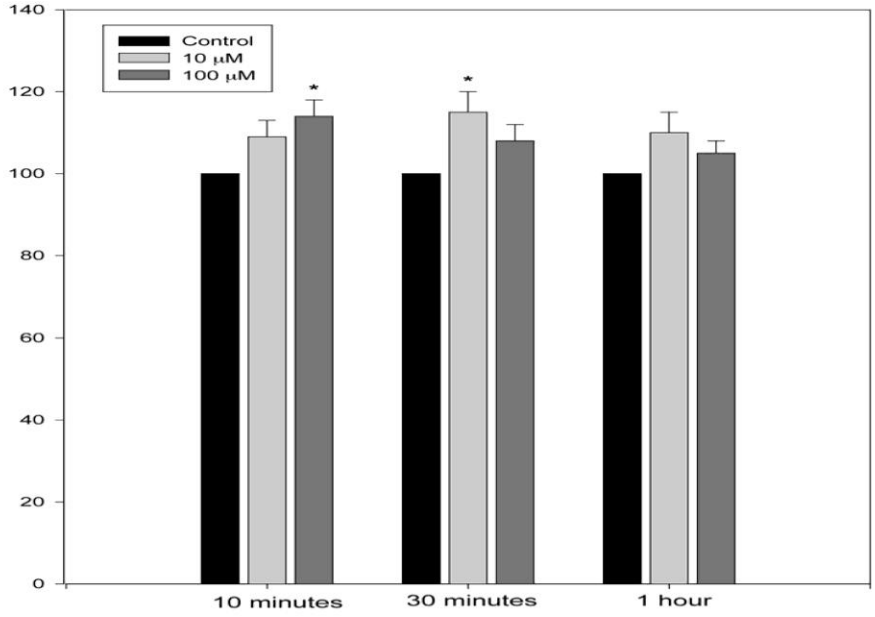

Fig. 2. Hsp90 induction in RBE4 cells treated with DU. RBE4 cells were treated with control media or 10 or 100 μM DU for 10 min, 30 min, or 1 h. The cells were then washed and harvested for Western blot analysis. Samples of 100 µg protein were probed with monoclonal antibody to Hsp90. Significant differences from control samples ($p > 0.05$) are indicated by the asterisk.

Volume Measurements in RBE4 Cells Treated with DU

Volume changes are expressed as change in voltage. RBE4 cells were treated with 10, 50, or 100 µ*M* DU (U_3O_8) and monitored for cell volume changes for 30 min. As can be noted in Fig. 3, the maximal change in voltage was an increase from approx 140 mV for controls to 140.75 mV in RBE4 cells treated with 100 µ*M* $_{U3O8}$. This translates to about a 0.5% change in cell volume *(17).* Because a 1% change in volume in our system approximates a 25% change in cell volume (assuming cellular cell height of 4 µ*M*), we conclude from these experiments that $_{U3O8}$ does not cause a drastic change in cell volume over the 30-min exposure period. Thus, RBE4 cells are capable of withstanding a high exposure to DU without a discernable change in cellular volume.

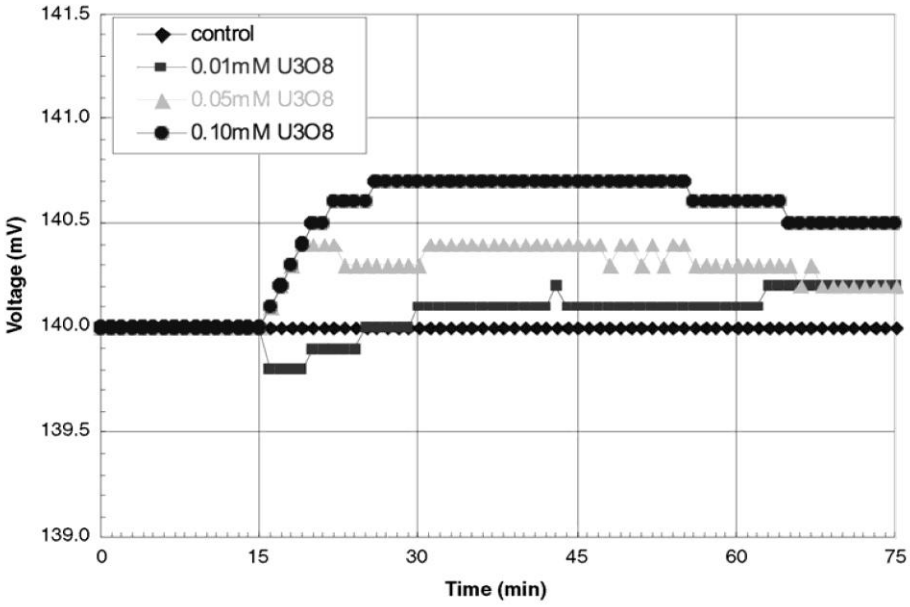

Fig. 3. RBE4 cell volume changes in response to DU treatment. Monolayer RBE4 cells were bathed in a HEPES-buffered solution of 300 mOsmol for 15 min, followed by 60 min exposure to U_3O_8 (0, 10, 50, or 100 µ*M*). RBE4 cell volume was measured with the electrical impedance method. A channel joins the RBE4 chamber to another chamber 250 µm above. Increase in the measured resistance in the channel above the cells reflects changes in cell volume. Because *V*=*IR* (where *V* is voltage, *I* is current, and *R* is resistance) and *I* was kept constant, changes in *V* are directly proportional to changes in *R*.

LDH and MTT Cytotoxicity Assays

The assays for LDH activity and MTT activity are widely utilized as indicators of cytotoxicity. Both demonstrated that 10 or 50 µ*M* U_3O_8 causes little or no toxicity to the RBE4 cells (Fig. 4). Additionally, caspase assays showed no induction of caspases 8 or 9 following U_3O_8 exposure (data not shown).

DISCUSSION

The possibility of DU transport to the brain from soft tissues embedded with DU fragments poses considerable concern. Based on the previous reports of increased DU levels in the brain tissue of rats with DU fragments embedded in the muscles of their extremities, we would expect efficient uptake into endothelial cells *(4,5,7).* Our studies confirm this; however, we also show that despite the elevated level of uranium taken up by these cells, toxicity measured by several biochemical indicators is minimal. A report by Kalinich et al. showed DU induction of apoptosis in mouse macrophages *(18).* Another recent study reported that in HepG2 cells, DU induced some changes in gene expression, including positive results for 10 different stress-related genes *(19).* By contrast, we found little or no evidence of DU toxicity to rat brain endothelial cells using four different assays.

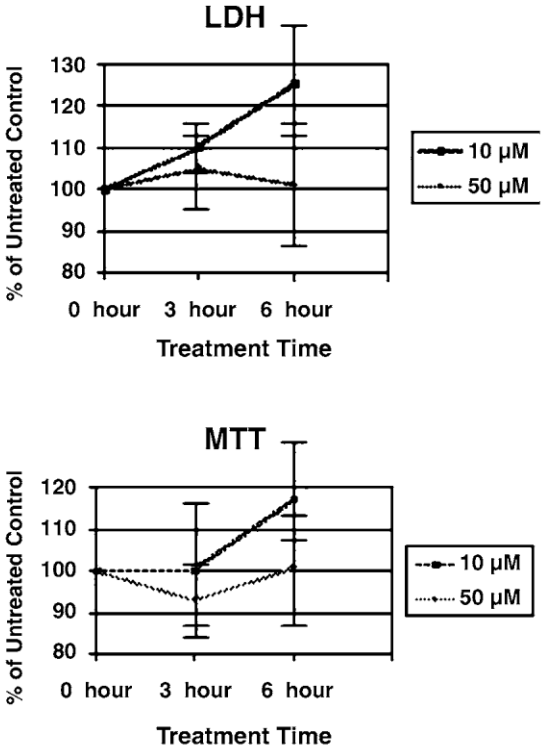

Fig. 4. Viability of RBE4 cells following DU treatment. RBE4 cells in 96-well culture plates were treated with 10 or 50 µ*M* U_3O_8 in HEPES buffer or HEPES alone control for 3 or 6 h. **(A)** Treatment media was removed and cells were lysed and combined with LDH substrate to analyze remaining LDH activity. **(B)** MTT reagent was added to treated cells to analyze remaining mitochondrial function. Both assays employed Promega kits for cell viability assessment and utilize colorimetric analysis at 490 nm in a spectrophotometer to measure enzymatic activity.

The 90-kDa heat shock protein (Hsp90) is one of the most abundant proteins in cells, constituting 1–2% of total intracellular protein. It is constitutively and ubiquitously expressed and is the most abundant molecular chaperone of the eukaryotic cytoplasm. Chaperones help to achieve and maintain the conformational status of cellular proteins and enzyme complexes. By influencing higher-order protein structure, Hsp90 is involved in the conformational regulation of key proteins in multiple signaling pathways, including nitric oxide synthases (NOS), kinases, phosphatases, and steroid hormone receptors. Hsp90 regulates the redox status of other proteins by assisting in the formation and breakage of disulfide bridges. The importance of Hsp90 interactions in the promulgation of neurotoxicity is incompletely understood, but its induction is a well-known stress response to treatment of cultured cells with heavy metals *(20).* Our data show slight inductions in Hsp90 at the earlier time-points of 10 and 30 min following exposure to 100 and 10 µ*M* DU, respectively. However, 1 h after exposure, there was no detectable difference between DU samples and controls. Considering the recent report of Prat et al. *(21),* in which Hsp90 was found to be downregulated in HEK293 cells by DU exposure, there might be a differential Hsp90 response depending on cell type and duration of exposure.

Cell volume change is a stress response that is found after heavy metal exposure, among other insults *(17,22).* We detected minimal volume change in RBE4 cells following DU exposures that was indistinguishable from controls, even when the concentration of uranium was increased to 100 µ*M.* By comparison, other heavy metals induce much larger cell volume changes *(23).*

Metabolically active cells are able to reduce the commercially marketed tetrazolium compound Owen's reagent (MTS) into formazan, presumably through NADPH or NADH. This reagent is therefore a good indicator of cell viability, especially as it relates to mitochondrial function *(24,25).* The release of LDH upon cell lysis is another widely used and commercially available indicator of cell viability *(26).* Our data show that there was no

evidence of any loss of mitochondrial function or LDH release, once again indicating high viability following DU treatment.

A very recent report described rats exposed to DU in their drinking water that showed significant behavioral changes *(27).* There was also some evidence of lipid peroxidation in the brain. Interestingly, the authors also noted more behavioral effects in male rats than female. These results suggested that, at least in rats, the DU that crosses the BBB has neurological effects. Further studies are merited to determine the effects in humans.
We conclude here that rat brain endothelial cells show efficient uptake of DU. Considering the ease with which DU entered RBE4 cells, the astrocytes beyond the endothelial layer are presumably also exposed to comparable levels of DU. Studies similar to those reported here are currently being conducted to investigate the responses of astrocytes to such exposure. Overall, these studies indicate that exposure of endothelial cells to DU does not profoundly affect their function. Whether more subtle effects are inherent to this type of exposure has yet to be determined.

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