TOXICOLOGICAL SCIENCES **99(2)**, 553–565 (2007)
doi:10.1093/toxsci/kfm171
Advance Access publication July 16, 2007

Neurotoxic Potential of Depleted Uranium—Effects in Primary Cortical Neuron Cultures and in *Caenorhabditis elegans*

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Received April 17, 2007; accepted June 13, 2007

Depleted uranium (DU) is an extremely dense metal that is used in radiation shielding, counterbalances, armor, and ammunition. In light of the public concerns about exposure to DU and its potential role in Gulf War Syndrome (GWS), this study evaluated the neurotoxic potential of DU using focused studies on primary rat cortical neurons and the nematode *Caenorhabditis elegans*. We examined cell viability, cellular energy metabolism, thiol metabolite oxidation, and lipid peroxidation following exposure of cultured neurons to DU, in the form of uranyl acetate. We concurrently evaluated the neurotoxicity of uranyl acetate in *C. elegans* using various neuronal–green fluourescent protein reporter strains to visualize neurodegeneration. Our studies indicate that uranyl acetate has low cytotoxic potential, and uranium exposure does not result in significant changes in cellular energy metabolism, thiol metabolite oxidation, or lipid peroxidation. Furthermore, our *C. elegans* studies do not show any significant neurodegeneration following uranyl acetate exposure. Together, these studies suggest that DU, in the form of uranyl acetate, has low neurotoxic potential. These findings should alleviate the some of public concerns regarding DU as an etiologic agent of neurodegenerative conditions associated with GWS.

Key Words: **depleted uranium; primary neurons; neurotoxicity; Gulf War Syndrome; *C. elegans*.**

Depleted uranium (DU) is a by-product of the enrichment of naturally occurring uranium for its most radioactive isotope, ^{235}U. The extremely dense and pyrophoric properties of DU make it an excellent metallic substrate for radiation shielding, counterbalances, and in armor and ammunition (Jiang and Aschner, 2006). As a heavy metal, internalized DU is cleared by the kidneys, and numerous studies have demonstrated nephrotoxicity after exposure to high levels of DU (Andrews and Bates, 1987; Carriere *et al*., 2005; Goldman *et al*., 2006; Kobayashi *et al*., 1984; Taulan *et al*., 2004). Other than the effects on the kidneys, DU exposure is thought to result in neurologic sequelae. Indeed, it has been hypothesized that DU may contribute to the etiology of Gulf War Syndrome (GWS) (Abu-Qare and Abou-Donia, 2002; Bem and Bou-Rabee, 2004; Doucet, 1994; Durakovic, 2003; Gronseth, 2005; Jamal *et al*., 1996; Jiang and Aschner, 2006). Follow-up studies on Gulf War veterans exposed to DU demonstrated decreased cognitive performance compared to unexposed veterans, which provided evidence for such a theory (McDiarmid *et al*., 2000). The increased usage and health concerns have led researchers to scrutinize the effects of DU exposure on the central nervous system (CNS).

The recent interest in the effects of DU exposure on the CNS has led to a number of studies with small animals. Such studies have shown that uranium (U) indeed crosses the blood–brain barrier (Abou-Donia *et al*., 2002; Barber *et al*., 2005; Briner and Murray, 2005; Fitsanakis *et al*., 2006; Houpert *et al*., 2004; Leggett and Pellmar, 2003; Lestaevel *et al*., 2005; Paquet *et al*., 2006; Pellmar *et al*., 1999a,b), accumulates in a dose-dependent manner in specific brain structures (Fitsanakis *et al*., 2006; Pellmar *et al*., 1999a), and results in increased lipid oxidation (Briner and Murray, 2005), nitric oxide generation (Abou-Donia *et al*., 2002), and sensorimotor deficits (Abou-Donia *et al*., 2002). These studies have attempted to correlate the observed neurobiological changes with potential functional changes in cognitive behavior (Abou-Donia *et al*., 2002; Belles *et al*., 2005; Briner and Murray, 2005; Houpert *et al*., 2005). To date, however, there remains a significant gap in understanding the specific effects of uranium on cells of the CNS, and the potential molecular changes involved upon DU exposure.

The cellular effects of DU have only been evaluated in a limited number of cell culture models. Studies in Chinese hamster ovary cells have demonstrated cytogenetic toxicity of uranium (Lin *et al*., 1993), and induction of hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) mutations and DNA adducts (Albertini *et al*., 2003; Stearns *et al*., 2005). Studies with immortalized human osteoblast cells to evaluate

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the effects of DU have corroborated this finding, further demonstrating that DU results in genotoxicity, and that it can be neoplastic (Miller *et al*., 1998a, 2001, 2002, 2003). Uranium has also been shown to induce activation of stress gene expression in human liver carcinoma cells (HepG2) (Miller *et al*., 2004). In the mouse macrophage cell line, J774, uranium treatment resulted in time- and concentration-dependent uptake of uranium, cytotoxicity, and induction of apoptosis (Kalinich *et al*., 2002). Concentration-dependent cytotoxicity was also observed in NRK-52E cells, another immortalized cell culture model representative of rat kidney proximal epithelium cells (Carriere *et al*., 2004). Researchers have also evaluated the transcriptomic and proteomic responses of HEK293 kidney cells, and renal tissue from rats exposed to DU, and found that there were several oxidative-response–related transcripts that were upregulated, and significantly increased peroxide levels that support the implication of oxidative stress (Prat *et al*., 2005; Taulan *et al*., 2004, 2006). In rat brain endothelial cells, the closest *in vitro* model to cells of CNS origin, researchers demonstrated that uranium did not result in significant cytotoxicity (Dobson *et al*., 2006).

To date, researchers have not undertaken focused studies to determine the effects of DU on cells of CNS origin. Numerous CNS cell models are available for study, including primary cultures and immortalized cell lines. Although primary cultures have a finite life span compared to immortalized cell lines, the former offer many advantages as cell lines will often show numerous changes in cell cycle and proliferation, morphology, and chromosomal variations. Furthermore, primary are cultured in the context of their naturally occurring neighboring cell types. In these studies, we have attempted to fill the gap in the knowledge of DU neurotoxicity by performing focused studies using primary rat cortical neurons to examine the acute neurotoxic potential of DU and the specific cellular effects in neurons. We are testing the hypothesis that DU results in significant concentration-dependent cytotoxicity, and oxidative stress, as has been previously seen in other cell culture models.

The nematode, *Caenorhabditis elegans*, is an excellent model organism that has been used in a number of toxicological studies (Anderson and Wild, 1994; Dhawan *et al*., 1999; Reichert and Menzel, 2005; Swain *et al*., 2004). The worms are easily grown and maintained, and have a rapid replication cycle, allowing for thousands of worms to be evaluated within a number of days (Brenner, 1974). The nematode is a model organism, with its complete genome determined, numerous genetic mutants freely available, and multicolor reporter constructs, e.g., green fluorescent protein (GFP), can be easily introduced into the system (Hobert and Loria, 2006; Link and Johnson, 2002; Miller, *et al*., 1999). Furthermore, there are only 302 neurons in the nematode, in which all the projection pathways have been determined (Gally and Bessereau, 2003; Wadsworth and Hedgecock, 1992). All of these *C. elegans* characteristics make it a powerful organism to evaluate the toxicological potential of a wide array of compounds. For our studies, *C. elegans* is an organism in which we can evaluate the *in vivo* effects of uranium on CNS cells. We tested the hypothesis that uranium exposure results in significant concentration-dependent neurotoxicity as can be visualized by neurodegeneration.

In light of the public concerns regarding DU, this study sought to evaluate the neurotoxicity of DU, in the form of uranyl acetate, using focused studies of a relatively homogeneous cell population of CNS origin. Here, we investigate the cytotoxic effects of U in primary rat neuronal cultures, subsequent changes in cellular metabolism, and concurrently evaluate the neurotoxicity of U in *C. elegans* using neuronal-GFP reporter strains.

MATERIALS AND METHODS

Materials. Uranyl acetate ($UO_2(CH_3COO)_2{\cdot}2H_2O$) was purchased from Ted Pella, Inc. (Redding, CA). All other chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated. Coverslips for cell culture were purchased from Carolina Biological Supply (Burlington, NC). All tissue culture media and supplements were purchased from Invitrogen (Carlsbad, CA), except for Hyclone Fetal Bovine Serum and Hyclone F12, which were purchased from VWR (Suwanee, GA). Nematode growth reagents and plasticware were purchased from VWR.

Cell culture conditions and uranyl acetate treatments. All experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and were performed according to Guidelines for Animal Experimentation as set forth by Vanderbilt University. Rat cortical neuron cultures were prepared from E17 rat pups, as previously described (McLaughlin *et al*., 1998). Briefly, E17 Harlan Sprague–Dawley rat embryos were decapitated, and the brains rapidly removed and placed in a 35-mm petri-dishes with cold Hank's balanced salt solution (HBSS). The cortices were dissected under a dissection microscope and then were placed in another dish containing HBSS to further remove blood vessels and meninges from cortical tissues. The isolated cortices were then transferred to a petri-dish containing 0.6% (wt/vol) trypsin in HBSS for 30 min. After two washes in HBSS, the cortical tissues were mechanically dissociated with a glass Pasteur pipette. Dissociated cortical cells were plated on poly-L-ornithine-treated glass coverslips in six-well plates, using a plating medium of glutamine-free Dulbecco's modified Eagle's medium–Eagle's salts (Invitrogen), supplemented with Ham's F12 (Hyclone, Logan, UT), heat-inactivated fetal bovine serum (Hyclone), and penicillin/streptomycin (Sigma), at a density of 700,000 cells per well. After 2 days *in vitro*, nonneuronal cell division was halted by a 1-day exposure to 10μM cytosine arabinoside (Sigma), and cultures were shifted to Neurobasal media (Invitrogen), supplemented with B27 (Invitrogen) and penicillin/streptomycin. Cells were maintained by changing the media every 2–3 days and grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cells were treated 3 weeks after isolation with DU (uranyl acetate), prepared as sterile solutions in treatment buffer, for 24 h, at 37°C in a humidified atmosphere of 5% CO_2 in air. Treatment buffer consisted of minimal essential media (Invitrogen) supplemented with 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 ml N2 media supplement (Invitrogen), 0.001% BSA (Sigma). N-methyl-D-aspartate (NMDA, Sigma) was used as a positive control for cytotoxicity at a final concentration of 100μM in conjunction with 10μM glycine.

Cell viability determinations. Primary rat cortical neuron viability was determined by fluorescence activated cell sorting (FACS) using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Both floating and attached cells were collected and stained with 2 μl of calcein and 8 μl of

ethidium homodimer in phosphate buffered saline (PBS) as previously described (Chen *et al*., 2002). The percentage of viable cells was analyzed by flow cytometry (BD Immunocytometry Systems, San Jose, CA). For each sample, at least 10,000 cells were counted on a BD FACScan (Becton Dickinson, San Jose, CA). Data analyses were performed with **WinMDI** (**Win**dows **M**ultiple **D**ocument **I**nterafce for Flow Cytometry) (http://facs.scripps.edu).

Cell viability and proliferation were evaluated by lactate dehydrogenase (LDH) (Sigma) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (Sigma) assays. LDH release was measured with an *in vitro* toxicology assay kit (Sigma) by assaying 40 µl sample medium spectrophotometrically (490:630 nm) according to the manufacturer's protocol, to obtain a measure of cytoplasmic LDH released from dead and dying neurons (Legrand *et al*., 1992). MTT is yellow until reduced to purple formazan in the mitochondria of living cells. The reduction of MTT to formazan occurs only when mitochondrial reductase enzymes are active, and thus conversion is a measurement of mitochondrial inhibition, and can be correlated to the number of viable (living) cells. LDH release and MTT analyses were determined according to manufacturer's instructions. LDH release results were confirmed qualitatively by visual inspection of the cells and, in several instances, quantitatively by cell counts by the method of Rosenberg and Aizenman (1989).

Thiol metabolite determination. Quantification of levels of glutathione (GSH) and its related products were performed by high-performance liquid chromatography (HPLC) as previously described (Jones, 2002; Jones *et al*., 1998; Nelson *et al*., 1999). Briefly, treated cells were washed with PBS, and resuspended in 0.5% perchloric acid with 0.2M boric acid and 10µM γ-Glu–Glu (internal standard), and sonicated with a Sonics Vibra-Cell, two times for 20 s at 25% power. Extracts were derivatized with iodoacetic acid and dansyl chloride. The acid soluble cysteine (Cys), cystine (CySS), GSH, and oxidized glutathione (GSSG) were analyzed by HPLC using fluorescence detection on a Waters 2695 Alliance HPLC system (Waters, Milford, MA). Samples were loaded onto an YMC Pack NH2 (amino) column (Waters) and were eluted with a gradient of sodium acetate. The solvent used for mobile phase was 80% methanol. The peaks were quantified by integration relative to the internal standard. Using this method, samples were analyzed for Cys, CySS, GSH, and GSSG content. Redox status for the GSH/GSSG redox couple (E_h GSH), and the Cys/CySS redox couple (E_h Cys) were calculated using the Nernst equation.

Total adenosine nucleotides determination. Changes in adenosine nucleotides were measured by isocratic reversed-phase HPLC as previously described (Yang *et al*., 2004). For HPLC analysis, treatment media was removed from the cell samples before adding 950 µl of chilled 0.3M perchloric acid with 1mM disodium ethylenediaminetetraacetate to each well to harvest cell extracts into microcentrifuge tubes. An aliquot of 2M potassium hydroxide (170 µl) was then added to each sample, followed by centrifugation at 9000 × g to remove precipitates of $KClO_4$. The supernatant was then stored at − 80°C until HPLC analysis on a Waters HPLC system (Waters), coupled with a dual λ-absorbance UV detector (Model 2487) equipped to a computer system with Waters Millennium software program (Workstation v. 4.0) for data processing. The mobile phase used was 0.1M ammonium dihydrogen phosphate (pH 6.0) with 1% methanol. Using the Symmetry Shield C-18 column and a flow rate of 0.6 ml/min, the peaks of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were eluted at retention times of 3.462, 3.868, and 5.694 min, respectively, with a variation window of 0.2 min in both standard and sample extracts. The peak height responses for all three nucleotides were recorded at 206 nm. The concentration of each nucleotide was determined in a 15-µl sample extract injected to HPLC and finally expressed in terms of nmol nucleotide per ml extract. The total adenosine nucleotides (TAN) content was calculated by TAN = ATP + ADP + AMP, while the energy charge potential (ECP) was calculated by the equation ECP = [ATP + 0.5 (ADP)]/TAN, as previously described (Yang *et al*., 2004).

F_2-IsoP quantitation. Quantification of F_2-isoprostanes (F_2-IsoP) levels was determined using a stable isotope dilution method with detection by gas chromatography/mass spectrometry and selective ion monitoring as previously described (Milatovic *et al*., 2005; Morrow and Roberts, 1991; Roberts and Morrow, 1994). Briefly, samples were extracted and saponified, a stable isotope internal standard added, and then prepared for gas chromatography through a series of purifications by C-18 and Silica Sep-Pak cartridges and thin layer chromatography (TLC). Gas chromatography was performed using a 15 m long, 0.25 mm diameter, 0.25-µm film thickness, DB1701 fused silica capillary column (Fisons, Folsom, CA). The injector temperature was 265°C and oven (column) temperature was programmed from 200°C to 300°C at 15°C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Ion source temperature was 250°C, electron energy was 70 eV, and filament current was 0.25 mA. For analysis, compounds were dissolved in undecane that was dried over a bed of calcium hydride. Negative ion chemical ionization mass spectrometry was performed using an Agilent Technologies G1789A GC/MSD instrument with a Hewlett–Packard computer system with ChemStation-NT. Total protein content was determined by BCA assay (Pierce, Rockford, IL) with bovine serum albumin as the standard (Smith *et al*., 1985).

Strains and maintenance. *Caenorhabditis elegans* strains were cultured on bacterial lawns of either NA-22 or OP-50, seeded on 8P or nematode growth medium (NGM) plates respectively, at 20°C according to standard methods (Brenner, 1974). *Caenorhabditis elegans* strain N2 (var. Bristol) is the wild-type strain, and was a gift of Dr Richard Nass (Vanderbilt University, Nashville, TN). The BY250 strain was developed and obtained from Dr Richard Nass (Vanderbilt University, Nashville, TN). Strain NW1229 (dpy-20(e1362) IV; evIs111) was obtained from the *C. elegans* Genetics Center (CGC, University of Minnesota, Minneapolis, MN).

Exposure of* C. elegans *to uranyl acetate. Embryos were obtained by hypochlorite treatment of gravid adults (Lewis and Fleming, 1995). After 17–24 h incubation in M9 buffer to obtain synchronized L1s, such that all nematodes are at the same point in their life cycle, the worms were washed once in 10 ml of dH_2O, and then diluted to 50 worms per µl. L1 worms were treated with DU (uranyl acetate), prepared from a 1M stock solution in water. Five thousand worms were used in each siliconized microcentrifuge tube (Denver Scientific Inc., Metuchen, NJ) per treatment assay, and incubated with gentle shaking at 800 rpm for 30 min on a VWR Digital Mini Vortex Mixer (VWR Scientific, Suwanee, GA). Worms were then spread on NGM/OP-50 plates and incubated for 24 h at 20°C before further evaluation. For quantitative analyses of uranyl acetate-induced changes in worm viability, total number of live worms was determined for each concentration by counting each plate under a Stemi-2000 dissecting microscope (Zeiss, Thornwood, NY).

Photomicroscopy. Cell morphology was visually inspected on a Zeiss Axiovert 40 inverted microscope (Zeiss, Thornwood, NY). Cortical culture images were captured on an inverted Nomarski microscope (Zeiss Axiovert 200M) with AxioCam and AxioVision 4.4 software (Zeiss), using fixed exposures for all image captures between different treatments. Nematode images were captured on a Zeiss upright LSM510 confocal microscope (Zeiss), using laser scanning fluorescence and DIC (Nomarski) imaging. Worms were photographed under oil immersion with a 40×/1.30 Plan-Neofluar objective using fixed exposure settings for all image captures between different treatments. Images were exported using the Zeiss LSM Image Browser. Images were quantified for their fluorescence using Adobe Photoshop 6.0 (Adobe, San Jose, CA) and NIH ImageJ software. The fluorescent intensities were subsequently used to test if the levels of fluorescence were decreased upon treatment with U. With BY250 worms, cell bodies and dendrites were also manually scored as present if fluorescence could be seen. Dendrites were scored as abnormal if they had breaks or were barely visible. The ratio of abnormal:normal dendrites was used to calculate ratios for the different treatments, which were then compared for significance as previously described (Nass and Blakely, 2003).

Data analysis. All results are given as mean ± standard error of the mean. Differences between groups were analyzed statistically with one-way ANOVA followed by *post hoc* tests for multiple comparisons with $p < 0.05$ considered statistically significant.

RESULTS

DU Has Minimal Effect on Cortical Neuron Viability and Morphology

Exposure of primary rat cortical neurons to DU, in the form of uranyl acetate, did not result in significant cytotoxicity, as measured by FACS of calcein and ethidium homodimer (Fig. 1A). Figure 1A illustrates the cytotoxicity data obtained with FACS using two simultaneous probes, calcein and ethidium homodimer. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, by the conversion of nonfluorescent cell-permeant calcein to its fluorescent green analog. Ethidium homodimer, normally excluded by intact plasma membranes of live cells, enters cells with damaged membranes and undergoes 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence. This lack of cytotoxicity was further corroborated by results of the MTT assay, in which there were no significant differences between increasing concentrations of uranium compared to control (Fig. 1B). One-way ANOVA followed by Bonferroni's *post hoc* test indicated that NMDA treatment of neurons led to a statistically significant decrease ($p < 0.05$) in cell viability (Fig. 1B). Similarly, there was also no statistically significant increase in LDH release from the cells to the media with increasing uranium concentrations (Fig. 1C). Only NMDA-treated neurons resulted in a statistically significant ($p < 0.05$) increase in LDH release compared to control (Fig. 1B), as determined by one-way ANOVA followed by Bonferroni's *post hoc* test.

Visual inspection of cortical neurons exposed to uranium did not indicate significant changes in cell morphology until cells were exposed to very high concentrations (100μM) of uranium (Fig. 2). Cell body and neurite morphological changes begin to be apparent at 100μM DU, although they are minimal when compared to the positive control NMDA (Fig. 2). In the absence of DU, cells appear phase bright with long uninterrupted processes extending from each neuron. At 10μM DU, some loss of phase bright appearance and mild swelling of cell bodies is evident. At 100μM DU, cells begin to lose morphological integrity, consistent with the increase in LDH release and neuritis become discontinuous and headed.

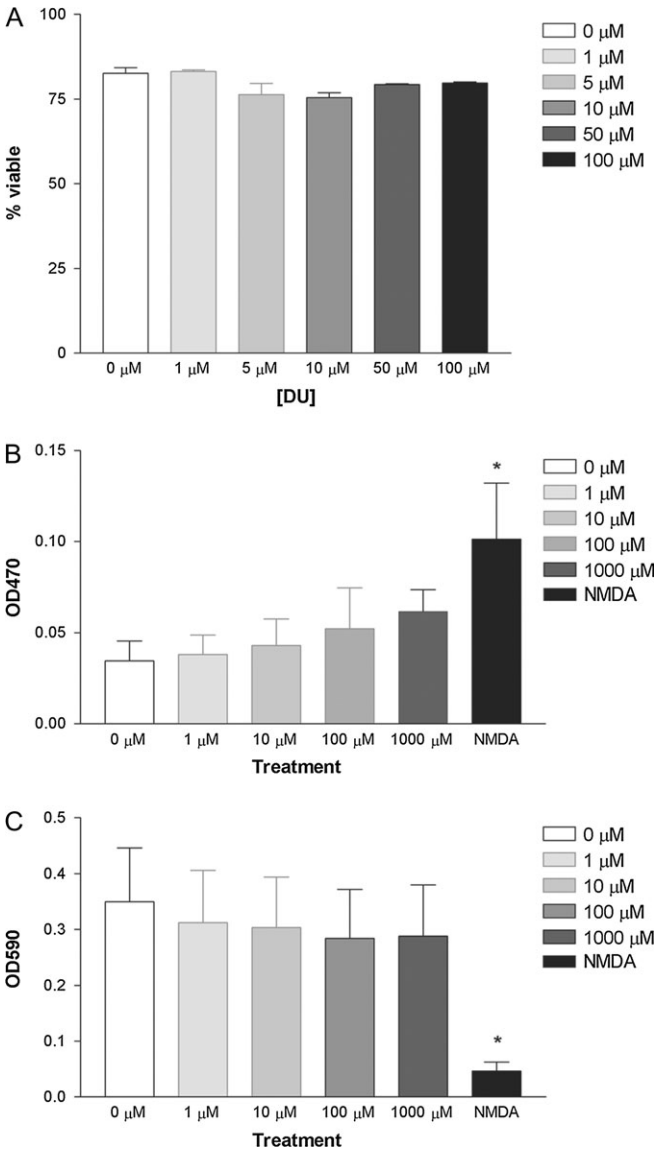

FIG. 1. Primary rat neuron cell viability measured by FACS, MTT, and LDH assays. These panels illustrate the viability of primary rat cortical neurons following 24-h continuous exposure to uranyl acetate as measured by (A) LIVE/DEAD, (B) MTT, and (C) LDH cytotoxicity assays. Error bars indicate standard error of the mean. *One-way ANOVA, followed by a Dunnett's test at 95% confidence indicated that the differences between control and treatment condition were statistically significant ($p < 0.05$). The data are from six independent sets of results ($N = 6$).

DU Has Minimal Effects on Thiol Metabolite Levels, Redox Potential, and High Energy Phosphates

Thiol metabolite analysis indicates that cysteine (Fig. 3A) and cystine (Fig. 3B) levels remain unchanged following uranyl acetate, or NMDA, exposure compared to control. One-way ANOVA followed by Dunnett's *post hoc* test indicated that GSH levels (Fig. 3C) are not significantly different upon uranium exposure compared to control ($p > 0.05$), but are significantly decreased by NMDA exposure ($p < 0.001$). NMDA exposure results in decreased GSH levels of 0.00052 ± 0.00003 nmol/mg protein compared to the control level of 0.0091 ± 0.0014 nmol/mg protein. One-way ANOVA followed by Dunnett's *post hoc* test indicated that GSSG levels (Fig. 3D) are significantly decreased by 50μM uranyl acetate ($p < 0.05$), 100μM uranyl acetate ($p < 0.001$), or NMDA exposure ($p < 0.001$) compared to control. Control GSSG levels were 0.0017 ± 0.00053 nmol/mg protein while 50μM uranyl acetate, 100μM uranyl acetate, or NMDA treatments levels were 0.00047 ± 0.00014 nmol/mg protein, 0.00022 ± 0.00004 nmol/mg protein, or 0.00012 ± 0.00003 nmol/mg protein, respectively (Fig. 3D).

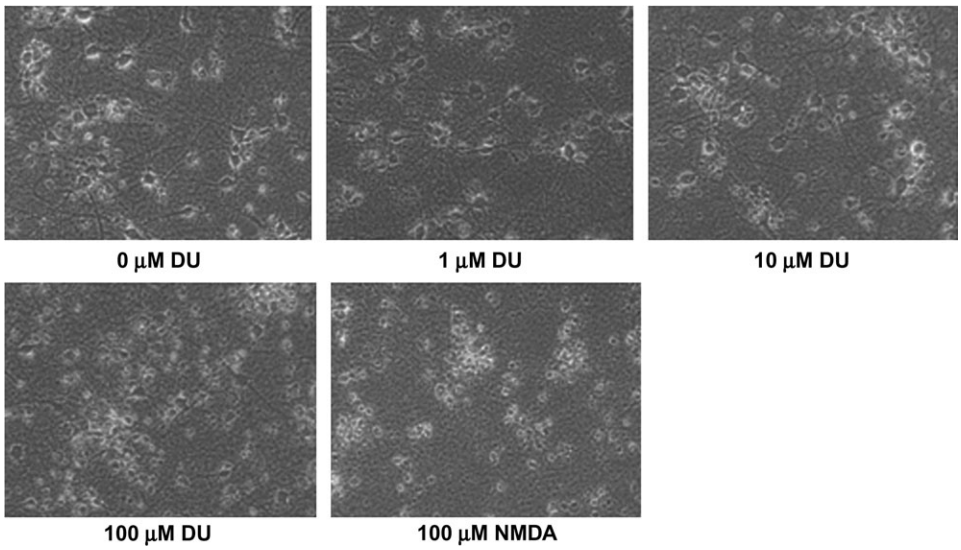

FIG. 2. Uranyl acetate exposure results in minimal morphological changes in primary rat cortical neurons. These panels are representative images to illustrate the cellular morphology of primary rat cortical neurons exposed to 0-, 1-, 10-, or 100μM uranyl acetate or the excitotoxic positive control (100μM NMDA with 10μM glycine). Changes in cell body and dendrite morphology begin to be noticeable at 100μM uranyl acetate, and are readily apparent in the positive NMDA control.

One-way ANOVA analysis followed by Dunnett's *post hoc* test indicated that the GSH/GSSG ratio of 100μM uranyl acetate exposed neurons was 31.16 ± 5.23, which was significantly higher ($p < 0.001$) than the control ratio of 6.98 ± 1.83 (Fig. 3E). There was no statistical difference between GSH/GSSG ratios (Fig. 3E) for 50μM uranyl acetate, or NMDA treatments compared to control ($p > 0.05$). Overall the Nernst potential for GSH (E_h GSH/GSSG, Fig. 3F) is − 176.0 ± 4.62 for control, and − 182.4 ± 3.08 in 50μM uranyl acetate exposed neurons, which is not statistically significant ($p > 0.05$). E_h GSH/GSSG for 100μM uranyl acetate exposure (Fig. 3F) is − 192.7 ± 2.94, and is statistically significant compared to control ($p < 0.05$). The E_h GSH/GSSG following NMDA exposure (Fig. 3F) is − 138.9 ± 6.46 and exhibits greater statistical significance ($p < 0.001$).

DU exposure of neurons did not result in a statistically significant decrease in TAN compared to control, although there was a statistically significant ($p < 0.001$) decrease in TAN in NMDA-exposed neurons to control (Fig. 4A), as determined by one-way ANOVA followed by Dunnett's test at 95% confidence interval. NMDA treatment also yielded an overall decrease in the ATP to TAN ratio from 0.75 ± 0.029 in controls to 0.42 ± 0.098 (Fig. 4B), which was statistically significant ($p < 0.05$). The ADP to TAN ratio does not result in statistically significant differences with increasing DU concentrations, or with NMDA treatment, compared to control (Fig. 4C). Like the ATP to TAN ratio, the ratio of AMP to TAN ratio is only significantly different ($p < 0.001$) in NMDA treated cells, but not in DU exposed (Fig. 4D). Overall, the ECP of the control primary rat cortical neurons is 0.84 ± 0.016, and did not result in statistically significant changes upon DU exposure, but was significantly decreased ($p < 0.05$) in the NMDA treated cultures 0.63 ± 0.050 (Fig. 4E).

DU Exposure Does Not Significantly Change F_2-IsoP Levels in Primary Rat Cortical Neurons

One-way ANOVA indicates that DU exposure did not result in a significant increase in the level of F_2-IsoP, products of lipid peroxidation, between controls and treatments (Fig. 5). After a 24 h 50μM DU exposure, neurons demonstrated F_2-IsoP levels of 55.5 ± 8.1 pg/mg total protein, while 24 h 100μM DU exposed neurons exhibited 51.0 ± 9.2 pg/mg total protein F_2-IsoP. The only statistically significant difference between samples occurred between the control (43.6 ± 3.3 pg/mg total protein) and the positive control NMDA treated neurons, which increased F_2-IsoP levels to 69.3 ± 5.9 pg/mg total protein ($p < 0.05$).

DU Exposure Does Not Cause Neurodegeneration in C. elegans

DU exposure does lead to increased uranium accumulation in the different *C. elegans* strains, with 100μM DU treated N2 worms exhibiting 10.9 ± 1.10 ng ^{238}U/μg total protein (data not shown). The NW1229 strain is a transgenic *C. elegans* strain that is a fusion of the GFP gene to the promoter of the *F25B3.3* gene, the *C. elegans* ortholog of the Ca^{2+}-regulated *ras* nucleotide exchange factor CalDAG-GEFII/RasGRP, which is ubiquitously expressed in the vertebrate nervous system (Ebinu *et al.*, 1998; Kawasaki *et al.*, 1998). The resulting transgenic strain produces exclusive pan-neural GFP expression, in which all neurons express GFP, and can be easily visualized with

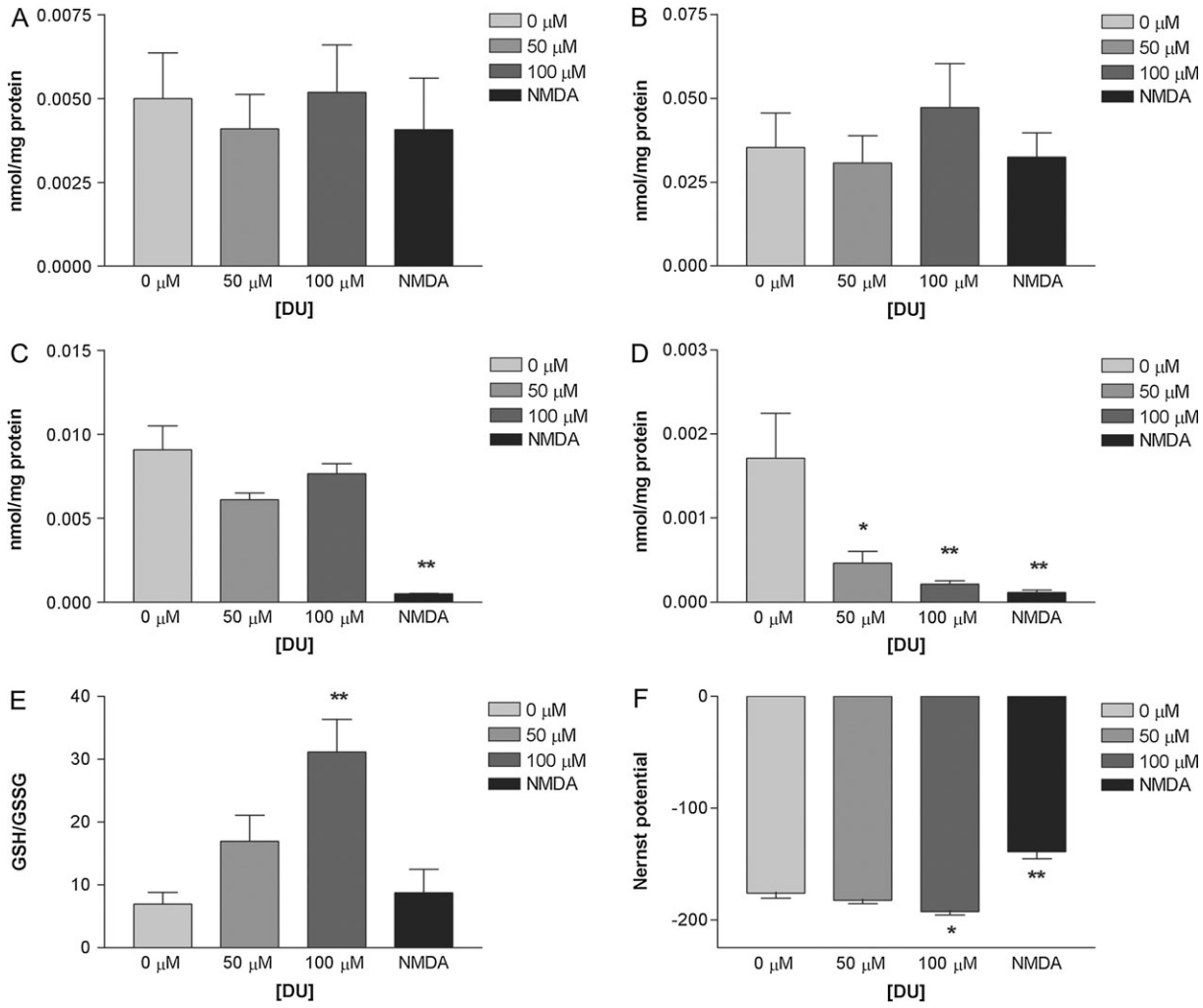

FIG. 3. Uranyl acetate does not cause significant alterations in thiol metabolite levels and redox potential. These panels illustrate the changes in thiol metabolites in primary rat cortical neurons exposed to different uranium concentrations or the positive control NMDA for 24 h, as measured by HPLC. The panels illustrate (A) cysteine (Cys) levels; (B) cystine (CysS) levels; (C) glutathione (GSH) levels; (D) oxidized glutathione (GSSG) levels; (E) GSH/GSSG ratio; (F) Nernst potential for GSH (E_h GSH/GSSG). Error bars indicate standard error of the mean. *One-way ANOVA, followed by a Dunnett's test at 95% confidence indicated that the thiol metabolite levels between control and treatment condition were statistically significant ($p < 0.05$). **One-way ANOVA, followed by a Dunnett's test at 95% confidence indicated that the thiol metabolite levels between control and treatment condition were statistically significant ($p < 0.001$). The data are from six independent sets of results ($N = 6$).

fluorescence (Altun-Gultekin *et al*., 2001). The BY250 *C. elegans* strain is a transgenic worm strain that has GFP under the control of the dopamine transporter promoter. Previously, BY250 worms have been used as a model for Parkinson's disease, and have been used to demonstrate dopamine neurodegeneration following exposure to the neurotoxin 6-hydroxydopamine (Nass and Blakely, 2003; Nass *et al*., 2002). In the pan-neural GFP worm strain NW1229, a high concentration exposure of 1mM DU did not result in significant neurodegeneration (Fig. 6). Similarly, using the BY250 strain of *C. elegans*, which exhibits dopamine neuron specific GFP expression, 1mM DU exposure did not lead to increased neurodegeneration of dopamine cell bodies or dendrites, as visualized by GFP fluorescence (Fig. 7).

DISCUSSION

Cell culture allows strict control of the cellular environment, which could influence cellular responses. Using the knowledge gathered from DU accumulation studies in the rodent models (Arfsten *et al*., 2005; Fitsanakis *et al*., 2006; Hahn *et al*., 2002; Miller *et al*., 1998b; Pellmar *et al*., 1999a) and from Gulf War veterans (Gwiazda *et al*., 2004; McDiarmid *et al*., 2000, 2001; Scott, 2003; Squibb and McDiarmid, 2006), we can extrapolate that CNS cells may potentially be exposed to nanomolar concentrations of U from leached uranium from embedded DU fragments. We also considered previous studies with U in cell cultures in the selection of our experimental uranium criteria and concentrations (Carriere *et al*., 2004; Kalinich and

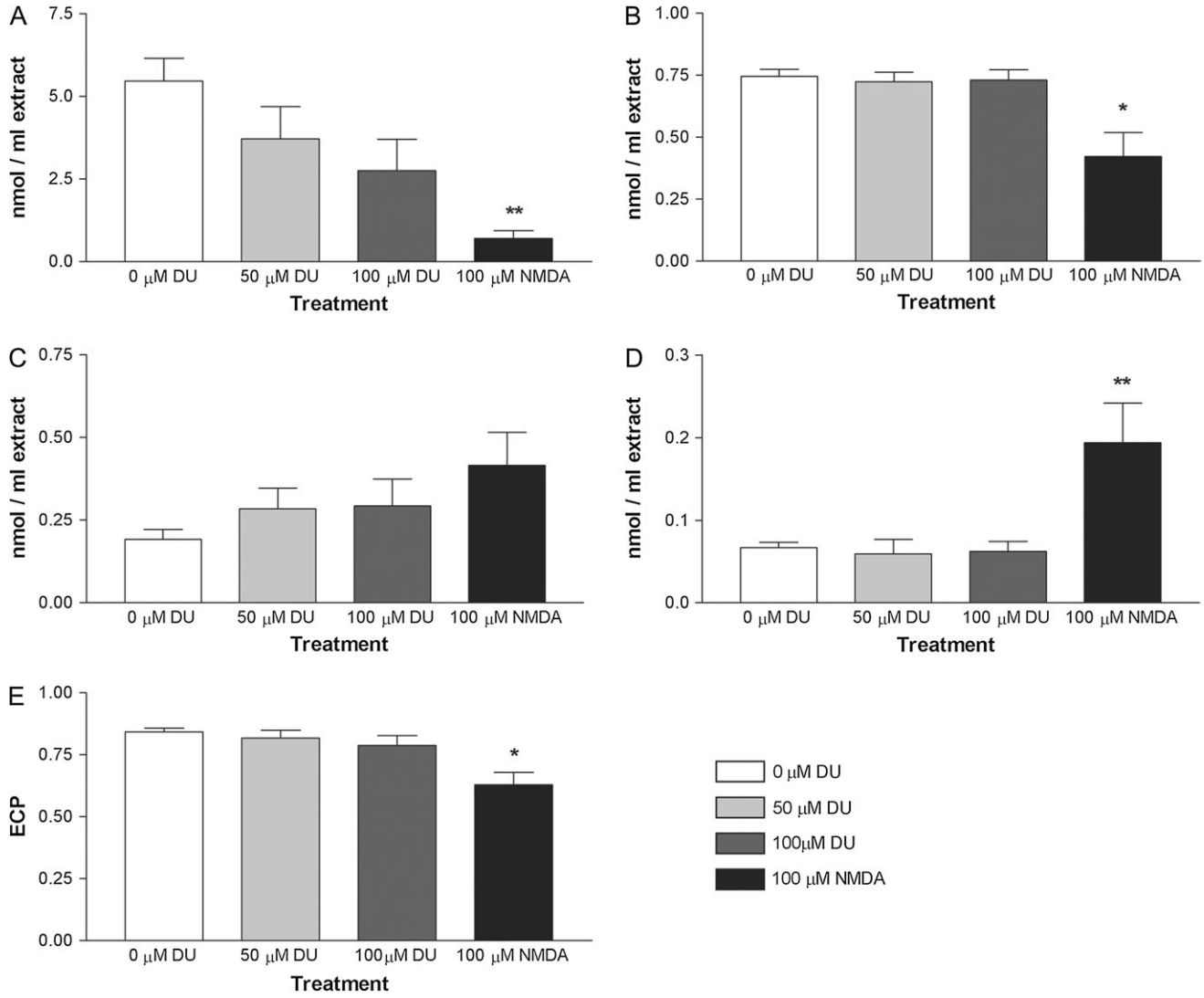

FIG. 4. Uranyl acetate does not cause significant alterations in high energy phosphates. These panels illustrate the changes in high energy phosphates in primary rat cortical neurons exposed to different uranyl acetate concentrations or the positive control NMDA for 24 h, as measured by HPLC. The panels illustrate (A) total adenosine nucleotides (TAN) levels; (B) ATP to TAN ratio; (C) ADP to TAN ratio; (D) AMP to TAN ratio; (E) energy charge potential (ECP). Error bars indicate standard error of the mean. *One-way ANOVA, followed by a Dunnett's test at 95% confidence indicated that the difference between control and treatment were statistically significant ($p < 0.05$). **One-way ANOVA, followed by a Dunnett's test at 95% confidence indicated that the differences between control and treatment condition were statistically significant ($p < 0.001$). The data are from six independent sets of results ($N = 6$).

McClain, 2001; Kalinich *et al*., 2002; Malard *et al*., 2005; Miller *et al*., 2001; Mirto *et al*., 1999; Prat *et al*., 2005; Vidaud *et al*., 2005). Uranyl acetate, prepared from DU, was selected to release the uranyl cation under more physiologically relevant conditions as the acetate form is more soluble and releases a more neutral anion, while a uranyl nitrate form would be less soluble and more oxidizing. The U concentrations used in our study to evaluate neuron viability were similar to previously used concentrations, and our highest concentrations (100μM) very unlikely to be attained *in vivo*.

The results of our LIVE/DEAD cell viability assays indicate that DU, in the form of uranyl acetate, does not result in significant decreased primary rat cortical neuron viability following 24-h exposures even at exceedingly high concentrations. Our cell sorting data (Fig. 1A) are further correlated by visual inspection of the neuronal cultures following treatment (Fig. 2). MTT results, which indicate mitochondrial inhibition, together with the LDH cytotoxicity data provide additional evidence for little change in cell viability. MTT data illustrate that U-treated samples do not show significant mitochondrial inhibition, while mitochondrial inhibition is seen in NMDA exposed neurons (Fig. 1B). Likewise, although it appears that there is increased LDH release into the medium as the concentration of uranium is increased, there is no statistically significant increase in cell death except with NMDA-treated neurons (Fig. 1C).

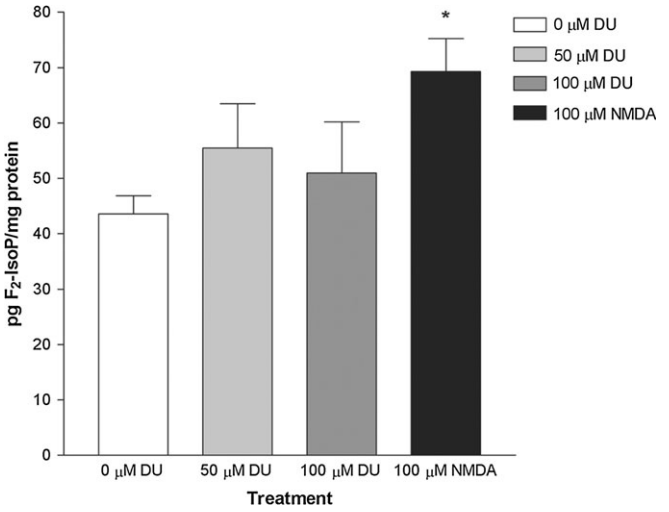

FIG. 5. F_2-IsoP levels following exposure to uranyl acetate remain unchanged. This bar chart illustrates the changes in F_2-IsoP levels in primary rat cortical neurons exposed to different uranium concentrations or the positive control NMDA for 24 h, as measured by mass spectrometry ($N = 12$). Error bars indicate standard error of the mean. *One-way ANOVA, followed by a Dunnett's test at 95% confidence indicated that the F_2-IsoP levels between control and NMDA were statistically significant ($p < 0.05$). The data are from 10 independent sets of results ($N = 6$).

Thiol redox metabolism and redox potential are essential to cell homeostasis (Jones, 2002). Disruption of redox status can occur through depleting redox components such as cysteine of GSH. It has been demonstrated that metals have profound oxidative effects on the major thiol antioxidant systems (Hansen *et al.*, 2006). Our thiol metabolite analysis indicates that cysteine (Fig. 3A) and cystine (Fig. 3B) levels are not significantly different in control, U, or NMDA-treated samples. GSH analysis did not show statistically significant differences in GSH upon DU exposure (Fig. 3C), but did indicate that GSSG levels were significantly decreased at both U concentrations, or following NMDA treatment, compared to controls (Fig. 3D). This difference in GSH and GSSG resulted in a statistically significant increase in the GSH/GSSG ratio for 100μM U treated neurons compared to control (Fig. 3E). This increase in GSH/_GSSG ratio was also reflected in the redox (Nernst) potential for GSH, which was significantly decreased for 100μM U-treated neurons, while significantly increased in the positive control treated samples. This difference suggests that DU acts in a different manner than NMDA, and may be reducing the oxidized pool but not affecting GSH synthesis. In conjunction with the cell viability data, the lack of significant changes in thiol metabolites and redox potential are not surprising, as the cell viability is not overtly decreased with higher DU concentrations. The thiol metabolite data only suggest that U does not result in significant changes in GSH that would lead to decreased neuron viability. The findings of little oxidation to the GSH system does not rule out the possibility that uranium could be affecting the thioredoxins, which could in turn activate toxic signaling and apoptotic pathways (Hansen *et al.*, 2006). Indeed, there is evidence for upregulation of specific thioredoxin-related proteins, and involvement of the thioredoxins, upon exposure to uranium in cultures and tissues followed by 2Dimensional-Difference In Gel Electrophoresis, and subsequent peptide mass fingerprinting studies (Malard *et al.*, 2005, data not shown).

TAN levels do not show a statistically significant dose-dependent difference in U-treated samples compared to controls, although there appears to be an overall decreasing trend in TAN as U concentration is increased (Fig. 4A). Neurons do exhibit a statistically significant decrease in TAN levels when exposed to the excitotoxicant NMDA (Fig. 4A). No significant differences were observed in ATP, ADP, or AMP levels, following U exposure. In our positive controls, NMDA exposure resulted in a significant decrease in ATP (Fig. 4B), and significant increase in AMP (Fig. 4D), compared to TAN levels. As such, ECP was not significantly different in control and U treated samples but was decreased in NMDA treated neurons. Although there is a trend of decreased high energy phosphates as uranium concentration is increased, the surviving neurons were not devoid of high energy phosphates. The relative abundance of ATP amongst the adenosine nucleotide pool and the lack of change in ECP can be interpreted as DU not damaging mitochondrial function. The high energy phosphate analyses suggest no significant mitochondrial dysfunction or increased toxicity by U to the primary rat cortical neurons, which further corroborates the MTT data, and correlates with our cytotoxicity data.

IsoPs are a unique series of prostaglandin-like compounds formed *in vivo* via a nonenzymatic mechanism involving the free radical-initiated peroxidation of arachidonic acid, and are hallmark *in vitro* and *in vivo* biomarkers of lipid peroxidation and generation of reactive oxygen species (Milatovic *et al.*, 2005; Milne *et al.*, 2005; Morrow and Roberts, 2002; Musiek *et al.*, 2005). As such, experimental methods measuring F_2-IsoP levels are an excellent measure of cumulative oxidative stress with changes in IsoPs have been observed as soon as 30 min after exposure to treatments (Milatovic *et al.*, 2005; Montine *et al.*, 2004; Morrow and Roberts, 2002). In our studies, the only statistically significant change in F_2-IsoPs after a 24-h exposure was between control and NMDA-treated samples (Fig. 5). F_2-IsoPs are not significantly different between control and U-treated samples even after 24 h exposures (Fig. 5). Taken together, we conclude that there is no significant increase in oxidative stress in primary rat cortical neurons following 50 or 100μM U exposure. These data also correlate with our cell viability data.

In animal models, DU has been shown to cross the blood–brain barrier and accumulate in specific regions of the brain (Abou-Donia *et al.*, 2002; Barber *et al.*, 2005; Briner and Murray, 2005; Fitsanakis *et al.*, 2006; Leggett and Pellmar, 2003; Lestaevel *et al.*, 2005; Pellmar *et al.*, 1999a). Some researchers have also studied the functional and behavioral

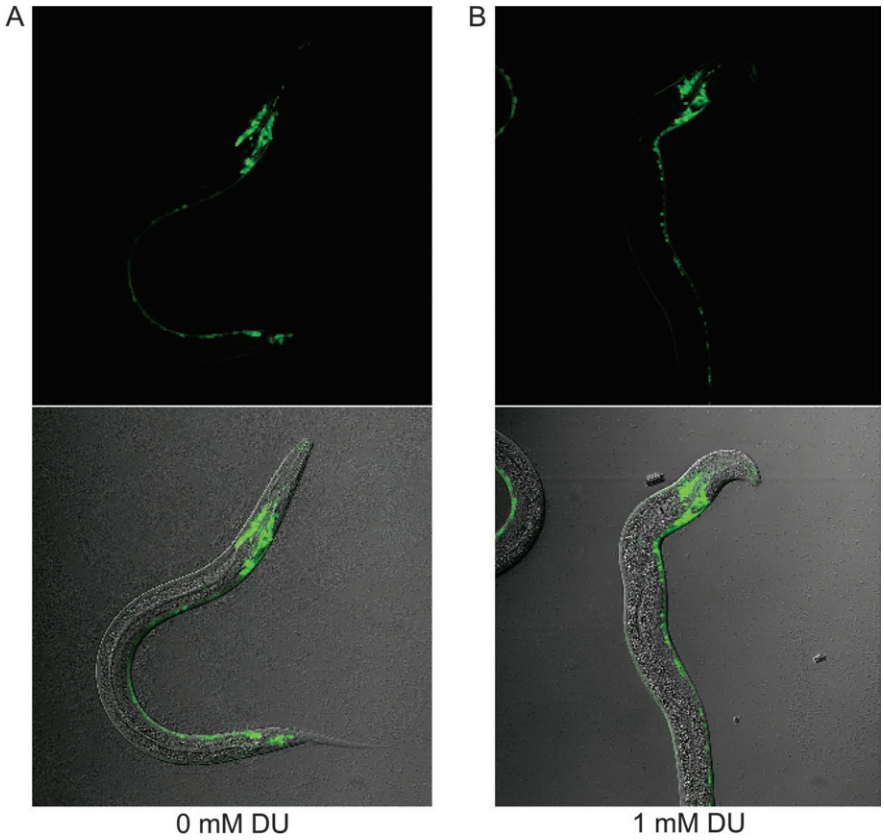

FIG. 6. Uranium exposure does not result in significant neurodegeneration in *Caenorhabditis elegans*. These images illustrate the pan-neural GFP reporter strain of *C. elegans* (NW1229) exposed to a (A) 0mM control and (B) 1mM treatment dose of uranyl acetate. The top row of images is under fluorescent excitation, while the bottom row of images is differential interference contrast with fluorescence overlayed. There is no visual evidence for neuronal degeneration following DU exposure.

changes associated with increased DU accumulation to investigate if the cognitive defects that were seen in Gulf War veterans could be correlated to specific neurotransmitters (Abou-Donia *et al*., 2002; Belles *et al*., 2005; Briner and Murray, 2005; Houpert *et al*., 2004, 2005; Pellmar *et al*., 1999b). While studies have shown increased oxidative stress in certain brain regions, it remains unclear whether DU accumulation in the brain results in any significant neurodegeneration. It would be logical to surmise that there is very little degeneration occurring considering the minimal functional changes that have been shown following DU administration. The nematode *C. elegans* provides an excellent model to visualize neurodegeneration, as there is no blood–brain barrier that neurotoxicants must traverse, and neurons can be easily labeled with fluorescent markers. The ease of growth, maintenance, and manipulation also allows researchers to evaluate the chemotoxic effects on a whole organism, which helps in further extrapolation of data to human health and disease. Considering these factors, we utilized two transgenic strains to determine if DU exposure led to any neurodegeneration. Nematodes do accumulate U with increasing U exposure (data not shown) and in the pan-neural GFP-expressing strain NW1229, our experiments demonstrate that there is no significant degeneration of neurons following U exposure (Fig. 6). Several pieces of data from previously published articles have suggested potential involvement of the dopaminergic system following DU exposure including increased uranium uptake in the midbrain of rats implanted with DU pellets (Houpert *et al*., 2004; Pellmar *et al*., 1999a), and increased lipid peroxidation and nitric oxide generation in the midbrain of rats exposed to DU (Abou-Donia *et al*., 2002). Confocal microscopic inspection of BY250 worms did not demonstrate significant degeneration of dopamine neurons following U exposure (Fig. 7).

Researchers have demonstrated increased accumulation of DU in specific brain regions following increased DU exposure as previously described (Jiang and Aschner, 2006). In our nematode experiments, we have evaluated the effects of U on *C. elegans* neurons in an attempt to evaluate neurons in brain regions that have been shown to accumulate DU in rats. Our data demonstrate that there is a dose-dependent increase in uranium accumulation (data not shown) but not a corresponding increase in neurodegeneration (Figs. 6 and 7). These results correlate with the results from our experiments with primary rat cortical neuron cultures which indicate that the neurons can tolerate U without significant cell death (Fig. 1A). Our data are

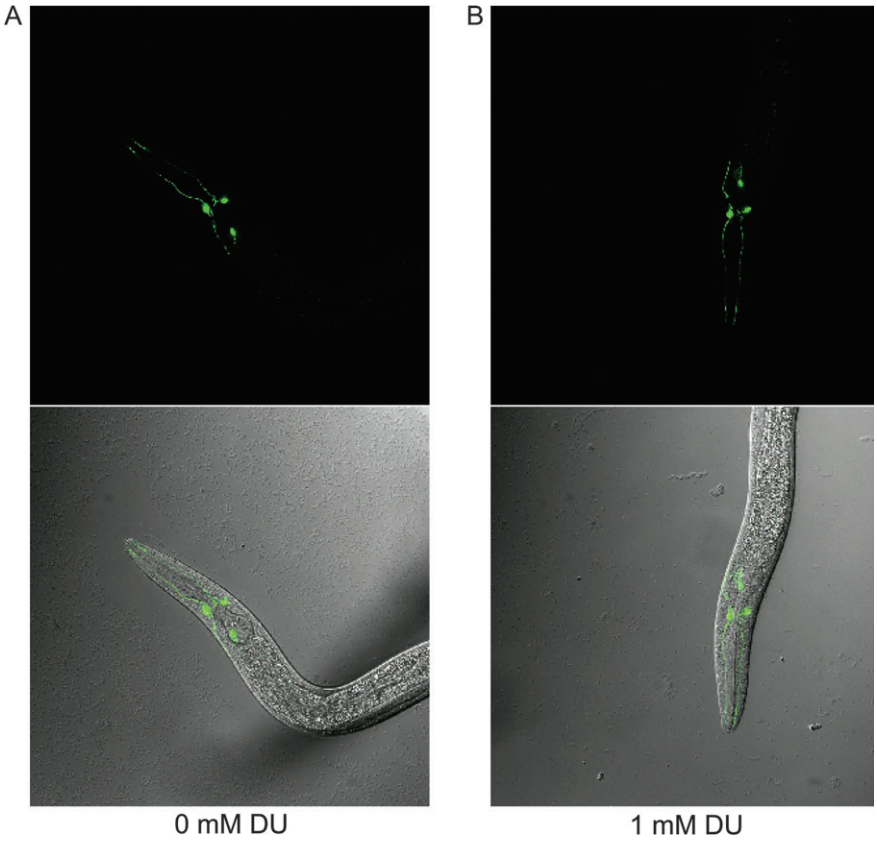

FIG. 7. Uranium exposure does not induce significant *Caenorhabditis elegans* dopamine neurodegeneration. These images illustrate dopamine neurons following exposure to a (A) 0mM uranium control and (B) 1mM uranyl acetate treatment in the GFP reporter worm strain BY250. The top row of images is under fluorescent excitation, while the bottom row of images is differential interference contrast with fluorescence overlayed. Dopamine neurons do not demonstrate degeneration following DU exposure.

similar to previous studies in rat brain endothelial cells, which demonstrated U has low cytotoxic potential (Dobson *et al.*, 2006).

The studies conducted here follow a reductionist approach to evaluate the toxicity of DU, in the form of uranyl acetate, in a specific cell population. The focused studies on primary cultured cortical neurons indicate that U results in little cytotoxicity and minimal cellular changes, suggesting low neurotoxic potential. As beneficial as this approach is to determine the effects within a specific cell population, this methodology cannot exclude the possibility that DU has neurotoxic effects on other cell populations within the CNS, such as astrocytes and/or microglia. Indeed, previous neurotoxicology studies demonstrated that astrocytes play an important role in detoxification of heavy metals (Aschner, 1997; Im *et al.*, 2006; Lindahl *et al.*, 1999; Tiffany-Castiglion and Qian, 2001). As such, the possibility exists that U accumulates within astrocytes, resulting in a variety of subsequent molecular events within the astrocytes, causing downstream detrimental effects on neurons. For example, one such event could be disruption of astrocytic glutamate uptake, causing increased extracellular glutamate that may lead to subsequent excitotoxicity of nearby neurons—a mechanism that has been observed following methylmercury exposure (Allen *et al.*, 2002; Aschner *et al.*, 2000; Juarez *et al.*, 2002; Qu *et al.*, 2003).

In summary, based on our data, there appears to be very little cytotoxicity in primary rat cortical neuron cultures upon exposure to uranyl acetate until cultures are exposed to high levels (> 100μM), which would be considered supraphysiological. Furthermore, from *in vitro* studies at these high levels of uranium, there are no significant changes in F_2-IsoP and thiol metabolite levels, and only minimal changes in TAN. Our *C. elegans* data using GFP reporter worm strains corroborates our cytotoxicity data in the primary cultures, and show that there is no significant neuronal neurodegeneration following uranium exposure. Although *in vitro* models may not fully recapitulate human health and disease, our focused studies indicate that neurons can tolerate high doses of uranyl acetate without significant oxidative injury or death. These studies have examined neuronal lethality after a relatively acute exposure, and it is also possible that there may be effects with longer term exposure or potential impairment of neuronal function that occur in the absence of acute lethality. Altogether,

although our reductionist approach cannot exclude the possibility of DU as a neurotoxic agent to other CNS cell populations, the results of our studies in primary rat cortical neurons and in *C. elegans* demonstrate low acute neurotoxic potential of uranyl acetate, and should alleviate some of the concern surrounding DU as a neurotoxin, and as a chemical that may be responsible for GWS. These results support the emerging agreement among workers in the field that DU neurotoxicity is not a component of primary GWS, though it may constitute a separate entity in the cluster of Gulf War illnesses.

FUNDING

D.O.D. grant (DAMD 17-01-1-0685) to M.A.

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