Brain Accumulation of Depleted Uranium in Rats Following 3- or 6-Month Treatment With Implanted Depleted Uranium Pellets

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Abstract:
Depleted uranium (DU) is used to reinforce armor shielding and increase penetrability of military munitions. Although the data are 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 mo. Prior to surgery, rats were randomly divided into five groups: Nonsurgical 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, brainstem (pons and medulla), midbrain, hippocampus, striatum, and cortex. The uranium content was measured in digested samples as its ^{238}U isotope by high-resolution inductively coupled plasma–mass spectrometry. After 3 mo postimplantation, DU significantly accumulated in all brain regions except the hippocampus in animals receiving the highest dose of DU ($p < 0.05$). By 6 mo, however, significant accumulation was measured only in the cortex, midbrain, and cerebellum ($p < 0.01$). Our data suggest that DU implanted in peripheral tissues can preferentially accumulate in specific brain regions.
Index Entries: Depleted uranium; heavy metal toxicity; Gulf War syndrome; blood–brain barrier transport; ICP-MS.

Article:

INTRODUCTION

Naturally occurring uranium (U) consists of three isotopes: ^{234}U (0.0055%), ^{235}U (0.720%), and ^{238}U (99.27%). During the production of U for nuclear fuel and weapons, the highly radioactive isotope, ^{234}U, is enriched, leaving the remaining U relatively depleted of both the ^{234}U and ^{235}U isotopes. This resulting mixture of U, known as “depleted uranium” (DU), is mainly the ^{238}U isotope (99.8%, with 0.001% ^{234}U and 0.20% ^{235}U), which is 40% less radioactive than natural U and is 1.7 times more dense than lead (Pb). Because of its unique physiochemical properties, DU has many civilian and military uses. For example, it is utilized in medical equipment radiation shields, aircraft counterbalances, and, until the 1980s, as a component in dental porcelains *(1)*. Potential health problems related to DU exposure were highlighted following the first Gulf War (1991) because of its use in reinforcing armor plating of military vehicles and as a component in armor-piercing munitions. Following service in the Gulf War, many veterans reported various health problems, including headaches, dizziness, fatigue, bone and joint pain, muscle weakness, memory loss, problems with sleep and concentration, skin rashes and sores, and various gastrointestinal problems *(2–4)*. Together these symptoms are known as Gulf War syndrome (GWS). Additional concern regarding health problems resulted following the war operations in Serbia, as well as the current use of DU in munitions and shielding in Iraq.

It is likely that no single chemical or environmental factor/ stressor is responsible for this syndrome, but it has been suggested that DU might play a role in the etiology of GWS. Military personnel in the battle arena are exposed to a variety of chemicals: organic fumes from petroleum fires, organophosphate pesticides, multiple vaccinations in combination with increased inhalation of small particulates (soot, ashes, sand), and high levels of stress *(4).* In general, American Gulf War veterans could have been exposed to DU from several sources: (1) inhalation/ ingestion of dust generated during repair, cleaning, or recovery of damaged tanks and armored vehicles with DU plating, (2) subcutaneous exposure resulting from injury from "friendly" fire, and (3) inhalation/ ingestion of dust generated from collection of DU-containing wartime memorabilia. Interestingly, persons with DU shrapnel fragments have high levels of DU in their urine *(5–10),* although this higher DU level does not appear to correlate with decreased neurological function *(11).* Because it is unknown whether DU is found in brain tissue of those with embedded DU fragments, it is important to establish whether DU can accumulate in the brains of animals implanted with DU pellets. Evidence of brain accumulation is necessary to link DU with the neurological symptoms reported by Gulf War veterans.

Others have implanted DU "shrapnel" in rats *(12,13)* and measured DU levels in brain, but the animals were not perfused prior to removal of the brain. Thus, it is unclear as to what degree the DU levels measured included DU from blood present in the brain samples. This is important to consider because blood volume in the cerebrovascular tree might represent up to 20% of the total brain volume. To better characterize DU accumulation in brain tissue, we implanted DU pellets in rats to determine whether brain DU levels were greater in treated animals compared to nonsurgical controls or rats receiving sham pellets made from tantalum (Ta), an inert metal used in various joint replacement surgeries in humans.

MATERIALS AND METHODS

Chemicals

Depleted uranium pellets (1 mm in diameter x 2 mm, part no. AOT PN 900397) were obtained from Aerojet Heavy Metals (Jonesboro, TN). Tantalum (Ta) pellets (1 mm in diameter x 2 mm, stock no. 77611, lot no. A16N11) were made to specification by Alpha Aesar (Ward Hill, MA).

Animals

The Wake Forest University School of Medicine Animal Care and Use Committee approved all of the animal procedures conducted in this study. Adult male Sprague–Dawley rats (Harlan, approx 250 g at study initiation) were housed two per cage in an approved laboratory facility under a 12 : 12 light : dark cycle with access to food and water ad libitum. Rats were randomly divided into 3- or 6-mo treatment cohorts. Each cohort was further divided into five groups: nonsurgical control (NS Control); 0 DU pellets/20 Ta pellets (Sham); 4 DU pellets/16 Ta pellets (Low); 10 DU pellets/10 Ta pellets (Medium); 20 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 12 mg/kg xylazine and 80 mg/kg ketamine prior to surgery. Each gastrocnemius was shaved with an electric razor, and the surgical area was cleaned with betadine. Rats were covered with a sterile surgical drape for the procedure, and an aseptic technique was followed. Pellets were implanted according to a modified method *(12).* Briefly, an initial incision (approx 3 cm long) was made parallel to the muscle. An 18-gage needle was used to produce a guide hole perpendicular to the muscle fibers for each pellet. Pellets were implanted to a depth of approx 1 cm. Following the implantation, the incision was closed with a 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 warmed, insulated box until fully conscious (about 45 min postsurgery). Animals were returned to their cages and monitored daily for a week for infection and pain, and weekly thereafter until sacrifice.

Tissue Collection

At the conclusion of the each respective study, rats were anesthetized with 12 mg / kg xylanine and 80 mg / kg ketamine. Following anesthetization, animals were perfused with 200 mL phosphate-buffered saline (PBS; pH

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 brainstem [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

Concentrated nitric acid (HNO3) (Suprapur, Merck) was added to the lyophilized samples in the following amounts: 1 mL HNO_3 for 0.05–0.159 g tissue, 2 mL HNO_3 for 0.16–0.259 g tissue, 3 mL HNO_3 for 0.26–0.359 g tissue, and 6 mL HNO_3 for 0.66–0.96 g tissue. Samples were allowed to sit at room temperature for 24 h prior to a 1-h digestion in a block heater at 110 °C. Samples were further digested in a microwave oven (Multiwave 3000; Anton Paar) at the following specifications: ramp 8 min from 0–130 °C, then holding at 130 °C for 3–6 min, depending on sample mass. Finally, samples were diluted to 0.6 *M* HNO_3 with 18.2 MQ water.

All brain regions were analyzed for DU content by inductively coupled plasma – mass spectrometry (ICP-MS) using a Thermo (Finnigan) model Element 2 instrument (Bremen, Germany), as previously published *(14)* except that radio-frequency power was set at 1300 W. Briefly, the sample was introduced using a CETAC ASX 510 autosampler (Omaha, USA) with a peristaltic pump (pump speed-1 mL/min). The instrument was 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 was adjusted daily to give a stable signal with maximum intensity for the isotopes ^{115}In and ^{238}U. The instrument was calibrated using 0.6 *M* HNO3 solutions of multielement 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 approximately every 10 samples. The limit of detection (LOD) was 0.4 ng ^{238}U/L, corresponding to 0.06–0.12 ng ^{238}U/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. Twoway ANOVA comparing treatment groups at either 3 or 6 mo 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 mo. 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 are presented as mean ± SEM, with $n = 5–7$ animals for each group in each cohort.

RESULTS

Treatment With DU Does Not Result in Overt Toxicity

One week postsurgery, visual inspections suggested that animals had fully recovered and were as ambulatory as nonsurgical controls (NS Controls). Although there was no significant difference in body mass by the end of the respective studies among groups for either the 3- or 6-mo cohorts, DU animals might have consumed greater quantities of food. As this was based only on how quickly rodent chow was replaced, it could also be possible that DU-treated animals were less efficient in feeding, leading to a greater waste of food pellets. Water consumption did not seem to differ from NS Controls for either cohort.

In the 3-mo cohort, Sham rats were initially statistically significantly smaller than the other groups (Fig. 1A). At wk 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 wk 2, 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, and Low and Medium were different compared to NS

Control ($p < 0.05$ for both). By wk 4 and 5, only NS Control ($p < 0.01$ and $p < 0.05$, respectively) differed compared to Sham. By wk 6, the body masses of the groups were indistinguishable.

Figure 1B indicates that although the mean body mass was not statistically significantly different after wk 5, the percentage gain (initial body mass compared to subsequent weeks) differed among groups. One-way ANOVA indicated differences among groups for wk 6–12 (*see* Fig. 1B). Thus, although variations in body mass among groups were apparent in the early time-points, differences in percentage mass increase among groups did not appear until wk 6. From wk 6 until the conclusion of the 3- mo experiment at wk 12, the mean percentage increase in body mass for NS Control was significantly lower compared to Sham ($p < 0.05$ for wk 6–8, $p < 0.01$ for weeks 9–12). Additionally, by wk 9 and continuing until wk 12, the percentage mass gain in the Low group was statistically decreased compared to Sham ($p < 0.05$ for all weeks).

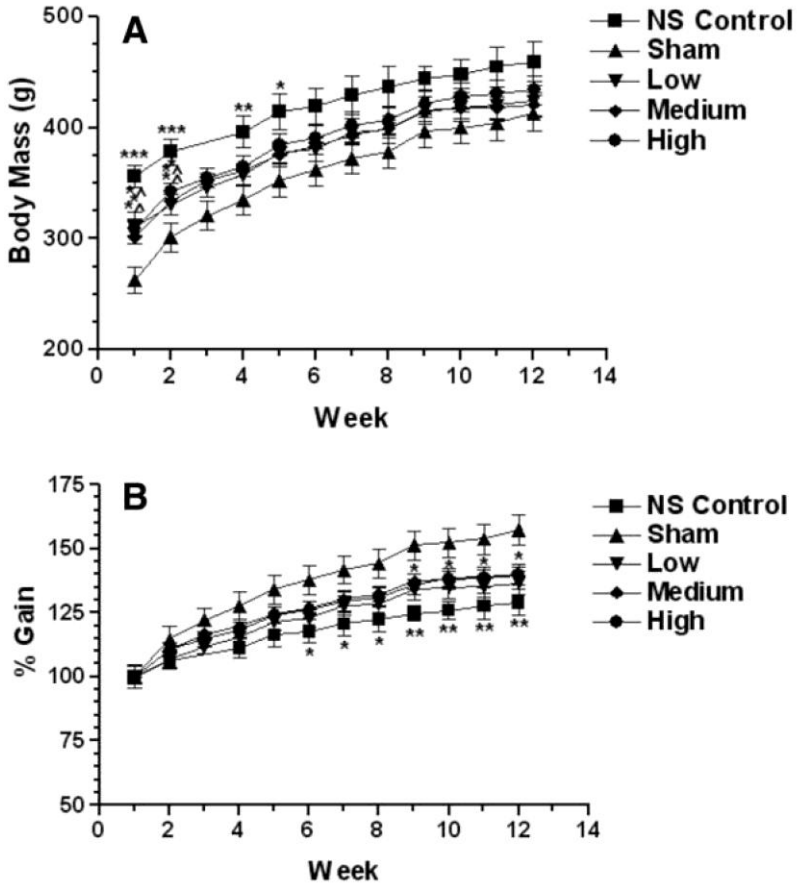

Fig. 1. Weight gain of rats treated for 3 mo with DU pellets. Groups for each cohort are as follows: non-surgical control (NS Control); 0 DU pellets/20 Ta pellets (Sham); 4 DU pellets/16 Ta pellets (Low); 10 DU pellets/10 Ta pellets (Medium); 20 DU pellets/0 Ta pellets (High). One-way ANOVA, followed by Tukey's posttest for multiple comparisons, at each time-point indicted that both treated rats and NS Control rats differed from Sham rats at various time-points. Indicators of statistically significant changes are as follows: $^{*}p < 0.05$ compared to Sham; $^{**}p < 0.01$ compared to Sham; $^{***}p < 0.001$ compared to Sham; $\wedge p < 0.05$ compared to NS Control. **(A)** Average body mass ± SEM for NS Control ($n = 5$), Sham ($n = 7$), Low ($n = 7$), Medium ($n = 7$), or High ($n = 7$) groups in the 3-mo cohort. **(B)** Average gain ± SEM of each group presented as a percent of the initial mass. **(C)** Average body mass ± SEM for NS Control ($n = 7$), Sham ($n = 7$), Low ($n = 7$), Medium ($n = 7$), or High ($n = 7$) groups in the 6-mo cohort. **(D)** Average gain ± SEM of each group presented as a percentage of the initial mass. *(continues)*

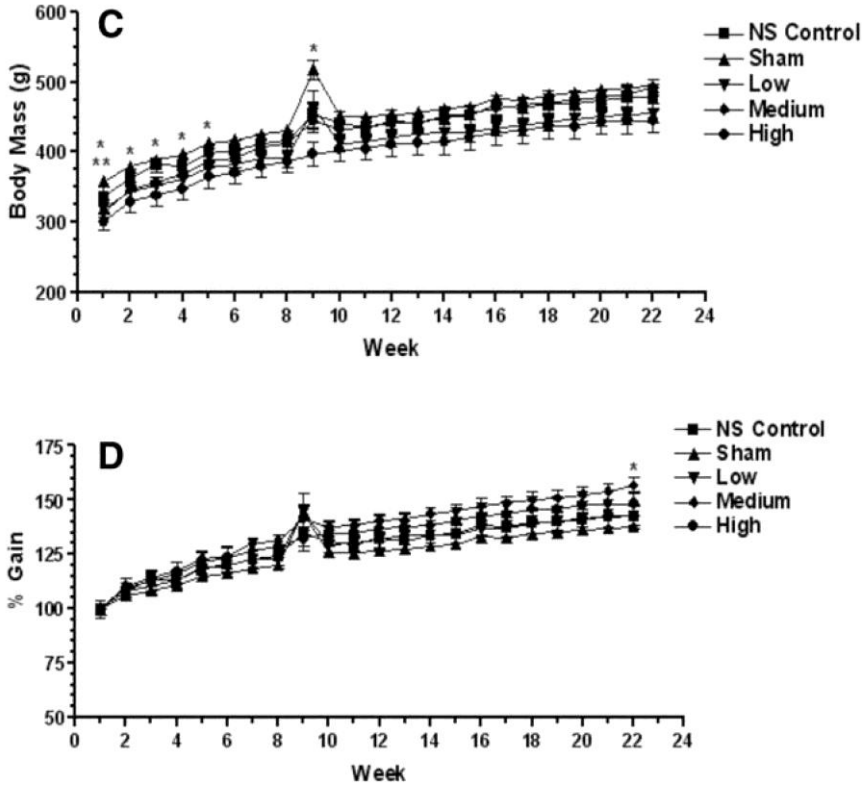

Fig. 1. (continued)

Figure 1C depicts the actual average body mass of the 6-mo group. As for the 3-mo cohort, early differences in body masses compared to Sham were observed. At wk 1, one-way ANOVA indicated ($p < 0.005$) that both Medium ($p < 0.05$) and High ($p < 0.01$) groups were lighter than the Sham group. One-way ANOVA indicated that only animals in the High group remained lighter for wk 2–5 ($p < 0.05$ for each week) and at wk 9 ($p < 0.01$). However, by the conclusion of the experiment at wk 22, the differences in body mass among the groups was indistinguishable.

When percentage gain relative to initial body mass was calculated (Fig. 1D), there was no statistically significant differences among groups until the last time-point at wk 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 different brain regions from rats in the 3-mo cohort (data not shown) indicated that the source of variation among the brain regions was the result of 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-mo cohort (data not shown). Although two-way ANOVA indicated that differences among group means were solely the result of the differences in regional wet masses ($p < 0.0001$), one-way ANOVA indicated no differences in the 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 mo, a dose-dependent pattern of DU accumulation was apparent in the cortex, striatum, brainstem and cerebellum (Fig. 2A). 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 was different from NS Control in the brainstem ($p < 0.05$) for the High group. The DU accumulation in the cerebellum was greater than NS Control only in the Medium group ($p < 0.05$).
By 6 mo (Fig. 2B), two-way ANOVA indicated variation resulting not only from treatment and regional ($p < 0.0001$ for both) differences but also from an interaction between the two ($p < 0.01$). Univariate analysis within the cortex and cerebellum revealed that both the 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 brainstem, although the midbrain from the high-dose group still had statistically significantly more DU than the Sham group ($p < 0.01$).

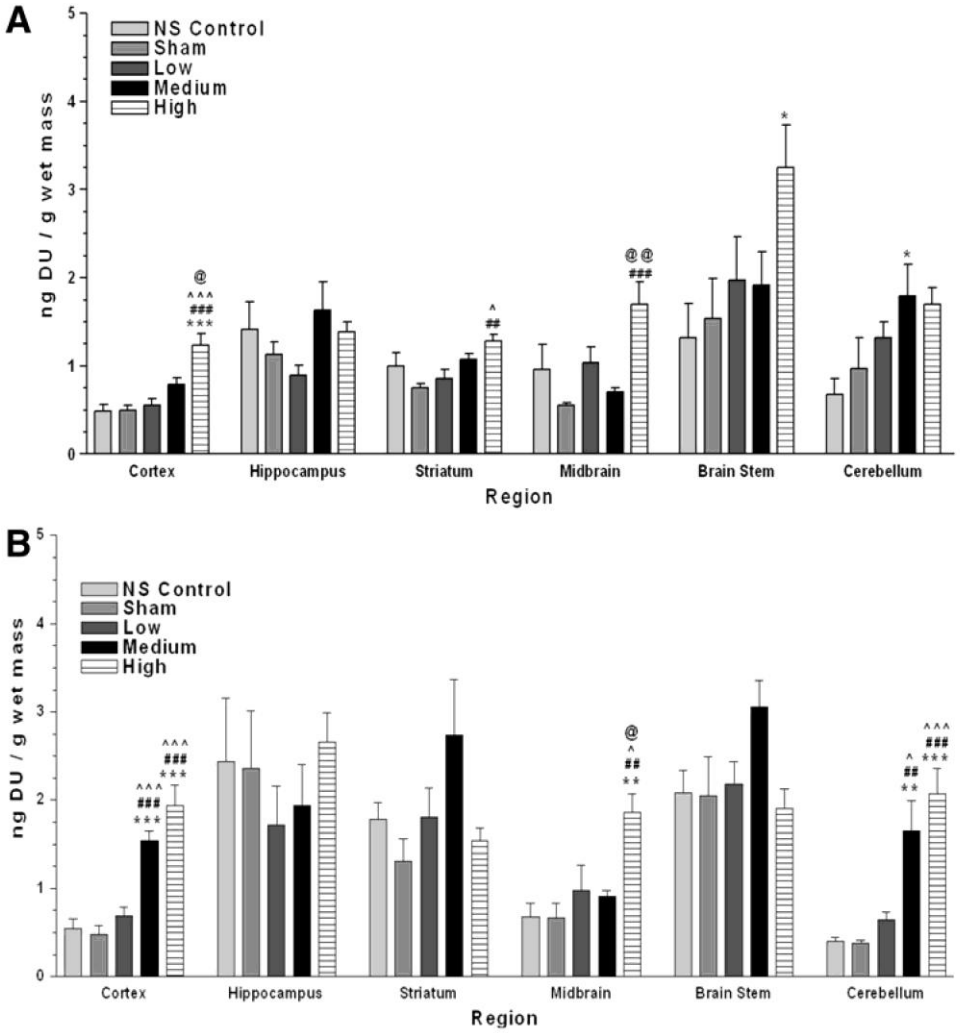

Fig. 2. Accumulation of DU in discrete brain regions. Treatment groups and sample size and number in each group for the two cohorts are defined in the legend for Fig. 1. Average amount (ng) of DU ± SEM for animals implanted with DU for either **(A)** 3 mo or **(B)** 6 mo. Two-way ANOVA with Tukey's posttest for multiple comparison indicated no treatment and region interaction at 3 mo. By 6 mo, two-way ANOVA indicated statistically significant variations among means because of treatment, region, and their interaction. However, one-way ANOVA at the respective time-points indicated statistically significant accumulation mainly in the high groups at both treatment durations. Statistically significant symbols are as follows: $^{*}p < 0.05$ compared to NS Controls; $^{**}p < 0.01$ compared to NS Control; $^{***}p < 0.001$ compared to NS Control; $^{\#\#}p < 0.01$ compared to Sham; $^{\#\#\#}p < 0.001$ compared to Sham; $^{\wedge}p < 0.05$ compared to Low; $^{\wedge\wedge\wedge}p < 0.001$ compared to Low; $^{@}p < 0.05$ compared to Medium; $^{@@}p < 0.01$ compared to Medium.

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 ($p < 0.01$), and midbrain and brainstem ($p < 0.05$ for both) of animals in the low-dose group. In general, however, the difference in the amount of DU accumulation in specific regions did not change significantly from 3 to 6 mo. This suggests that by 3 mo, DU deposition had either reached a maximum, protective mechanisms to prevent further metal accumulation had been activated, or that transport of the metal across the blood–brain barrier reached a steady-state equilibrium with DU uptake equaling clearance.

DISCUSSION

Depleted uranium is widely used by the US military (and others) for armor reinforcement and as a component in penetration munitions. The long-term effect of DU on soldiers and civilians is unclear. However, US veterans returning from areas where DU was used in the combat theater have, along with health care providers, expressed concern that health problems related to GWS might be the result of this heavy metal. In order to link DU to neurological and neuropsychological signs and symptoms, it is first necessary to demonstrate that DU actually crosses the blood–brain barrier (BBB) and accumulates in brain tissue. It is important to demonstrate the ability of DU to cross this barrier, as the restrictive nature of the BBB normally confers some degree of protection to the brain from the accumulation of foreign and potentially harmful toxicants.

Here, we verify that DU from pellets implanted in muscle indeed crosses the BBB and accumulates to statistically significant levels in discrete brain regions, namely the midbrain, striatum, cortex, and cerebellum. Similar to our results, others report DU accumulation in the cortex, mid-brain, and cerebellum, but not the hippocampus *(12),* although animals were not perfused prior to removing the brain and determining DU tissue levels *(12–15).* Although DU is higher in the urine of humans with imbedded DU fragments *(5,6,8,9,16),* no data have been reported concerning DU blood levels in this population. Because as much as 20% of the brain volume is accounted for by intracranial blood, it is important to determine whether previously reported uranium levels in brain tissue were the result of actual tissue accumulation or of blood DU contamination. With this in mind, we determined DU levels in the brains of animals that had first been perfused with saline.

In general, the level of DU in the NS Control and Sham groups for various brain regions agreed with previously published results *(12)* using the shrapnel model of exposure. However, our DU levels in brain regions from exposed rats are significantly lower than those previously reported. For example, our data indicate that in the high group at 3 mo, the total brain DU content is approx 10 ng / g tissue, compared to approx 30 ng / g tissue *(12).* By 6 mo, this difference is even greater in the High group: approx 12 ng / g tissue here, or approx 125 ng / g tissue *(12).* This could be the result of either of the following: (1) DU levels previously reported were higher because of residual DU from blood rather than brain tissue levels and (2) the other isotopes of U contribute significantly to the amount of metal measured by Pellmar et al. *(12),* as opposed to the accumulation of ^{238}U, measured here. The latter is possible, but unlikely because ^{234}U and ^{235}U contribute only 0.0055% and 0.720%, respectively, to the composition of U. Although DU blood levels were not measured in our study, this does not detract from the significance of our results, namely that peripherally implanted DU accumulates in the brain. Moreover, the phenomenon of heavy metal accumulation reaching a steady-state plateau is not without precedent in the literature. For example, rats treated with manganese show a maximum of a twofold to threefold accumulation of the metal in the brain, regardless of how long the treatment extends or the dose of the metal *(17–19).*

In both the Pellmar *(12)* study and ours, overall baseline DU levels, even in Sham animals with Ta pellets, are higher in the hippocampus as well as striatum and midbrain, relative to other brain regions. All three of these regions are known to be high in transferrin receptor (TfR) and the divalent metal transport 1 (DMT-1). Although the mechanism of transport for DU is unknown, some have suggested that DU is associated with the TfR *(20,21),* and both TfR and DMT-1 have been implicated in the transport of iron and manganese, as well as

other metals *(22–26).* As our instrumental analysis specifically determines ^{238}U, this strongly suggests an increased basal accumulation of DU in these discrete brain regions.

Finally, our data confirm the hypothesis that DU can cross the BBB and deposit in the brain parenchyma. Unfortunately, determining the cause(s) of GWS is compounded because of the variety of stressors and chemicals to which veterans and civilian populations are exposed during a war situation. Currently, only one study exists in the literature that examines the behavior of animals following treatment with DU. Briner and Murry *(27)* reported that male rats exposed to depleted uranium acetate in drinking water demonstrated increases in rearing and line crossing, suggesting involvement of the dopaminergic system *(28).* Much more work needs to be done in vivo to determine if behaviors in animals correlate with symptoms in human populations with DU fragments and if these symptoms are consistent with the regional DU accumulation patterns reported.

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