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MIPR NUMBER 95MM5530

TITLE: Health Risk Assessment of Embedded Depleted Uranium:
Behavior, Physiology, Histology and Biokenetic Modeling

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REPORT DATE: October 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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19980710 043

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1997	3. REPORT TYPE AND DATES COVERED Final (1 Dec 94 - 30 Sep 97)	
4. TITLE AND SUBTITLE Health Risk Assessment of Embedded Depleted Uranium: Behavior, Physiology, Histology, and Biokinetic Modeling			5. FUNDING NUMBERS 95MM5530	
6. AUTHOR(S) Terry C. Pellmar, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Armed Forces Radiobiology Research Institute Bethesda, Maryland 20889-5603			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Oct 97). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) This study assesses the health risks associated with embedded depleted uranium (DU) fragments by evaluating the behavioral, physiological and histological consequences of intramuscularly implanted DU pellets in a rodent model. Animals, distributed into 5 experimental groups [1) control (20 1-mm x 2-mm chemically inert tantalum (Ta) pellets), 2) high dose (20 1-mm x 2-mm DU pellets), 3) medium dose (10 DU and 10 Ta pellets), 4) low dose (4 DU and 16 Ta pellets) and 5) nonsurgical controls] were analyzed at the 30 day, 6 month and 12 month time points. Examination of the pellets <i>in situ</i> reveals fibrous tissue adhering to the DU but not the Ta pellets. Uranium levels are high and dose-dependent in kidney, urine, and bone. Despite high uranium levels in kidney, no renal toxicity was evident. Body weight in high- and medium-DU dose animals was significantly lower than controls. Unexpectedly, uranium was found in the brain of DU-implanted animals. No behavioral neurotoxicity was evident. However, excitability of hippocampal neurons was modified in the DU-implanted animals at 12 months. These data suggest that renal toxicity may be less of a hazard than anticipated but that cognitive deficits need to be considered. The 18-month time point will be completed this fall. A biokinetic model will be constructed from the uranium distribution data for predictions of uranium loads to body organs.				
14. SUBJECT TERMS Depleted Uranium, Toxicology, Behavior, Physiology, Histology, Biokinetic Model			15. NUMBER OF PAGES 44	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

FOREWORD

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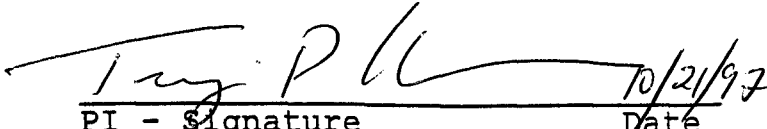
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HEALTH RISK ASSESSMENT OF EMBEDDED DEPLETED URANIUM: *BEHAVIOR, PHYSIOLOGY, HISTOLOGY AND BIOKINETIC MODELLING*

INTRODUCTION

Natural uranium consists of three isotopes: ^{238}U (99.276%), ^{235}U (0.718%) and ^{234}U (0.0056%). During the uranium enrichment process two products are produced, "enriched uranium" and "depleted uranium" (DU), that contain different relative ratios of these three isotopes. Enriched uranium contains the higher amount of the fissionable isotope ^{235}U and is used for nuclear reactor fuel and nuclear weapons. DU has a lower ^{235}U content and is a highly dense material. The DU used by the US in kinetic energy penetrators is alloyed with titanium (0.75% by weight) to retard oxidation. This DU alloy is of concern because the U.S. military currently uses this metal for munitions and armament. During Operation Desert Storm, a number of U.S. military personnel were wounded by shrapnel fragments consisting of DU^{6,7}. Since surgical removal can produce excessive tissue damage, these DU fragments were treated as conventional shrapnel and left in place in the wounded soldiers. The radiographs of injured soldiers show multiple embedded fragments ranging in size from 1 mm to over 5 mm in diameter. Fragments as large as 20 mm have been noted in other patients. Uranium bioassays taken over a year after injury indicate that uranium was present in the urine well in excess of natural background, up to 30 $\mu\text{g U/l}$ of urine. DU fragments present a radiologically and toxicologically unique situation with unknown health risks. Congress has mandated the study of these risks.

This study evaluates the consequences of both short-term and long-term exposure to DU fragments in the rat model. Using an interdisciplinary approach, we are assessing neurotoxicity, nephrotoxicity, histopathology of the tissue surrounding the fragment and pathology including evaluation of neoplastic changes in several body tissues. In addition, based on our animal data, we will develop a biokinetic model that describes the distribution of uranium from embedded fragments as a function of time.

Uranium toxicity: Although the toxicity of embedded DU is unknown, numerous studies have addressed the consequences of inhalation, ingestion and parenteral administration of other forms of uranium^{27,38,45,62}. After uranium is absorbed, it circulates in the blood as the uranyl ion forming uranium-carbonate and uranium-albumin complexes^{8,26,31}. As the uranium-carbonate complex passes through the kidney, it is filtered rapidly at the glomerulus where 60%-80% of absorbed uranium is excreted in the first 24 hours after acute exposure. The uranium not excreted is reabsorbed by the proximal tubules where it produces acute toxic effects. Uranium also enters the bone where it competes with calcium to form complexes with phosphate ions, thus becoming part of the bone matrix^{3,10,16,42}. This bone matrix then serves as a storage site from which uranium is slowly released back into circulation^{23,61}. The liver, muscle, and kidney are other major sites of uranium disposition, with a possible long-term storage mechanism in the kidney^{19,23,27,51,62}. At low doses, uranium may not readily distribute to the central nervous system (CNS)⁴⁵. With higher doses (8 mg/kg/day orally for 4 weeks), however, brain uranium levels are comparable to those in liver and in bone⁴⁵, major sites for uranium accumulation.

Acute morphological and biochemical changes of the kidney result from uranium exposure^{8,26,31,42}. The glomerular epithelial architecture is altered²⁵ and cellular necrosis occurs in the proximal tubules near the corticomedullary junction in the kidney^{2,17,18}. In addition, polyuria, enzymuria, glucosuria, and increased excretion of amino acids result^{8,9,26,63}. Acute renal failure can be the cause of death with exposure to high

doses of either soluble or insoluble forms of uranium^{43,57}. Environmental stressors such as restricted diets or changes in housing conditions significantly enhance uranium toxicity^{1,4}.

Few studies have addressed the chronic toxicity of uranium and the results available are conflicting. Galibin and colleagues¹⁴ reported severe renal toxicity in rats that inhaled the slightly soluble uranium compound, ammonium diuranate (1 or 8 mg/m³) for 128 days. Urine protein and blood, non-protein nitrogen were elevated. In the proximal tubules, there were sloughed dead cells and abnormal regenerating cells. These animals recovered, although the total number of tubules was reduced, with an accompanying increased proportion of connective tissue in the kidney. In contrast, Leach et al.^{29,30} found no renal toxicity in rats repeatedly exposed for a period of 12 months to uranium dioxide dust (5 mg/m³) (or in dogs or monkeys exposed for 5 years). Yet uranium concentrations in the kidney were as high as 1.1 µg U/g kidney wet weight in the rat (8.3 in the dog and 17.0 in the monkey), levels reported to cause acute renal toxicity (e.g.,²³). Thus the chronic effects of uranium exposure remain, for the most part, unresolved⁸.

The threshold concentration of kidney uranium levels in man that results in kidney chemical toxicity is in dispute^{8,26,52}. While the Nuclear Regulatory Commission has set the level at 3 µg/g kidney for renal damage in man, there is evidence from both human and animal reports that this level could be much lower. For example, chronically exposed uranium mill workers, whose kidney uranium levels probably did not exceed 1 µg U/g⁵⁴, showed mild renal dysfunction with increased urinary excretion of beta₂-microglobulin and various amino acids. In rats exposed subchronically to low doses (cumulative dose: 0.66 or 1.32 mg/kg) of uranyl fluoride, kidney uranium levels as low as 0.7 to 1.4 µg U/g wet kidney produced cellular and tubular necrosis of the proximal tubule, proteinuria, and enzymuria⁹. These changes in rat renal function, however, were temporary, with complete recovery within 35 days after exposure. These studies are important because they indicate that renal injury can occur at kidney uranium levels well below the 3.0 µg U/g limit.

Neurological effects have been reported with uranium exposure. In uranium workers excreting up to 200 µg U/l in their urine, normal mental function was disrupted²⁴. One case study linked the handling of a uranium bar and a subsequent increase in stool uranium with foot cramps, leg pain and abnormal gait¹⁵. With oral and subcutaneous administration of relatively high doses of uranyl acetate (210 mg/kg and 10 mg/kg, respectively), rats exhibited tremors¹¹. The uranyl ion has been demonstrated to enhance muscle contraction with acute local concentrations of 200-400 µM^{13,32}. At the neuromuscular junction in the mouse, multiple sites of action were identified, including increased duration of the muscle action potential, broadening of the compound nerve action potential, increased amplitude and quantal content of the endplate potential and increased frequency of the miniature endplate potentials³². These studies indicate that embedded DU fragments could lead to neural damage, affecting both motor and cognitive function. The CNS effects of uranium toxicity can result from secondary mechanisms since hormonal changes, electrolyte disruption and immune responses can all influence nervous system activity⁴⁷.

Local Tissue Response and Capsule Formation: Foreign bodies in tissue elicit an immune response that can result in encapsulation. Even when encapsulated, DU fragments provide a local, chronic source of alpha-radiation. Within 10-15 cells of the fragment, the dose rate is expected to be approximately 8.5 Gy/yr. This radiation could result in injury or damage to local muscle or nerve tissue (axonal injury, demyelination)^{48,58}. In addition, capsule formation around a DU fragment in close proximity to a nerve could increase the risk of compression injury to those nerves.

Encapsulation could limit the chemical toxicity of the DU fragments by decreasing the rate of release of the metal, as has been observed with lead³⁵. Encapsulation can also result in the formation of pseudocysts. Pseudocysts were formed that contained fluid with very high concentrations of soluble lead and insoluble

lead dioxide particles^{33,35} and with "black pigment...firmly adherent..." to portions of the inner wall of the capsule³³. If these cysts should rupture, the rapid release of this fluid could cause period spikes in circulating lead levels and result in acute lead toxicity 5 to 40 years after the initial injury^{33,35,59}. Similar type lesions may form around DU fragments. Intracapsular fluid may contain high concentrations of both soluble and insoluble DU. Tonry⁵⁵ demonstrated that DU disks formed both a soluble fraction and black insoluble particulates when emersed in simulated lung fluid. After a large fragment (approx. 20 mm) was removed from a U.S. soldier 17 months after he was wounded, the surgeon²⁸ noted that the fragment was encased in a fibrous capsule. When the capsule was breached, approximately 1-2 ml of a black fluid "gushed forth" from the cystic space.

DU can cause both local and systemic toxicity through a variety of mechanisms. Our study defines many of the potential sites of pathology that can result from long-term exposure to DU fragments and will provide a rationale for treatment of our wounded soldiers. The first six months of the study established the doses of DU to be used in future experiments (aim 1). This dose ranging study determined the number of DU pellets required to obtain uranium levels in the range of 0.7 to 1.4 $\mu\text{g/g}$ wet weight of kidney. This level of uranium has been reported to produce early signs of renal damage as measured by both biochemical and histopathological changes⁹ and would define the high dose in our toxicological studies. The low dose was chosen to produce no measurable acute toxicity. Subsequent experiments use the established doses to evaluate neurotoxicity, nephrotoxicity and histopathology and determine uranium distribution for biokinetic modeling.

Neurotoxicity is assessed by (a) a battery of behavioral tests to assess functional consequences and (b) conduction velocity studies in motor nerves to uncover any peripheral neuropathies. Behavioral tests have frequently been employed to detect and characterize potential neurotoxic effects in rodents and have been used extensively in animal toxicity studies⁴⁴. The neurobehavioral battery consists of (i) a functional observational battery (FOB), which is a series of tests designed to assess the neuromuscular, autonomic, and sensory integrity of the rat^{12,36,37,39,40}, (ii) an automated test of locomotor activity and (iii) the passive avoidance test used to evaluate memory. Electrophysiological experiments monitor nerve conduction velocity and integrity of the neuromuscular response. Nerve conduction velocity studies have been used clinically for many years to diagnose peripheral neuropathies and can even detect subclinical neuropathy induced by lead exposure^{20,41,49}.

Markers of renal function in the urine and plasma are used to assess nephrotoxicity. Altered creatinine clearance and proteinuria can indicate glomerular damage although tubular changes can also contribute. Increased urine content of enzymes such as lactate dehydrogenase (LDH) and N-acetyl- β -glucosaminidase (NAG) have been interpreted to reflect tubular damage⁴⁶. In addition, appearance of glucose in the urine, can indicate alterations in tubule reabsorption. These markers have demonstrated sensitivity with acute uranium nephrotoxicity^{8,9,31,63} and should indicate any toxicity that might result from long-term exposure to DU fragments.

Capsule formation and the sporadic release of pseudocyst fluid-contents can significantly influence the time course and concentration of uranium distributed through the body. The encapsulation process and pseudocyst formation is characterized at the time of euthanasia (1, 6, 12, 18 months after implantation), surrounding tissues are histologically examined and any capsular fluid is analyzed for its uranium content. In addition, tissues that are known to accumulate soluble uranium or uranium particulates (liver, bone, kidney, spleen)^{19,27,29,30,61,62} are histologically evaluated.

Although the distribution of uranium in the rat has been characterized for a variety of routes of internalization (inhalation, ingestion, and parenteral administration of soluble compounds), this information is not

available for embedded fragments. We are measuring uranium in urine, plasma, kidney, bone (tibia and skull), liver, spleen, brain, and skeletal muscle that is proximal and distal from the embedded pellets. Uranium is transported in plasma and urine and is stored in kidney and bone^{19,27,61,62}. Uranium has been detected in the liver and spleen of animals^{19,29,30} as well as in human subjects²³. The skeletal muscle is being sampled to determine the local concentrations of uranium. The brain was chosen because of the paucity of data and the need to assess whether any neurological effects observed were due to the direct or indirect interaction of uranium in the body. These data will allow a rat biokinetic model for implanted DU fragments to be developed.

METHODS

Approach: This report describes the data obtained in the first three years of a study of 325 rats which will provide toxicity data for 3 DU doses (low, medium, high) at 4 time points (1, 6, 12, 18 months). Each rat is thoroughly evaluated for changes in behavior, peripheral nerve function, CNS excitability, renal function and tissue histology including capsule formation. In addition, data on tissue uranium levels from a subgroup of rats are used to develop a biokinetic model to predict uranium distribution.

Rats are randomly assigned to 5 treatment groups: 1) rats implanted with low-dose DU, 2) rats implanted with medium-dose DU, 3) rats implanted with high-dose DU, 4) rats implanted with tantalum (Ta) to control for fragment implantation, and 5) a non-implanted sham-surgical control group. In the low-dose and medium-dose groups, Ta is substituted for a fraction of the DU pellets in order to keep the total number of implanted fragments constant. Half of the total number of pellets are implanted in each thigh.

Based on the variance of control data for neurological effects, a group size of 15 rats is necessary to see significant changes of 20% or greater at the $p < 0.05$ level. Additional animals (20 rather than 15) have been implanted for the 18-month time point with the expectation of approximately 25% natural mortality⁶⁴⁻⁶⁶. This will provide 15 animals for analysis of neurological and biochemical endpoints in all groups at all 4 time points. Five of the rats in each experimental group provide tissue for uranium quantification. At the time of euthanasia, tissues from 7 animals per group at each time point will be assessed for uranium content and the remainder will be evaluated for histopathology. Two-way analysis of variance is used to test statistical significance of any changes. Newman-Keuls test is used for multiple comparisons. In all analyses, statistical significance is accepted at the $p < 0.05$ level.

As Of October 1, 1997, we have completed the 1-, 6-, and 12-month animals. The 18-month animals are still being assessed. In some of the analyses, the total number of animals is less than the original group size of 15. Some animals have been euthanized during the course of the protocol because of pathological conditions. For a variety of reasons, some animals have been excluded from some of the assessments. These reasons include technical problems with the analytical procedures for the samples (e.g. urine or serum samples), deaths from anesthesia while recording conduction velocities, difficulties with dissection of the hippocampus for electrophysiological analysis etc. Data that were greater than two standard deviations different from the mean of the group were also excluded. This reason for exclusion was unusual and only accounts for an occasional data point. Under all circumstances, the number of samples remaining in the groups was sufficient to provide confidence in the statistical analysis.

Subjects: Sprague-Dawley rats (8-10 weeks of age) are maintained in an AAALAC-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23). Upon arrival, rats are quarantined and screened for diseases. Except during urine collection, all animals are

housed in plastic microisolator rat cages with hardwood chips as bedding. Commercial rodent chow and water are provided *ad libitum*. Rats are on a 12-hr light/dark cycle.

Fragments: DU fragments, consisting of 99.25% DU and 0.75% titanium by weight, were obtained from Oak Ridge National Laboratories, Oak Ridge, TN. The uranium isotopes present is ^{238}U (99.75%), ^{235}U (0.20%) and trace levels of ^{234}U . This is the same DU alloy used in U.S. military munitions. Tantalum (Ta) fragments were obtained from Alfa Products, Ward Hill, MA. Ta was chosen as the control substance because it is a biologically inert metal²² with a similar mass to uranium and is frequently used in human prostheses^{21,53}. Each fragment (both DU and Ta) is approximately 1 mm diameter x 2 mm long.

Experimental groups: Rats were divided into five experimental groups: (1) non-surgical controls (2) Ta controls (3) high dose of DU (4) medium dose of DU and (5) low dose of DU. The preliminary dose ranging study determined that 20 DU pellets produced urine uranium levels of $262 \pm 99 \mu\text{g U/l}$ and kidney levels of $1.22 \pm .31 \mu\text{g/g}$ after two weeks of exposure. This dose was chosen as the high dose because it was well tolerated by the rats but expected to produce kidney toxicity. Consequently, all surgically implanted animals have a total of 20 pellets of either Ta or DU or a mixture. Low dose DU rats are implanted with 4 DU pellets and 16 Ta pellets. Medium dose DU rats are implanted with 10 DU and 10 Ta pellets.

Surgery: The DU and Ta pellets are cleaned and chemically sterilized prior to implantation. The pellets are immersed in industrial detergent, rinsed in absolute alcohol, soaked in 50% nitric acid solution for 3 min and then rinsed with water. This procedure completely removes the oxide formation on the surface of the DU pellet⁵⁵. Anesthesia is induced with ketamine hydrochloride (80 mg/kg) in combination with xylazine hydrochloride (4 mg/kg), given i.p.

Fragments are implanted within the gastrocnemius muscle spaced approximately 8-10 mm apart on the lateral side of each leg. The surgical sites are shaved and cleansed with betadine, a topical disinfectant, prior to surgery. Scalpel incisions are made through the skin and pellets are inserted into the muscle with a trochar (16-gauge needle with plunger). Incisions are closed with absorbable sutures and surgical cement.

Rats are closely monitored following surgery until they are ambulatory and an analgesic (Demerol, 10 mg/kg, i.m.) is administered if needed. A veterinarian regularly examines the surgery sites for signs of inflammation, infection and local DU toxicity.

Behavioral neurotoxicity: The functional observational battery (FOB) consists of behavioral evaluations (home-cage, handling and manipulative) and several physiological measures. The parameters to be recorded are listed below and grouped according to the following functional domains: 1) Autonomic: lacrimation, salivation, palpebral closure, piloerection, defecation, urination, 2) Sensorimotor reactivity: tail pinch response, tactile response, click response, approach response; 3) Neuromuscular: gait, foot splay, forelimb and hindlimb grip strength, righting reflex, and 4) CNS Excitability: arousal, posture, ease of removal from cage, handling reactivity, convulsions, and locomotor activity.

The observer is blind as to the identity of each group. The behavioral battery commences with brief home cage observations during which time the observer describes the posture, and the existence of tremors or convulsions, and palpebral closure. The rats are then removed from their cage and rated for ease of removing and handling. While handling the rat, presence of piloerection and the degree of lacrimation and salivation are observed. The animals are then placed in an open-field with a perimeter barrier on clean absorbent white paper for 3 min. The number of rears, the gait, level of alertness, stereotypy (repetitive movements e.g., head weaving), unusual behaviors (e.g., writhing), and the number of fecal boli and urine pools are recorded.

Sensorimotor responses also are determined and include: approach response to a blunt probe, touch on the rump (tactile response), click response (auditory response), and pinch on the tail using forceps. Next, neuromuscular responses are determined and include: righting reflex, forelimb and hindlimb grip strength using digital strain gauges³⁷, and landing foot splay¹². The animals are weighed and rectal temperature determined using a digital thermometer. The FOB is conducted during the light portion of the light-dark cycle. Details of the FOB tests can be found in Moser et al.⁴⁰ and McDaniel and Moser³⁶.

Approximately, 1 hr after the FOB, the rats are monitored for horizontal and vertical locomotor behavior. Motor activity is recorded for 1 hr using automated photocell activity cages (Digiscan Analyzer, Omnitech Electronics, Columbus, OH). On the day following the FOB and motor activity tests, animals are trained on a passive avoidance test. This test is used to determine whether DU affects memory function. The tests are conducted in a passive avoidance apparatus (San Diego Instruments, San Diego, CA) that consists of two chambers (one lighted, one darkened) separated by a sliding door. The animal receives a training trial during which time it is initially placed into the lighted chamber. The natural tendency is for the rat to enter the darkened chamber. When it does, it receives a mild foot shock. During this acquisition phase, the rats are tested for eight trials or until criterion is met. The criterion is two consecutive trials during which the rat does not cross into the darkened chamber. Each trial is 3 min in duration with a 1 min intertrial interval. Seventy-two hours later the rat is placed into the lighted chamber and retested. A comparison is made with the initial training session to see if memory of the task has been retained.

Conduction velocities: One week following the behavioral testing, the rats are evaluated electrophysiologically. Rats are anesthetized with ketamine (80 mg/kg) with xylazine hydrochloride (4 mg/kg) i.m. (supplemented as necessary). The right sciatic nerve is exposed and bipolar stimulating electrodes are positioned along the nerve in the thigh close to the sciatic notch and in a second location close to the knee.

A recording electrode is inserted into the medial gastrocnemius muscle to monitor the compound muscle action potential. Nerve temperature is monitored and maintained near 37 °C with a heat lamp. Nerves are stimulated at a frequency of 0.2 Hz. Stimulus intensity is varied between approximately 10 and 100 V (0.1 ms duration) to determine the input-output relationship and the supramaximal stimulation parameters to use. Five muscle responses are averaged and the latency, duration and amplitude of the potentials are measured. Conduction velocities are calculated by dividing the distance between the stimulating electrodes by the average latency difference between the time of onset of the compound muscle action potentials.

Duration of the muscle action potential reflects the synchrony of discharge. In general, the distal stimulating electrode will produce a faster, larger response than the proximal electrode. Greater dispersion and greater decrease in amplitude than normal would suggest nerve damage. For example, demyelinating disorders cause dispersion of the muscle action potential by slowing the nerve conduction velocities^{5,50}. If dispersion occurs over a short segment, compression neuropathy may be indicated⁵.

All stimulation and recording are controlled by a 486 PC using standard electrophysiological software (Axon Instruments). Data are analyzed with routines written in AxoBasic (Axon Instruments) and statistical analysis is done with RS/1 (BBN Software Products) routines. Two-way analysis of variance (for time and dose) is used to compare differences among the experimental groups.

Hippocampal slice electrophysiology: At the termination of the conduction velocity experiment, the rat is euthanized by decapitation. The brain is quickly removed from the skull and submerged in iced oxygenated artificial cerebrospinal fluid (ACSF). Both hippocampi are dissected out and sliced on a McIlwain tissue chopper (425 μ m thick). Tissue is incubated at room temperature in oxygenated ACSF (see below) for 1 hr to allow recovery from the slicing procedure. During this interval, tissues are isolated from the rats for analysis of histopathology and DU content.

A single slice of rat hippocampus is then be placed in a submerged slice chamber and perfused at a rate of 1-2 ml/min with warmed (30 °C) oxygenated ACSF. ACSF has the following composition (in mM) 124 NaCl, 3 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 10 glucose, 26 NaHCO₃, pH 7.4, equilibrated with 95% O₂/5% CO₂. Extracellular recordings are obtained with glass microelectrodes filled with 2 M NaCl placed in s. radiatum and s. pyramidale of field CA1 to record the population synaptic potential (pPSP) and the population spike (PS) respectively. A stainless steel, concentric, bipolar stimulating electrode is positioned in s. radiatum of field CA1 to activate afferents. Constant current stimuli (0.1 - 1.5 mA, 300µsec) are applied at a frequency of 0.2 Hz. Except when generating input/output (I/O) curves, the stimulus current is held constant at an amplitude that elicits approximately 30% maximal response.

To obtain I/O curves, stimulus intensity is varied from approximately 0.1 to 1.5 mA in 13 steps. Three responses at each current step are recorded and averaged. I/O curves are generated following a 30-min equilibration period. The three I/O curves (stimulus vs PS, stimulus vs pPSP, pPSP vs PS) are analyzed with the data analysis software RS1 (BBN Software Products, Cambridge MA). The responses at each stimulus intensity are averaged for all experiments at each time point. A sigmoid curve is computer fit to the points. Differences between curves are tested for significance by comparing the residual sum of squares for the curve fit to the data of each experimental condition with the residual sum of squares for the curve fit to all the data. Significance is accepted at $p < 0.05$.

Sample collection: Following behavioral testing, blood and urine samples are obtained from all rats for analysis of renal function. To safely collect the blood samples, rats are immobilized by placing them in a Plexiglas restrainer. During each collection, 0.3-0.5 ml of blood is obtained from the tail vein using a 22-gauge needle. The blood is then centrifuged for 5 min at 3,000 X g. The serum is analyzed for uranium levels and/or for biochemical indices of renal function. Serum is stored at -70 °C until ready for analysis.

Urine samples are collected by housing the rats in individual metabolism cages (23.5 cm diameter X 12 cm high) where they have continuous access to food and water. However, since these housing procedures have been shown to induce stress and thus increase the toxicity of uranium⁴, the rats are acclimated to the metabolic cages for 5 days before the study begins. The metabolic cages are disinfected and decontaminated between each animal use. The 24-hr urine collection sample is obtained from each rat and the volume recorded (10-20 ml). Urine collection at 4 °C is unnecessary since enzyme activity has been shown to be stable at room temperature for up to 24 hours⁶³. After collection, urine is filtered to remove any debris and stored in plastic containers at 4°C until analyzed (less than one week).

Evaluation of renal function: Measurement of urine volume and osmolarity, urine levels of NAG, LDH, glucose, total protein, creatinine and blood levels of glucose, urea and creatinine are used as indicators of renal function. In addition, since weight loss may be indicative of nephrotoxicity, all the rats are weighed weekly throughout the study. Osmolarity of the urine is measured with a vapor pressure osmometer (Model 5100B, Wescor, Inc.). A Kodak Ektachem 700 Analyzer is used to determine plasma and urine levels of creatinine, glucose and urea. Total urine protein is measured with a dye-binding assay (Coomassie Blue, BioRad) sensitive down to 1 µg. The activity of NAG is measured by the methods of Tucker et al.⁵⁶ using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as the fluorescent substrate (excitation wavelength=356 nm; emission wavelength=446 nm). The dilution of the urine for this assay eliminates the effects of any inhibitors present⁵⁶. For LDH measurements, 1 ml of urine is dialyzed for 4 hr at 4 °C with 1 liter of deionized water. LDH is quantitated with a colorimetric assay that measures a reaction product, which is proportionate to LDH activity (Oxford Biomedical Research Inc). Only 50-100 µl of fluid (urine or plasma) are required for each of these assays.

Although, urine volume and osmolarity can vary greatly with fluid intake, these measures provide physical

indicators of renal function. For example, acute kidney failure drastically decreases urine volume, while moderate renal toxicity can increase urine output, as is seen with uranium exposure (e.g., ¹¹). Osmolarity can reflect the ability of the kidney to concentrate (or dilute) the urine. Plasma urea also changes with renal insufficiency. Since the rate of urea formation is proportionate to the rate of protein metabolism, other factors such as hepatic injury or altered protein intake can affect the measured urea in plasma. A small concentration of protein is normally present in the urine. Increases in total urine protein could result either from glomerular leakage or failure of tubule reabsorption. Urinary enzymes are sensitive, non-invasive markers of toxicity primarily in the kidney tubules⁴⁶. NAG is a lysosomal enzyme found in proximal renal tubule cells. LDH is a cytosolic enzyme of the tubular epithelium.

Creatinine clearance is a commonly used measure of glomerular filtration rate in the rat despite a significant but constant tubular secretion. The use of an intrinsic metabolite has an obvious advantage over inulin or mannitol, which (although not secreted) must be infused. Interpretation must be cautious since tubular injury with uranium could cause an underestimate of the glomerular filtration rate regardless of the marker used⁸. Creatinine clearance (C_c) is calculated from the equation: $C_c = U_c \cdot V_u / P_c$ where U_c and P_c are the creatinine concentrations in urine and plasma, respectively, and V_u is the rate of urine production (ml/min).

Appearance of glucose in the urine occurs when the tubule reabsorption maximum from the filtrate is exceeded. This can occur with hyperglycemia or with a decrease in tubular reabsorption capacity. Measurement of both urine and plasma glucose help to distinguish between these two possibilities. Changes in reabsorption is reflected in the calculated fractional excretion (FE): $FE = (U_g / P_g) (U_c / P_c)$; where U_g and P_g are the glucose concentrations in urine and plasma, respectively.

The proposed assays provide a broad spectrum of measures of kidney toxicity. Many of these substances have been shown to be very sensitive in acute uranium toxicity^{8,31}. Glucose is one of the most sensitive indicators^{8,9} showing increased urine glucose, without concurrent increases in plasma. LDH and to a lesser extent NAG increase following uranium exposure^{8,31}. A transient increase in urine volume and the appearance of protein in the urine also occur with acute uranium toxicity³¹. These measures are used together as indicators of kidney toxicity and carefully interpreted and correlated with histopathology. Two-way ANOVA is used to test the statistical significance of any changes.

Histopathology. Immediately following euthanasia on the day of electrophysiological analysis, tissue samples from bone (tibia, skull), hippocampus, sciatic nerve, kidney, liver, spleen and fragment capsule with associated skeletal muscle is obtained for histological examination or uranium measurement. Based on the literature, these are the most likely tissues to show increased levels of uranium^{19,27,29,30,61,62}. Standard procedures for handling biologic specimens are used in the preparation of the samples. Tissues are perfused, embedded, mounted and stained with hematoxylin and eosin stain (H & E)³⁴. Specialized stains are used to demonstrate specific lesions or further delineate lesions not well defined by the H & E stain. For example, silver stains are used on neural tissue to delineate nerve fiber disruption or degeneration³⁴.

The pathologist evaluating the tissue is blind to the experimental group from which the tissue was obtained. The pathologist generates a 0 to 4 scoring system to evaluate the degree of microscopic changes observed; where 0=no change, 1=minimal change, 2=mild change, 3=moderate change, and 4=marked or severe change. All tissue changes observed in the rats implanted with DU are contrasted and compared to the identical tissues taken from the controls. If there are significant changes noted in a particular system, for example the renal system, a detailed statement of criteria for 0-4 scores is stated by the pathologist at the time of interpretation.

Uranium measurement Tissue samples are frozen and shipped by overnight courier on dry ice to

Battelle, Pacific Northwest Laboratories for analysis of uranium content. The samples are stored at -70° C until the wet ashing procedure. Wet ashing consists of 12 cycles of treatment of the samples (over 3 days) with 2 ml of 16 N nitric acid followed by several hours of heating, brief cooling, addition of 0.5 ml of 30% hydrogen peroxide and reduction of the volume to approximately 0.5 ml. After this, samples are heated to dryness, dissolved in 2 ml of 4 M nitric acid with warming and filtered through 0.45 μ m syringe filter units. For analysis, 0.5 ml of sample or identically handled standards are dissolved in 2 ml of Uraplex reagent. The samples are analyzed with a Kinetic Phosphorescence Analyzer (KPA-11, Chemchek Instruments Inc, Richland WA). Background measurements are made using 4 M nitric acid. Calibration curves are established prior to sample analysis. Measurements include analysis of relative standard deviations and correlation coefficients of the luminescence decay curve.

RESULTS

Uranium distribution: Tissues and fluids from five rats of each of the five experimental groups for the 1-day, 30-day, 6-month and 12 month time points have been analyzed for uranium content by Battelle Pacific Northwest Laboratories. Urine was not collected from the rats at the 1-day time point because the complications introduced by the surgery and the procedures for the collection of the fluid. At all time points uranium distributed primarily to bone and kidney in a dose-dependent manner (Figures 1, 2, 3, 4). Even at day one, uranium was present in these tissues (Figure 1). By day 30, the levels in kidney and bone had increased and uranium was evident in the urine (Figure 2). In addition, there was evidence of uranium distributing to the spleen and to the brain of the high dose DU animals. At 6 months, the levels, for the most part, continued to rise (Figures 3). By 12 months after implantation of the pellets, levels in the kidney appeared to level off while levels in tibia and bone continued to rise (Figure 4,5). Levels in the brain rose through the 6-month time point (Figure 5). At 12 months, the sample of brain tissue was changed and the concentrations are not likely to be comparable. At the earlier time points, an entire hemisphere was sampled. At 12 months, only the cortex was measured. An analysis of brain regions was done on a different series of animals and a dose dependent distribution of uranium to cerebellum, frontal cortex and midbrain was observed. Hippocampal levels of uranium were very high but the uncertainty in the data was large because of the small sample size. It was not possible from these data to calculate the levels that would be present in the entire hemisphere to compare with earlier time points. Distribution to the spleen in the high dose and medium dose DU animals had leveled out around 60 $\mu\text{g/gr}$ but for the low dose animals were much below that at 12 months (17 $\mu\text{g/gr}$) (data not shown). Levels in the liver were comparable to controls at the 30-day time point but increased slightly at 6 months and had not substantially changed at 12 months (data not shown). Muscle levels were, in general, quite variable. Some of the muscle samples that were in close proximity to the DU pellets showed exceptionally high levels of uranium. It is our belief that these high levels resulted directly from fragments of the implanted pellets in the analyzed samples. This "contamination" could have occurred during the removal of the pellets at time of necropsy or might have happened by flaking and redistribution *in vivo*. Further analyses are expected to clarify this issue. Even though kidney levels have appeared to level out by 12 months, the urine levels of uranium continue to increase. At 12 months the levels were: 2.98 ± 0.76 ng/ml in Ta controls, 224.30 ± 32.11 ng/ml in 4 DU rats, 330.14 ± 48.03 ng/ml in 10 DU rats and 1008.71 ± 86.92 ng/ml in 20 DU rats (Figure 6)

Histopathology: Tissues have been excised and fixed for histopathological analysis. These tissues (bone: tibia and skull, kidney, spleen, liver, brain, and muscle: proximal and distal). No histological evidence of toxicity has been observed. There is no evidence of cellular necrosis, inflammation or fibrosis. During excision of the pellets, it was observed that the DU pellets but not the Ta pellets were associated with adherent tissue. Even in the 12-month animals, a capsule had not fully formed around the pellets and dark fluids were not observed near the fragments.

Nephrotoxicity: The urine and serum samples have been analyzed for biochemical markers of kidney toxicity. Osmolarity, 24-hour volume, pH and urine levels of glucose, protein, NAG, LDH, urea nitrogen, serum glucose and serum urea nitrogen were not significantly altered at either the 30-day, 6-month or 12 month time points. The data for urine osmolarity, volume, glucose, protein, LDH and NAG are shown in Figures 7-9. Creatinine clearance was not significantly different among the experimental groups (Figure 10). Fractional excretion (FE) of glucose (glucose clearance/creatinine clearance) was similarly not significantly affected by the experimental procedures. All groups showed a FE between 0.0008 and 0.0038 and the differences between groups were not statistically significant at any time point ($p > 0.2$) (Figure 10).

Neurotoxicity: Animals were evaluated for body weight and for changes in the functional observation battery (FOB), locomotor activity, and passive avoidance learning. The rats were weighed weekly and all steadily gained weight. Animals in the high and medium DU dose groups have shown significant differences in the body weight compared to Ta controls at a number of time points (Figure 11) ($p < 0.05$). The low DU dose group did not show any significant differences in body weight compared with the controls.

The FOB did not reveal any significant differences among the experimental groups. No significant differences were observed in body temperature at 30 days, 6 months or 12 months. Sensorimotor, neuromotor and autonomic responses as well as locomotor activity showed no significant differences across the experimental groups at either time point. Grip strength of the hind- and forelimbs was not significantly altered by DU exposure (Figure 12). Conduction velocity measurements from the nerves of the hindlimb also did not reveal any differences among the experimental groups at either time point (Figure 13).

Passive avoidance was used as a measure of learning ability. Any rats that failed to cross to the darkened compartment were dropped from the study. As a consequence, the number of subjects reported for this endpoint is less than the number of animals in the study. For all time points (30 days, 6 months and 12 months), there were no significant differences among the five experimental groups for their performance on the passive avoidance test ($N=7-10$ /group at 30 days; $N=4-9$ /group at 6 months; $N=8-15$ /group at 12 months). All animals learned to avoid the mild foot shock within 2-3 trials. The latency to initial crossover was also not significantly different among groups (Figure 14).

Because of the observed distribution of uranium to the brain only in the high DU animals at 30-days, hippocampal electrophysiology was assessed at 6 and 12 months. Only the high DU dose group was assessed at 6 months, while at 12 months all of the dose groups were evaluated. At 6 months, the population spike in the hippocampus of the 20-DU implanted animals was significantly smaller than that in the controls (Figure 15, circles DU, squares control). In contrast, the differences in the size of the synaptic potentials were not statistically significant (Figure 16). The input-output relationship reflecting the ability of the synaptic potential to elicit the population spike (called E/S coupling)(Figure 17) indicated that in the high-dose DU animals this process was significantly impaired ($p < 0.05$). At 12 months, all of the DU groups showed significant differences from control. The data are shown (Figures 18-20) for the high dose group to allow comparison with the 6-month data. Unlike at 6 months the population spike was unaffected by the presence of DU (Figure 18). On the other hand, the synaptic potentials were significantly enhanced (Figure 19). E/S coupling was significantly impaired (Figure 20). This was statistically significant for the low, medium and high dose groups at 12 months (low and medium dose data not shown).

Miscellaneous observations:

In the course of this study a total of 40 animals to date have been euthanized or have died due to pathological conditions causing the loss of some data. Of these 3 were in the 6-month time point, 6 were in the 12-month time point and 31 were in the 18-month time point. Because of the natural life span of these rats, we expected 25% mortality by the 18-month time point.

The animals showed a variety of conditions that contributed to their deaths. Six rats had problems with their teeth. Their upper teeth broke off and the lower teeth began to grow abnormally. All of these rats had DU pellets implanted (one low dose, two medium dose and two high dose before 12 months and another low dose at 15 months after implantation). These animals had difficulty eating, showed weight

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loss and were euthanized.

A number of animals were euthanized for extreme weight loss with no apparent disease (3 Ta-controls, 1 non-surgical control, 2 low dose). Many additional animals died without obvious pathology. Necropsy reports indicated abnormalities normally associated with aging and obesity. Four of the rats died from cardiac insufficiency. These were evenly distributed among the experimental groups (1 high, 1 medium, 1 low and 1 Ta control). Three animals showed paralysis of the hind limbs. Two of these animals were in the medium dose group while one was a non-surgical control. Two rats were euthanized because of labored breathing (1 high, 1 Ta control). One rat (non-surgical control) had a confirmed lymphosarcoma. One rat had gall bladder stones (low dose). One rat had blood stool (Ta control). One animal was euthanized because of a hematoma in the abdomen (high dose). A hematoma was found upon termination of the experiment in another rat as well (medium dose). Three rats were euthanized because of their extensive skin lesions that did not respond to topical antibiotics (1 medium dose, 1 Ta-control, 1 non-surgical control). Five more animals (2 non-surgical, 1 low, 1 medium, and 1 high) were observed to have sores or cysts upon termination of the experiment. Tumors were the reason for euthanasia of two animals (one medium, one Ta control). These were large growths found on the shoulder in one rat and in the right leg of another rat. Tumors were observed in three additional animals including one rat that showed mammary tumors at 9 months of age (low dose). Of the animals that died in their cages, two were non-surgical controls (average age 13 months); five were Ta-controls (average age 16 months); nine were low dose group (average age 15 months); two were medium dose group (average age 19 months); and three were high dose group (average age 15 months).

CONCLUSIONS

The data presented here describe the distribution of uranium from intramuscularly embedded pellets to body tissues over six months and characterize the physiological and behavioral consequences of this exposure. The most striking findings of this study to date are 1) the absence of measurable nephrotoxicity despite very high levels of uranium in the kidney and urine and 2) the distribution of uranium to the brain and the changes in neuronal excitability in the hippocampus.

Uranium levels in the kidney of the experimental animals implanted with DU exceed the levels considered to be toxic in both animal models and man. The medium and high dose groups averaged kidney levels greater than 3 $\mu\text{g/g}$, the level set by the Nuclear Regulatory Commission for renal damage. At 12 months, the high, medium and low doses of DU pellets resulted in 5.1 ± 0.3 , 3.3 ± 0.2 and 1.5 ± 0.3 $\mu\text{g U/g kidney}$, respectively. At 6 months uranium levels were 6.9 ± 1.7 , 4.7 ± 0.8 and 2.3 ± 0.6 $\mu\text{g U/g kidney}$, respectively. The uranium levels in the kidney appear to have stabilized after 6 months. In contrast, the concentrations of uranium in the urine have continued to increase. Urine levels of uranium in all of the dose groups at 12 months exceed that found in Desert Storm veterans (Keogh, personal communication). At 6 months the rats in the low dose group still showed uranium (46 ± 13 $\mu\text{g/l}$) comparable to that population. Despite these high levels, our data do not demonstrate any statistically significant signs of nephrotoxicity. At 12 months, LDH and glucose reveal hints of a DU-dependent effect, but this was not statistically significant and could be random variation within the population. The data from the 18-month animals will be carefully assessed for a continuation of this trend.

Chemical form, route of administration, and the dose of uranium exposure can all affect the toxicological consequences and distribution of uranium. It is possible that chronic exposure to uranium allows tolerance to higher concentrations of the metal. The studies of Leach et al.^{29,30} demonstrate an absence of renal toxicity in rats following chronic inhalation exposure to uranium dioxide producing kidney levels up to 1.1 $\mu\text{g/g}$. In contrast, Diamond et al.⁹ observed acute, but reversible, renal toxicity in rats at levels as low as 0.7 $\mu\text{g/g}$ following i.v. injection of uranyl fluoride. The absence of nephrotoxic effects in our present study does not preclude the possibility that with longer exposures to the uranium, toxicity will develop.

At the 30-day time point, uranium was observed to distribute to the brain in the high DU dose animals. This was in agreement with the literature⁴⁵ in which uranium did not accumulate in the brain at the lower doses of DU. At 6 months, however, substantial amounts of uranium are accumulating in the central nervous system. Although behavioral measures did not reveal any adverse effects of this uranium, electrophysiological assessment of hippocampal neuronal activity demonstrated that excitability was impaired. It is possible that the electrophysiological changes are too subtle to produce a behavioral manifestation. Alternatively, it is possible that the behavioral tests we have performed are not sufficiently sensitive to reveal an existing cognitive deficit. It also needs to be emphasized that these animals had levels of uranium in their urine that significantly exceeded the levels found in any of the veterans.

The electrophysiological data suggest that neural function of the hippocampus is disrupted by exposure to DU and that these effects adapt over time. At 6 months, the ability of the action potential to elicit a synaptic potential (E/S coupling) was significantly reduced. As a consequence of this effect, the population spike (reflecting the number of neurons in a population firing an action potential) was also reduced. In contrast, the synaptic potential of the population was not effected. The average amplitude of the elicited excitatory postsynaptic potential did not appear to be altered in DU implanted animals at 6 months. At 12

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months, E/S coupling continued to be reduced. However, at this time the synaptic potential had significantly increased. Now, despite the impaired ability of the synaptic potential to elicit an action potential, the population spike was not different from controls. These results suggest the possibility that the neural tissue has adapted to the DU-induced decrease in E/S coupling by enhancing the synaptic potential to maintain the normal output of the hippocampus. It is possible that because of this adaptation, behavioral function is conserved. Alternatively, the electrophysiological changes may have complex implications for the overall function of this area of the brain.

Bone, like kidney, is well accepted as a primary reservoir of uranium. Unlike kidney, uranium appears to be continuing to accumulate in the tibia and skull of the DU embedded rats. At 6 months, it had appeared as if the uranium concentration in skull had saturated. Yet, at 12 months, the levels have continued to rise. Uranium in the tibia has progressively increased over the course of this study. It would seem that both bone growth and bone maintenance provides the opportunity for incorporation of uranium into the bone matrix.

Other organs accumulate the uranium to varying degrees. At thirty days, concentrations in the liver were not above background while concentrations in the spleen and muscle were significantly higher. At 6 months levels in the liver had become statistically significant but were still exceeded by levels in the spleen. At 12 months, levels in spleen and liver appear to have leveled off. Muscle levels raise the possibility that neuromuscular deficits will develop through heavy metal effects. Spleen levels cause concern that immunological consequences could arise.

The significant changes in body weight with exposure to embedded DU also may be a cause for concern. It is uncertain, based on the data in the present study, if the changes are due to decreased intake of food, increased excretion of nutrients, or altered hormonal or metabolic status. These issues should be addressed in future studies.

Although histological analysis of tissues from animals in this study revealed no abnormalities apart from normal aging, Dr. Alexandra Miller has observed increases in oncogenes in these same animals (Miller, personal communication). Miller has collected samples of muscle and kidney from the rats implanted with DU. She has observed a time- and dose- dependent increase the expression of oncogenes in these tissues and is continuing to study the carcinogenic potential of depleted uranium.

This report only includes assessment of DU toxicity through the 12-month time point. The data from the 18-month animals are still being collected. By the end of November 1997, we will have sacrificed all of the experimental animals. We are providing the uranium distribution data to our collaborator Dr. Richard Leggett at Oak Ridge. He is working on the biokinetic model that will be usable for clinical prediction of uranium loads to body organs based on urine uranium levels. By May 1998, this project should be complete.

Our studies demonstrate the potential health hazards associated with exposure to depleted uranium shrapnel. The data suggest that chronic exposure to the uranium may not be as toxic to the kidneys as had been anticipated from acute exposure studies. In contrast, the distribution of uranium to the brain and the electrophysiological changes that result raise serious concerns about cognitive deficits that may increase over time. Furthermore, the distribution of uranium to the spleen may suggest potential immunological effects. Since the use of depleted uranium armaments is expanding, it is increasingly important to be aware of health risks associated with exposure in order to formulate an appropriate protocol for handling casualties with DU shrapnel.

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APPENDIX

Figure 1
Uranium Distribution in Rat Tissue -
1 day post DU Pellet Implantation

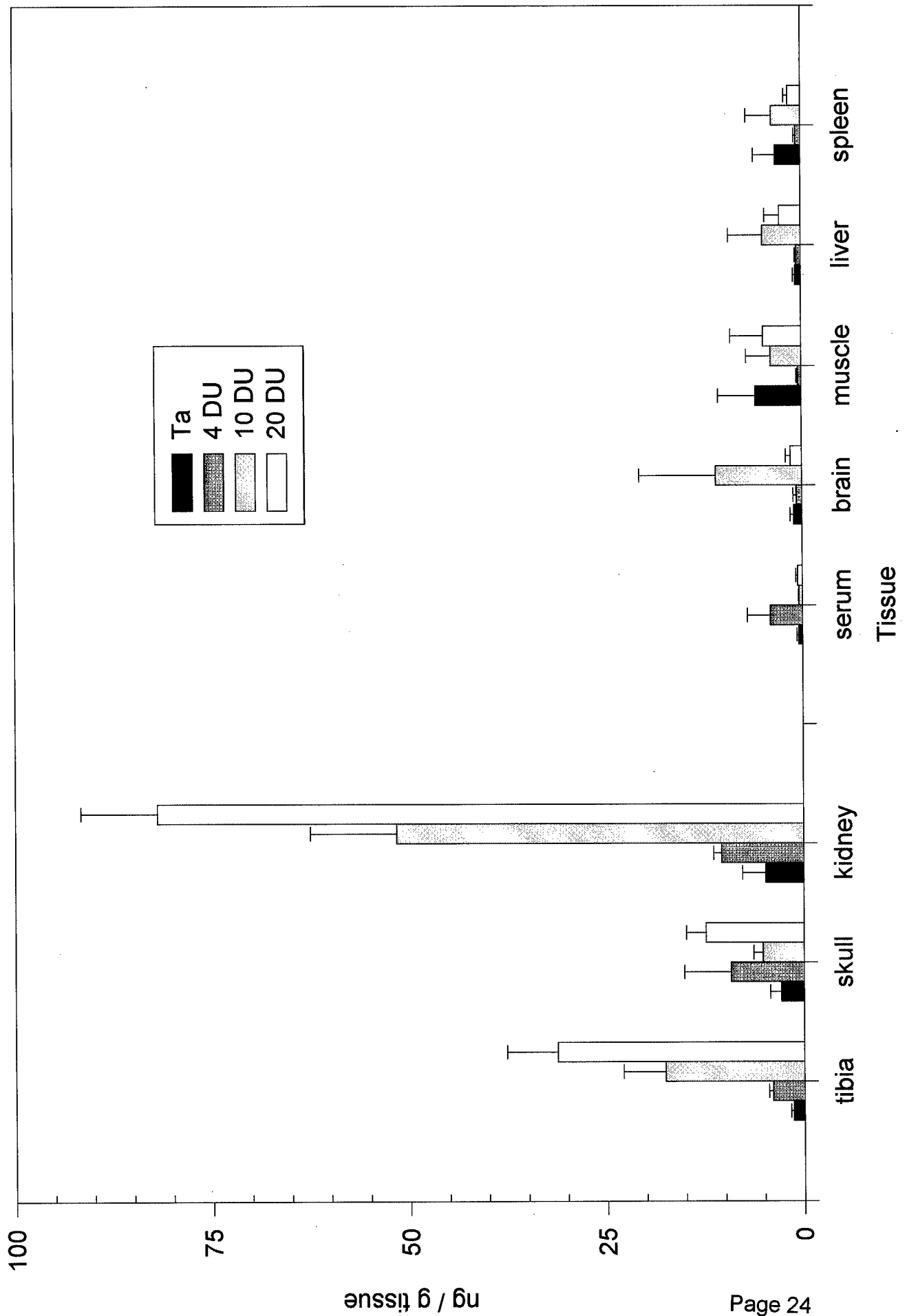


Figure 2
Uranium Distribution in Rat Tissue -
30 days post DU Pellet Implantation

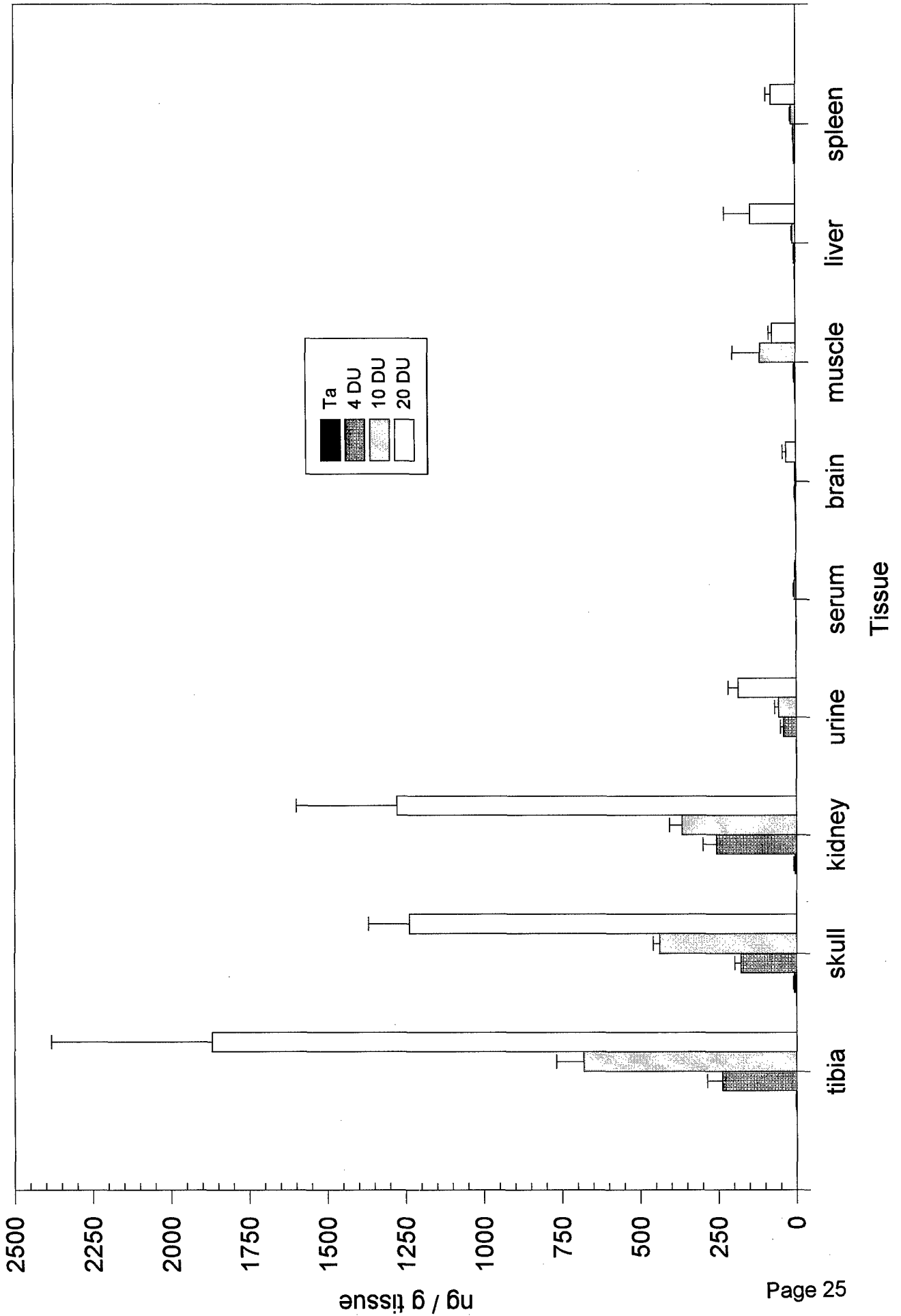


Figure 3 **Uranium Distribution in Rat Tissue -**
6 Months post DU Pellet Implantation

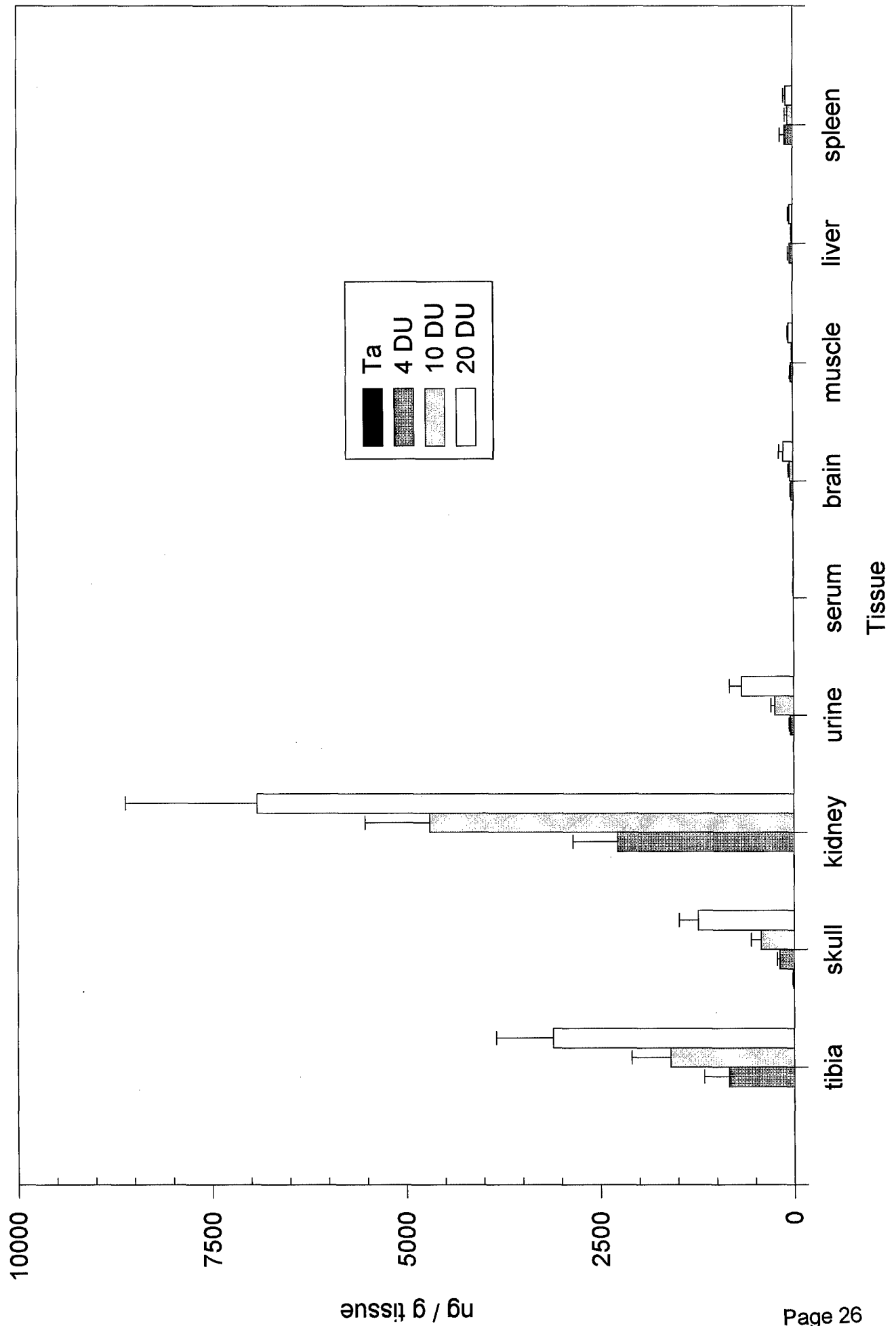


Figure 4

Uranium Distribution in Rat Tissue -
12 Months post DU Pellet Implantation

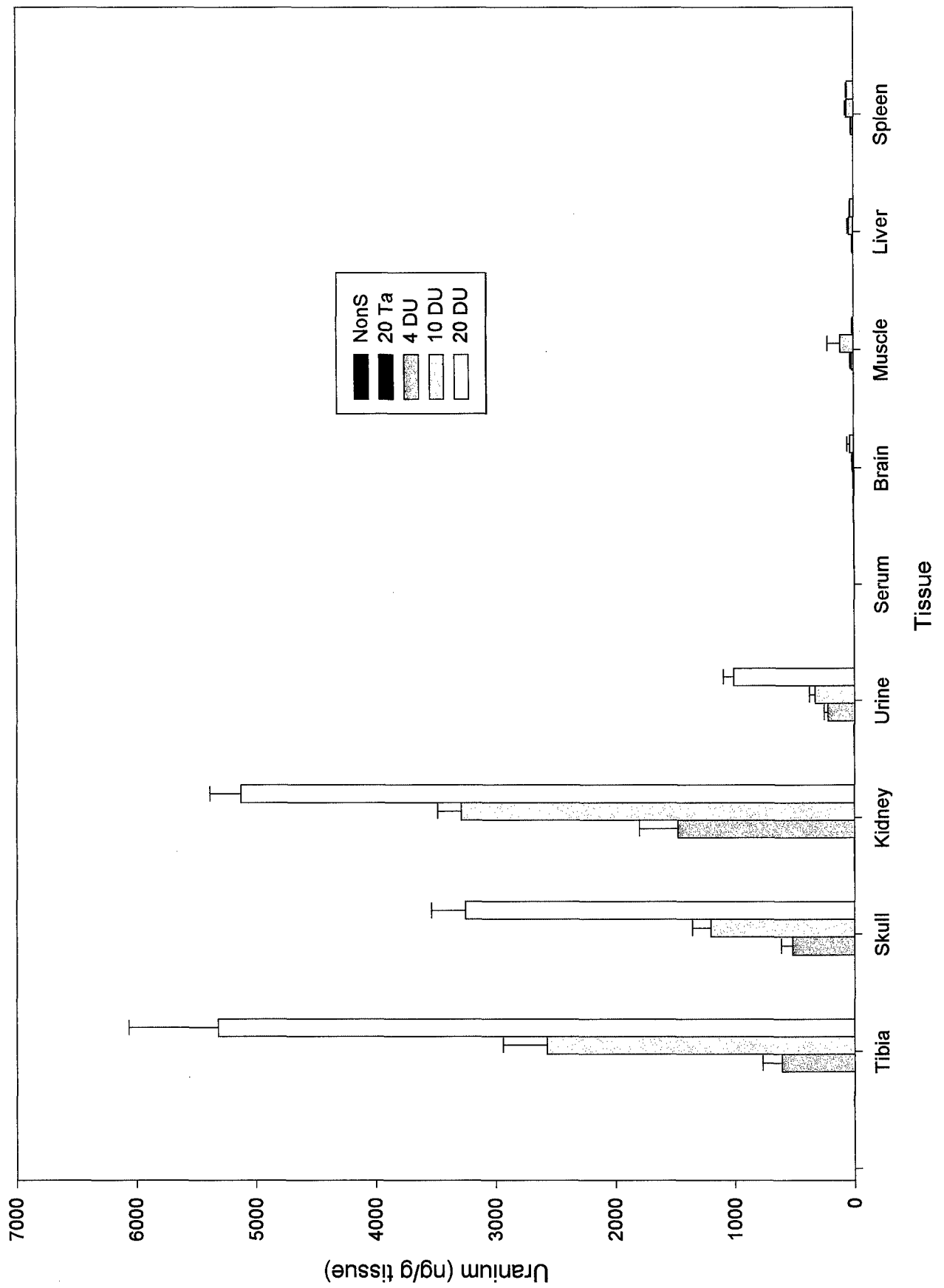


Figure 5

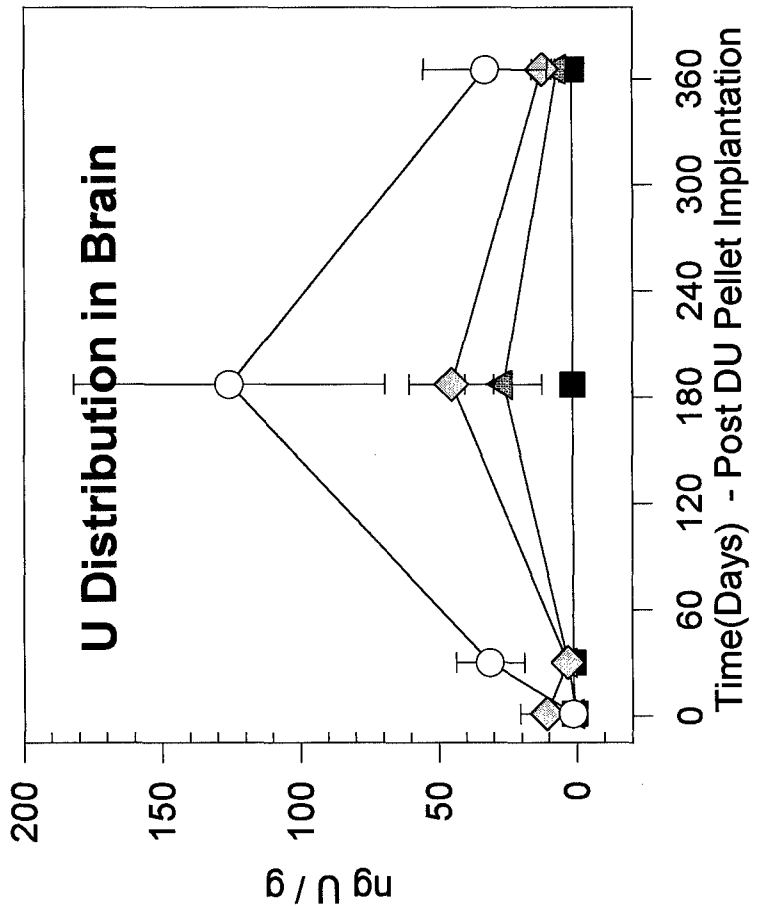
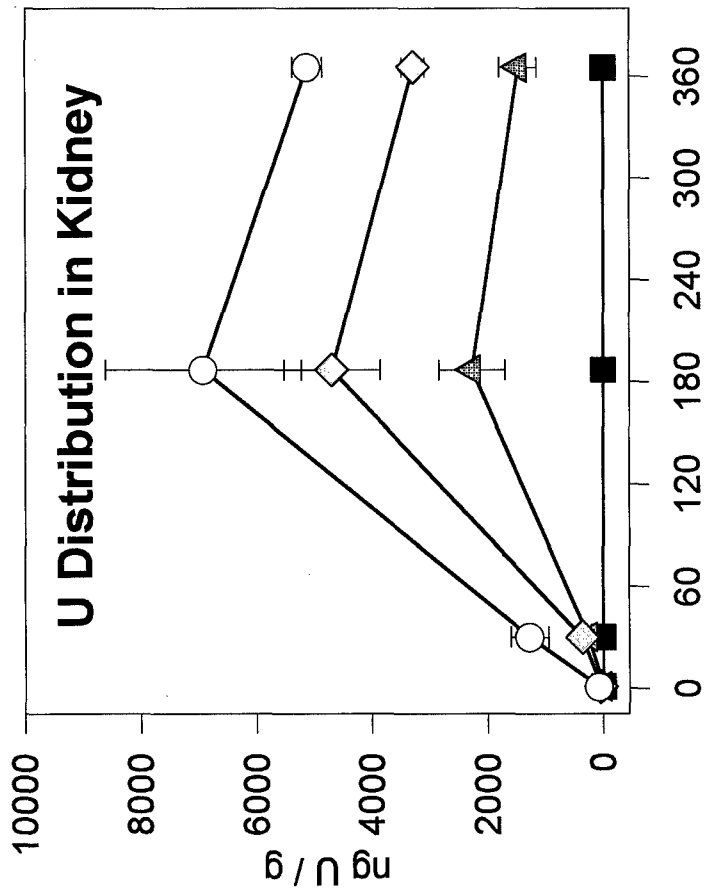
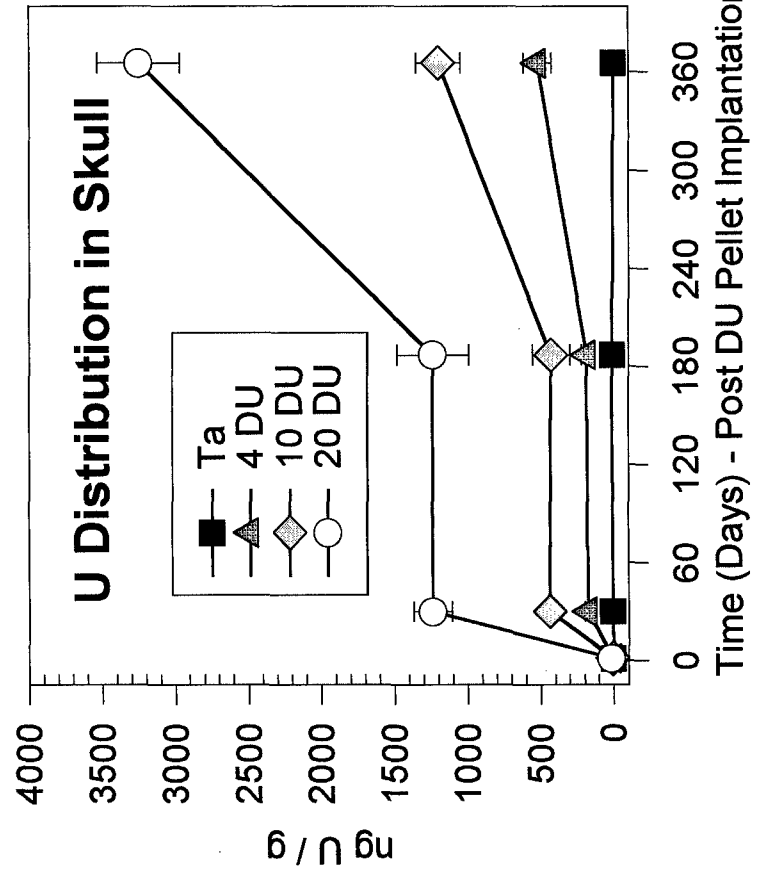
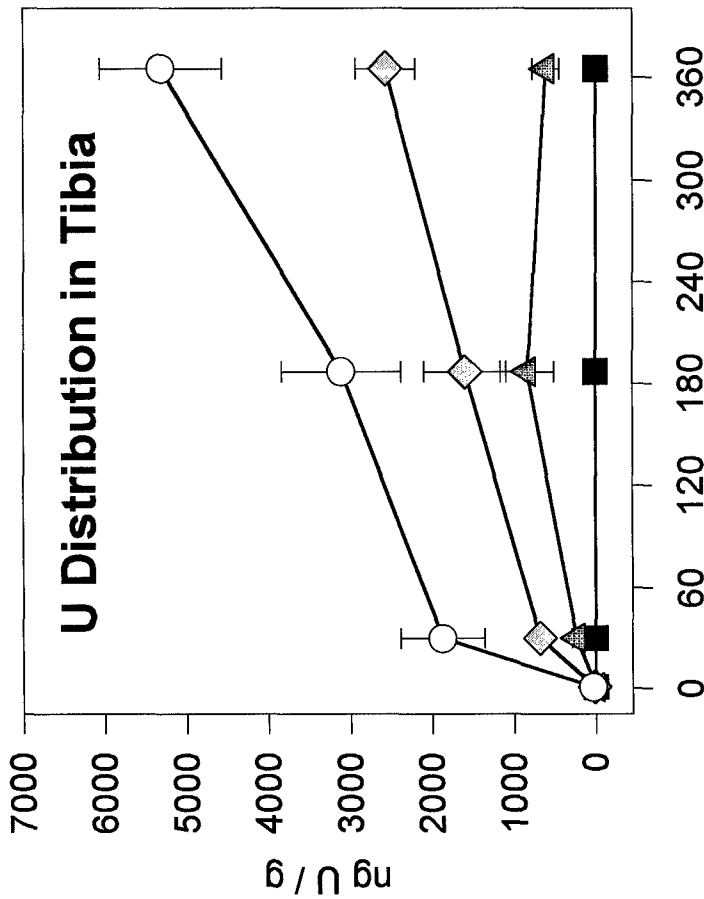


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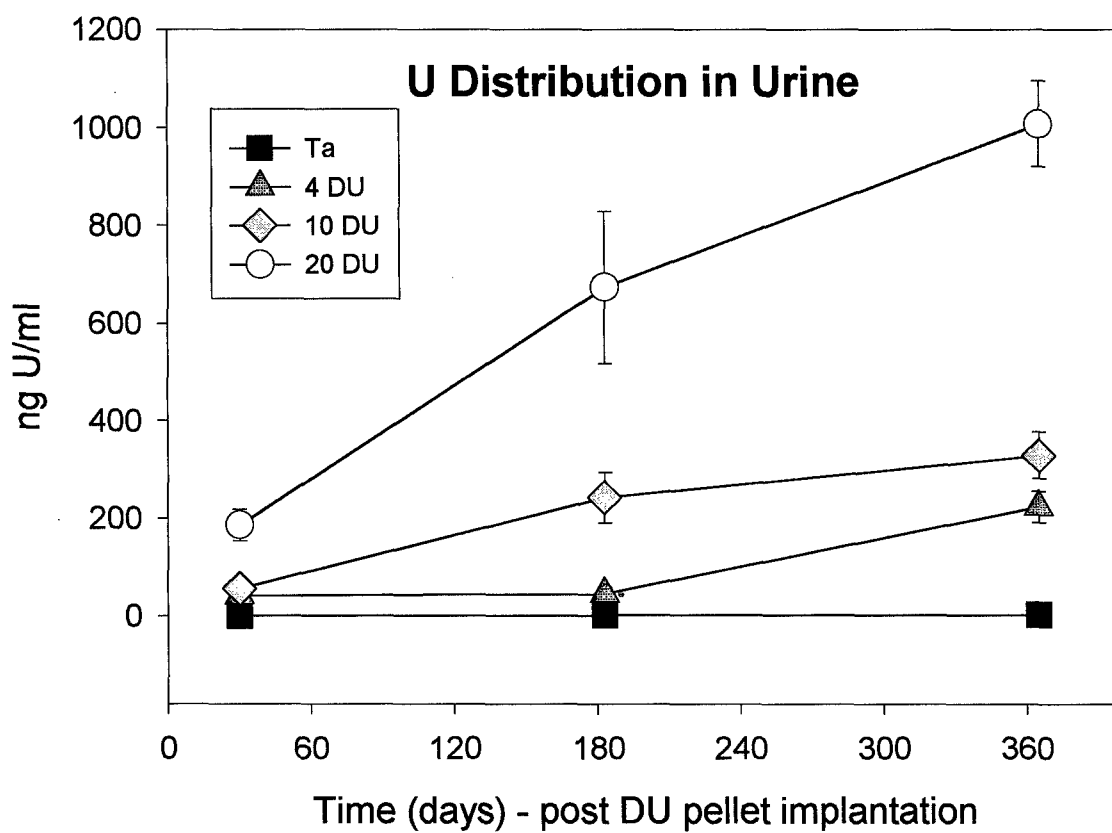
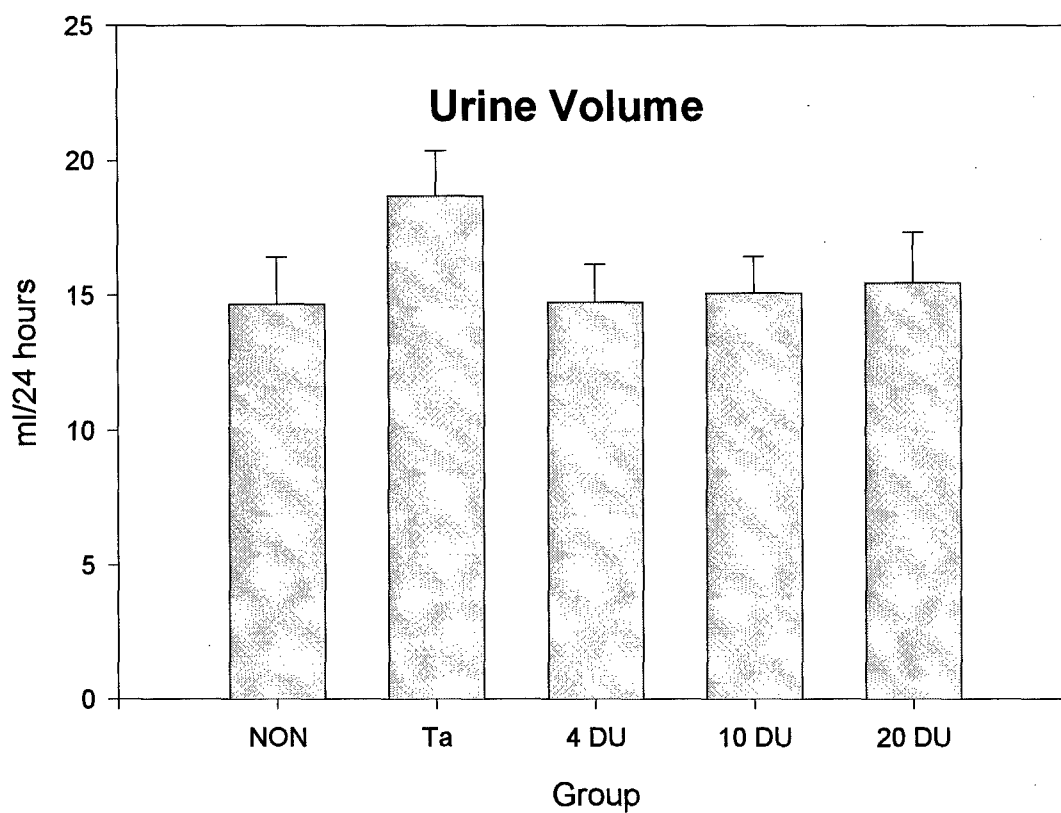
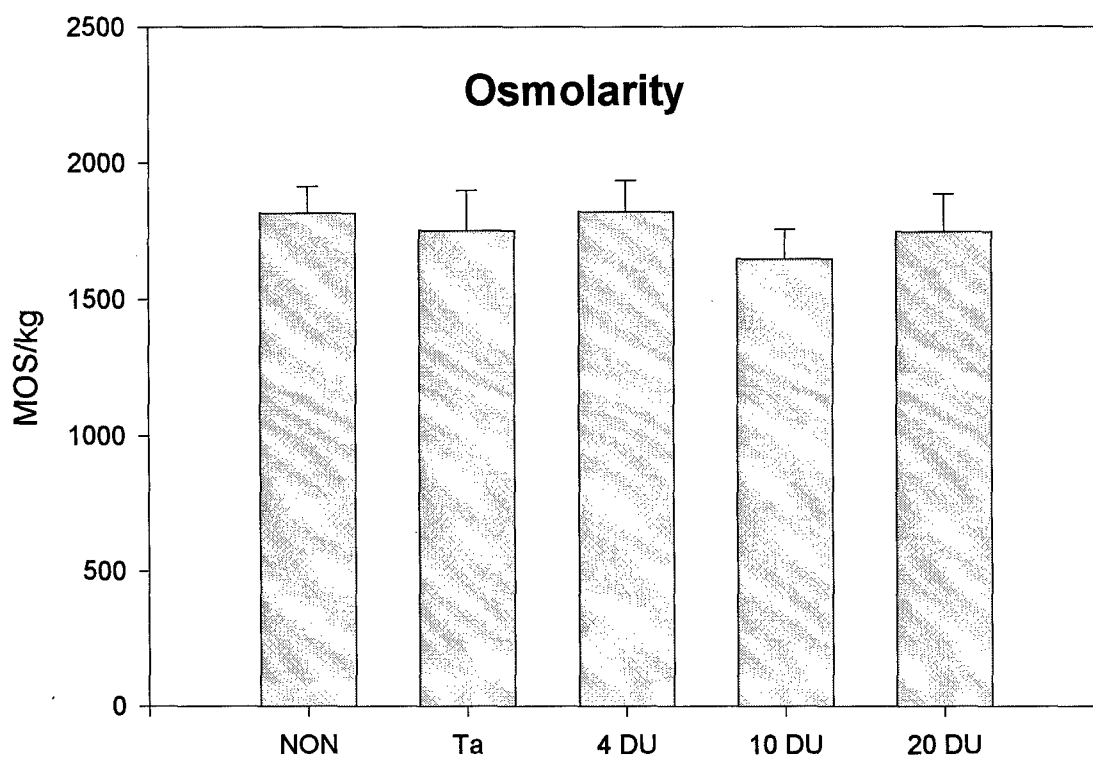


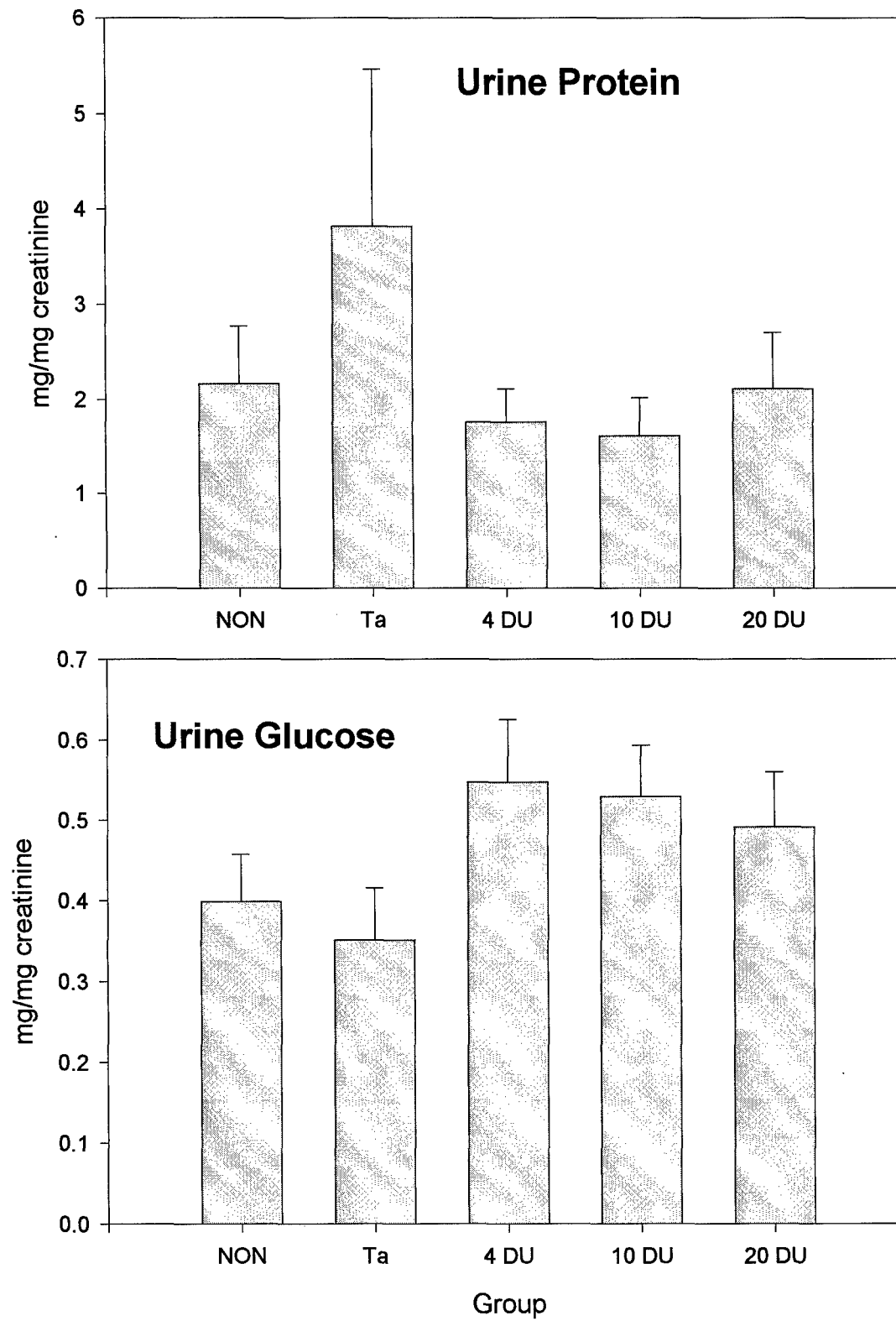
Figure 7



12 Month Time Point

N=11-15/ Group

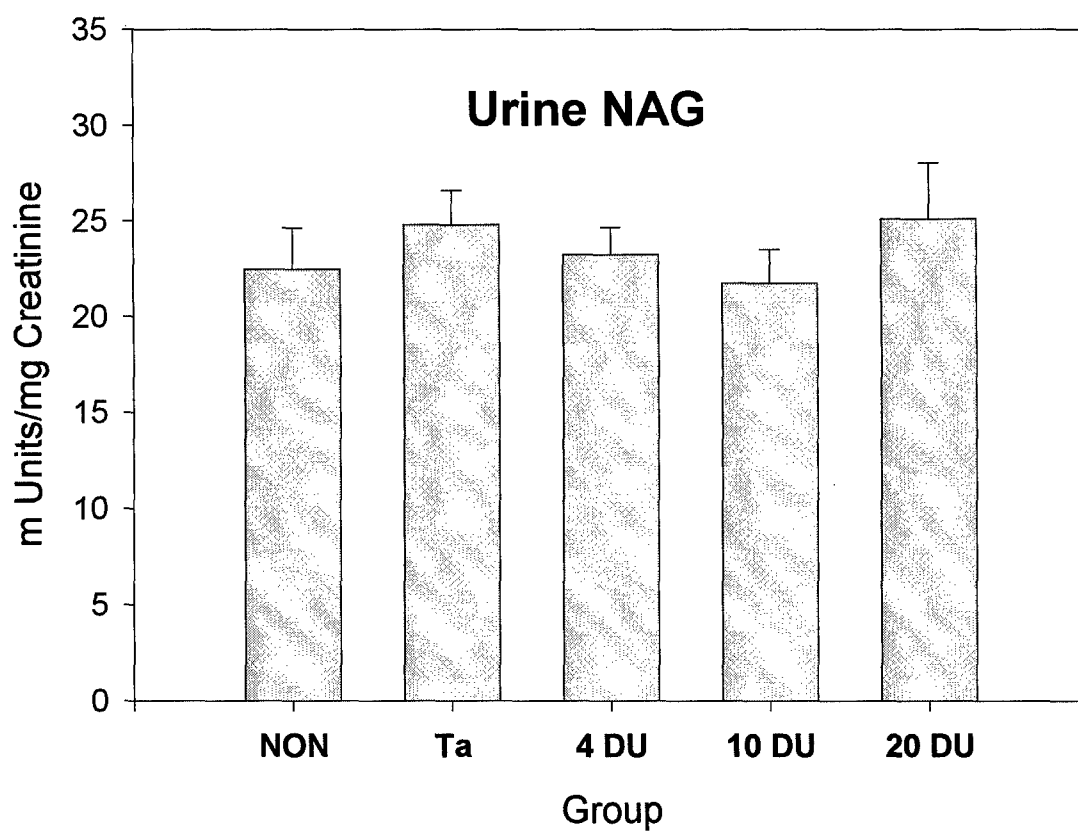
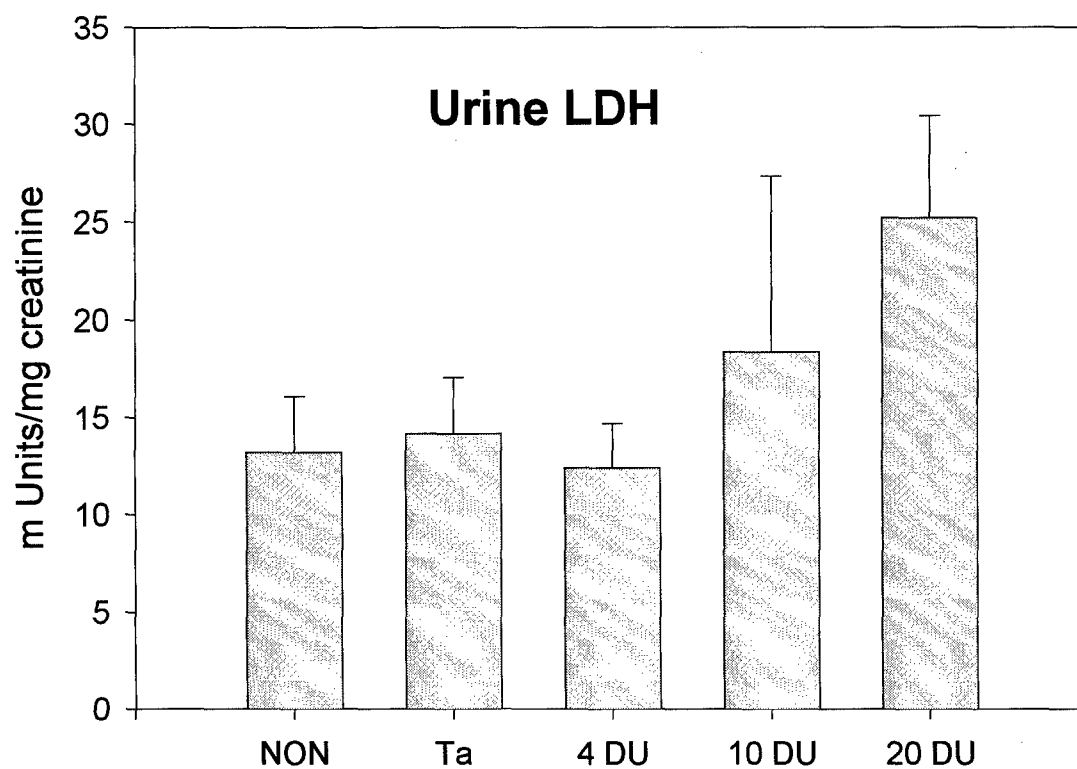
Figure 8



12 Month Time Point

N=11-15/ Group

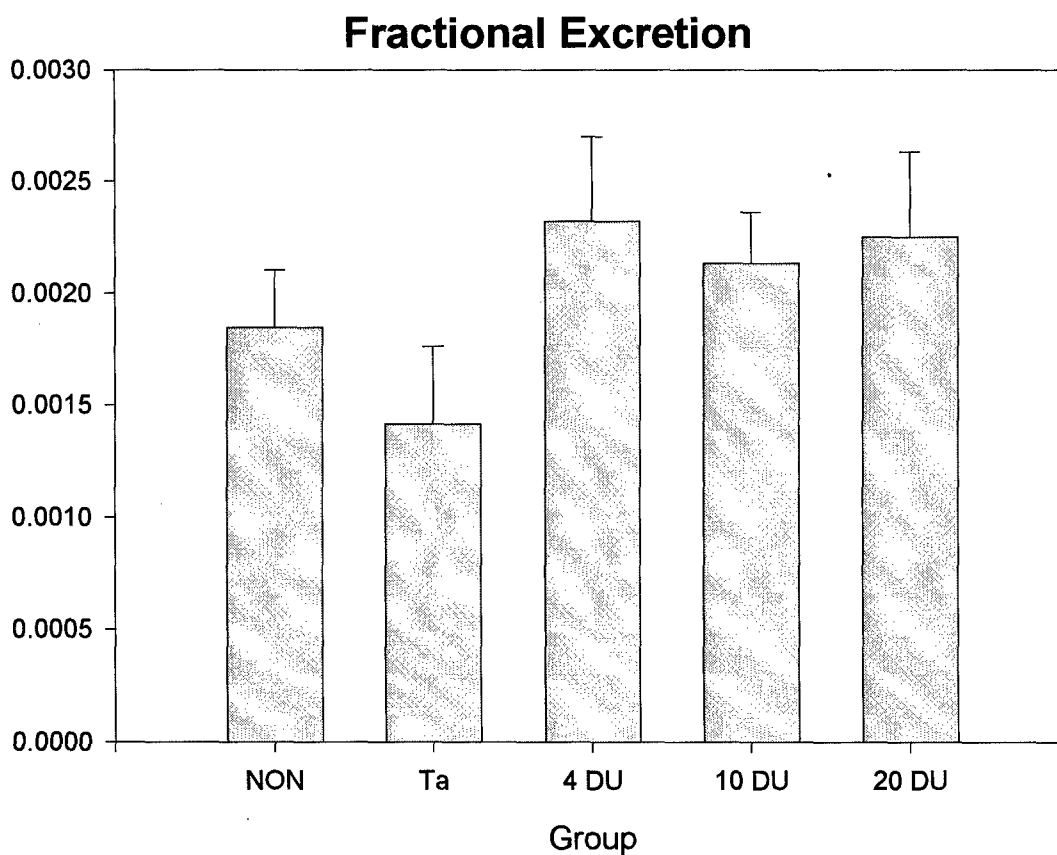
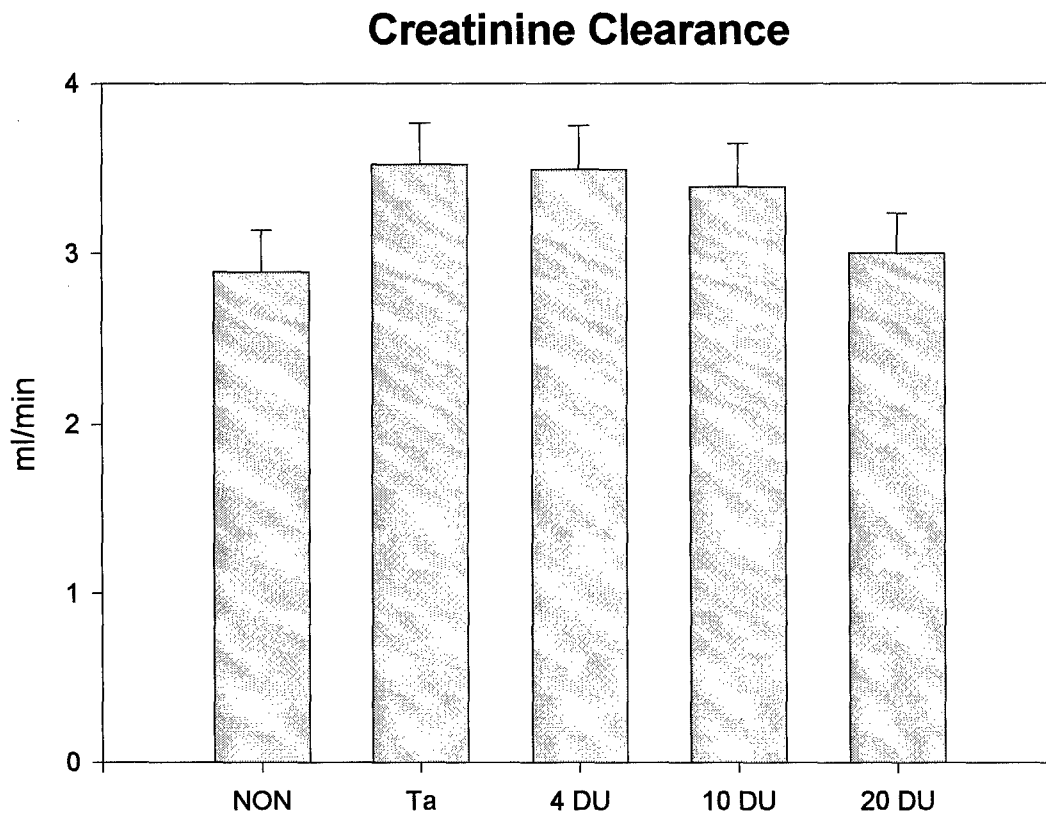
Figure 9



12 Month Time Point

N=11-15/ Group

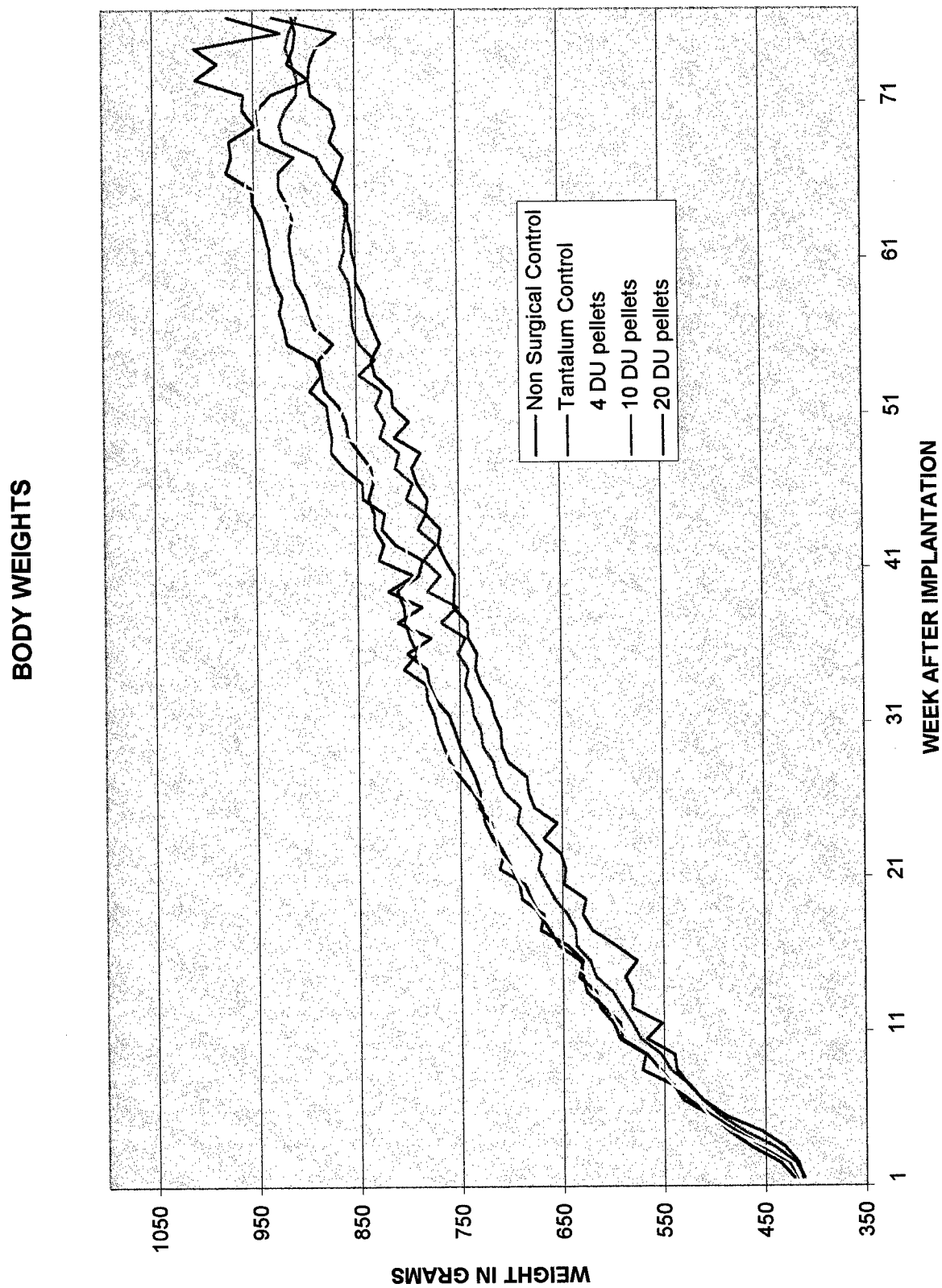
Figure 10



12 Month Time Point

N=11-15/ Group

Figure 11



Grip Strength

Figure 12

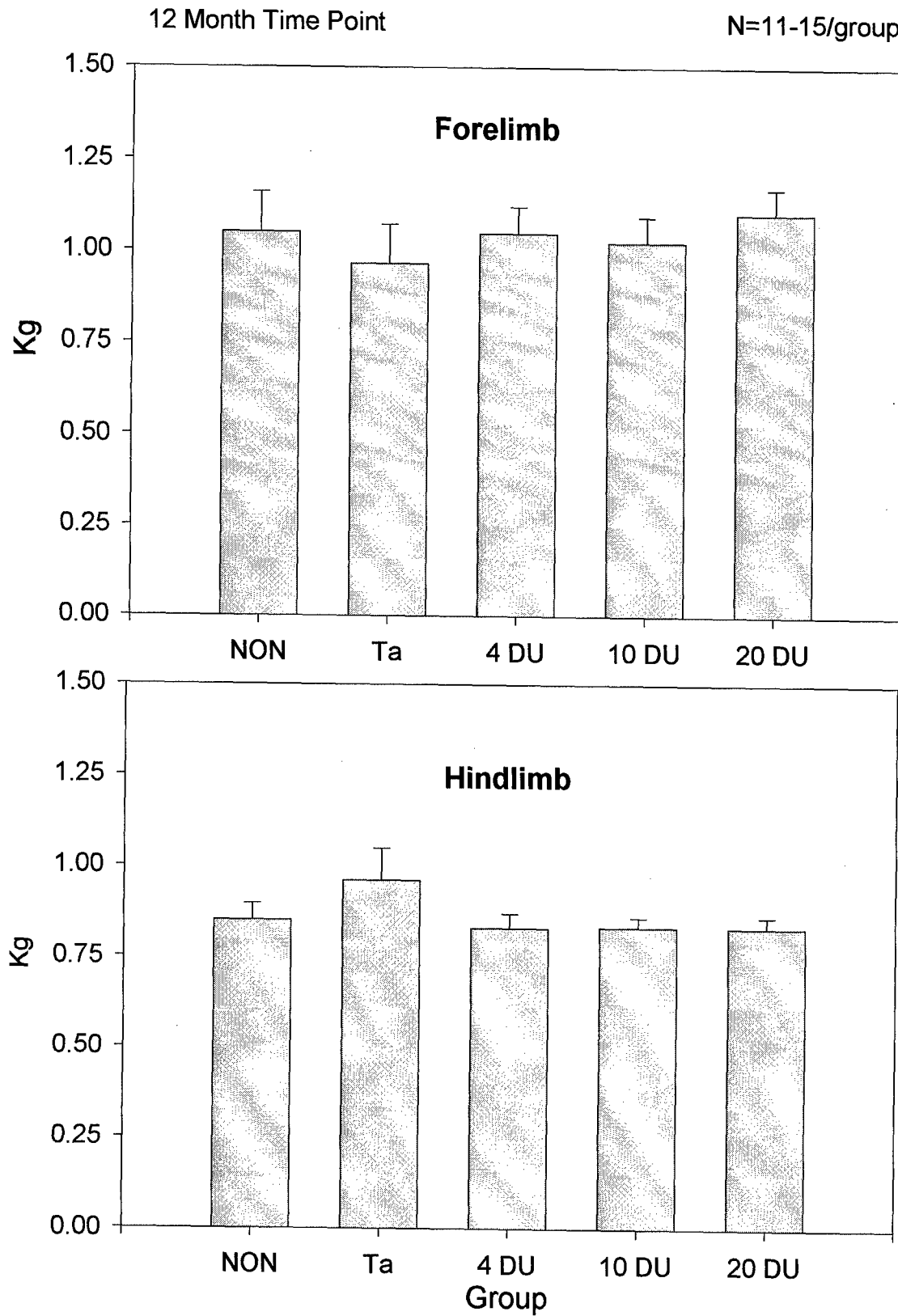


Figure 13

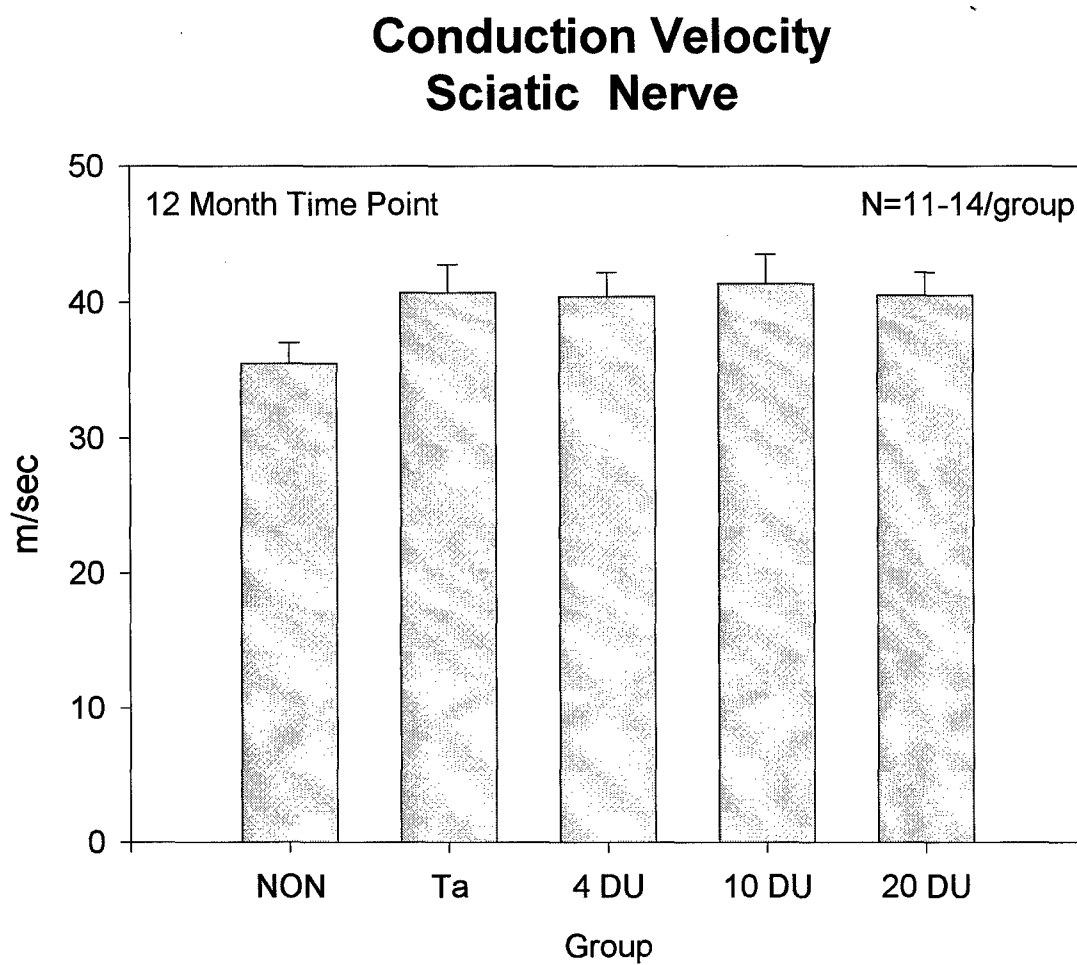
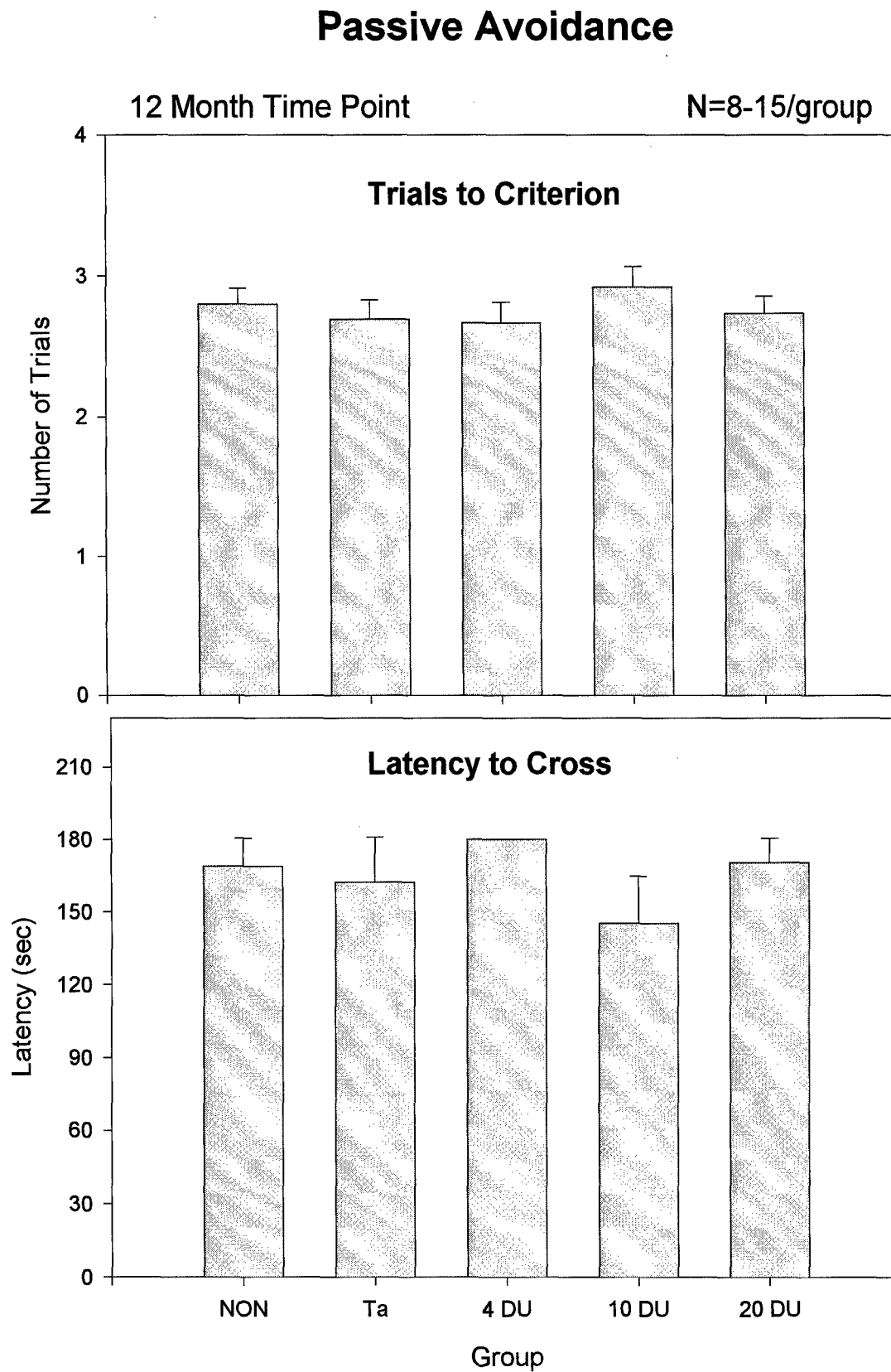


Figure 14



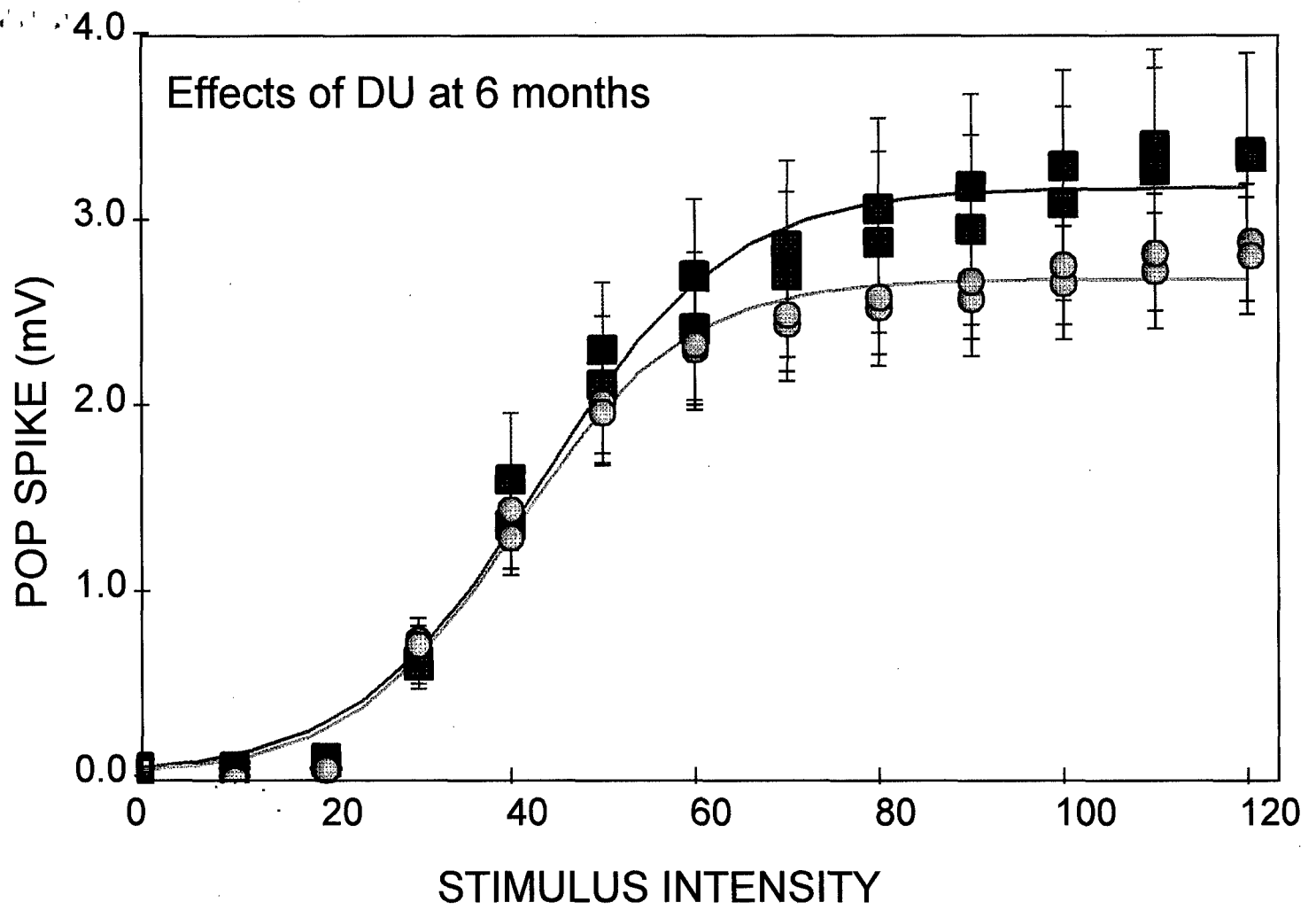


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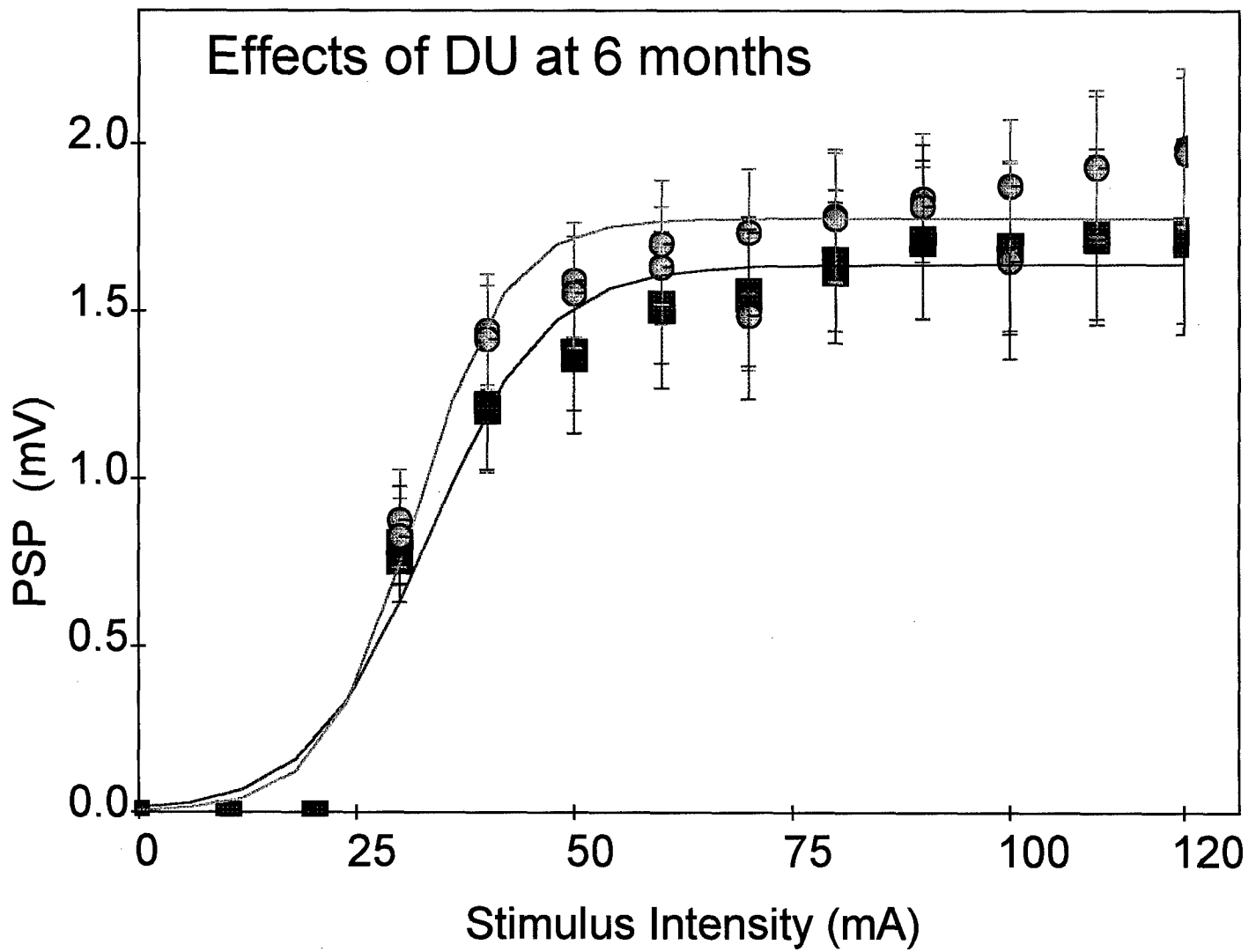


Figure 16

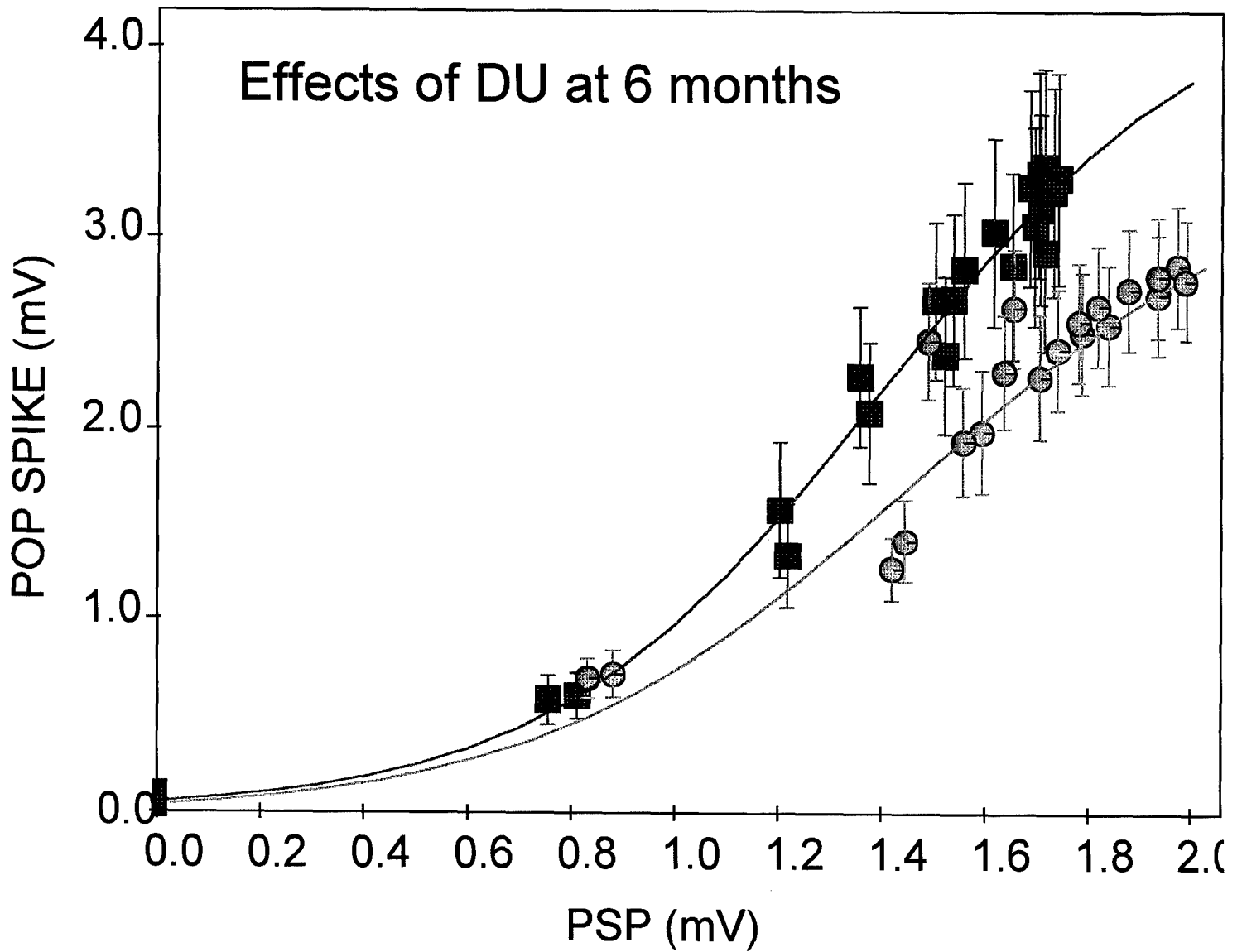


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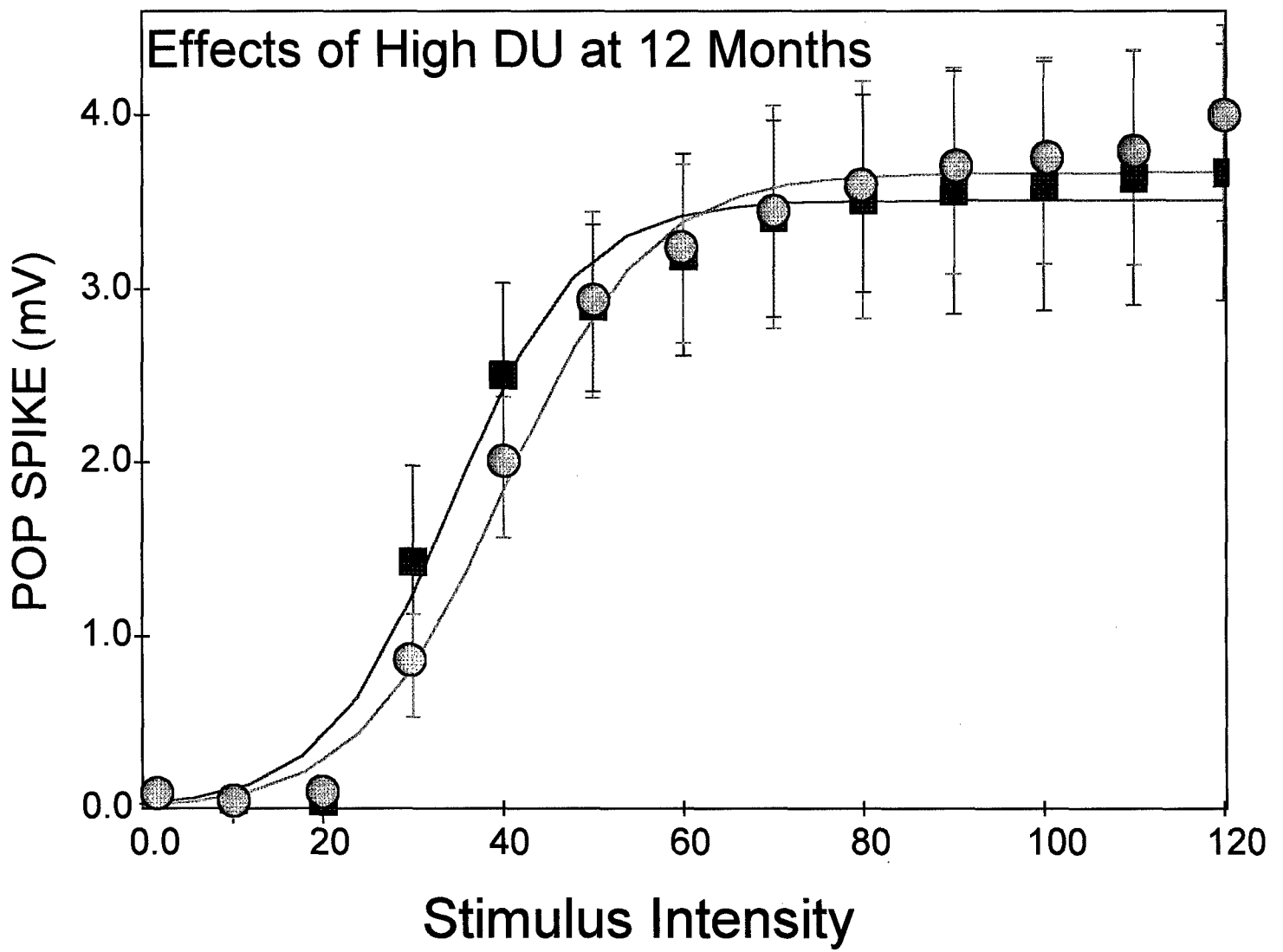


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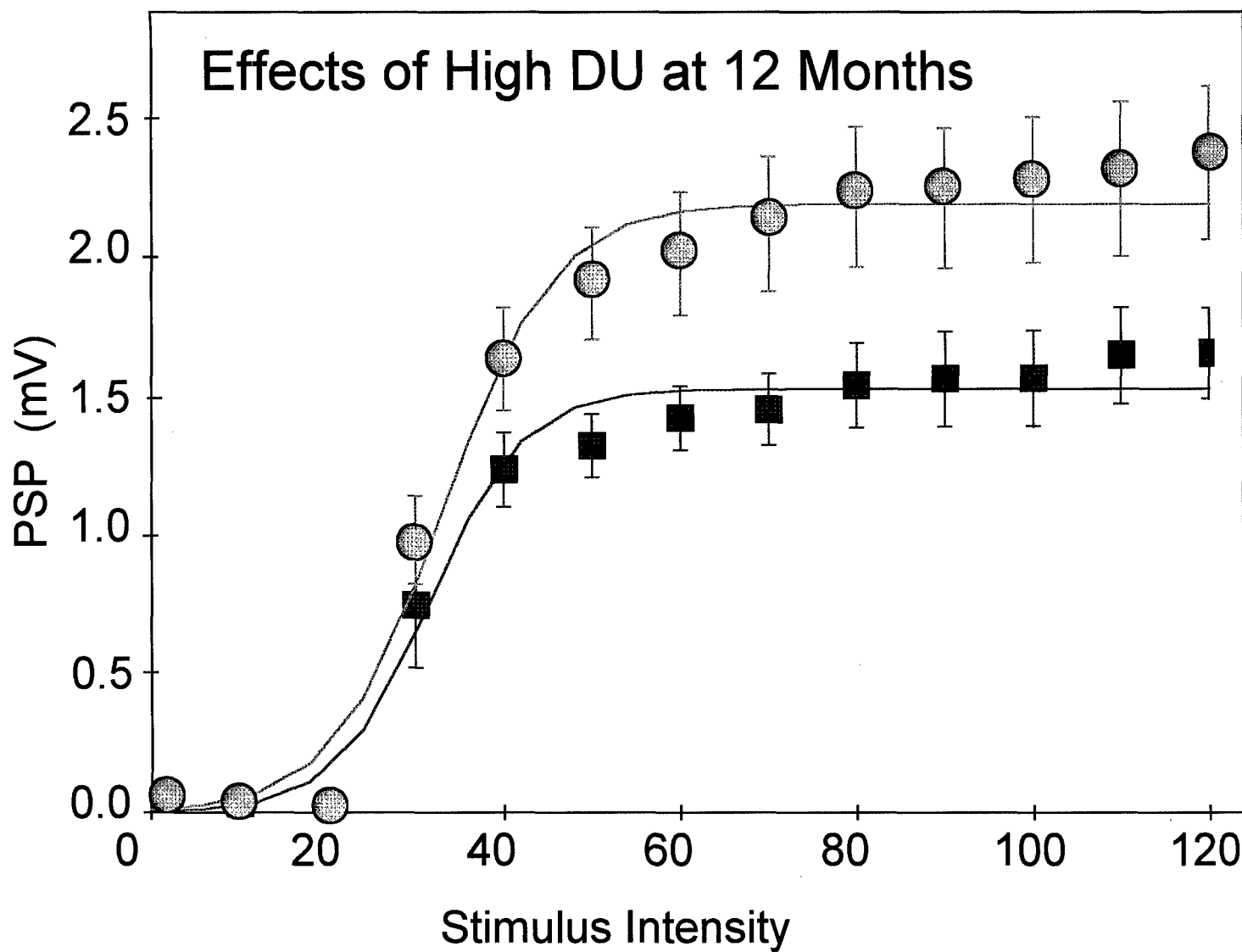


Figure 19

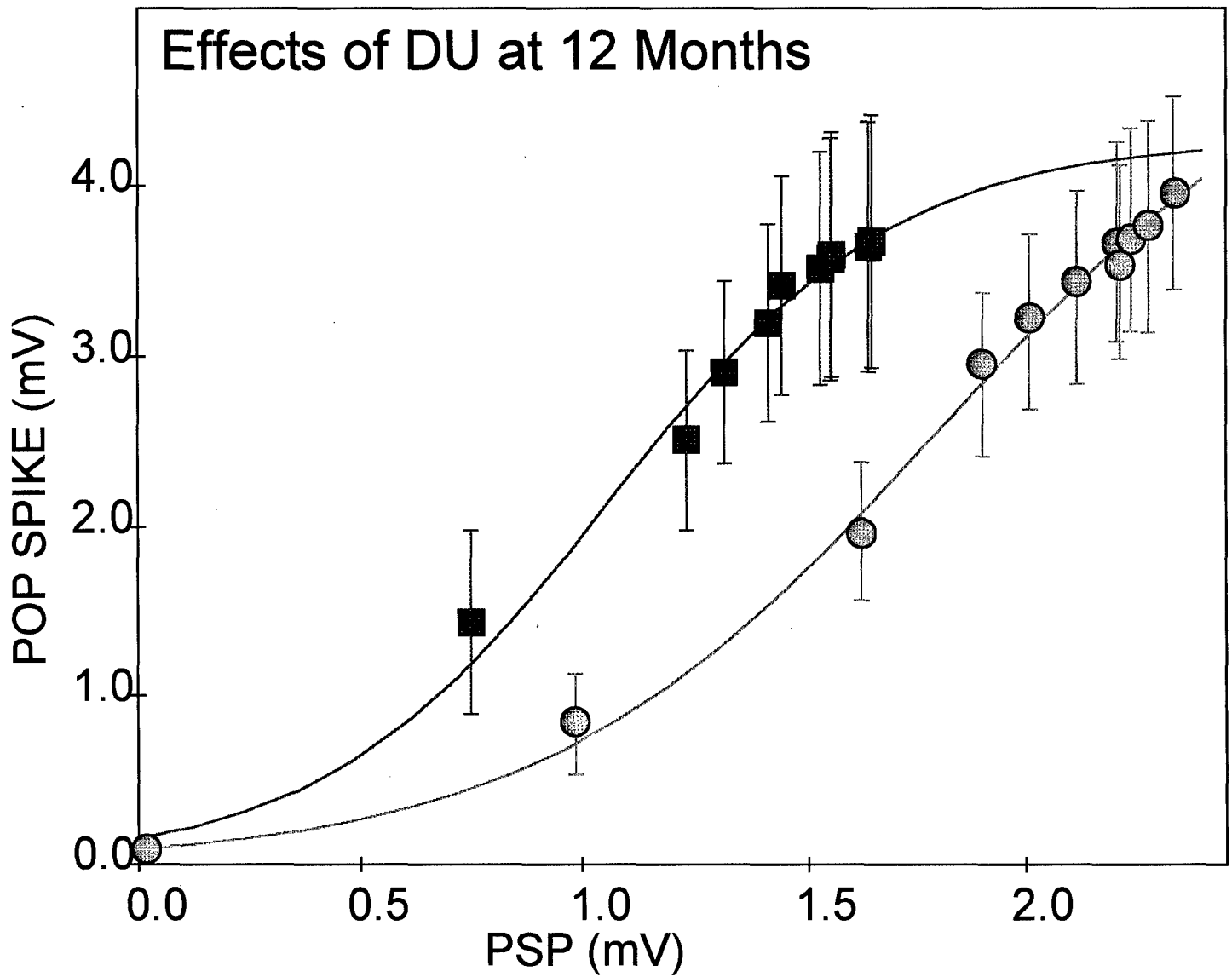


Figure 20

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Michael Landauer

John Hogan (resigned February 1997)

Rosemary Castillo (hired September 1996, resigned February 1997)

Michael Bixler (resigned July 1996)

Christopher Emery (hired May 1997)

Chris Emond (hired March 1997)



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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

17 Jan 03

MEMORANDUM FOR Administrator, Defense Technical Information
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for accession numbers [REDACTED], ADB227335, ADB236551, and ADB283653 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

A handwritten signature in cursive script, reading "Phyllis Rinehart", is positioned above the typed name.

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management