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Uranyl acetate-induced sensorimotor deficit and increased nitric oxide generation in the central nervous system in rats

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Abstract

We investigated the effects of uranyl acetate on sensorimotor behavior, generation of nitric oxide and the central cholinergic system of rats. Male Sprague–Dawley rats were treated with intramuscular injection of 0.1 and 1 mg/kg uranyl acetate in water, daily for 7 days. Control animals received equivalent amount of water. The treatment was stopped after the seventh injection because the animals in the 1-mg/kg group appeared lethargic. The animals were maintained for an additional observation period of 30 days. The study was initiated as a dose-finding study that covered doses of 10 and 100 mg/kg, as well. However, all the animals in the 100-mg/kg treatment group died after the third and fourth injections, and all animals given 10 mg/kg died after the fifth and sixth injections. On Day 30 following the cessation of treatment, the sensorimotor functions of the animals in the 0.1- and 1-mg/kg treatment groups were evaluated using a battery of tests that included measurements of postural reflexes, limb placing, orientation to vibrissae touch, grip time, beam walking and inclined plane performance. The animals were sacrificed the same day and the cerebral cortex, brainstem, cerebellum and midbrain were dissected. The levels of nitric oxide as marker for increased oxidative stress, and the integrity of the cholinergic system as reflected in acetylcholinesterase (AChE) activity and m2 muscarinic acetylcholine receptors ligand binding, were determined. The data from behavioral observations show that there was a dose-related deficit at the 0.1- and 1-mg/kg treatment groups for inclined plane performance. Both doses reduced grip time, but there was no significant difference between the two doses. Similarly, both beam-walk score and beam-walk time were impaired at both doses as compared with the controls. A significant increase in nitric oxide was seen at 0.1 mg/kg dose in cortex and midbrain, whereas brainstem and cerebellum showed an insignificant decrease at both the doses. Similarly, there was no significant change in nitric oxide levels in kidneys and liver of the treated animals as compared with the controls. There was a significant increase in AChE activity in the cortex of the animals treated with 1 mg/kg uranyl acetate, but not in other brain regions. Ligand binding densities for the m2 muscarinic receptor did not show any change. These results show that low-dose, multiple exposure to uranyl acetate caused prolonged neurobehavioral deficits after the initial exposure has ceased.

Keywords: Uranyl acetate; Depleted uranium (DU); Nitric oxide; Acetylcholinesterase; Muscarinic acetylcholine receptor; Neurotoxicity; Sensorimotor

1. Introduction

Natural uranium is composed of ^{238}U, ^{235}U and ^{234}U isotopes. Of these, only isotope ^{235}U is useful for the production of electricity. The concentration of ^{235}U is increased during the manufacture of nuclear fuel. One by-product of this enrichment process is depleted uranium (DU), which typically contains $\sim$70% less ^{235}U and $\sim$80% less ^{234}U than does the natural uranium. A number of commercial and military uses have been developed that have taken advantage of DU's high density and low cost (ATSDR, 1999; IOM, 2000).

The US weapon system utilized DU for offensive and defensive purposes for the first time in the Gulf War (OSAGWI, 1998). Consequently, a significant number of US military personnel were exposed to varying levels of DU; among these, there are $\sim$173 people who may have been exposed to high levels of DU. Twenty-nine soldiers still carry DU fragments scattered throughout their muscle and soft tissues as a result of friendly fire accident (McDiar-

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mid et al., 2000; IOM, 2000). Because of the low level of radioactivity associated with DU, it is believed that most of its toxic effects could be due to its chemical properties (Hartmann et al., 2000; Priest, 2001). Inhalation or ingestion of soluble or moderately soluble uranium such as uranyl fluoride or uranium tetrafluoride causes accumulation of uranium in various organs. When insoluble compounds such as uranium oxides (U_3O_3 and UO_2) are inhaled, uranium is generally deposited in the lungs where it can remain for long periods of time (IOM, 2000). In humans, approximately two-thirds of injected uranium is eliminated from plasma within 6 min after injection with 99% is eliminated from plasma by 20 h (Struxness et al., 1956; Lussenhop et al., 1958). The kidneys excrete more than 90% of soluble uranium, with less than 1% excreted in the feces. Both animal (Diamond et al., 1989; Gilman et al., 1998) and epidemiological studies (Kathern et al., 1989; Singh et al., 1987) demonstrate that kidney and bone marrow are the primary reservoirs for uranium regardless of the route of exposure. The toxic effects of DU on bone marrow are caused by reduction in the active osteoblast population (Ubios et al., 1998). However, recently, Pellmar et al. (1999) have shown that following implantation with DU pellets in rats, a significant amount of uranium is also deposited in muscle, brain, testes, spleen, liver heart, lung and lymph nodes. In a subsequent study, observed abnormal electrophysiological changes in the hippocampal slices, obtained 12 months after implantation with DU pellets, were observed (Pellmar et al., 1999). Domingo et al. (1987) reported acute cholinergic toxicity following a single oral dose of uranyl acetate dihydrate in rats. Kathern and Moore (1986) found acute disruption of mental function following accidental exposure to a cloud of soluble uranium compounds. Furthermore, McDiarmid et al. (2000) recently showed that PGW veterans who have retained fragments of DU since Gulf War continue to excrete uranium in their urine 7 years after the initial exposure. These veterans also had a significant cognitive deficit that correlated with their urinary levels of uranium. Paradoxically, there are no signs of kidney damage to these individuals. This is not surprising, since animal studies have shown that acute kidney damage is recovered if mortality does not occur (Diamond et al., 1989). Therefore, it is possible that long-term low-level exposure to DU causes toxic effects to other organs such the brain, which may lead to neurological deficit.

The chemical toxicity associated with DU exposure has been ascribed to its effects similar to other heavy metals such as mercury, cadmium, arsenic and lead (ATSDR, 1999). However, the molecular targets and mechanism of toxicity may be different. The amount of uranium present in uranyl acetate is approximately the same (0.3–0.4% ^{235}U, 99.6–99.7% ^{238}U) as that present in DU, and thus could be a representative compound to evaluate the toxicity of DU. However, in view of the differences in the physical and chemical characteristics of various forms of uranium salts, their target organ localization may be different. Currently, DU is unavailable commercially or through any other sources for research purposes. Therefore, in the present study, we used uranyl acetate to evaluate the sensorimotor performance and brain regional nitric oxide levels, acetylcholinesterase (AChE) activities and m2 muscarinic acetylcholine receptor (mAChR) ligand binding following intramuscular administration. These results show that treatment with low-dose uranyl acetate resulted in long-term sensorimotor impairment and increased generation of nitric oxide in certain brain regions.

2. Materials and methods

2.1. Materials

Uranyl acetate dihydrate was obtained from EM Sciences (Fort Washington, PA). According to the information provided by the supplier, the amount of uranium present in this preparation of uranyl acetate is approximately same (0.3–0.4% ^{235}U, 99.6–99.7% ^{238}U) as that present in the DU. Sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride), sodium nitrite, butyrylthiocholine iodide and acetylthiocholine iodide were obtained from Sigma (St. Louis, MO). [^{3}H]AF-DX384 [2,3 dipropylamino] (specific activity, 100 Ci/mmol) was obtained from NEN (Boston, MA). All other reagents were of highest purity available commercially.

Male Sprague–Dawley rats (200–250 g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in Duke University Medical Center vivarium on a 12-h dark–light cycle. The animals were allowed food and water ad libitum. All the treatments and procedures of the animals were carried out strictly according to the guidelines recommended by the Duke University Medical Center institutional animal care and use committee.

2.2. Methods

2.2.1. Treatment of animals

Rats were randomly assigned to control and experimental groups. These experiments were initially planned as part of a dose-finding study with a dose range of 0.1–100 mg/kg. By the end of the fourth and fifth days, all the animals in the 10- and 100-mg/kg groups were dead. Therefore, only the 0.1- and 1-mg/kg groups are described here. The animals were divided into following groups of five animals each:

(i) Control: The animals received daily intramuscular injection of water for 7 days;
(ii) Uranyl acetate 0.1 mg/kg: Animals in this group were treated daily for 7 days with an intramuscular injection of uranyl acetate dissolved in water; and
(iii) Uranyl acetate 1.0 mg/kg: Animals in this group were treated daily for 7 days with an intramuscular injection of uranyl acetate dissolved in water.

The treatment was stopped after the seventh day as the animals in the 1.0-mg/kg appeared lethargic. The animals were maintained for 30 days thereafter because we are interested in studying the long-term neurobehavioral effects following exposure to uranyl acetate. Following behavioral testing on Day 30, the animals were anesthesized with 0.2 ml of ketamine/xylocane and blood was drawn in heparinized syringe. Animals were sacrificed and the brains, liver and kidneys removed and washed thoroughly with ice-cold normal saline. Brain regions, cortex, midbrain, cerebellum and brainstem were dissected on ice and rapidly frozen in liquid nitrogen. Plasma was separated and frozen at $-80\ ^{\circ}C$ for biochemical studies.

2.3. Behavioral testing battery

A battery of standardized, well-understood and well-described behavioral tests assessing sensorimotor function were employed. These behavioral tests evaluate sensorimotor reflexes, motor strength and coordination. The animals were handled for 2 min each day for 5 days before beginning the evaluations. All behavioral testings were performed by an observer blind to the animal's treatment status and were carried out in a sound-proof room with subdued lighting (less than 10.76 lm/m2, ambient light) between 7 and 9 a.m.

2.3.1. Postural reflexes

2.3.1.1. Description. Postural reflexes were measured according to the methods described by Bederson et al. (1986) and Markgraf et al. (1992). Rats are held gently by the tail, 1 m above the floor and observed for forelimb extension. Normal rats extend both forelimbs. Consistent flexion of the forelimb is an abnormal response. Rats with consistent forelimb flexion were then placed on a large sheet of plastic-coated paper that can be gripped with the forepaws. With the tail held, gentle lateral pressure is applied behind the rat's shoulder until the forelimb slides several inches. The maneuver is repeated five times in each direction. Normal rats resist lateral pressure by gripping the coated paper.

2.3.1.2. Scoring. Grade 0, rats without evidence of consistent forelimb flexion when held above the floor; Grade 1, rats with consistent forelimb flexion; Grade 2, otherwise Grade 1 rats that do not resist lateral pressure on at least three of five trials in either direction.

2.3.2. Limb placing

2.3.2.1. Description. Visual, tactile and proprioceptive forelimb placing responses were examined as described by Markgraf et al. (1992). For visual placing, rats were held in the examiner's hands 10 cm above the tabletop with forelimbs hanging free. The rats were then slowly tilted towards the table. Intact rats reached towards the table with both forepaws. For tactile placing, the dorsal and then lateral portions of the forepaws were touched to the table edge. Intact rats immediately place the paw on the surface of the table. Proprioceptive placing was tested by pushing the forepaw onto the table edge.

2.3.2.2. Scoring. For each test: Grade 0, the placing response is immediate; Grade 1, the placing response is slow or delayed; Grade 2, the placing response does not occur within 2 s.

2.3.3. Orienting to vibrissae touch

2.3.3.1. Description. The rat was placed atop an inverted polycarbonate cage and allowed 1 m for habituation. Its vibrissae were then touched with a cotton-tipped swab (Whishaw et al., 1985).

2.3.3.2. Scoring. Grade 0, the rat orients to the side of the probe on at least two of three trials from each side; Grade 1, fails to orient on at least two of three trials on either side.

2.3.4. Inclined plane

2.3.4.1. Description. Rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised (Yonemori et al., 1998). The board was slowly rotated to the vertical position. Two trials are performed for each testing session.

2.3.4.2. Scoring. The angle that the rat begins to slip downward was recorded. The results of the two trials were averaged for each testing session.

2.3.5. Forepaw grip time

2.3.5.1. Description. The rat's forepaw strength was assessed by having them grip a 5-mm-diameter wood dowel that was held horizontally and raised so that the rat supports its body weight as described by Andersen et al. (1991). Two trials were performed for each testing session.

2.3.5.2. Scoring. Time to release grip was recorded in seconds. The results of the two trials were averaged for each testing session.

2.3.6. Beam walking

2.3.6.1. Description. The testing apparatus was a $2.5 \times$ 122-cm wooden beam elevated 75.5 cm above the floor with wooden supports as described by Goldstein (1993). A $20 \times 25 \times 24$ cm goal box with a 9.5-cm opening is located at one end of the beam. A switch-activated source of bright light (75-W tungsten bulb) and white noise are located at the start–end of the beam and served as avoidance

stimuli. The rats were first trained with a series of three approximate trials. Rats were readily trained to perform the beam-walking task. For each testing trial, the rats were placed at the start–end of the beam, near the sources of light and noise. Each testing session consisted of a series of five trials.

2.3.6.2. Scoring. Beam-walking ability was measured with a seven-point scoring system scale as previously described by Goldstein (1993): (1) the rat is unable to place the affected hindpaw on the horizontal surface of the beam; (2) the rat places the affected hindpaw on the horizontal surface of the beam and maintains balance for at least 5 s; (3) the rat traverses the beam while dragging the affected hindpaw; (4) the rat traverses the beam and at least once places the affected hindpaw on the horizontal surface of the beam; (5) the rat crosses the beam and places the affected hindlimb on the horizontal surface of the beam to aid less than half its steps; (6) the rat uses the affected hindpaw to aid more than half its steps and; (7) the rat traverses the beam with no more than two footslips. In addition, the latency until the animal's nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fell off of the beam or did not enter the goal box were assigned latencies of 90 s.

2.3.7. Statistical analyses

Comparisons across treatment groups for postural reflexes, limb placing and vibrissae touch orientation were analyzed with nonparametric analysis of variance (ANOVA; Kruskal–Wallis test). Data for the remaining behavioral tests were compared among groups by one-way ANOVA. A repeated-measures ANOVA was used to compare the effects of increasing doses of uranyl acetate. If a significant difference was found, Fisher's LSD tests were applied to permit post-hoc, pairwise comparisons. For presentation in the figures to facilitate comparisons, raw behavioral data were recalculated as a percentage of control.

2.4. Biochemical assays

2.4.1. Determination of nitric oxide in the brain regions

Nitric oxide levels were quantitated according to the spectrophotometric method of Green et al. (1982) as described by Canals et al. (2001) by measuring nitrite, a stable oxidation endproduct of nitric oxide. Briefly, the brain regions, liver and kidneys were homogenized in 10 mM phosphate buffer, pH 7.4, containing 0.15 M KCl. The homogenate was centrifuged at $5000 \times g$ for 5 min. The supernatant was diluted 20-fold with the homogenization buffer and the color was developed by mixing an equal volume of Griess reagent [equal volumes of 1.5% sulfanilamide in 5% phosphoric acid and 0.15% *N*-(1-naphthyl)ethylenediamine dihydrochloride]. The reaction was allowed to proceed at room temperature for 20 min and the absorbance recorded at 562 nm using a multiwell microplate reader. The levels of nitric oxide were determined with a standard curve generated from sodium nitrite and the concentrations are expressed as micromolars per milligram of protein.

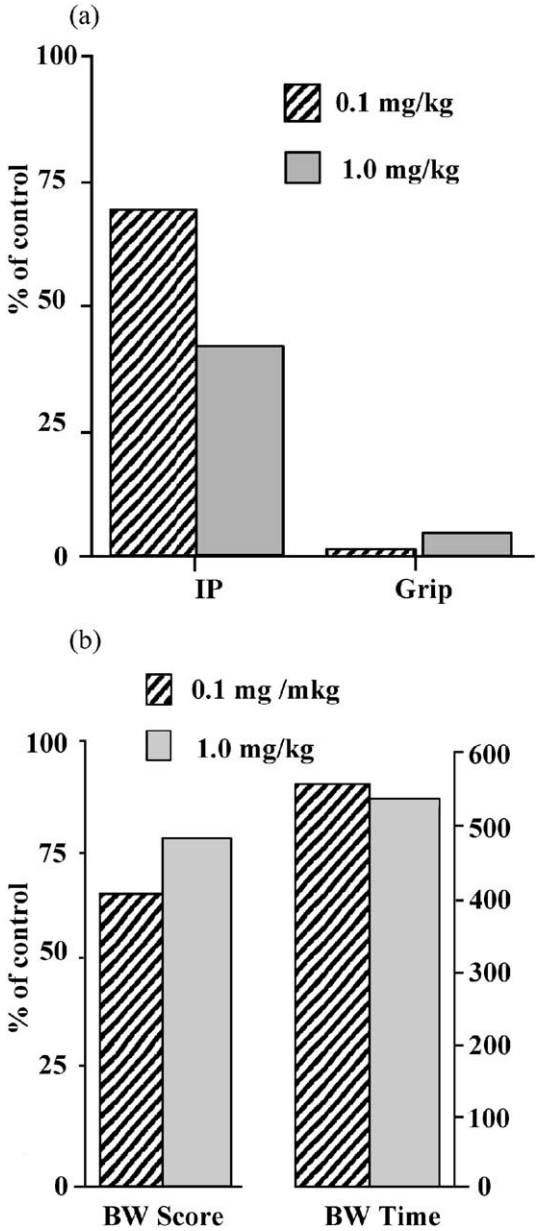

Fig. 1. Effect of intramuscular treatment with uranyl acetate on sensorimotor performance. The animals were pretreated with intramuscular injection of 0.1 and 1.0 mg/kg uranyl acetate daily for 7 days as described in Materials and Methods. Treatment with uranyl acetate was discontinued thereafter and the animals were maintained for 30 days following discontinuation of the treatment. On Day 30, the animals were tested for beam-walk score (BW Score), beam-walk time (BW Time), inclined plane (IP) and grip response (Grip). (a) Inclined plane (IP) and grip response (Grip); (b) beam-walk score and beam-walk time. The data were computed and detailed statistical evaluations were carried out on the raw data as described in the Results section. The data are presented as mean ± S.E. (percent of control).

2.4.2. Cholinesterase determination

AChE in brain regions and BChE in plasma activities were determined according to the method of Ellman et al. (1961) modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader as previously described (Abou-Donia et al., 1996; Khan et al., 2000). Protein concentration was determined by BCA method according to Smith et al. (1985). The enzyme activities are expressed as micromoles of substrate hydrolyzed per minute per milligram of protein for brain regions and nanomoles of substrate hydrolyzed per minute per milligram of protein for plasma (percent of control).

2.5. mAChR binding assay

For the assay of mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000 × *g* for 10 min and the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml as described by Huff et al. (1994), Slotkin et al. (1999) and Khan et al. (2000). The m2 mAChR binding was carried out by using an m2-selective ligand, [^{3}H]AF-DX384, at room temperature for 60 min. Nonspecific binding was carried out in the presence of 2.22 μM atropine. Ligand-bound membranes were trapped on glass filters presoaked with 0.1% polyethyleneimine using rapid vacuum filtration. The results are expressed as specific binding (dpm) per milligram of protein (percent of control).

2.6. Statistical analysis

The results were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test, and results were plotted using Excel graphics for Macintosh. P value $< .05$ was considered significant.

3. Results

3.1. Clinical condition

All the animals in the 100-mg/kg treatment group died between Days 3 and 4, and those in the 10-mg/kg treatment group died by Day 5 or 6 of the treatment. One animal died in the 1-mg/kg treatment group on Day 7, and therefore, the treatment was discontinued thereafter. The body weight of the surviving animals was recorded every week. There was a significant decrease in the body weight of animals in the 1-mg/kg treatment group during the first week of treatment that remained lower up to the second and third weeks. These animals recovered body weight gain by the fourth week following the cessation of treatment. There was no difference in body weight between the animals treated with 0.1 mg/kg uranyl acetate and controls at any time point.

3.2. Behavioral assessment

3.2.1. Postural reflexes, limb placing, orienting to vibrissae touch

There was no effect of any treatment at any dose on postural reflexes, limb placing or tactile orientation (data not shown).

3.2.2. Inclined plane, forepaw grip, beam walking

Data presented in Fig. 1a and b give comparisons for performance on the inclined plane, forepaw grip, beam-walk

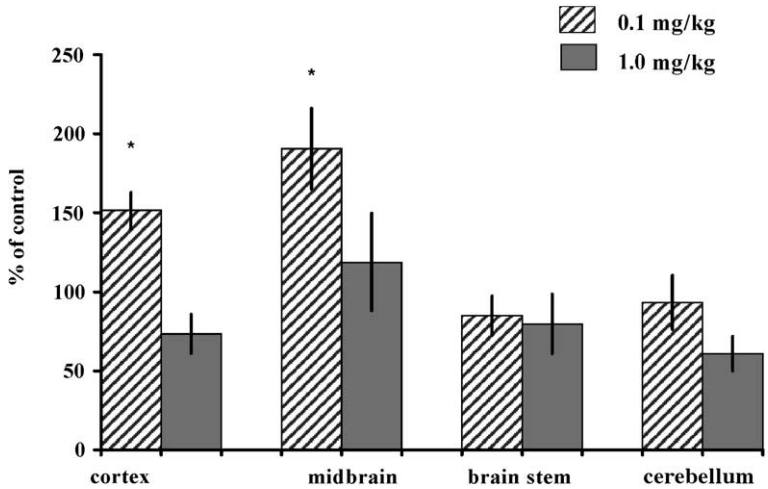

Fig. 2. Effect of intramuscular treatment with uranyl acetate on the levels of nitric oxide in brain regions. The animals were pretreated with intramuscular injection of 0.1 and 1.0 mg/kg uranyl acetate daily for 7 days as described in Materials and Methods. Treatment with uranyl acetate was discontinued thereafter and the animals were maintained for 30 days following discontinuation of the treatment. On Day 30, the animals were sacrificed and brain regions were dissected on ice. Nitric oxide concentration was determined by derivatization to nitrite as described in Materials and Methods. The data were computed and detailed statistical evaluations were carried out on the raw data as described in the Results section. The control values were 50 ± 5, 20 ± 2, 50 ± 9 and 45 ± 9 nM/mg protein for cortex, midbrain, brainstem and cerebellum, respectively. The data are presented as mean ± S.E. (percent of control). * $P < .05$.

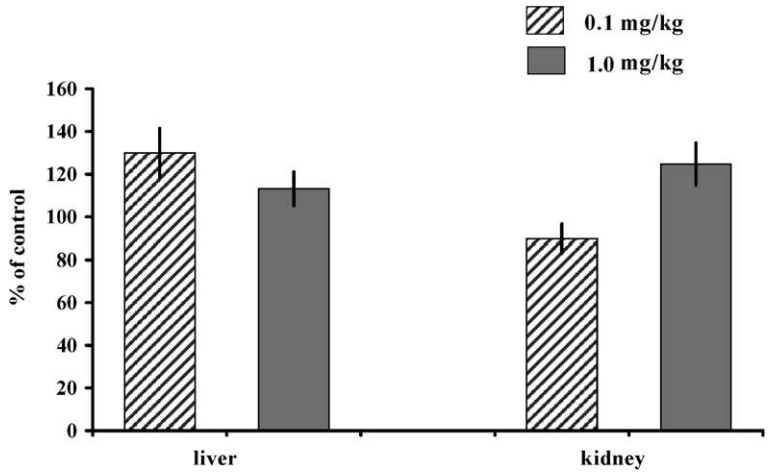

Fig. 3. Effect of intramuscular treatment with uranyl acetate on the levels of nitric oxide in liver and kidney. The animals were pretreated with intramuscular injection of 0.1 and 1.0 mg/kg uranyl acetate daily for 7 days as described in Materials and Methods. Treatment with uranyl acetate was discontinued thereafter and the animals were maintained for 30 days following discontinuation of the treatment. On Day 30, the animals were sacrificed and livers and kidneys were dissected out. Nitric oxide concentration was determined by derivatizing to nitrite as described in Materials and Methods. The data were computed and detailed statistical evaluations were carried out on the raw data as described in the Results section. The control values were 80 ± 10 and 30 ± 2 nM/mg protein for liver and kidney, respectively. The data are presented as mean ± S.E. (percent of control).

score and beam-walk time as a percent of control 30 days after cessation of the treatment with 0.1 and 1 mg/kg uranyl acetate. Because we were interested in long-term neurobehavioral consequences following exposure with uranyl acetate, the behavioral assessments were carried out only on Day 30 following the cessation of the treatment. For inclined plane, a dose–effect relationship was seen [1 mg/kg < 0.1 mg/kg < control; ANOVA, $F(2,11) = 40$, $P < .00001$; Fisher's LSD, $P < .0004$ for each dose compared with control; Fisher's LSD, $P = .002$ for 1.0 vs. 0.10 mg/kg].

For grip time, both doses of uranyl acetate impaired performance, but there was no significant difference between the doses [1.0 mg/kg = 0.1 mg/kg < control; ANOVA, $F(2,11) = 21$, $P = .0002$; Fisher's LSD, $P < .0002$ for each dose compared with control; Fisher's LSD, not significant when compared between the doses].

For beam-walk score (Fig. 1b), treatment with both doses of uranyl acetate resulted in poorer performance scores than the controls, but the differences between the controls and those treated with uranyl acetate were not significant [ANOVA, $F(2,11) = 1$, $P = .035$].

For beam-walk time (Fig. 1b), both doses of uranyl acetate resulted in markedly prolonged latencies as compared with controls, but the differences were not significant

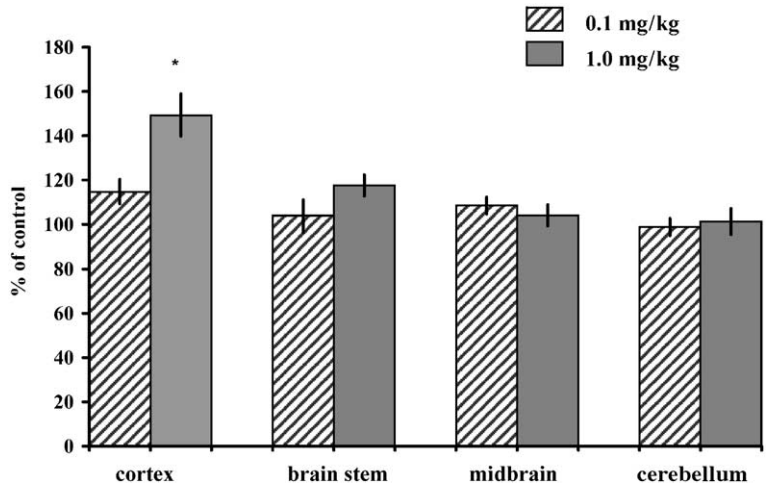

Fig. 4. Effect of intramuscular treatment with uranyl acetate on AChE activity in brain regions. The animals were pretreated with intramuscular injection of 0.1 and 1.0 mg/kg uranyl acetate daily for 7 days as described in Materials and Methods. Treatment with uranyl acetate was discontinued thereafter and the animals were maintained for 30 days following discontinuation of the treatment. On Day 30, the animals were sacrificed and brain regions were dissected on ice. The details of the treatment and determination of enzyme activity are elaborated in Materials and Methods. The control enzyme activities were 0.4 ± 0.06, 0.7 ± 0.05, 0.6 ± 0.03 and 0.2 ± 0.01 μmol/min/mg protein for cortex, midbrain, brainstem and cerebellum, respectively. Data presented as mean ± S.E. (percent of control).
* $P < .05$.

[ANOVA, $F(2,11)=3$, $P=.07$]. These results suggest that treatment with uranyl acetate significantly affected the inclined plane performance in a dose-dependent manner and grip time whereas beam-walking time and beam-walk score showed a decrease, but statistically nonsignificant.

3.2.3. Effect of treatment with uranyl acetate on levels of nitric oxide in brain regions and kidney and liver

These data on the effects of uranyl acetate on the levels of nitric oxide in cortex, brainstem, midbrain and cerebellum 30 days after treatment with seven daily intramuscular injections of 0.10 and 1.0 mg/kg uranyl acetate are presented in Fig. 2. The control values were 50 ± 5, 20 ± 2, 50 ± 9 and 45 ± 9 nM/mg protein for cortex, midbrain, brainstem and cerebellum, respectively. There was a significant increase in the level of nitric oxide in the cortex ($\sim 150\%$ of control, $P<.007$) and midbrain ($\sim 190\%$ of control, $P<.01$) in the animals treated with 0.1 mg/kg uranyl acetate. A decrease was observed in cortex and midbrain in rats given the 1.0-mg/kg dose. Brainstem and cerebellum showed a nonsignificant decrease at either doses. These results suggest that uranyl acetate causes differential effects on nitric oxide levels in different brain regions. There was no significant change in nitric oxide levels in liver and kidney (Fig. 3).

3.2.4. Effect of treatment with uranyl acetate on AChE activity in brain regions

The effects of uranyl acetate on AChE in cortex, brainstem, midbrain and cerebellum are presented in Fig. 4. The control enzyme activities were 0.4 ± 0.06, 0.7 ± 0.05, 0.6 ± 0.03 and 0.2 ± 0.01 μmol/min/mg protein for cortex, midbrain, brainstem and cerebellum, respectively. There was a significant increase in AChE activity in the cortex of the animals treated with 1.0 mg/kg uranyl acetate ($\sim 150\%$ of control, $P<.02$), but not with 0.1 mg/kg dose. Other regions did not show any change at either doses. Similarly, plasma cholinesterase activity did not show any change at either doses (data not shown). Thus, from these data, it appears that uranium exposure may have differential response in different brain regions.

3.2.5. Effect of treatment with uranyl acetate on m2 mAChRs in the cortex and brainstem

Cholinergic system in the CNS plays an important role in a variety of neurobehavioral functions as well as in learning and memory. AChE and muscarinic receptor are important elements of this complex system. We studied the receptor ligand binding for m2 mAChRs in the cortex and brainstem. Data presented in Fig. 5 on cortical and brainstem m2 muscarinic receptor ligand binding from the animals treated with either 0.1 or 1.0 mg/kg uranyl acetate suggest that the ligand binding density did not show any significant change 30 days after treatment with uranyl acetate was stopped.

4. Discussion

The purpose of these studies was to evaluate the long-term neurobehavioral effects of low-dose exposure to uranyl acetate and to explore the molecular mechanism(s) that could underlie these effects. We used uranyl acetate, as its composition of uranium isotopes (^{234}U, ^{235}U, ^{238}U) is the same as that present in DU, and therefore, the chemical toxicity associated with uranyl acetate exposure will reflect the possible toxic effects following DU exposure. The present results suggest that low-dose multiple exposure with uranyl acetate causes long-term neurobehavioral deficits after the initial exposure has ceased. These findings are consistent with recently published report (McDiarmid et al.,

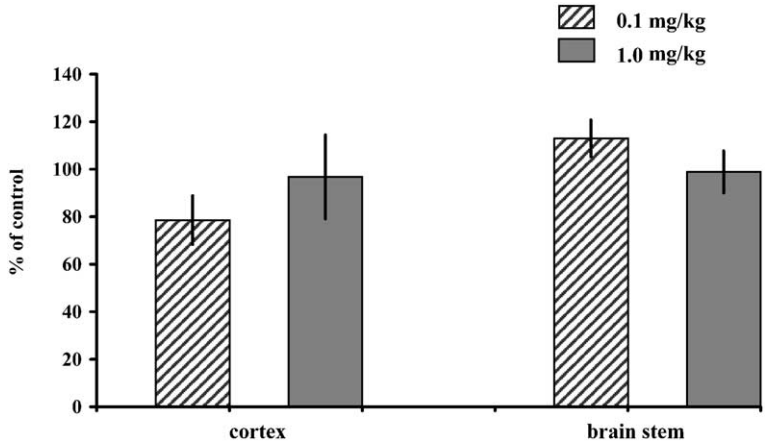

Fig. 5. Effect of intramuscular treatment with uranyl acetate on m2 mAChR ligand binding in the cortex and brainstem. The animals were pretreated with intramuscular injection of 0.1 and 1.0 mg/kg uranyl acetate daily for 7 days as described in Materials and Methods. Treatment with uranyl acetate was discontinued thereafter and the animals were maintained for 30 days following discontinuation of the treatment. On Day 30, the animals were sacrificed and brain regions were dissected on ice. The details of the treatment, membrane preparation and [^{3}H]AF-DX384 binding assay are elaborated in Materials and Methods. The control ligand bindings were 1226 ± 110 and 353 ± 19 fmol/mg protein. Data presented as mean ± S.E. (percent of control).

2000) showing a strong correlation between the levels of DU and cognitive deficiencies in the PGW veterans who carry fragments of DU in their soft tissues.

The neurobehavioral data show that treatment with low-dose uranyl acetate resulted in a significant sensorimotor impairment that were reflected in inclined plane performance, forepaw grip time, beam-walk scores and beam-walk times 30 days after the treatment was stopped (Fig. 1a and b). There was a dose-dependent impairment of inclined plane performance. Grip time, beam-walk time and beam-walk score were significantly impaired at the lowest dose tested. However, there was no dose-dependent response, possibly related to "floor effect". The behavioral measures may reflect dysfunction at multiple anatomical areas of the CNS, peripheral nervous system or muscle. These effects are mediated by complex array of multiple molecular pathways. For example, beam-walking performance is an integrated form of behavior necessitating pertinent levels of consciousness, memory, sensorimotor and cortical, subcortical, cerebellar, spinal cord, peripheral, neuromuscular junction and muscular functions. However, it has been suggested that an injury to cortex is reflected by a deficit in beam-walk task (Whishaw et al., 1985). In a recent study, Hoane et al. (2000) observed that pretreatment with antagonists of *N*-methyl-D-aspartate (NMDA) receptor, MK-801 and antioxidant *N-tert*-butyl-α-phenylnitrone (PBN) facilitated a faster recovery in forelimb placing deficit induced by large lesions of the sensorimotor cortex. Therefore, it is possible that uranyl acetate-induced behavioral deficits involve regulation of multiple pathways in the CNS that may cause overstimulation of the receptors modulated by excitatory amino acids such as NMDA receptor. Further studies are required to evaluate the relative contribution of these structures in the sensorimotor impairments observed in these studies.

Free radicals are highly reactive species that can exist in independent state, or because of their unpaired electrons, they can modify proteins, lipids and DNA and initiate a cascade of events that can damage the cellular milieu in which they are generated. Nitric oxide produced in the CNS is a highly reactive species that has been implicated in a variety of neurodegenerative diseases (Gobbel et al., 1997; Squadrito and Pryor, 1998; Bogdan, 2001). However, there are secondary radicals that are generated by interaction of two reactive oxygen species (ROS), e.g., peroxynitrite that is generated by reaction between superoxide anion and nitric oxide (Dalton et al., 1999). Typically, equilibrium exists between generation of ROS and the antioxidant defense system, which maintains homeostatic control over the cell's oxidative state. Oxidative stress occurs when this balance is altered to favor ROS (Simonian and Coyle, 1996; Bains and Shaw, 1997), resulting in a buildup of oxidatively modified molecules that can disrupt normal cellular activity. It has been recognized that abnormal oxidative metabolism and environmental stimuli such as UV radiation and transition metal ions can induce excessive generation of free radicals (Bains and Shaw, 1997; Ye et al., 1999). Our data suggest that DU could induce an increase in the generation of nitric oxide in the cortex and midbrain of the animals treated with 0.1 mg/kg uranyl acetate, whereas at 1 mg/kg treatment, there was statistically insignificant decrease in cortex and midbrain as well as in brainstem and cerebellum. Because our data are derived from single time-point evaluations, the possibility also exists that the increased levels might have occurred earlier than 30 days, which may have persisted for longer period. Also, in view of the recognized fact that the expression of nitric oxide synthase is regulated by both physiological and pathological changes, it is possible that pathways acutely affected by uranyl acetate may be causing increased expression of nitric oxide synthase, leading to the increased levels of nitric oxide. A number of heavy metals are known to be sequestered in the astrocytes; it is possible that at 0.1 mg/kg dose of uranyl acetate, the components of nitric oxide synthase are activated at transcriptional as well as translational levels, as observed by Spranger et al. (1998) in astrocytes and Eckhardt et al. (1999) in beta cells of pancreatic islets. A possible explanation for this biphasic response in cortex and midbrain could be that higher concentrations of uranyl acetate might inactivate the components of protein machinery of nitric oxide synthase(s) in these brain regions. Similar increases and decreases in nitric oxide levels have been observed in murine macrophages by lead, nickle and cobalt (Tian and Lawrence, 1996).

Oxidative stress can induce neuronal cell death in a variety of circumstances (Halliwell, 1992). There have been several studies that provide strong evidence that oxidative stress can be a final and common pathway in various forms of neuronal cell death including a number of acute and chronic neurological and neurodegenerative diseases (Halliwell, 1992; Simonian and Coyle, 1996). Certain heavy metals such as cadmium, nickle, vanadium and cobalt are known to cause oxidative damage by regulating the generation of free radicals such as superoxide anion and nitric oxide (Tian and Lawrence, 1996; Donaldson and LaBella, 1983; Oster et al., 1993). It has recently been shown that nitric oxide selectively releases various metal ions from different metal binding proteins (Zangger et al., 2001). Extracellular nitric oxide has also been shown to react with reduced glutathione, thus causing a decrease in the available pool of an important cellular-reducing agent (Clancy et al., 1994). Therefore, it is possible that uranyl acetate exposure may cause damage to the CNS by perturbing the redox system and abnormal generation of highly reactive nitric oxide and peroxynitrite. Furthermore, it has been shown that nitric oxide could regulate the permeability of the blood–brain barrier (BBB) (Shukla et al., 1996; Minami et al., 1998). The BBB regulates the entry of molecules into the CNS based on their size, hydrophobicity and charge. This selective nature of BBB helps maintain the homeostasis of the CNS environment to ensure proper brain functioning (Rubin and Staddon, 1999). If the BBB is compromised, various humoral, immunological, ionic and chemical factors may gain access to the CNS. These changes may induce a

cascade of biochemical, electrophysiological and inflammatory responses that may lead to neurological disorders. Thus, it is also possible that the long-term health effects following exposure with uranyl acetate may be a consequence of changes in the BBB permeability.

We studied the effect of treatment with uranyl acetate on the CNS cholinergic system because of central role of AChE and muscarinic receptors in memory and other neurologic functions (Taylor and Brown, 1998; Nostrandt et al., 1997; Wess, 1996). There was no significant effect of treatment with uranyl acetate on AChE activity in any brain region except cortex, where treatment with 1 mg/kg uranyl acetate resulted in a significant increase in AChE activity. The increased AChE could cause a continuous depletion of required acetylcholine at the synapse, leading to defective neural transmission. Additionally, while not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling (Bigbee et al., 2000; Guizzetti et al., 1996; Sternfeld et al., 1998). Therefore, it is possible that following treatment with uranyl acetate, there may be subtle changes in the CNS that are reflected in long-term increased synaptic modeling and repair. Exposure to heavy metals such as mercury causes regulatory changes in cholinergic pathway (Coccini et al., 2000). In particular, an increase in m2 muscarinic receptor ligand binding density has been observed in hippocampus and cerebellum following low-dose exposure to mercury in drinking water (Von Burg et al., 1980; Castoldi et al., 1996; Coccini et al., 2000). Although we did not find long-term changes in m2 AChR receptor ligand binding density in brainstem and cortex in response to treatment with uranyl acetate, there may be acute changes in cholinergic system following treatment that were not evaluated in the present study.

In summary, our results suggest that low-dose exposure with uranyl acetate caused significant sensorimotor deficits and alteration in nitric oxide levels in the brain regions in rats long after the treatment with uranyl acetate was seceded. The changes in neurobehavioral performance may involve a combination of mechanisms related to central and peripheral or neuromuscular system.

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