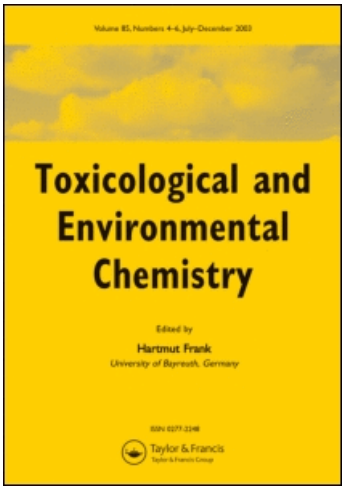

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Functional and morphological alterations induced by uranyl nitrate in rat submandibular gland

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RESEARCH ARTICLE

Functional and morphological alterations induced by uranyl nitrate in rat submandibular gland

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Exposure to uranium (U) is an occupational hazard to workers who continually handle it and an environmental risk to the population at large. Adverse effects of U on different tissues, particularly kidneys, have been reported. The aim of the present study was to investigate whether U might produce damage to the rat submandibular gland (SMG). Uranyl nitrate (UN) was used to evaluate the secretory responses to norepinephrine (NE) or to the parasympathomimetic agent, methacholine (MC), along with some morphological and histological parameters. In addition, the presence of U in saliva was determined by atomic absorption spectrometric techniques. Results indicate that intraperitoneal (i.p.) injection of a single dose of UN (2 or 4 mg kg^{-1}) induced a functional decrease in the induced secretory responses in the rat SMG, demonstrating for the first time that U interferes with salivary secretion. Moreover, salivary responses to MC as well as to NE were decreased after UN administration, in time- and dose-dependent manner, displaying a higher diminution after 7 days post injection. In agreement with the functional studies, the injection of UN produced morphological alterations of SMG, consisting in a reduction of mean acinar area and a marked vacuolization. Data indicate that UN produced an adverse effect in a dose- and time-dependent manner on SMG function and morphology. Furthermore, it was shown that U was incorporated in saliva and therefore, these findings may contribute to create a useful, noninvasive method to detect the exposure to U.

Keywords: salivary gland; uranium; salivary secretion

Introduction

The adverse effects of uranium (U) salts and 18 other metals on animals were reported for the first time by Gmelin (1824). Even before the discovery of radioactive compound, U was known to be toxic; and reports over a hundred years ago demonstrated its nephrotoxic effects (Lee, Kim, and Lee 2003; Lee et al. 2004; Van Crugten, Somogyi, and Nation 2000).

Uranium is a naturally occurring, ubiquitous, heavy metal found in various chemical forms in all soils, rocks, seas, and oceans, which is also present in drinking water and food, and used primarily in nuclear power plants. Uranium remaining after removal of the

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enriched fraction of U^{235} is referred to as depleted uranium (DU). DU has about 60% of the radioactivity of U, and may also result from the reprocessing of spent nuclear reactor fuel. Therefore, exposure to U is an occupational hazard to workers who continually handle it and an environmental risk to the population at large (WHO 2001).

Uranium enters the body by different routes, mainly through ingestion, inhalation, and dermally via contamination of wounds. The target organs in acute U intoxication are kidneys and bone (WHO 2001). Several investigators showed the distribution and excretion of U after inoculation of uranyl nitrate (UN), $UO_2(NO_3)_2$, confirming kidneys and bone as preferred accumulation sites (Newman et al. 1948; Novikov 1972; Priest et al. 1982).

Damage to the kidney includes vacuolization of tubular cells, formation of hyaline casts, and tubular necrosis, producing alterations in renal function that lead to death (Martínez et al. 2003). By intravenous (i.v.) injection of varying doses of U compounds, it was further demonstrated that this metal promptly leave the bloodstream (Newman et al. 1948). Within 40 min post administration, about half is excreted in urine, one-quarter to one-third deposited in skeleton, and the remainder almost equally spread between kidney and other soft tissues. At this time, less than 1% remains in blood. Gradually, U deposited in other soft tissues and skeleton tends to move into the kidney to be excreted in the urine. However, studies did not evaluate the potential contribution of salivary glands in the U secretion. Paquet et al. (2006) reported a wide distribution of this tracer in the majority of the tissues after chronic exposure. In fact, exposure to U was found to induce pathological changes in several organs and tissues (Domingo 2001; Guglielmotti, Ubios, and Cabrini 1985; Leggett 1989; Lin et al. 1993; Martínez, Cabrini, and Ubios 2000; Tasat et al. 2007; Ubios, Guglielmotti, and Cabrini 1990).

The submandibular gland (SMG) is one of the major salivary glands, together with the sublingual and the parotid glands. The two branches of the autonomic nervous system control secretion of saliva. The parasympathetic nervous system exerts its function through the activation of muscarinic cholinergic receptors on salivary glands, while the sympathetic nervous system acts on α_1- and β-adrenergic receptors. Salivary glands are known to act as an alternative excretory organ of metals including mercury (Joselow, Ruiz, and Goldwater 1968), strontium (Setala 1962), lead (Craan, Nadon, and P'an 1984), among others. However, no data seem available on the role of salivary glands as a possible U excretory organ, or on the alterations that may be produced by this metal on the functional activity of these glands.

Therefore, this study was performed in order to investigate whether UN incorporation into animals might produce damage to rat SMG. A range of injection schedules of UN was carried out to evaluate the secretory responses to norepinephrine (NE) or to the parasympathomimetic agent, methacholine (MC), together with some morphological and histological parameters. Further, the presence of U in saliva was determined after metallic administration.

Materials and methods

Animals

Adult male Sprague-Dawley rats, weighing 200–230 g were purchased from the Division of Laboratory Animal Production, Faculty of Veterinary Sciences, University of La Plata, Buenos Aires. Rats were maintained in our animal health care facility at 22–24°C and 50–60% humidity, on a 12 h light/dark cycle, with food and water available *ad libitum*.

Rats were randomly separated into three groups and UN was administered intraperitoneally (i.p.) dissolved in Tris maleate buffer (vehicle; Sigma Chemicals, St Louis, MO, USA). Animal procedures were in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA, 1996.

Functional studies

Rats were received i.p. injections of UN, 2 or 4 mg kg^{-1} or vehicle (control) (Guglielmotti, Ubios, and Cabrini 1985). Each half was treated differentially with MC chloride (FLUKA, Berlin, Germany) or NE bitartrate (Sigma Chemicals, St Louis, MO, USA) to induce salivary secretion.

Salivary responses were determined in anesthetized rats (ether induction followed by chloralose 100 mg kg^{-1}, 0.5 mL NaCl 0.9%, i.v.). The ducts of each SMG were cannulated as previously described (Bianciotti et al. 1994). Different doses of the sialogogues MC (1, 3, or 10 μg kg^{-1} in saline) or NE (1, 3, 10, or 30 μg kg^{-1} in saline) were sequentially injected via the right femoral vein to induce salivation, because resting flow of saliva is not observed in rats. MC and NE dose response curves (DRC) on salivary secretion were performed after UN or vehicle administration. The saliva secreted from both SMG during 3 min after administration of each dose of the sialogogues was collected on aluminum foil and weighed. The results were expressed as milligram saliva.

Morphological studies

For histomorphometrical studies, rats were killed by cervical dislocation 2, 4, and 7 days after UN injection to harvest SMG: six glands of each group were fixed in 10% formaldehyde and embedded in paraffin. The material was cut into 4 μm sections using four slices of each gland, and measurements were made in five randomly selected fields of each gland and, 10 acini per gland were randomly selected and the mean area calculated. Seven glands from untreated animals served as controls and were similarly processed. A semi-automatic analyzing system (Kontron) was employed to quantify glandular components. The SMG structures' diameter was the parameter evaluated following the method described elsewhere (Weibel, Kistler, and Scherle 1964).

Measurements of uranium

UN (2 and 4 mg kg^{-1}) was injected i.p. to the rats. A few minutes later, MC (10 μg kg^{-1}) or NE (30 μg kg^{-1}) stimulated saliva secretion was collected and U excreted was measured by atomic absorption spectrophotometry by the National Commission of Atomic Energy of Argentina. Before the measurement, the U was deproteinized with HCl 0.8 N. The results were expressed in part per million (ppm) UN.

Statistics

Data are presented as mean ± standard error of the mean (SEM). The control and treated groups were compared using one-way analysis of variance (ANOVA) that was followed by Newman-Keul's Multiple Comparison Test or Dunnett's, as indicated. All statistical analyses were performed with GraphPad Prism Version 5.00 software (San Diego, CA, USA). Differences with p values < 0.05 were considered statistically significant.

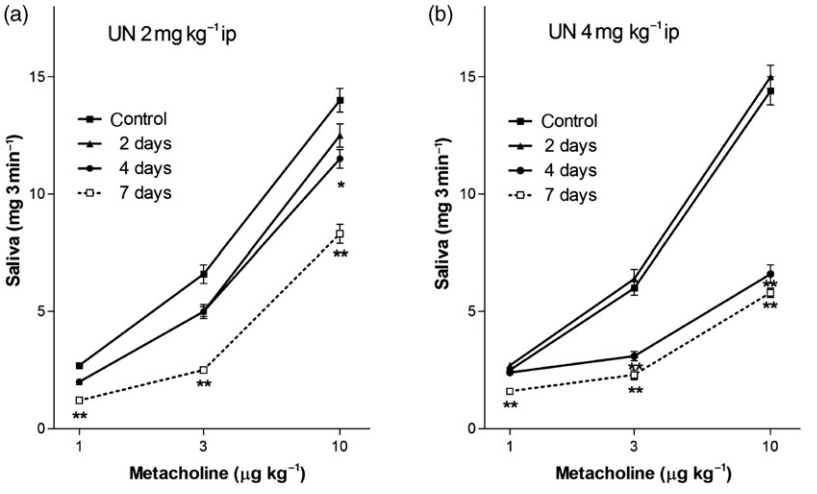

Figure 1. Effect of UN on the secretory responses induced by a cholinergic agonist.
Notes: UN (2 or 4 mg kg^{-1}) administrated i.p. on the MC-induced salivary secretion studied 2, 4, and 7 days post treatment. Values are means ± SEM. Six to eight animals per group, ANOVA post test Newman-Keuls', $*p < 0.05$ and $**p < 0.01$ *vs.* respective control.

Results

Effect of UN on SMG functionality

Figure 1 (a) and (b) show the secretory responses induced by MC after administration of 2 or 4 mg UN kg^{-1}, respectively.The salivation of rats exhibited a gradual decrease in response to MC after UN injection, which was dose- and time-dependent, as evidenced by the shift to the right of the curves. Both doses modified gland functionality after 4 days post injection. The highest decrease in salivary secretion was observed at 7 days post injection, regardless of the UN dose injected. The maximal response to MC (10 μg kg^{-1}) evaluated at 7 days post injection was diminished by 40 and 60% after 2 and 4 mg UN kg^{-1}, respectively (Figure 3a and b).

The doses of UN employed decreased the efficacy of NE to induce salivary secretion at 4 and 7 days post injection. In addition, a significant decrease in salivation was observed in 2 and 4 mg UN kg^{-1}-treated animals after 2 days post injection, employing NE concentrations of 1 and 3 μg kg^{-1}, and concentrations between 1 and 10 μg kg^{-1}, respectively (Figure 2a and b). The maximal response to NE (30 μg kg^{-1}) was markedly reduced 7 days post injection of 2 and 4 mg UN kg^{-1}, displaying a decrease of 45 and 82%, respectively (Figure 3c and d). Therefore, the gradual decrease in salivation in response to NE after UN injections showed a dose and time dependence.

Morphological and histological studies

In order to determine whether the altered glandular functionality was associated to an impartment in gland morphology, the characteristics of the acini, the glandular units involved in saliva production were investigated. Quantitative morphological analysis indicated that the area of acini from 2 and 4 mg UN kg^{-1}-treated glands was significantly reduced, compared with their respective control glands. The reduction of the mean acini area was significant 4 days post injection of 2 mg UN kg^{-1} or 2 days post injection of

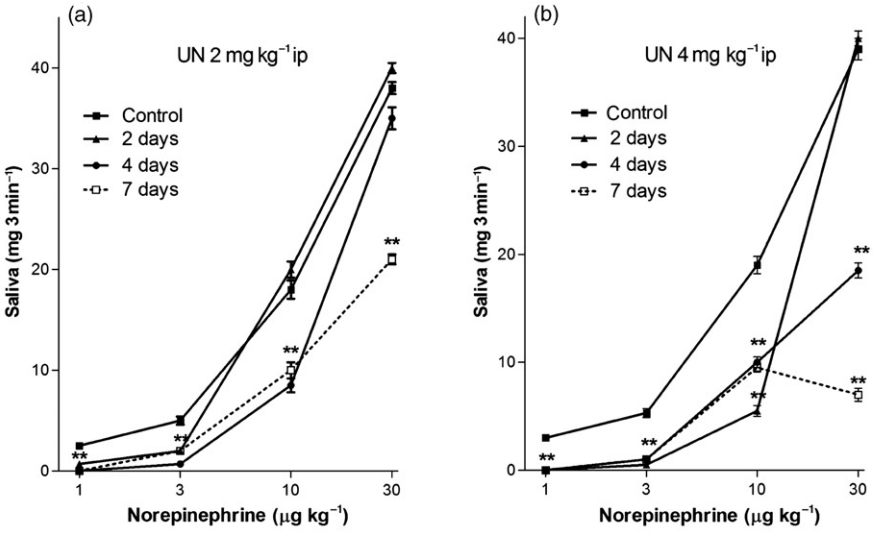

Figure 2. Effects of UN on the secretory responses induced by a sympathetic agonist.
Notes: UN (2 or 4 mg kg^{-1}) administrated i.p. on the NE-induced salivary secretion studied 2, 4 and 7 days post treatment. Values are means ± SEM. Six to eight animals per group, ANOVA post test Newman-Keuls', $^{**}p < 0.01$ *vs.* respective control.

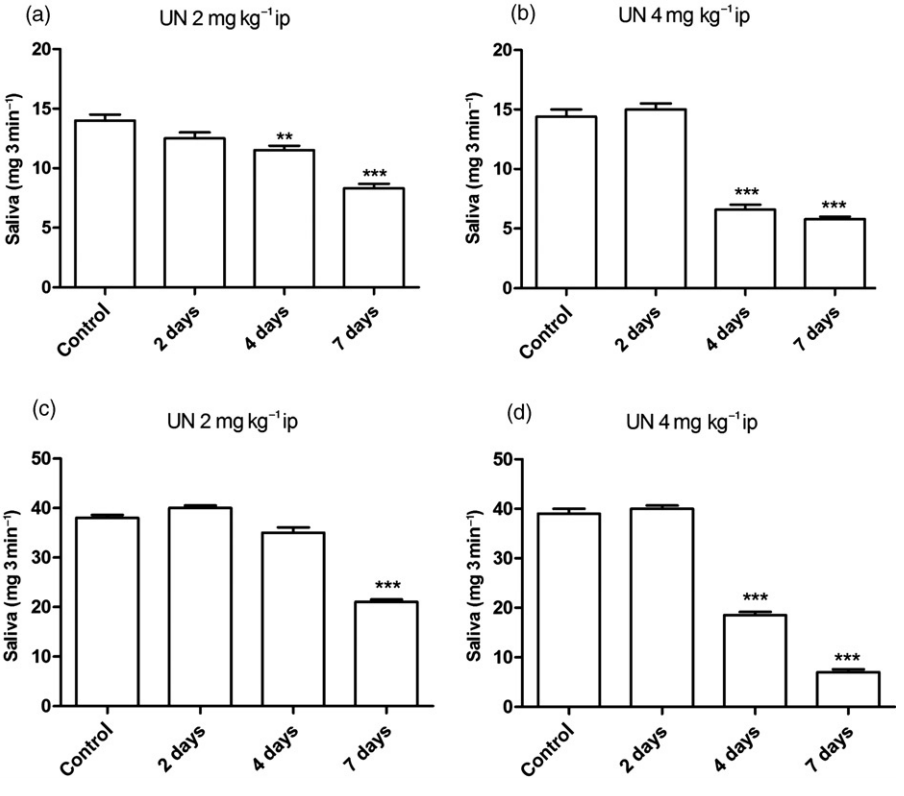

Figure 3. Alterations of maximal response induced by cholinergic and sympathetic agonist by UN.
Note: The maximal response induced by 10 μg kg^{-1} of MC and 30 μg kg^{-1} of NE evaluated at 2, 4, and 7 days post treatment (UN 2 and 4 mg kg^{-1} administrated, i.p.). Values are means ± SEM. Eight to ten animals per group, ANOVA post test Dunnett, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* respective control.

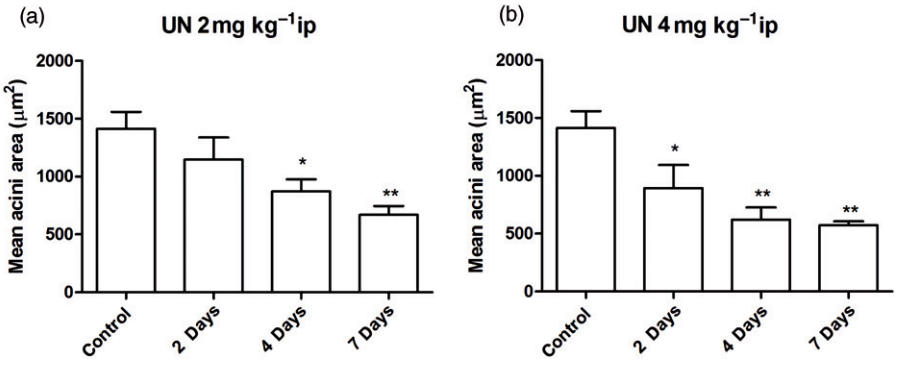

Figure 4. Effect of UN on mean area of acini mean area determinate in SMG 2, 4 and 7 days post UN (2 and 4 mg kg^{-1} by i.p. administrated, (a) and (b), respectively).
Notes: Values are means ± SEM. ANOVA post test Dunnett, *$p < 0.05$, **$p < 0.01$ *vs.* respective control.

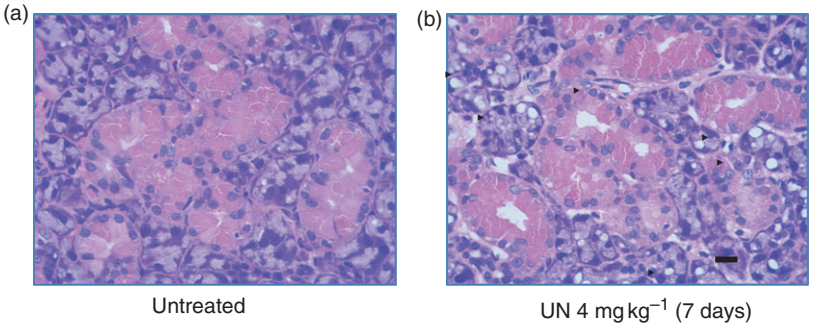

Figure 5. Effect of UN on gland histology. (a) Normal histological appearance of SMG from untreated rat; (b) SMG after 7 days of UN treated 4 mg kg^{-1}, exhibiting alterations in the structure organization of acini and vacuolization (►). Scale bar 20 μM. 630×-fold magnification. Data represent six animals per group.

4 mg UN kg^{-1}, demonstrating a maximal diminution after 7 days at both UN doses and thus indicating time dependency (Figure 4a and b). In agreement with this, histological studies demonstrated a marked alteration in the structural organization of acini and an increased number of vacuoles in salivary glands (Figure 5a and b).

Dosage of uranium in saliva

Whether U might be secreted to saliva was determined by atomic absorption spectrometric techniques. Results showed that both doses of UN injected produced the presence of the metal in salivary secretion in response to either MC or NE. In addition, no significant differences in secreted U were observed between the two concentrations of UN administered (Table 1). On the other hand, nondetectable U levels were shown in control rats injected with vehicle.

Table 1. Dosage of uranium in saliva.

	Metacholine ($10\,\mu g\,kg^{-1}$)		Norepinephrine ($30\,\mu g\,kg^{-1}$)	
Time (min)	$2\,mg\,UN\,Kg^{-1}$	$4\,mg\,UN\,Kg^{-1}$	$2\,mg\,UN\,Kg^{-1}$	$4\,mg\,UN\,Kg^{-1}$
15	0.45 ± 0.05	0.40 ± 0.05	0.44 ± 0.05	0.47 ± 0.04
30	0.29 ± 0.09	0.375 ± 0.05	0.35 ± 0.04	0.30 ± 0.10
45	0.32 ± 0.07	0.36 ± 0.05	0.32 ± 0.05	0.34 ± 0.05
60	0.31 ± 0.06	0.365 ± 0.05	0.34 ± 0.04	0.33 ± 0.02

Notes: Uranium excreted through saliva after UN administration (2 and $4\,mg\,kg^{-1}$) via i.p. Every 15 min and until 60 min the salivary secretion was induced by NE ($30\,\mu g\,kg^{-1}$) or MC ($10\,\mu g\,kg^{-1}$) to obtain the saliva. Values are means ± SEM.

Discussion

Natural U exposure is derived from mining, milling, and processing of U ore, as well as from ingestion of groundwater that is naturally contaminated with U (Brugge, de Lemos, and Oldmixon 2005). Furthermore, DU is a byproduct of the enrichment process of U for its more radioactive isotopes to be used in nuclear energy. DU is pyrophoric and a dense metal with unique features when combined in alloys; DU is used by the military in armor and ammunitions. Concerns about possible health consequences to troops and civilian populations residing in conflict areas where DU munitions were used have raised many important environmental health questions. Therefore, in addition to occupational exposure to soluble and insoluble U compounds, military use of DU is likely to have significant impact on environmental levels (WHO 2001).

The primary health outcomes of concern documented with respect to U are renal, developmental, reproductive, diminished bone growth, and DNA damage. However, the reported health effects derived from experimental animal studies and human epidemiology are still limited regarding other organs (Jiang and Aschner 2006).

This study demonstrated for the first time that U interferes with salivary secretion, decreasing stimulated salivation that was associated to morphological alterations of the SMG, especially acini production. Our findings indicate that i.p. injection of a single dose of UN (2 or $4\,mg\,UN\,Kg^{-1}$) induced functional decrease in the secretory responses induced by either MC or NE in rat SMG. Moreover, salivary responses to MC as well as to NE were decreased after UN administration, in time- and dose-dependent way, displaying the higher diminution after 7 days post injection. As reported in various tissues, the degree of U toxicity depends on the amount absorbed and the resultant plasma levels irrespective of the route of administration (Juile 1973). Therefore, health consequences are determined by the level and duration of exposure (WHO 2001).

In agreement with the functional studies, the injection of UN produced a dose-and time-dependent morphological alteration of SMG, consisting in a reduction of the mean acinar area. The acini are the secretory units of the salivary glands (Cook and Young 1989), indicating that the impairment in salivary function was associated with alterations in acini. Accordingly, a vast body of literature supports the role of the reduction and morphological disturbances of acini in alterations of SMG functionality (Correia et al. 2008). In addition, histological studies showed a marked vacuolization after UN administration, similar to pathologies in which the salivary secretion is decreased (Ekuni et al. 2010).

The structural damage observed in the SMG could be related to that observed in kidneys of rats exposed to UN (Gilman et al. 1998). Abnormalities including focal interstitial inflammation and fibrosis, coupled with nephritic atrophy and glomerular scarring were described for kidneys after U exposure (Bencosme et al. 1960; McDonald-Taylor, Singh, and Gilman 1997). In addition, damage to the kidney includes vacuolization and necrosis of tubular cells was noted (Martínez et al. 2003).

Of the U that is absorbed into the blood, approximately 67% is filtered by the kidney and excreted in the urine within the first 40 min (Brugge, de Lemos, and Oldmixon 2005; Paquet et al. 2006; WHO 2001). Therefore, the intake of U can be determined from the amounts excreted daily in urine, evaluated using sensitive mass spectrometric techniques. Results indicate that SMG of the rat is capable of excreting U after either stimulation with cholinergic or sympathetic sialogogues agents soon after UN administration. Accordingly, the amount of U found in urine is high after which for the first 2 h intoxication was high (Guglielmotti et al. 1989). It is well-known that other metals such as mercury (Joselow, Ruiz, and Goldwater 1968), strontium (Setala 1962), lead (Craan, Nadon, and P'an 1984), thalium (Richelmi et al. 1980) lithium (Spring and Sprites 1969), and magnesium (Gow 1965), as well as bromides and iodides (Stephen et al. 1973) are also excreted through saliva.

In summary, these results demonstrated the adverse, dose- and time-dependent effects of UN on SMG function and morphology. Further, it was found that U is incorporated in saliva through an unknown mechanism. Therefore, these findings may contribute to create a useful, noninvasive method that could improve the diagnostic and follow-up for the exposure to U.

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