Uranium (U) is a heavy metal used in the nuclear industry and for military applications. U compounds are toxic. Their toxicity is mediated either by their radioactivity or their chemical properties. Mammalian kidneys and bones are the main organs affected by U toxicity. Although the most characteristic response to U exposure is renal dysfunction, little information is available on the mechanisms of its toxicity at the molecular level. This report studied the genotoxicity of U. Apoptosis induction in normal rat kidney (NRK-52\textsuperscript{E}) proximal cells was investigated as a function of exposure time or concentrations (0–800\textmu M). In parallel, DNA damage was evaluated by several methods. In order to distinguish between the intrinsic and the extrinsic pathways of apoptosis, caspases-8, -9, -10 assays were conducted and the mitochondrial membrane potential was measured. Three methods were selected for their complementarities in the detection of genetic lesions. The comet assay was used for the detection of primary lesions of DNA. \gamma-H2AX immunostaining was achieved to detect DNA double-strand breaks. The micronucleus assay was used to detect chromosomic breaks or losses. DNA damage and apoptosis were observed in a concentration-dependent manner. This study demonstrated that U is genotoxic from 300\textmu M and induces caspase-dependent apoptosis cell death from 200\textmu M mainly through the intrinsic pathway in NRK-52\textsuperscript{E} cells. These results suggest that the DNA damage caused by U is reversible at low concentration (200–400\textmu M) but becomes irreversible and leads to cell death for higher concentrations (500–800\textmu M).

**Key Words:** uranium; genotoxicity; apoptosis; DNA damage; renal cells; comet assay.

Natural uranium (U) exposure derives from ore mining, milling, and processing, as well as from naturally contaminated groundwater. Release of U in the environment presents a threat to human health. Indeed, acute exposure was observed in miners exposed to high U concentrations in uranium mines. Exposure by ingestion of contaminated drinking water was observed, for example, in Finland (Kurttio et al., 2006) in regions where bedrock consists mainly of U-rich granitoids and granites which lead to soil contamination. In the case of long term exposure, uranium accumulates in the bone and kidneys (Leggett and Pellmar, 2003). In kidney, the most characteristic response to short- or long-term exposure is renal dysfunction. This dysfunction mainly involves proximal tubule renal epithelial cells (Leggett, 1989; Zamora et al., 1998).

**In vivo**, few studies have been conducted in rat and mice and demonstrated that depleted U (DU) (Montel et al., 2006; Wan et al., 2006) or natural uranium (Taulan et al., 2004) alter gene expression, particularly that of genes involved in inflammation, oxidative stress, and apoptosis but few studies described the mechanism of apoptosis and genotoxic effect induced by U. **In vitro**, in kidney proximal cells, U (VI) induces 50% loss of viability (CL\textsubscript{50}) at a concentration of 500\textmu M in NRK-52\textsuperscript{E} normal rat cells (Carrière et al., 2004) and 800\textmu M in LLC-PK\textsubscript{1} porcine cells (Mirto et al., 1999). Kalinich et al. (2002) observed that DU induces apoptosis in mouse macrophages. However, the pathway of apoptosis induction has never been studied. Two pathways can be involved in apoptosis induction: the extrinsic and the intrinsic pathways. The extrinsic pathway, also called death receptor pathway involves ligation of death receptors, resulting in caspase-8 activation which activates other caspase effectors such as caspase-3. The intrinsic pathway is activated by various stimuli such as drugs or cytotoxic compounds and results in mitochondrial alteration leading to mitochondrial membrane depolarization and activation of caspase-9. Active caspase-9 has been shown to cleave and activate directly the effector protease, caspase-3 (Twomey and McCarthy, 2005).

Apoptosis cell death can be the consequence of DNA damage, which can be caused by metals either indirectly through the generation of free radicals, or by direct interaction between the metal and DNA. Several methods are classically used to study the genotoxicity of a substance. Among them, the cytokinesis-block micronucleus assay is used to identify genetic damage such as chromosome breaks or losses (Fenech, 2000). The comet assay is widely used to detect simple, double DNA strand breaks, and alkali-labile sites (Collins, 2004). Moreover, phosphorylated H2AX histone (\gamma-H2AX) immunostaining reveals nuclear foci generated by double-strand breaks, in the course of their reparation (Aten et al., 2004).
Indeed, it has been shown that U produces DNA strand breaks in the presence of vitamin C ex vivo (Yazzie et al., 2003). It was also demonstrated that DU can induce oxidative DNA base damage ex vivo (Miller et al., 2002). Moreover, DNA damage was observed in rat after inhalation of DU (Monleau et al., 2006). In vitro, natural U induces DNA damage in Chinese hamster ovary EM9 (Stearns et al., 2005), CHO cells (Lin et al., 1993), and human osteoblast cells (Miller et al., 2002, 2003), as shown by the formation of micronuclei and DNA adducts as well as sister chromatide exchange and chromosome aberrations.

This report investigated the genotoxicity of DU for renal cells, which can be the cause of the renal dysfunctions observed after an acute contamination. Cell mortality (apoptosis, necrosis) was assessed by microscopic observations, and the pathway of apoptosis induction was determined by enzymatic tests (caspases) and mitochondrial membrane potential (MMP) measurement. The micronucleus assay, the comet assay, and γ-H2AX immunostaining were achieved to assess U genotoxicity. Finally, reactive oxygen species (ROS) production was measured after U exposure to evaluate the role of oxidative stress in apoptosis and DNA damage induction.

**MATERIAL AND METHODS**

**Materials.** Depleted uranyl nitrate was obtained from Labosi (Illkirsh, France). Since its radioactivity is weak, we consider that mainly its chemical toxicity is studied in this work. All other chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

**Cell culture and U exposure.** Normal rat kidney proximal cells (NRK-52E, CRL-1571) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). They were cultured in Dulbecco’s minimum essential medium (MEM) as described previously (Carrie et al., 2004). Negative controls were exposed to MEM to which was added 5mM of rhodamine 123. 480 THIEßBAULT ET AL.

**Cell culture and U exposure.** Normal rat kidney proximal cells (NRK-52E, CRL-1571) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). They were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2mM L-glutamine. Cells were maintained at 37°C in a humidified 5% CO2,95% air atmosphere incubator. A 10mM U-bicarbonate stock solution was prepared by dissolution of uranyl nitrate in 100mM NaHCO3. The stock solution was stirred for 30 min to ensure the total dissolution of the crystals and filtered through 0.2-μm filter units. For each experiment, cells were exposed to U (0–1000μM) diluted in minimum essential medium (MEM) as described previously (Carrière et al., 2004). Negative controls were exposed to MEM to which was added 5mM of sodium bicarbonate, pH 7.4. For the micronucleus test, 1.5 × 107 cells/cm2 were seeded, 7 × 103 cells/cm2 were seeded for MMP measurement, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, and ROS production measurement, and 3 × 104 cells/cm2 were seeded for apoptosis and γ-H2AX detection. Once at 80% confluency, cells were exposed to various U concentrations.

**Cytotoxicity.** The colorimetric MTT assay (Mosmann, 1983) was used to evaluate uranyl cytotoxicity. This test is based on the ability of the mitochondrial succinate dehydrogenase enzyme of living cells to reduce MTT to a blue formazan compound. A 5 mg/ml MTT stock solution was prepared by dissolving MTT powder in phosphate buffered saline (PBS). After U exposure (24 h), 10 μl of MTT solution was added to each well and incubated for 2 h at 37°C. Wells were emptied and blue formazan crystals were dissolved with 100 μl of MTT solubilization solution (10% Triton X-100, 0.1N HCl in 2-propanol). After 5 min of dissolution at room temperature under agitation, absorbance at 570 nm was measured using a Statfax-2100 microplate reader (Awareness Technology, Inc., Palm City, FL).

**Apoptosis versus necrosis determination.** For apoptosis versus necrosis determination, after U exposure, cells were washed twice with PBS and incubated for 10 min in 50 μg/ml acridine orange, then 50 μg/ml ethidium bromide in PBS, then washed three times. Stained slides were observed under a fluorescence microscope (BX41, Olympus, Rungis, France) equipped with a fluorescent isothiocyanate (FITC) and a DAPI filter (band pass filter, 350–390 nm; long pass filter, 456 nm) at ×600 magnification. Acridine orange stains viable cells, ethidium bromide stains cell dead by necrosis or apoptosis. A morphological observation by fluorescence microscopy distinguishes between apoptotic cells and necrotic cells. Apoptotic cells differ substantially from necrotic cells in their morphology. Necrotic cells are lysed and emptied their contents resulting in cells ghosts, whereas apoptotic cells present specific feature of condensed and fragmented chromatin.

**Apoptosis assay.** For the determination of MMP, cells were incubated for 10 min at 37°C with 100μM of rhodamine 123. After incorporation of the fluorescent probe, cells were exposed to U up to 24 h. At the end of the incubation period, they were washed twice with PBS, harvested by scraping, centrifuged, and resuspended in 1 ml of PBS. The fluorescence intensity of each cell suspension was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm with a Gemini-X fluorescence spectrophotometer (Molecular Device, Saint Gregoire, France). Cell caspase-3 and -8 activities were determined with the colorimetric assay kits according to the protocols suggested by the manufacturer (Sigma-Aldrich). After U exposure, cells were washed with cold PBS, harvested by scraping, centrifuged (600 × g for 5 min at 4°C), and incubated for 20 min in cell lysis buffer (250mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 25mM dithiothreitol). Cell lysates were centrifuged (16,000 × g for 15 min at 4°C). Ten microliters of the supernatant was incubated with 80 μl of the caspase assay buffer and 10 μl of the colorimetric caspase-3 (Acetyl-asp-glu-val-asp-p-nitroanilide) or caspase-8 (Acetyl-leu-glu-thr-asp-p-nitroanilide) substrate. Plates were incubated for 90 min at 37°C and absorbance was read at 405 nm with a Statfax-2100 microplate reader (Awareness Technology Inc.). The caspase-9 activity was determined with the caspase-9 fluoresceine assay according to the protocol suggested by the manufacturer (Sigma-Aldrich). After U exposure, cells were washed with PBS and incubated for 1 h at 37°C with 5% CO2 with a fluorochrome inhibitor of caspases ×30 solution. Cells were harvested by scraping, washed, and collected by centrifugation (600 × g for 5 min at 4°C). Cells were resuspended in 400 μl of PBS. The fluorescence intensity of cell suspensions was measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm with fluorescence spectrophotometer (Gemini-X, Molecular Devices). The results of caspase-3 and caspase-8 activities were normalized to the total cell protein concentration.

**Genotoxicity assays.** Micronucleus formation was assessed by the cytokinesis-blocked assay. After 24 h of exposure to U, medium was replaced by fresh medium containing 4 μg/ml cytochalasin b in order to arrest cytokinesis. This concentration was chosen because it gives a high percentage of multinucleated cells and a low baseline micronucleus frequency (Surralles et al., 1994). Cells were then washed twice with PBS, fixed for 20 min at room temperature with methanol/acetic acid (3:1, vol/vol), and stained 10 min with 10 μg/ml of acridine orange in PBS. Stained slides were observed under a fluorescence microscope (Olympus, BX41) equipped with a FITC filter (excitation filter, 450–490 nm; emission filter, 515 nm) at ×600 magnification. For each slide, 500 cells were scored (mononucleated, binucleated, trinucleated, tetranucleated cells) to determine the Nuclear Division Index (NDI): NDI = (M1 + 2M2 + 3M3 + 4M4)/N where M1 to M4 represent the number of cells with one to four nuclei and N is the total number of scored viable cells (Fenech, 2000). The comet assay was used to determine DNA damage. Under alkaline conditions, it detects DNA single- and double-strand breaks and alkali-labile sites (Tice et al., 2000). The procedure of Singh et al. (1998) was applied with minor modifications. A base layer of 1% agarose was placed on microscope slides. Tested cells suspended in 0.75% low melting point agarose were spread on the base layer and cover slipped. Slides were placed on ice packs until the
agarose layer hardens. Coverslips were removed and slides were placed for 1 h at 4°C in lysis solution (2.5M NaCl, 100mM ethylenediaminetetraacetic acid [EDTA], 10mM Trizma Base, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10.0) to remove cellular proteins. Slides were then transferred to an electrophoresis chamber containing a 300mM NaOH, 1mM Na2EDTA alkaline solution (pH 13). They were kept in this solution for 30 min to allow the unwinding of the DNA and the expression of alkali-labile damage. A fixed current of 300 mA was applied for 20 min, corresponding to a voltage of 12 V. Slides were neutralized with 0.4M Tris–HCl at pH 7.5 and stained with 50 μl of a 20 μg/ml ethidium bromide aqueous solution. Comets were analyzed under a fluorescence microscope (Olympus, BX41) equipped with a DAPI filter (excitation filter, 350–390 nm; emission filter, 456 nm) at ×20 magnification. For each condition, two slides were analyzed and 300 comets per slide were scored. Comets were manually analyzed by using Image J software. For each comet, tail moment was measured as comet tail moment (μm) = comet tail intensity/comet head intensity × comet tail length.

DNA double-strand breaks were analyzed by γ-H2AX immunostaining. After U exposure, cells were washed with PBS, fixed for 15 min at room temperature with 3% paraformaldehyde, and permeabilized for 10 min at room temperature with a BTP buffer (2% bovine serum albumin, 0.1% Triton X-100, PBS). Cells were incubated with the anti-γ-H2AX antibody (Interchim, Montluçon, France) and with an anti-mouse IgG-FITC secondary antibody (Invitrogen, Cergy-Pontoise, France). Cell nuclei were stained by incubation for 20 min at room temperature with 5 μg/ml Hoechst 33342 solution. Slides were fixed and observed under a fluorescence microscope (Olympus, BX41) at ×600 magnification equipped with a FITC and a DAPI filter. For each sample, two slides were observed and 300 cells per slide were analyzed.

**Determination of intracellular ROS production.** For ROS quantification, cells were stained with 2’,7’-dichlorodihydrofluorescein diacetate acetyl ester (H2-DCF-DA, Invitrogen, Cergy-Pontoise, France) according to Oh and Lim (2006). This dye is a stable nonpolar compound which diffuses readily into the cells and yields H2-DCF. Intracellular OH or ONOO- react with H2-DCF when cells contain peroxides, to form the highly fluorescent compound DCF. Thus, DCF fluorescent intensity is proportional to the amount of peroxides which are produced by the cells. Cells were incubated for 30 min at 37°C with 80μM H2-DCF-DA. After incubation of the fluorescent probe, cells were exposed to U and washed twice with PBS. Cells were then harvested by scraping, centrifuged, and resuspended in 1 ml of PBS. The fluorescence intensity was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm in a Molecular Devices Gemini-X fluorescence spectrophotometer (Molecular Devices). The possible interaction between metal and H2-DCF-DA probe was evaluated by incubation of NRK-52E cells with 80μM H2-DCF-DA for 30 min at 37°C then 2 h with different U concentrations. Fluorescence intensity was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm in a Molecular Devices Gemini-X fluorescence spectrophotometer (Molecular Devices). No interaction between stain and metal was found in our conditions.

**Statistical analysis.** All data were expressed as means ± SD. One-way analysis of variance with Dunnett posttest was used to assess statistical significance of differences between cells exposed to U and control cells. p-values were considered to be statistically significant as *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 with n ≥ 3 in all tests.

**RESULTS**

**Cytotoxicity. Apoptosis versus Necrosis**

A concentration-dependent toxicity was observed, with a 50% loss of viability (Cl50) reached at 550μM after 24 h exposure of NRK-52E cells to U as previously described (Carrière et al., 2004). No cytotoxicity was measured below 300μM of U or before 16 h when cells were treated with a uranyl concentration of 600μM of U (Carrière et al., 2004).

In order to determine the mechanism of cell death, NRK-52E cells were exposed for 24 h to increasing concentrations of U. Marked morphological changes were noticed after U intoxication. Necrotic versus apoptotic cells were counted by fluorescence microscopy and typical features of apoptosis such as cell shrinkage were observed. The percentage of cell mortality is reported on Figure 1A. Concentration-dependent apoptosis induction was measured up to 700μM of U. This increase of apoptosis was significant from 400μM of U. We quantified about 12% of cell mortality for U concentrations of 600 and
700μM with a percentage superior to 80% of apoptosis. In the opposite, 800μM of U induced 80% of cell mortality, which was quasi exclusively necrosis. Kinetics of apoptosis versus necrosis occurrence was studied for 300 and 600μM of U. For 300μM of U, we observed little cytotoxicity (about 2.5% of cell mortality after 30 h of exposure), 100% of dead cells were apoptotic (Fig. 1B). For 600μM of U, apoptosis cell death appeared until 24 h, with a percentage of cell mortality of about 12% (Fig. 1C). After 30 h of exposure, cell mortality reached 45%, 100% of these dead cells were necrotic.

Mechanism of Apoptosis Induction

Activity of caspase-3 was measured after 24 h of exposure to U (0–700μM) (Fig. 2A). A concentration-dependent increase of activity was observed for U concentration ranging from 200 to 400μM (statistically significant, threefold increase).

The MMP, early indicator of cellular intrinsic apoptosis was measured in NRK-52E cells exposed for 24 h to 0–700μM of U. In NRK-52E cells, the decrease in MMP was strongly correlated with apoptosis cell death occurrence (Fig. 3A). This decrease was significant from 200μM of U. Indeed, the MMP decreased by a factor 60 when apoptosis cell death reached its maximum. Kinetics of apoptosis occurrence and MMP decrease was studied for sublethal (300μM of U, Fig. 3B) and lethal (600μM of U, Fig. 3C) concentrations. A correlation between apoptosis and MMP was also observed.

To determine which apoptotic pathway was involved, caspase-8 and caspase-9 activities were measured. Caspase-9 was strongly activated after exposure of cells to concentration

![Image](https://academic.oup.com/toxsci/article-abstract/98/2/479/1713787/1)

**FIG. 2.** Evaluation of caspase-3 (A), caspase-9 (B), and caspase-8 (C) activation in NRK-52E cells after U exposure. Cells were treated during 24 h (caspase-3) or 20 h (caspase-8 and caspase-9) to 0–700μM of U. Caspase-3 and caspase-8 activities were evaluated by colorimetric method and caspase-9 was evaluated by fluorescence method. Caspase activities are presented as mean ± SD (n = 3). P values were considered to be statistically significant as *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 with n ≥ 3 in all tests.

**FIG. 3.** MMP (open circle) and apoptosis (filled square) were evaluated in NRK-52E cells by measuring fluorescence intensity of rhodamine 123 probe. NRK-52E cells were exposed to 0–700μM of U during 20 h (A) or to 16–24 h with 300μM (B) or 600μM (C) of U. Percentage of cells or fluorescence intensity are presented as mean ± SD (n = 3). P values were considered to be statistically significant as *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 with n ≥ 3 in all tests.
of U higher than 500μM (Fig. 2B). Caspase-9 activity increased about three, five, and eightfold for 500, 600, and 700μM of U, respectively, as compared to nontreated cells. This concentration-dependent increase was statistically significant. On the other hand, caspase-8 activation appeared for 600 and 700μM of U, the enzyme activity slightly increased as compared to nontreated cells (Fig. 2C). However, this increase was not statistically significant.

DNA Damage Induced by U

DNA strand breaks and alkali-labile sites induced by U treatment (0–700μM) of NRK-52E cells were investigated using the comet assay (Fig. 4). A concentration- and time-dependent increase of DNA damage was observed. DNA breaks appeared from 300μM of U with a comet tail moment of 1.73 ± 1.1 μm, i.e., 3.5 times longer than in the control. After exposure to 700μM of U, the comet tail moment measured

FIG. 4. Induction of DNA damage in NRK-52E cells after U treatment. Formation of comet after exposure to 500μM of U for 18 h (A) or 24 h (B). Induction of DNA damage in NRK-52E cells treated during 24 h with 0–700μM of U (C) Induction of DNA damage in NRK-52E cells treated with 500μM U for 0–24 h (D).

About 100 cells were counted on three independent pictures captured on three independent slides. Results are indicated as mean ± SD (n = 3). P values were considered to be statistically significant as *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 with n ≥ 3 in all tests.
49.4 ± 15 µm, i.e., 68 times longer than in control cells (Fig. 4C). DNA damage occurred after 18 h of exposure to 500µM of U. The comet tail moment increased slightly between 18, 20, and 22 h of exposure, it was two to four times longer than in control. A strong increase was then observed after 24 h of exposure, the tail of the comet was eight times longer than in nontreated cells (Fig. 4D).

The occurrence of γ-H2AX foci, generated in cell nuclei in response to DNA double-strand breaks, was analyzed after exposure of NRK-52E cells to 200–600µM of U (Fig. 5). In control cells (Fig. 5A), a slight γ-H2AX signal was detected, as it is the case in dividing cells. The quantity of nuclear foci by cell increased with U concentration (Figs. 5B and 5C). The maximal signal was obtained after exposure to 500µM of U during 24 h, with 47 foci by cell when only six foci by cell were counted in nontreated cells (Table 1). The quantity of nuclear γ-H2AX foci in cells exposed to 400, 500, or 600µM of U was not significantly different.

Interestingly, no significant formation of micronuclei was observed after U treatment and whatever U concentration (Table 2). Still the positive control (etoposide 25µM) ensured that the assay was valid.

### DISCUSSION

It is well known that kidney and bone are the major targets of U toxicity (Leggett, 1989; Mirto et al., 1999). In kidney, nephrotoxic damage occurs mainly in the renal proximal tubular epithelium, but very few studies described molecular processes leading to these pathological effects. The present study investigated the genotoxic effects of U on normal rat kidney (NRK-52E) proximal cells.

### TABLE 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nuclear foci/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated cells</td>
<td>6.1 ± 2.4</td>
</tr>
<tr>
<td>U 200µM</td>
<td>14.6 ± 4.6</td>
</tr>
<tr>
<td>U 300µM</td>
<td>20.2 ± 7.6</td>
</tr>
<tr>
<td>U 400µM</td>
<td>35.4 ± 11.4</td>
</tr>
<tr>
<td>U 500µM</td>
<td>46.9 ± 12.7</td>
</tr>
<tr>
<td>U 600µM</td>
<td>39.1 ± 14.9</td>
</tr>
</tbody>
</table>

*Cells were exposed for 24 h with the indicated concentrations. After immunostaining, the quantity of nuclear foci was manually counted in each cell nucleus. About 100 cells were counted on three independent pictures captured on three independent slides. Results are indicated as mean ± SD (n = 3).

### UInduces the Production of ROS

To identify a possible cause of DNA damage, we evaluated the global production of ROS in cells exposed to U. After exposure to 0–600µM of U during 20 h, ROS production increased from 300µM of U (Fig. 6A). We observed a significant increase of ROS production from 400µM, of 2.5-fold and 7.5-fold for 400 and 600µM of U, respectively, as compared to nontreated cells (Fig. 6A). When cells were exposed to U (600µM) up to 24 h, a maximum of ROS production was observed after 18 h of exposure (Fig. 6B). For longer exposure periods, it decreased but did not reach again the level observed in nontreated cells. No significant increase of ROS production was detected after exposure to 300µM of U during 24 h (Fig. 6B).

### TABLE 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>NDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated cells</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td>U 100µM</td>
<td>0.04 ± 0.06</td>
</tr>
<tr>
<td>U 200µM</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>U 400µM</td>
<td>0.06 ± 0.07</td>
</tr>
<tr>
<td>U 600µM</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Etoposide</td>
<td>57.6 ± 14.6</td>
</tr>
</tbody>
</table>

*Cells were incubated for 24 h with indicated concentrations. After acridine orange staining, the quantity of binucleated cells and micronuclei were manually counted. About 100 cells were counted on four independent pictures captured on three independent slides. Results are indicated as mean ± SD (n = 3).
In this study, the cytotoxicity profile is similar to previous studies which observed CI50 for 500 μM of U in NRK-52E cells (Carrière et al., 2004) and 870 μM of U in renal epithelial cells LLC-PK1 (Mirto et al., 1999). However, in other types of cells, the cytotoxicity of DU was lower, the CI50 was 200 μM of DU in CD4+ T cells (Wan et al., 2006) and 100 μM in mouse J774 macrophages (Kalinich et al., 2002).

After exposure to U, we observed apoptosis cell death, occurring for low concentrations of U, below the CI50 concentration. Five to 10% of the cells were apoptotic, i.e., twice to three times more than in control cell culture. For higher concentrations of U, cells underwent necrosis. Microscopic observations, MMP, and caspase activation demonstrated that U induces apoptosis in a time- and concentration-dependent manner. Our results are consistent with previous studies conducted in vitro. Apoptosis (25% of cells) and necrosis (50% of cells) were observed for 200 μM of DU in CD4+ T cells (Wan et al., 2006). In mouse J774 macrophages, apoptosis was observed from 1 μM (Kalinich et al., 2002); when cells were exposed to 100 μM of DU, the percentage of apoptotic cells was about 40%. In kidneys of C57Bl/6j mice (Taulan et al., 2004) and in human renal cells (Prat et al., 2005), expression of apoptosis-related genes was modified after U exposure. Here, we show for the first time that apoptosis induced by U is caspase dependent and activated via the intrinsic pathway: after U exposure, caspase-8 is only slightly activated, whereas caspase-9 is strongly activated and MMP decreases. Intrinsic apoptotic pathway is initiated by the majority of apoptotic stimuli, including irradiation and cytotoxic drugs (Twomey and McCarthy, 2005) but also metals such as arsenic and cadmium (Pulido and Parrish, 2003). It is often the consequence of DNA damage (Twomey and McCarthy, 2005).

We detected single- and double-strand breaks and/or alkali-labile sites in DNA after exposure of NRK-52E cells to sublethal and lethal concentrations of U. This DNA damage increased with U concentration. When cells were exposed to 500 μM of U, the comet tail length was relatively stable after 18–22 h of exposure, but strongly increased after 24 h. γ-H2AX staining increased as U concentration increased, until 400–500 μM of U. For higher concentrations the quantity of γ-H2AX foci was stable, suggesting that cells had attained the maximum of their double-strand breaks detection capacity. Interestingly, whatever the concentration, no micronucleus was detected. This suggests that damage did not lead to non-repairable chromosome break or loss (Fenech, 2000). These results are in accordance with previous studies which described DNA damage in human colon cells treated with 500 to 1000 μM of U (Knobel et al., 2006). Miller et al. (2003) described that DU is genotoxic in human osteoblast cells. In rat, a genotoxic effect appeared only at the highest dose of DU inhalated (375 mg/m³) in broncho-alveolar cells but not in rat kidney isolated cells (Monleau et al., 2006).

DNA damage can be the consequence of a direct interaction of U with DNA. This direct interaction has already been described: it was recently demonstrated that U can form DNA adducts in Chinese hamster ovary EM9 cells after direct exposure (Stearns et al., 2005) and in human colon cells (Knobel et al., 2006). DNA damage can also be indirectly caused by induction of ROS, which would be generated after U treatment. It is the case in vivo as suggested by Monleau et al. (2006), who show that DNA damage can be a consequence of inflammatory reaction and ROS production. Indeed, we also observed a strong ROS production in cells after exposure to lethal concentrations of U. ROS concentration then decreased but did not reach basal value, suggesting that the oxidative stress persists during U treatment. ROS generation, which is often seen in metal

![FIG. 6. Induction of double DNA strand breaks in NRK-52E cells after exposure to 0 μM (A), 200 μM (B), or 600 μM (C) of U.](https://academic.oup.com/toxsci/article-abstract/98/2/479/1713787)

toxicity, seems to be an important pathway of metal-induced apoptosis (Pulido and Parrish, 2003): it is thought to affect directly mitochondrial membranes, or to increase DNA damage thus leading to the amplification of the apoptosis phenomenon (Stearns et al., 2005).

Our data suggest that at low concentrations, U induces small DNA damage and cellular modifications which can be efficiently repaired by cells. Some of these DNA damage might be misrepaired and lead to apoptosis cell death of a minor population of cells (5–10%). For higher concentrations, U induces strong cellular modifications and DNA damage, which lead to necrosis. These results are consistent with the observations that after U exposure, cultured human normal renal cells return to normal status at low exposure but enter irreversible cell death after high concentrations contaminations (Prat et al., 2005).

In favor of our results, it is possible to hypothesize a mechanism which suggests the different stages involved in cell death after U intoxication (Fig. 7). U enters in cells and induces MMP decrease, which increases ROS production. These events lead to activation of caspase-9 and caspase-3 and apoptotic cell death. When cells are treated with stronger U concentrations (700, 800 μM), intrinsic (caspase-9) and extrinsic (caspase-8) apoptosis pathways are activated. Moreover, U induces ROS, which produces DNA damage and leads to oxidation of lipids: cells enter in necrosis cell death. This study showed for the first time the genotoxic effect of U in normal rat kidney (NRK-52E) proximal cells. The genotoxic effect was observed for lethal (600 μM of U) and sublethal (300 μM of U) concentrations. However cells are able to repair single and/or double DNA strand breaks for sublethal concentrations but enter in apoptotic pathway for lethal concentrations (600 μM of U).

Although the link between ROS generation, DNA damage, and the occurrence of apoptosis is not clearly established, it is now evident that this phenomenon is deeply implicated in U toxicity. These results suggest that there is a threshold from which DNA damage is too important and lead cells to apoptosis. For U concentrations below this threshold (400–500 μM), DNA damage is less important and makes it possible for cell to develop an adaptive response. A previous study (Mirto et al., 1999) described that acute toxicity is observed in rat kidney from 300 μM of U, but not for lower concentrations. In our cell model, the acute toxicity of U is observed from 400–500 μM.

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