Influence of Uranium Speciation on Normal Rat Kidney (NRK-52^{E}) Proximal Cell Cytotoxicity

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Uranium is a naturally occurring heavy metal. Its extensive use in the nuclear cycle and for military applications has focused attention on its potential health effects. Acute exposures to uranium are toxic to the kidneys where they mainly cause damage to proximal tubular epithelium. The purpose of this study was to investigate the biological consequences of acute in vitro uranyl exposure and the influence of uranyl speciation on its cytotoxicity. NRK-52^{E} cells, representative of rat kidney proximal epithelium, were exposed to uranyl−carbonate and −citrate complexes, which are the major complexes transiting through renal tubules after acute in vivo contamination. Before NRK-52^{E} cell exposure, these complexes were diluted in classical or modified cell culture media, which can possibly modify uranyl speciation. In these conditions, uranium cytotoxicity appears after 16 h of exposure. The CI_{50} cytotoxicity index, the uranium concentration leading to 50% dead cells after 24 h of exposure, is 500 μM (±100 μM) and strongly depends on uranyl counterion and cell culture medium composition. Computer modeling of uranyl speciation is reported, enabling one to draw a parallel between uranyl speciation and its cytotoxicity.

Introduction

Uranium is found naturally in the earth's crust. Its use in the nuclear cycle and more recently for military purposes has raised concerns regarding its potential hazard to animal and human health. Natural uranium is a heavy metal composed of three radioactive isotopes, uranium-238 (U-238, 99.27%), uranium-235 (U-235, 0.72%), and uranium-234 (U-234, 0.006%).

The effects of uranium contamination on human health have been largely studied in vivo (*1*−*4*). Natural uranium, mostly chemotoxic, highly affects kidney functions (*5*). After an accidental acute exposure, uranium enters the human body by ingestion, inhalation, or through the skin and 80% of the initial dose is excreted in the urine during the first 24 h after contamination. A small part of the initial dose is retained in the body, deposited in bones and kidneys (*5*). While transiting in the body, uranium is transported through the blood, complexed either with large proteins such as transferrin and albumin, or with low molecular weight ions, mainly carbonate and citrate (*6*−*8*). To pass through kidneys and reach the urine flow, uranyl complexes have to filter through renal glomeruli, whose structure allows only low molecular weight carbonate and citrate uranyl complexes to pass. These complexes are then in direct contact with proximal tubule epithelium and are toxic for epithelial cells.

After an accidental exposure to uranium, the actual treatment consists of chelation therapy, which is aimed at binding the metal and subsequently favoring its excretion as a metal−chelate complex (*9*). Up to date, such treatments exist but remain poorly efficient (*10*−*14*). To develop an efficient chelating strategy, it is of key importance to understand uranium speciation at the vicinity of kidneys and to determine which uranyl complexes are responsible for the totality or at least a part of its toxicity.

In vitro models allow uranium speciation control by modification of cell culture medium composition. They thus are the more potent systems to study the relation between uranium speciation and its cytotoxicity. Up to date, all in vitro uranium toxicology studies were made on the porcine kidney proximal tubule cancerous cell line LLC-PK1 (*15*−*17*). The NRK-52^{E} cell model is the only cell line assumed to be representative of a noncancerous rat kidney proximal tubule epithelium (*18*−*20*) and is, thus, the most representative model for human and animal kidney cells submitted to accidental uranium exposure.

In vitro, uranyl−bicarbonate diluted MEM[1] is assumed to be the only uranium formulation cytotoxic to LLC-PK1 cells (*15*, *16*). This formulation potentially leads to a multitude of carbonate or phosphate or calcium plus carbonate uranyl complexes, one or a small number of them being cytotoxic (*21*−*27*). Such uranium speciation can be modeled with software such as J-Chess (*28*), using optimized databases constructed from thermodynamic data (*29*).

The aim of this work is to correlate uranyl speciation with its cell effects and particularly to determine which

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[1] Abbreviations: MEM, minimum essential medium; ICP-MS, inductively coupled plasma mass spectroscopy; MTT, methylthiazol tetrazolium; BASSIST, base applied to speciation in solution at interface and solubility.

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Table 1. MEM and DMEM Cell Culture Media Composition[a]

	DMEM	MEM
inorganic salts (mM)		
$CaCl_2 \cdot 2H_2O$	1.8	1.8
$MgSO_{4(anhyd)}$	0.811	0.811
KCl	5.365	5.365
NaCl	109.51	116.36
$NaHCO_3$	44.0	26.2
$NaH_2PO_{4(anhyd)}$	0.908	1.017
others (g/L)		
glucose	1	1
phenol red·Na	0.015	0.011
vitamins (g/L)		
myo-inositol	0.0072	0.002
pyridoxal·HCl	0.004	0.001
riboflavin	0.0004	0.0001
choline chloride, folic acid, niacinamide, D-pantothenic acid·1/2Ca, thiamine·HCl	0.004	0.001
amino acids (g/L)		
L-alanine		0.0089
L-arginine·HCl	0.084	0.126
L-asparagine·H_2O		0.015
L-aspartic acid		0.0133
L-cystine·2HCl	0.0626	0.0313
L-glutamic acid		0.0147
glycine	0.03	0.0075
L-histidine·HCl·H_2O	0.042	0.042
L-isoleucine, L-leucine	0.105	0.052
L-lysine·HCl	0.146	0.0725
L-methionine	0.03	0.015
L-phenylalanine	0.066	0.032
L-proline		0.0115
L-serine	0.042	0.0105
L-threonine	0.095	0.048
L-tryptophan	0.016	0.01
L-tyrosine·2Na·$2H_2O$	0.072	0.0519
L-valine	0.094	0.046

[a] MEM and DMEM are classical, commercially available cell culture media. Compositions were obtained from Sigma-Aldrich.

uranyl complexes are cytotoxic. NRK-52^E cells were exposed to various uranium solutions prepared either as uranyl−carbonate or −citrate complexes, diluted in various cell culture media. This study shows that cell culture medium composition and consequently uranyl speciation has a dramatic influence on its cytotoxicity. Modeling uranyl speciation with J-Chess software reveals that uranyl−carbonate and uranyl−calcium carbonate complexes are implicated in uranyl cytotoxicity.

Materials and Methods

Chemicals. Uranyl acetate and classical salts were obtained from Sigma, and uranyl nitrate was obtained from Labosi. MEM and Dulbecco's modified Eagle's medium (DMEM), classical cell culture media, were obtained from Sigma. These media contain inorganic salts, amino acids, vitamins, and glucose, and their composition is detailed in Table 1.

Cell Culture. Normal rat kidney NRK (CRL-6509) and NRK-52^E cells (CRL-1571) were derived from rat proximal tubule, human kidney HK-2 cells (CRL-2190) were derived from human proximal tubule, and Madin Darby canine kidney MDCK cells (CCL-34) were derived from canine renal tubule. All of these cell lines were used from the first to the 20th passage since the cells were purchased from the supplier (American Type Culture Collection). Cells were grown in 25 cm^2 flasks in DMEM containing 1 g/L glucose (NRK, MDCK) or 4.5 g/L glucose (NRK-52^E, HK-2) and supplemented with 2 mM L-glutamine, penicillin/streptomycin (50 U/mL and 50 μg/mL, respectively), and 10% (v/v) fetal bovine serum. Cells were maintained at 37 °C in a 5% CO_2/air incubator and passed at confluence.

Uranium Exposure. Cells were subcultured in 96 well plates and seeded at 6000 cells per well. Uranium exposure was made on confluent monolayers, i.e., 3 days after subculture.

Uranyl stock solutions were prepared by dissolution of uranyl nitrate or uranyl acetate crystals in either 100 mM $NaHCO_3$ or 100 mM Na_2CO_3 or 100 mM Na_2citrate or 50 mM citric acid plus 50 mM Na_2CO_3. In all of these stock solutions, the final uranyl concentration was 10 mM. The preparation procedure was strictly studied to avoid precipitation: the stock solutions were stirred for 30 min to ensure the total dissolution of uranium and then filtered through 0.22 μm filter units. The pH of uranyl stock solutions was evaluated using a Sartorius pH meter equipped with a micro Ag/AgCl electrode. They were adjusted to pH 7.2 with 1 M HCl or 1 M HEPES buffer (pH 8.5). These stock solutions were diluted in serum and antibiotic free cell culture medium and then filtered at 0.22 μm. For the dilutions, MEM, DMEM, or MEM of modified composition was used. All uranyl solutions were prepared just before exposure and were not stocked.

Uranium concentration in these solutions was measured by ICP-MS using a quadrupole apparatus (X7 series, Thermo Electron Corporation). The apparatus was calibrated with a SPEX CertiPrep (Metuchen, NJ; supplied by JOBIN YVON, Longjumeau, France) uranium standard range (0−5 ppb). Yttrium was used as an internal standard (1 ppb). Before analyses, samples were acidified with ultrapure 65% nitric acid (NORMATOM quality grade, VWR Prolabo) and diluted in ultrapure water.

Cell Viability Test. The colorimetric MTT assay (*30*) was used to evaluate uranyl cytotoxicity. This test is based on the ability of the mitochondrial succinate dehydrogenase enzyme of living cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a blue formazan compound. The MTT stock solution was prepared by dissolving MTT crystals (Sigma or Euromedex) in PBS to a final concentration of 5 mg/mL and then filtered at 0.22 μm.

After the uranium exposure period (1−24 h), 10% (v/v) MTT stock 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 MTT solubilization solution (10% Triton X-100, 0.1 N HCl in 2-propanol). After 5 min of dissolution at room temperature under gentle agitation, absorbance at 570 nm was measured using a Stat Fax-2100 microplate reader (Awareness Technology Inc.).

Uranium Speciation Modeling. The speciation of the uranium in various cell culture media was modeled using the J-Chess computer simulation program (Version 2) (*28*). For these simulations, we used the BASSIST thermodynamic database (*29*). This database has been developed by the French Atomic Energy Agency and contains selected values from the International Nuclear Energy Agency and the Paul Sherrer Institute. Uranium speciation was simulated for a solution containing 400 μM uranyl and 4 mM $NaHCO_3$, in either classical or modified MEM and DMEM media, over the pH range 0−10.

Results

Uranium Cytotoxicity Assessment on the NRK-52^E Cell Model. To date, the only published studies on the in vitro cytotoxicity of uranium involved the porcine proximal tubule epithelial cells LLC-PK1 (*15*, *16*). Preliminary studies of the effects of uranium were made using four other cell lines, NRK-52E derived from rat proximal tubule epithelial cells, NRK derived from non-epithelial cells of rat kidney proximal tubules, HK-2 epithelial cells from human kidney proximal tubule, and MDCK a canine distal tubular epithelial cell line, exposed to uranyl−$NaHCO_3$ solution in MEM. Concentration-dependent cytotoxicity was observed only with the NRK-

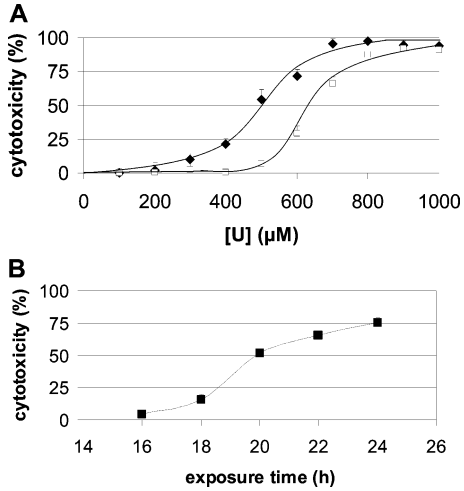

Figure 1. Uranyl cytotoxicity in the NRK-52^E cell model. NRK-52^E (A, losanges) or HK-2 (A, squares) cells were exposed during 24 h to 100−1000 μM uranyl−$NaHCO_3$ and diluted in MEM cell culture medium. Uranyl cytotoxicity kinetics was evaluated by exposing NRK-52^E cells to 600 μM uranyl for 0−24 h (B). For these experiments, cytotoxicity was evaluated with the MTT assay.

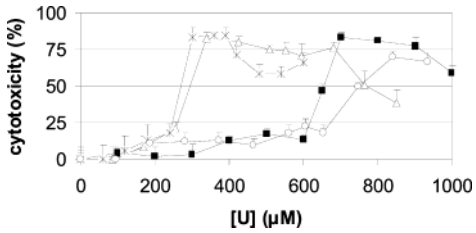

Figure 2. Influence of the counterion on uranyl cytotoxicity. NRK-52^E cells were exposed for 24 h to increasing uranyl concentrations. Uranyl exposure solutions were prepared from 10 mM uranyl/100 mM $NaHCO_3$ (square), 10 mM uranyl/100 mM Na_2CO_3 (circle), 10 mM uranyl/100 mM Na_3citrate (triangle), or 10 mM uranyl/25 mM Na_3citrate/25 mM Na_2CO_3 (cross) stock solutions. These stock solutions were pH adjusted and diluted in MEM cell culture medium.

52^E and HK-2 cells; the concentrations leading to a 50% loss of viability (CI_{50}) were 500 and 650 μM, respectively (Figure 1A). These studies also revealed that no cytotoxicity was observed below a uranyl concentration of 300 μM or with exposure times shorter than 16 h (Figure 1B); furthermore, lactate dehydrogenase or sulforhodamine B yielded similar values for the cytotoxicity. The cytotoxicity profiles and CI_{50} values were identical irrespective of whether uranyl nitrate or uranyl acetate was used to prepare the uranium solutions; therefore, uranyl nitrate was used in all subsequent studies.

Influence of Citrate and Carbonate as Uranyl Counterion. After accidental exposure, in vivo, uranyl ion reaches the proximal tubule epithelium mainly associated with carbonate or citrate as low molecular weight complexes (*6*−*8*). To evaluate the relative cytotoxicity of these complexes, uranyl stock solutions were prepared by dissolving uranyl nitrate crystals with either $NaHCO_3$, Na_2CO_3, Na_2citrate, or citric acid plus Na_2CO_3 (v/v) solutions, which then served as counterions for uranyl. It was considered that uranyl nitrate complexes dissociated in solution and that uranyl reassociated with the complexing agent, i.e., carbonate or citrate. Cytotoxicity was assessed using solutions in MEM medium containing different concentrations of uranium at pH 7.2. Uranyl−citrate was more cytotoxic than uranyl−carbonate; the concentrations leading to a 50% loss of viability after 24 h of exposure were 300 (±25 μM) and 700 μM (±50 μM), respectively. Interestingly, the cytotoxicity of uranyl−citric acid plus Na_2CO_3 and uranyl−Na_2citrate was approximately the same (Figure 2). We observed that NRK-52^E cells were more sensitive to uranyl when the cell monolayer was not confluent; inversely, NRK-52^E cells that were confluent for a few days were nearly insensitive to uranyl. This explains the slight differences in CI_{50} from one experiment to another (Figures 1A and 2).

For higher uranyl−Na_2citrate and uranyl−citric acid plus Na_2CO_3 concentrations (>800 μM), uranyl cytotoxicity decreased, and ICP-MS analysis of such solutions after 0.22 μm filtering proved that this was not due to uranyl precipitation. Uranyl speciation certainly changed, leading to noncytotoxic uranyl complex forms.

Influence of Carbonate Concentration in Cell Culture Medium. Salt concentrations of classical cell culture media are described in Table 1. As these media are used to dilute uranyl−$NaHCO_3$ stock solutions, their composition governs uranyl speciation. In uranyl−$NaHCO_3$ stock solution, carbonate was 10 times as concentrated as uranyl, i.e., a 600 μM uranyl solution contained 6 mM carbonate. Therefore, this concentration of carbonate cannot be neglected as compared to the 26 mM carbonate concentration in MEM cell culture medium.

We evaluated the influence of carbonate concentration in MEM medium on uranyl−$NaHCO_3$ cytotoxicity. For these experiments, we prepared a solution containing the same concentrations of inorganic ions and glucose as MEM, omitting amino acids and vitamins, which did not influence uranyl cytotoxicity (our own unpublished data). When varying $NaHCO_3$ concentrations in the MEM medium, we observed that uranyl cytotoxicity was maximal when $NaHCO_3$ concentration was between 20 and 26 mM (Figure 3A). When $NaHCO_3$ was below 20 mM, uranium precipitated and was no longer cytotoxic. When the $NaHCO_3$ concentration was above 26 mM, no precipitation and no cytotoxicity were observed, indicating that uranium was certainly stabilized in a noncytotoxic complexed form. In this $NaHCO_3$ concentration range, the pH of uranyl exposure solutions, after 24 h in the 5% CO_2 incubator, was between 7.6 and 7.8.

Another way of evaluating the influence of $NaHCO_3$ concentration on uranyl cytotoxicity is to dilute the uranyl−$NaHCO_3$ stock solution in DMEM cell culture medium instead of MEM. This medium contains 44 mM $NaHCO_3$, in contrast to MEM, which only contains 26 mM $NaHCO_3$. Uranyl−$NaHCO_3$ stock solutions diluted in DMEM were not cytotoxic to NRK-52^E cells (Figure 3B). If DMEM $NaHCO_3$ concentration was adjusted to 26 mM, which corresponds to the $NaHCO_3$ concentration in MEM medium, uranium cytotoxicity was recovered (Figure 3B). Cytotoxicity profiles were exactly the same, and CI_{50} after 24 h of exposure was 550 μM for uranyl−$NaHCO_3$ diluted either in 26 mM carbonate DMEM or in MEM (Figure 3B).

Influence of Cell Culture Medium Phosphate, Calcium, and Magnesium Concentration. Even if $NaHCO_3$ concentration in exposure solutions is very high, it is not the only salt that potentially complexes the uranyl ion. Uranium speciation must also include the

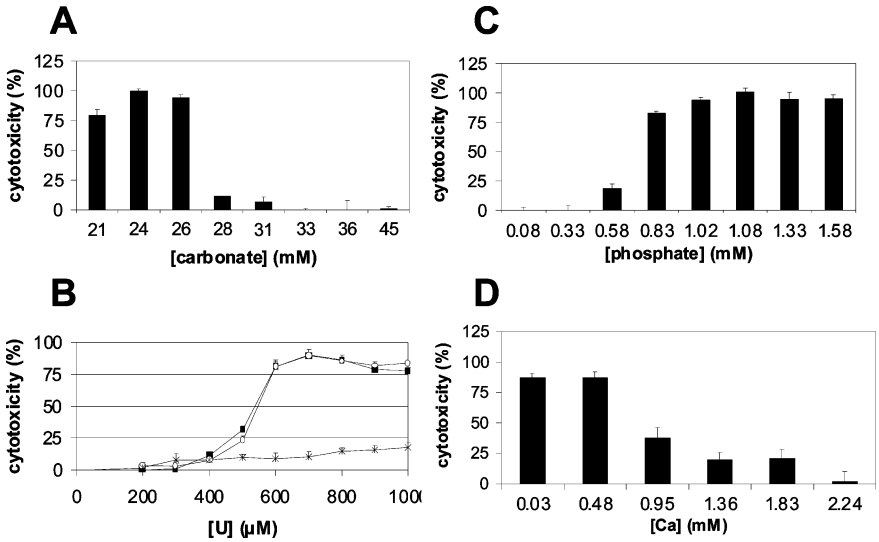

Figure 3. Influence of cell culture medium composition on uranyl cytotoxicity. NRK-52^E cells were exposed for 24 h to uranyl prepared from a 10 mM uranyl/100 mM $NaHCO_3$ stock solution. To investigate the influence of $NaHCO_3$ concentration in MEM on uranyl cytotoxicity, uranyl−$NaHCO_3$ stock solution was diluted to 700 μM in MEM adjusted to contain 21−45 mM $NaHCO_3$ (A). This stock solution was also diluted in classical MEM cell culture medium containing 26 mM $NaHCO_3$ (B, squares), in classical DMEM containing 44 mM $NaHCO_3$ (B, crosses), or in DMEM adjusted to contain 26 mM (2.2 g/L) $NaHCO_3$ (B, circles). To investigate NaH_2PO_4 concentration influence on uranyl cytotoxicity, uranyl stock solution was diluted to 700 μM in DMEM adjusted to contain 0.08−1.58 μM NaH_2PO_4 (C). To investigate $CaCl_2$ concentration influence on uranyl cytotoxicity, uranyl stock solution was diluted to 700 μM in MEM adjusted to contain 0.07−2.24 mM $CaCl_2$ (D).

already described uranyl−phosphate and uranyl−calcium carbonate complexes (*21*−*27*).

The influence of NaH_2PO_4 concentration in MEM medium on uranyl−$NaHCO_3$ cytotoxicity was examined. MEM medium was prepared with NaH_2PO_4 concentrations varying from 0.08 to 1.58 mM. From 0.08 to 1.02 mM NaH_2PO_4, uranyl−NaHCO3 cytotoxicity decreased with NaH_2PO_4 concentration. No cytotoxicity was observed when MEM contained 0.08−0.33 mM NaH_2PO_4, and cytotoxicity was maximal for 1.08−1.58 mM NaH_2PO_4 (Figure 3C).

Classical MEM cell culture medium contains 1.80 mM $CaCl_2$. MEM was prepared with $CaCl_2$ concentrations varying from 0.03 to 3.6 mM. Doubling the $CaCl_2$ concentration led to precipitation; this medium was not used to dilute uranyl stock solution. When MEM contained 0.48−2.24 mM $CaCl_2$, uranyl−$NaHCO_3$ cytotoxicity was inversely proportional to $CaCl_2$ concentration (Figure 3D). Cytotoxicity was maximal for 0.48−0.03 mM $CaCl_2$, and no cytotoxicity was observed for 2.24 mM $CaCl_2$.

Cytotoxicity of uranyl−$NaHCO_3$ was constant as $MgSO_4$ concentrations in MEM varied from 114 μM to 9.74 mM (classical MEM contains 811 μM $MgSO_4$). Modifications of $CaCl_2$, NaH_2PO_4, and $MgSO_4$ concentrations in MEM medium had no effect on the pH of exposure solutions.

Modeling of Uranium Speciation. Uranyl speciation was modeled using the J-Chess software, which calculates the concentration and precipitation/dissolution of the uranium complexes at thermodynamic equilibrium. Uranyl complexes concentrations were compared in order to determine which complexes cause a part or all of uranium cytotoxicity. For these modelings, the optimized BASSIST database of thermodynamic stability constants was used (*29*). Simulation could be done either enabling or disabling precipitation reactions. In all of the conditions used enabling precipitation, J-Chess calculated that uranyl exposure solutions were subject to calcite and hydroxyapatite precipitation. These precipitates were also assumed to be present in classical MEM medium, although this commercially available medium does not show any precipitates. The only condition in which uranium precipitated was when MEM containing 5 mM $NaHCO_3$ was used to dilute uranyl−$NaHCO_3$ stock solutions. In this condition, we actually observed precipitates in exposure solutions. This solution was excluded from our simulations. All of the modeling reported here was done with the J-Chess precipitation facility disabled.

A first series of simulations concerned uranium from the uranyl−$NaHCO_3$ stock solution, diluted in MEM cell culture medium before exposure of NRK-52^E cells. In this condition, according to J-Chess software, four uranyl complexes were predominant at pH 7, namely, $UO_2(CO_3)_3^{4-}$, $UO_2Ca_2(CO_3)_3$(aq), $UO_2Ca(CO_3)_3^{2-}$, and $UO_2(CO_3)_2^{2-}$, the four of them representing 99.96% of the total uranium concentration (Figure 4A and Table 2). Among the four major complexes, $UO_2(CO_3)_3^{4-}$ had the highest concentration, representing 60.3% of uranium in solution (Table 2). Uranyl free ion (UO_2^{2+}) was only observed below pH 5, and uranyl−phosphate complexes concentrations could be neglected below pH 2 and above pH 6. In the case of uranyl−citrate stock solutions diluted in MEM medium, $UO_2CIT_2^{4-}$ represented 100% of uranyl species between pH 2 and pH 9 (Figure 4B).

We then imposed variations in MEM composition that were used to dilute uranyl stock solution, as done experimentally (Figure 3). Simulation results are detailed in Table 2.

When uranyl−$NaHCO_3$ stock solution was diluted in MEM containing 0.5 mM $CaCl_2$ or 1.6 mM NaH_2PO_4, concentrations of $UO_2(CO_3)_3^{4-}$ and $UO_2(CO_3)_2^{2-}$ increased and concentrations of $UO_2Ca_2(CO_3)_3$(aq) and $UO_2Ca(CO_3)_3^{2-}$ decreased. These variations were reverse when diluting uranyl−$NaHCO_3$ stock solution in MEM containing 2.2 mM $CaCl_2$ or 0.1 mM NaH_2PO_4. The concentration of $UO_2PO_4^-$ and UO_2HPO_4(aq) minor complexes

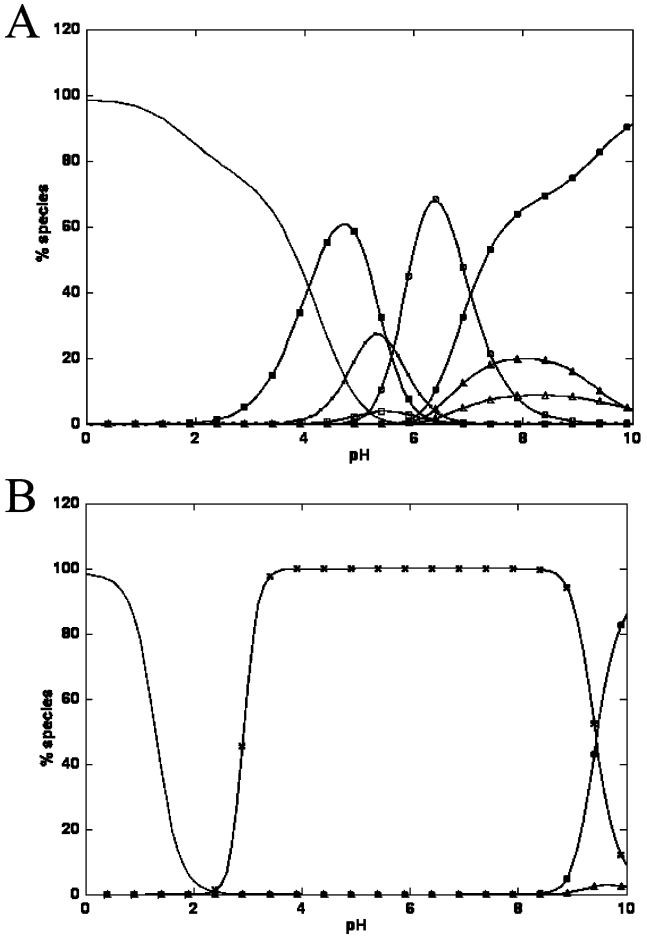

Figure 4. Computer simulation of uranyl speciation. Speciation was simulated with J-Chess software in conditions previously used to study cytotoxicity. Calculations are based on BASSIST optimized database with the J-Chess precipitation facility disabled. Speciation of uranyl−$NaHCO_3$ (A) and uranyl−Na_3-citrate (B) diluted in MEM cell culture medium is reported as a function of uranyl exposure solution pH. Percentage of uranyl complexed on the $UO_2(CO_3)_3^{4-}$ (full circles), $UO_2(CO_3)_2^{2-}$ (empty circles), $UO_2Ca_2(CO_3)_3$(aq) (full triangles), $UO_2Ca(CO_3)_3^{2-}$ (empty triangles), UO_2CO_3(aq) (punctated), $UO_2PO_4^-$ (full squares), UO_2HPO_4(aq) (empty squares), $UO_2CIT_2^{4-}$ (cross) chemical form, or as the UO_2^{2+} free ion (grey line) were evaluated with J-Chess software.

increased with $UO_2(CO_3)_3^{4-}$ and $UO_2(CO_3)_2^{2-}$ concentrations.

When uranyl−$NaHCO_3$ stock solution was diluted in MEM containing 44 mM $NaHCO_3$, uranyl complexed species evolution was the same as when using MEM containing 2.2 mM $CaCl_2$ or 0.1 mM NaH_2PO_4 except for $UO_2(CO_3)_3^{4-}$, whose concentration increased, and $UO_2PO_4^-$ and UO_2HPO_4(aq), whose concentrations decreased.

Discussion

In this study, the NRK-52^E cell line proved to be a pertinent model for in vitro investigation of uranyl renal cytotoxicity. As compared to results obtained by others on the LLC-PK1 cell line, NRK-52^E cells were more sensitive to uranium. We observed a dose- and time-dependent cytotoxicity response; uranyl−$NaHCO_3$ concentration leading to 50% dead cells (CI_{50}) was 500 μM (±100 μM) for 24 h of exposure, whereas it was 850 μM for LLC-PK1 cells (*15*). Furthermore, these cells proved to be sensitive to uranyl−citrate exposure, when LLC-PK1 cells were not (*15*); uranyl−citrate CI_{50} was 300 μM (±50 μM). The absence of uranyl−citrate cytotoxicity on LLC-PK1 cells may result from uranyl stock solutions preparation procedure. We observed that NaOH caused uranyl stock solution precipitation; NaOH was used to adjust the pH of uranyl stock solution used for LLC-PK1 exposure (*15*). In this work, the pH values of uranyl stock solutions were adjusted with HCl 1 N or HEPES buffer. MDCK and NRK cells are not sensitive to uranium exposure, and LLC-PK1 are less sensitive than HK-2, which in turn are less sensitive than NRK-52^E cells. These cytotoxicity differences probably come from cell lines characteristics. Uranium metabolism is possibly different in these cell lines, leading to more accumulation in NRK-52^E cells, thus leading to increased mortality. Less sensitive cells may also be able to produce a protecting agent whose action could render uranyl less cytotoxic.

Cytotoxicity of uranyl−$NaHCO_3$ was independent of the initial uranyl counterion, namely, nitrate or acetate. Conversely, it was dependent on the composition of the cell culture medium in which uranyl stock solution was diluted, particularly carbonate, calcium, and phosphate concentrations. Uranyl−$NaHCO_3$ was not cytotoxic when diluted in MEM containing high concentrations of $NaHCO_3$, high concentrations of $CaCl_2$, or low concentrations of NaH_2PO_4. Uranyl cytotoxicity increased when $NaHCO_3$ concentration increased, $CaCl_2$ concentration lowered, or NaH_2PO_4 concentrations increased in MEM. These differences are certainly explained by the modification of uranium speciation in such media, leading to increased or decreased concentrations of uranyl cytotoxic complexes.

J-Chess computer simulation indicates that when diluted in MEM medium at pH 7, the uranyl−$NaHCO_3$ solution mainly led to $UO_2(CO_3)_3^{4-}$ complex but also lower concentrations of $UO_2Ca_2(CO_3)_3$(aq), $UO_2Ca(CO_3)_3^{2-}$, and $UO_2(CO_3)_2^{2-}$ and very low concentrations of $UO_2PO_4^-$ and UO_2HPO_4(aq). When lowering the $CaCl_2$ concentration or increasing the NaH_2PO_4 concentration in MEM, which experimentally leads to high cytotoxicity, the $UO_2(CO_3)_3^{4-}$

Table 2. Uranyl Speciation in Cell Culture Medium Simulated with J-Chess Software[a]

	MEM	$NaHCO_3$	$CaCl_2$		NaH_2PO_4	
		44 mM	0.5 mM	2.2 mM	0.1 mM	1.6 mM
Cytotoxicity	+	-	+	-	-	+
$UO_2(CO_3)_3$[4-]	60.3%	↑ 65.2%	↑ 85.2%	↓ 52.2%	59.0%	60.6%
$UO_2(CO_3)_2$[2-]	6.2%	↓ 4.0%	↑ 8.9%	5.4%	6.2%	6.3%
$UO_2Ca_2(CO_3)_3$(aq)	26.9%	↓ 24.4%	↓ 3.1%	↑ 35.4%	↑ 28.0%	26.6%
$UO_2Ca(CO_3)_3$[2-]	6.6%	6.4%	↓ 2.7%	7%	6.7%	6.6%
UO_2PO_4[-]	< 0.1%	↓ < 0.1%	↑ < 0.1%	↓ < 0.1%	↓ < 0.1%	↑ < 0.1%
UO_2HPO_4(aq)	< 0.1%	↓ < 0.1%	↑ < 0.1%	↓ < 0.1%	↓ < 0.1%	↑ < 0.1%

[a] Uranyl speciation is simulated in classical or modified MEM medium, containing various $NaHCO_3$, $CaCl_2$, and NaH_2PO_4 concentrations. The percentage of uranium complexation is detailed, with arrows indicating a significant increase (↑) or a decrease (↓) in percentage as compared to classical MEM medium. The first line gives the relative cytotoxicity of each uranyl exposure solution obtained experimentally.

concentration is increased. Conversely, the $UO_2(CO_3)_3^{4-}$ concentration is decreased in conditions leading to less cytotoxicity, i.e., high $CaCl_2$ or low NaH_2PO_4 concentrations in MEM. This is an indication of its cytotoxicity. $UO_2(CO_3)_2^{2-}$ evolves the same way. Thus, $UO_2(CO_3)_3^{4-}$ and $UO_2(CO_3)_2^{2-}$ complexes are possibly implicated in uranium cytotoxicity. On the contrary, $UO_2Ca_2(CO_3)_3(aq)$ and $UO_2Ca(CO_3)_3^{2-}$ concentrations increase when uranium is prepared in conditions leading to less cytotoxicity. Consequently, these complexes might trap uranium in a noncytotoxic chemical form. Finally, $UO_2PO_4^-$ and $UO_2HPO_4(aq)$ complexes might also contribute to uranyl cytotoxicity, since their concentrations increase in conditions leading to increased uranium cytotoxicity. However, their concentration is very low, and consequently, if they contribute to uranium cytotoxicity, their contribution must be weak and their toxicity very high.

In the case of cell exposure to uranyl−citrate, uranium is totally trapped as $UO_2CIT_2^{4-}$; this complex is thus certainly responsible for uranium cytotoxicity. Uranium speciation is exactly the same if it is prepared in a citrate/carbonate (mol/mol) solution. It is interesting to note that the higher negatively charged uranyl complexes, namely, $UO_2(CO_3)_3^{4-}$ and $UO_2CIT_2^{4-}$, are the more concentrated at pH 7 and, according to our conclusions, the more cytotoxic uranium chemical forms.

These preliminary results need to be confirmed by in situ intracellular uranium speciation. Furthermore, it is possible that the complexes assumed to be responsible for uranium toxicity in this in vitro cell system are not toxic in a functioning renal tubule. Our results are thus somehow speculative but are a first step into the comprehension of the influence of uranium speciation on its cytotoxicity.

The next question concerns the way these complexes enter renal cells and the link between their cell internalization and the occurrence of toxicity. This knowledge will enable one to develop efficient decorporating treatments.

In summary, our renal cell model was sensitive to uranyl−bicarbonate and −citrate intoxication, with CI_{50} after 24 h of exposure of 500 (±100 μM) and 300 μM (±50 μM), respectively. Uranium speciation was found to highly influence its cytotoxicity; in our conditions, $UO_2(CO_3)_3^{4-}$ and $UO_2CIT_2^{4-}$ are assumed to be the more cytotoxic complexes, whereas $UO_2Ca_2(CO_3)_3(aq)$ and $UO_2Ca(CO_3)_3^{2-}$ might trap uranium in a nontoxic or nonbioavailable chemical form.

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