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Fluoride Ion Toxicity in Human Kidney Collecting Duct Cells

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Background: **Several halogenated anesthetics induce a urinary concentrating defect, partly related to fluoride ion toxicity in collecting duct cells. The aim of this study was to investigate the effects of fluoride ion in human kidney cells.**

Methods: **Immortalized human collecting duct cells were used. In a first set of experiments, the toxicity threshold concentration was determined by exposing cell cultures for 24 h to increasing concentrations of fluoride ion in the medium: 0, 1, 5, and 10 mM. The second set of experiments was a time-effect study in which cells were exposed to 5 mM fluoride for 2, 6, and 24 h. Assessment of toxicity was based on several endpoints: cell number, protein content, ^{3}H-leucine incorporation in newly synthesized proteins, extracellularly released lactate dehydrogenase, Na-K-ATPase pump activity, and electron microscope studies.**

Results: **After 24 h of exposure, fluoride ion decreased cell number (−23%, $P < 0.05$), total protein content (−30%, $P < 0.05$), and ^{3}H-leucine incorporation (−43%, $P < 0.05$) and increased lactate dehydrogenase release (+236%, $P < 0.05$) at a threshold concentration of 5 mM. Fluoride ion also inhibited Na-K-ATPase activity at 5 mM (−58%, $P < 0.05$). Major morphologic alterations of mitochondria, including crystal formation, were detected from 1 mM fluoride concentration. Time-effect studies showed that, after only 6 h of exposure at 5 mM, fluoride decreased cell number (−13%, $P < 0.05$), ^{3}H-leucine incorporation (−48%, $P < 0.05$), and Na-K-ATPase activity (−20%, $P < 0.05$) and increased lactate dehydrogenase release (+145%, $P < 0.05$). Crystal deposits in mitochondria again were a more sensitive marker of cell injury, detectable after only 2 h of exposure.**

Conclusions: **These results suggest that the mitochondrion is a target of fluoride toxicity in human collecting duct cells, and its alteration is partly responsible for the sodium and water disturbances observed in patients. (Key words: Ions: fluoride. Kidney, collecting duct: toxicity.)**

SEVERAL halogenated agents, such as methoxyflurane, enflurane, and perhaps sevoflurane, can induce dose-related renal dysfunction, characterized by polyuria due to impaired urine-concentrating ability.[1–6] Methoxyflurane, enflurane, and sevoflurane are known to be metabolized into inorganic fluoride. It is well established that inorganic fluoride ion is responsible for the renal dysfunction occurring with fluorinated anesthetics.[1,7–10] In the distal nephron, two sites play a role in the urine concentrating mechanism: the ascending limb of the Henle's loop and the collecting duct. Functional impairment of either of these two tubule segments may inhibit the urine concentrating mechanism. Renal-tissue solute concentration studies have shown that medullary sodium concentration is reduced by fluoride infusion,[11] indicating that fluoride could be toxic in the ascending limb of Henle's loop.[11,12] On the other hand, the decreased response to exogenous vasopressin injection, at least in some patients,[2–4] suggests that the collecting duct could be the target of toxicity. Fluoride may render the collecting duct unresponsive to vasopressin.[8,13–15] This antidiuretic hormone acts mainly *via* the cAMP generation, which induces shuttling of water channels between the apical and basolateral poles of collecting duct principal cells. Wallin and Kaplan established that fluoride ion

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could decrease vasopressin-induced cAMP generation, thus inhibiting water reabsorption.[14]

Studies on the cellular mechanisms of fluoride cytotoxicity have been hampered by a lack of *in vitro* models using cells of human origin. We took advantage of the availability of a simian-virus-40 (SV40)-immortalized human cell line recently established in our laboratory[16] to analyze the effects of fluoride ion in the human collecting duct. In particular, we studied Na-K-ATPase activity and morphologic changes occurring after fluoride exposure.

Materials and Methods

Cell Culture

The human collecting duct cell line was established from a cell preparation from the nontumorous part of a kidney that had been removed for a localized carcinoma. Cells were immortalized by transfection with a plasmid containing a defective SV40 virus genome[17,18] and cloned twice. The resulting cell line has retained, in more than 50 passages, the main characteristics of the principal cells of the human collecting duct. These include specific hormonal responsiveness and, in particular, a marked increase in cAMP content after incubation with arginine-vasopressin, the expression of a specific antigen localized at the basolateral side of the cell and the expression of principal cell-specific water channels.‡‡ Moreover, neither the antigens in the renal proximal tubule nor the Tamm-Horsfall protein, which is exclusively synthesized *in vivo* in the thick ascending limb of Henle's loop, were expressed.

Cells were cultured to subconfluency in 6- or 12-well trays (Costar, Cambridge, MA) for 6 days, at 37°C, in an atmosphere containing 20% O_2, 75% N_2, and 5% CO_2. The culture medium was a hormonally defined medium composed equally (vol/vol) of Dulbecco's modified Eagle medium and Nutrient mixture F12 (Gibco, Paisley, Scotland) supplemented with 5 $\mu g \cdot ml^{-1}$ transferrin (Sigma, St. Louis, MO), 30 nM sodium selenate (Sigma), 2 mM glutamine (Sigma), $5 \cdot 10^{-8}$ M dexamethasone (Sigma), 5 $\mu g \cdot ml^{-1}$ insulin (Sigma), and 20 mM Hepes (Gibco BRL, Cergy Pontoise, France), to which 2% newborn calf serum (Gibco BRL) was added. The medium *p*H was 7.4. The medium was changed every other day. Toxicity studies were performed after 7 days of culture. For the final 2, 6, and 24 h, the medium was replaced with a fresh medium containing or not containing sodium fluoride (Sigma).

‡‡ Valenti I: Personal communication. 1995.

Experimental Protocol

In a first set of experiments, the toxicity threshold was determined by incubating the cells with increasing concentrations of sodium fluoride for 24 h. Parameters of cell viability, Na-K-ATPase activity, and cell ultrastructure were assessed at three concentrations of fluoride: 1, 5, and 10 mM plus control (0 mM).

To analyze the sequence of events involved in fluoride ion toxicity to this cell population, a time-effect study was performed at 5-mM fluoride ion concentration. This concentration was chosen because, in the first set of experiments, it was the lowest concentration responsible for both mitochondrial alterations and impairment of cell viability parameters and Na-K-ATPase activity. Four durations of exposure were tested: 2, 6, and 24 h plus control (time 0).

Evaluation of Fluoride Toxicity on Cell Viability

The cell number was evaluated by counting in a Malassez cell. Cell damage was assessed by measuring extracellular lactate dehydrogenase (LDH) activity, which is a sensitive tool in detecting nonspecific cell damage.[19] LDH activity was determined using an enzymatic rate method with a Synchron Cx5 (Beckman, Brea, CA), with an analytic range of 20–2,500 $IU \cdot l^{-1}$ and precision (SD) of 15 $IU \cdot l^{-1}$.

Cell protein content was measured under every experimental condition. After removing the medium, the wells were washed with phosphate buffer saline. The cells were dissolved in 1 ml NaOH solution (1 M), 20 μl of which was compared to standards according to manufacturer's instructions (Pierce, Rockford, IL) to evaluate protein concentration by spectrophotometric determination.

Protein synthesis was measured by the incorporation of ^{3}H-leucine (Amersham, Les Ulis, France). Cells grown in six-well trays were incubated with 10 μCi ^{3}H-leucine for 5 min. The medium was removed quickly, and the wells were washed three times with 2 ml of ice-cold phosphate buffer saline. Cells were dissolved in 2 ml of NaOH solution (1 M), and 1 ml was recovered for counting (liquid scintillation counter LKB, Les Ulis, France). Values were expressed in counts $\cdot$ $min^{-1} \cdot$ mg $protein^{-1}$.

Evaluation of Na-K-ATPase Activity

One of the main characteristics of the collecting duct is its intense water and sodium chloride transport. Sodium reabsorption is driven by the Na-K-ATPase pump, located at the basolateral side of the cell, which decreases intracellular sodium concentration and thus secondarily activates the inwardly directed sodium channel located in the luminal domain. Na-K-ATPase also induces a potassium influx into the cell and is thus responsible for the maintenance of high potassium cell concentration. Ouabain selectively inhibits the Na-K-ATPase pump[20,21] and, consequently, sodium reabsorption in collecting ducts. Na-K-ATPase activity was assessed by measuring rubidium cell influx (^{86}RbCl, Amersham) in the presence or absence of ouabain. Rubidium was used as a tracer of potassium influx because of its high affinity to the potassium binding site on the pump. Na-K-ATPase activity was determined as the ouabain-sensitive rubidium influx (*i.e.*, the part of influx inhibited by ouabain).[22]

Experiments were carried out on cells grown in 12-well trays. The cells were washed three times with the incubation buffer (NaCl 140 mM, KCl 5.5 mM, Hepes 15 mM) prewarmed to 37°C. *p*H was adjusted to 7.40 with small aliquots of NaOH solution. Cells were incubated for 5 min with 1 ml/well of incubation buffer containing 1 $\mu Ci \cdot ml^{-1}$ rubidium, in the presence or absence of ouabain (1 mM; Sigma). At the end of the incubation period, the medium was removed quickly, and the wells were washed three times with 1 ml of ice-cold saline ($9\ g \cdot l^{-1}$). The cells were dissolved in 500 μl of NaOH solution (1 mM), and 400 μl was recovered for counting (liquid scintillation counter LKB). Values were expressed in $nM \cdot mg\ protein^{-1} \cdot 5\ min^{-1}$.

Electron Microscope Studies

Cells grown in plastic Petri dishes and exposed or not to fluoride ion as described above were fixed in glutaraldehyde diluted in a 0.1-M sodium cacodylate buffer, *p*H 7.4, for 3 h at 4°C. They were postfixed in 1% osmium, dehydrated in a grade series of ethanols, infiltrated, and embedded in Epon 812. Ultrathin sections of confluent monolayers were cut transversally, counterstained with uranylacetate and lead citrate, and viewed under an EM 109 Zeiss electron microscope. Three concentration-effect and two time-effect experiments were analyzed, and a total of 43–109 cells were examined in each experimental condition.

Statistical Analysis

Three to five experiments were carried out in triplicate at each concentration and each time tested for the evaluation of cell toxicity parameters and Na-K-ATPase activity (n = 9–12). Data are expressed as mean ± SD. Comparison of several means was performed using analysis of variance and Neuman-Keuls test.

Comparison of percentages was performed using Fisher's exact test with the Bonferroni correction. All *P* values were two-tailed. A *P* value less than 0.05 was necessary to reject the null hypothesis. Statistical analysis was performed on a computer using PCSM software (Deltasoft, Meylan, France).

Results

Concentration-Effect Study

Viability Parameters and Na-K-ATPase Activity. At the least concentration tested (1 mM), fluoride ions failed to alter any parameter of cell injury and Na-K-ATPase pump (table 1). At greater concentrations (5 and 10 mM), fluoride ions induced dramatic concentration-dependent changes in extracellular LDH release, which were accompanied by a parallel and significant decrease in the cell number and protein content per well. Fluoride ions also reduced protein synthesis (assessed by ^{3}H-leucine incorporation) to 43% (5 mM) and 13% (10 mM) of control values. As the other biologic parameters studied, Na-K-ATPase (table 1) was lower at 5 and 10 mM fluoride (41% and 39% of mean control value, respectively).

Ultrastructural Effects. Electron microscope studies showed that collecting duct cells grown in control conditions formed an epithelial sheet, with normal appearance of mitochondria (fig. 1A). After 24 h of exposure with 1 mM fluoride ions, major alterations in mitochondria were visible (fig. 1B). In 16 of 67 cells, mitochondria contained crystal structures, with minor disorganization of the crests. Eight-one percent of 43 cells at 5 mM (fig. 1C) and all 70 cells examined at 10 mM (fig. 1D) showed crystal formations in mitochondria. Furthermore, crystals increased in frequency, size, and density in a concentration-dependent manner (figs. 1B–D and table 2). Swelling of mitochondria was also observed from 1 mM (fig. 1B).

Time-Effect Study

Viability Parameters and Na-K-ATPase Activity. After 2 h of exposure to 5 mM fluoride, no impairment

Table 1. Fluoride Toxic

Cell number (% of control
Protein content (mg/well)
LDH activity (IU · l
^{3}H-Leucine incorporation (cpm · min^{-1} · mg protei
Na-K-ATPase (nmol · 5 min^{-1} · mg^{-1})

Values are the mean ± SD o

* $P < 0.05$ *versus* control val

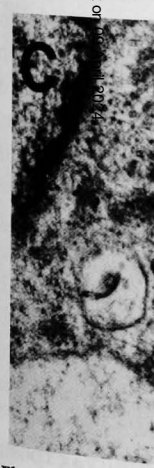

Fig. 1. Electron microg
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Table 1. Fluoride Toxicity in Collecting Duct Cells: Concentration Effect

	Fluoride Concentration (mM) (24 h exposure)			
	0	1	5	10
Cell number (% of control)	100	98 ± 17	77 ± 20*	47 ± 14*
Protein content (mg/well)	1.05 ± 0.10	0.95 ± 0.15	0.70 ± 0.20*	0.45 ± 0.15*
LDH activity ($IU \cdot l^{-1}$)	44 ± 3	46 ± 8	148 ± 25*	230 ± 24*
^{3}H-Leucine incorporation ($cpm \cdot min^{-1} \cdot mg\ protein^{-1} \times 10^3$)	53 ± 10	69 ± 14*	30 ± 7*	7 ± 3*
Na-K-ATPase ($nmol \cdot 5\ min^{-1} \cdot mg^{-1}$)	147 ± 20	174 ± 73	61 ± 9*	57 ± 12*

Values are the mean ± SD of four experiments performed in triplicate (n = 12).

* $P < 0.05$ *versus* control values.

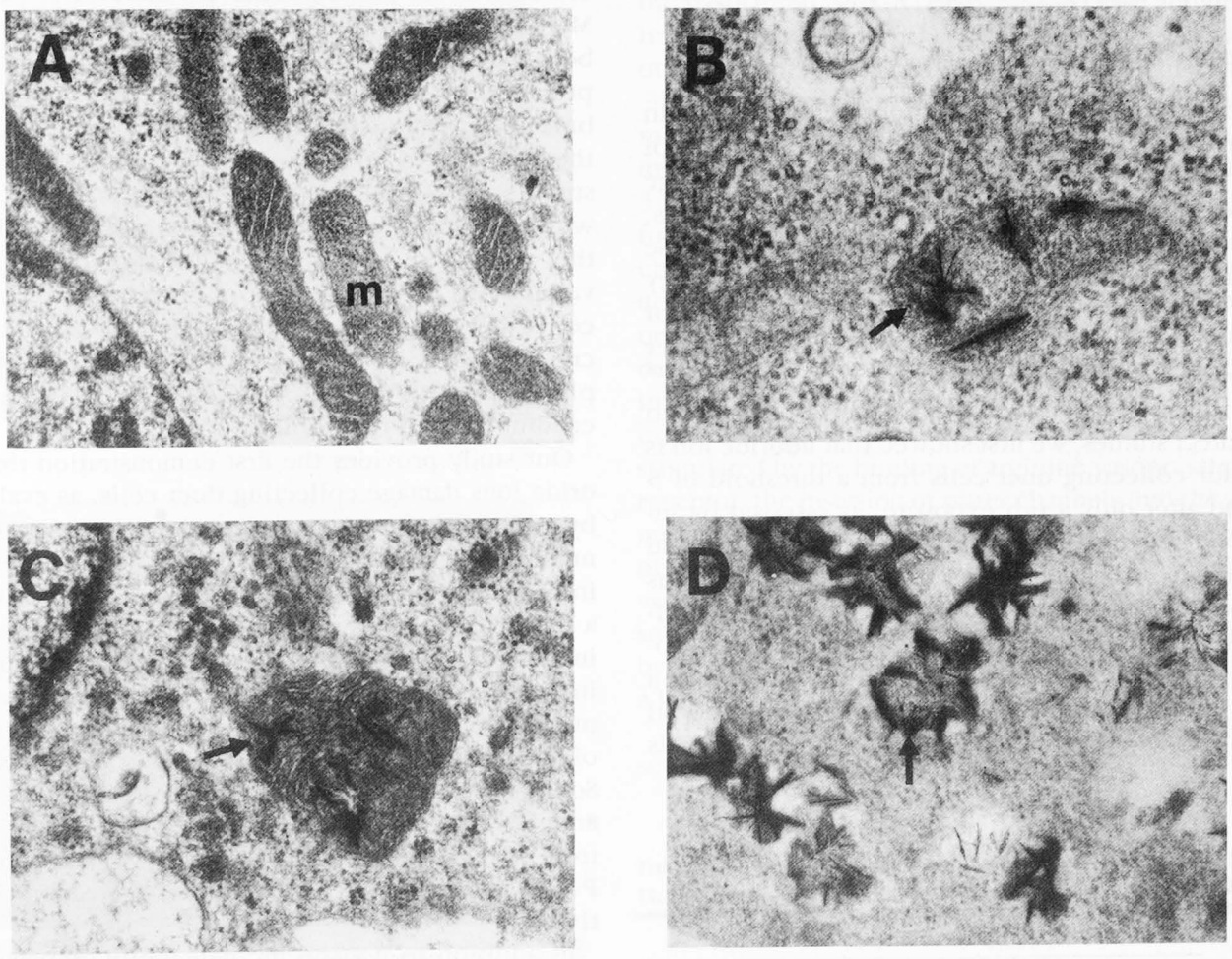

Fig. 1. Electron micrographs of mitochondria (m) in cells grown in the absence (*A*) or presence of 1 (*B*), 5 (*C*), or 10 mM (*D*) fluoride. In the presence of fluoride ion, crystals (arrows) were localized within mitochondria (*B* and *C*), whereas in its absence, mitochondria had a normal appearance (*A*). Note the increase in size and density of crystals at 10 mM (*D*), which resulted in almost complete disappearance of mitochondria structure (*D*). Magnification 5,000×.

of cell viability parameters and Na-K-ATPase pump was detected (table 3). After 6 h, fluoride ion was responsible for an increasing LDH release, decreasing cell number and ^{3}H-leucine incorporation (table 3). Like the other biologic parameters studied, Na-K-ATPase pump activity was not modified after a 2-h exposure but was lower after 6 and 24 h (80% and 46% of control group, respectively).

Ultrastructural Effects. At 5 mM fluoride concentration, crystals were detected after 2 h of exposure in only 14 of 109 cells (fig. 2A and table 4). In 22 other cells, after 2 h, minor alterations were observed, including disorganization of the crests and swelling (fig. 2B). Thus, 33% of the whole cell population contained damaged mitochondria after 2 h at 5 mM.

After 6 h of exposure, most of the cells (43 of 61) contained mitochondria with crystal formations (table 4).

Discussion

In this study, we investigated fluoride ion toxicity in human kidney collecting duct cells. Pure culture of these cells requires the microdissection of a large number of collecting ducts from fresh human kidneys, which is an almost impossible task. To circumvent these difficulties, we used an immortalized human kidney collecting duct cell line exhibiting major features of collecting duct principal cells, responsible for water and sodium reabsorption in this renal tubule segment.[16] Using this cell material in concentration-effect and time-effect studies, we first showed that fluoride ion is toxic for collecting duct cells from a threshold of 5 mM and after only a 6-h exposure, as assessed by increased LDH release and decreased cell number and ^{3}H-leucine incorporation. Na-K-ATPase activity was significantly reduced after 6 h. Protein content significantly dropped after 24 h. Second, electron microscope studies suggested that the mitochondrion is a target of fluoride ion toxicity, because mitochondrial alterations are observed at subcytotoxic levels. Crystals appeared in these organelles at a concentration of fluoride ion (1 mM, 24 h) and after a short exposure (2 h, 5 mM), which did not affect cell viability or Na-K-ATPase activity.

Fluoride ion responsibility in the renal toxicity of halogenated agents has long been suspected. Both inorganic fluoride and oxalic acid have been proposed as nephrotoxins because they are liver metabolites of methoxyflurane, enflurane, and sevoflurane, but oxalic acid has been exonerated because the clinical syndromes after oxalic acid and halogenated agents are different, and oxalic acid particularly induces anuric renal failure. On the other hand, several studies concluded that the dose-related nephrotoxicity observed after administration of halogenated agents was principally due to their liver transformation into inorganic fluoride.[3,7–10,23–25] The severity of renal dysfunction is correlated to plasma fluoride concentration.[1,12,15] Moreover, renal histologic abnormalities are similar for both fluoride ions and methoxyflurane.[1] However, the precise cellular target of fluoride ions in the renal tubule remains a matter for debate. Polyuria after methoxyflurane, or fluoride ion infusion in experimental studies, is ADH-resistant.[1,8,14] These findings, combined with a normal free-water-clearance, suggest a defect in the ability of the collecting duct to respond to vasopressin[8,11,12,14,15] and, consequently, a decreased collecting duct permeability. Its association with a decreased medullary solute concentration cannot be explained except by an associated impairment of the ascending limb of the Henle's loop.[8,11,12]

Our study provides the first demonstration that fluoride ions damage collecting duct cells, as evaluated by an increase in LDH release and a decrease in cell number, protein content, and ^{3}H-leucine incorporation in newly synthesized proteins after 6 h of exposure to a fluoride concentration of 5 mM. As for ^{3}H-leucine incorporation, we first observed a slight, but significant increase at 1 mM, with no significant effect on the cell number or total protein content. The biphasic effect of fluoride has been observed in other cell activities: Soni *et al.* showed that low fluoride doses enhance aminopyrine *N* demethylase, NADPH activity, and electron transport components, including cytochrome P450, whereas higher fluoride doses decrease these activities.[26]

In addition to nonspecific cell injury, exposure of human collecting duct cells to fluoride ions also induced major impairment of a pump known to be responsible for sodium transport across the cell, namely

Table 2. Number of Cells with Crystal Deposits According to Fluoride Concentration: 24 h Exposure

	Concentration (mM)			
Cells	0	1	5	10
n (%)	0/72 (0)	16/67 (24)	35/43 (81)	70/70 (100)

Table 3. Fluoride Toxicity in Collecting Duct Cells: Time Effect

	Time (h) (5 mM exposure)			
	0	2	6	24
Cell number (% of control)	100	101 ± 16	87 ± 8*	77 ± 20*
Protein content (mg/well)	1.06 ± 0.14	1.07 ± 0.35	0.86 ± 0.13	0.70 ± 0.20*
LDH activity ($IU \cdot l^{-1}$)	53 ± 2	54 ± 2	130 ± 33*	148 ± 25*
^{3}H-Leucine incorporation ($cpm \cdot min^{-1} \cdot mg\ protein^{-1} \times 10^3$)	60 ± 13	57 ± 12	31 ± 10*	30 ± 7*
Na-K-ATPase ($nmol \cdot 5\ min^{-1} \cdot mg^{-1}$)	132 ± 29	133 ± 27	106 ± 21*	61 ± 9*

Values are mean ± SD of three or four experiments performed in triplicate (n = 9 or 12).

* $P < 0.05$ *versus* control values.

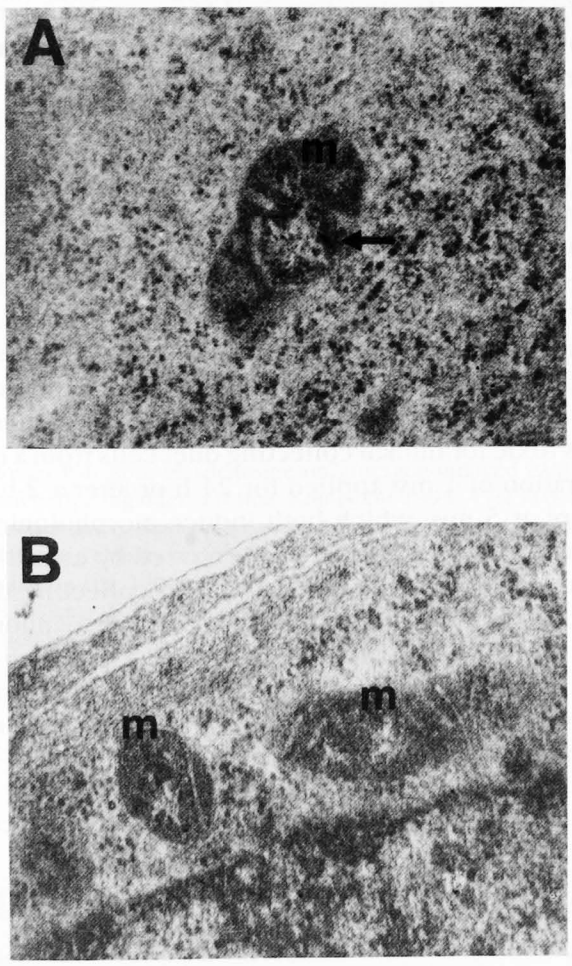

Fig. 2. Electron micrographs of mitochondria (m) in cells grown in the presence of 5 mM fluoride for 2 h. Note the crystal deposit (arrow) within one mitochondrion (*A*) and the abnormal appearance of two other mitochondria (*B*). Magnification 5,000×.

the basolateral Na-K-ATPase. Na-K-ATPase activity was impaired after only 6 h of exposure to 5 mM, as the other parameters of cell injury previously discussed.

In the current study, morphologic analysis showed mitochondrial abnormalities at a concentration of 1 mM applied for 24 h and after only 2 h of exposure at 5 mM. They suggest that this organelle may be a target for fluoride ion toxicity in human kidney cells. Mitochondria play a central role in providing energy to the cell, mainly as ATP molecules. Its impairment by fluoride could explain the fluoride-induced concentrating defect in two ways. First, although water reabsorption in the collecting duct is a passive phenomenon, it requires energy indirectly for the generation of cAMP stimulated by the binding of arginine-vasopressin to its receptor, the insertion of water channels into the apical membrane of the cell, and the subsequent trafficking of these channels to the basolateral domain in arginine-vasopressin stimulated cells. Second, sodium reabsorption is a secondarily active phenomenon, driven by the Na-K-ATPase pump, whose function requires ATP.

To assess the clinical relevance of our observations, several points should be addressed.

Table 4. Number of Cells with Crystal Deposits According to Time Exposure: 5 mM Fluoride Ion

	Time (h)			
Cells	0	2	6	24
n (%)	0/79 (0)	14/109 (13)	43/61 (70)	35/43 (81)

First, we acknowledge that exposing collecting duct cells to fluoride *in vitro* is not the same as administering fluoride-containing anesthetic agents *in vivo*. The aim of our experimental protocol was to investigate the mechanism of toxicity of fluoride ion, the major toxic metabolite of some halogenated agents.

Second, we tested fluoride concentrations between 1 and 10 mM. In clinical studies, plasma concentrations depend on the concentration and the anesthetic agent administered and on the duration of anesthesia.[24,27,28] The plasma toxicity threshold, established with methoxyflurane, usually is considered to be 50 μM.[24] However, this threshold remains debatable, because a 25% reduction in maximum concentrating ability was observed in volunteers with a plasma fluoride concentration of less than 33 μM.[3] On the other hand, the effect of fluoride appears to depend more on its intrarenal than plasma concentration. A significant increase in urine flow rate occurred in rats with a plasma fluoride concentration of only 32 μM, but with a medullary concentration reaching 5.8 mM in the papilla and 2.1 mM in the outer medulla, after 1 μM fluoride infusion for 3.5 h (*i.e.*, a total dose of 210 μmol).[12] These results indicate both a renal handling of fluoride and a gradient of this ion within the kidney.[12] Furthermore, a recent study suggested that renal metabolism of halogenated agents into fluoride exists, partly explaining the discrepancy between plasma values and renal toxicity.[29] This may explain why urinary fluoride concentrations in patients often are very high, even if plasma concentrations are relatively low.[11,28] The precise factors that determine the importance of the gradient between kidney and plasma fluoride concentrations remain unknown but probably include the duration and intensity of exposure. Therefore, we consider that the concentrations of fluoride tested in the current study may occur in the kidney during anesthesia with halogenated anesthetics.

Third, in the current study, the duration of exposure of the cells to fluoride was 2–24 h, which is the expected duration of anesthesia in most surgical procedures. Furthermore, prolonged fluoride elimination follows methoxyflurane, enflurane, and sevoflurane administration. Mazze *et al.* showed that plasma inorganic fluoride concentration peaked 6 h after the end of prolonged enflurane anesthesia in volunteers and that there were still detectable concentrations of fluoride in the urine 42 h later.[3] With sevoflurane, peak plasma fluoride concentration occurred at the 7th hour of anesthesia, and the highest urinary concentration was observed during the first 24 h of the postoperative period.[28] Moreover, in the same study, on the 4th postoperative day, urine fluoride concentration was still 192 μM. These data suggest that fluoride ions have sustained urine elimination after enflurane or sevoflurane anesthesia and that a few-hour exposure of the kidney cells is clinically relevant.

Fourth, despite the human origin of the cells under study and their morphologic, antigenic, and hormonal characteristics suggesting a high level of differentiation close to that of collecting duct principal cells *in vivo,* extrapolation of our results to clinical situations may be controversial. In particular, the source of cells is the cortical collecting duct, which is exposed to lower concentrations of fluoride because of its cortical situation. On the other hand, it cannot be ruled out that the *in vitro* collecting duct could be more susceptible to toxins than the *in vivo* collecting duct. However, it must be stressed that our morphologic observations are in keeping with those of Mazze *et al.*,[8,30] who observed mitochondrial swelling and degeneration after prolonged methoxyflurane anesthesia in rats. It is worth noting that cultures of renal proximal-tubule cells have been instrumental in unraveling the mechanisms of drug-induced nephrotoxicity.[31] Our culture of human collecting duct cells could represent a reproducible and reliable model system for studying the metabolic effect of fluoride ion and the mechanism of its toxicity.

In conclusion, our study demonstrates that fluoride ion is toxic for human collecting duct cells from a concentration of 1 mM applied for 24 h or after a 2-h exposure at 5 mM, which both induce morphologic alterations of mitochondria characterized by crystal formation. We conclude that, in human collecting duct cells, this cellular organelle could be a target of fluoride toxicity. We suggest that the ensuing lack of high-energy phosphates is partly responsible for the urinary concentrating defect occurring after administration of biotransformed inhaled anesthetics.

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