

Renal Tubular Site of Action of Fluoride in Fischer 344 Rats

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Methoxyflurane is capable of producing high-output renal failure in some patients and animal models, probably through metabolic liberation of free fluoride. The tubular site of action of fluoride was examined in Fischer 344 rats using clearance techniques. Free water reabsorption ($T_{H_2O}^C$) and free water excretion (C_{H_2O}) were measured during mannitol or water diuresis in control rats and in rats given methoxyflurane or pretreated with sodium fluoride. Pretreatment produced statistically significant increases in urinary flow (from 10.5 ± 1.4 to 20.1 ± 1.9 $\mu\text{l}/\text{min}/100$ g b. wt.), in glomerular filtration rate (from 814 ± 31 to $1,039 \pm 53$ $\mu\text{l}/\text{min}/100$ g b. wt.), in per cent sodium excretion (from 0.107 ± 0.008 to 0.155 ± 0.015 per cent), and in per cent water excretion (from 1.27 ± 0.15 to 2.00 ± 0.20 per cent). Free water excretion remained relatively unaltered in rats pretreated with fluoride, perhaps due to elevated glomerular filtration rate and/or reduced proximal tubular reabsorption combined with inhibition of reabsorption in the ascending loop. Percentage free water reabsorption, on the other hand, was markedly reduced by the pretreatment, from 2.66 ± 0.21 to 0.66 ± 0.09 per cent. The observations are consistent with the hypothesis that fluoride inhibits tubular reabsorption primarily in the medullary portion of the ascending limb of Henle's loop, perhaps by inhibition of an active chloride pump located in this nephron segment. (Key words: Anesthetics, volatile, methoxyflurane; Ions, fluoride; Kidney, tubular function.)

ELEVATED SERUM FLUORIDE levels produced by the metabolism of methoxyflurane have been reported to cause renal insufficiency in man and experimental animals.^{1,2} The renal defect is characterized by polyuria unresponsive to antidiuretic hormone (ADH) and by variable effects on sodium and potassium excretion. Blood urea nitrogen (BUN), plasma sodium, and osmolarity tend to increase,

and there is an associated weight loss. The renal failure is usually reversible. However, electrolyte imbalances, renal shutdown, and death have been reported.^{1,3,4}

Several observations suggest that inorganic fluoride is the causative agent of the methoxyflurane-induced nephrotoxicity. First, the onset and severity of the renal dysfunction are related to the plasma fluoride levels. Second, methoxyflurane has been shown to be metabolized in man and animals to oxalic acid and free fluoride.² In addition, injection or infusion of sodium fluoride (NaF) in rats and dogs has produced polyuria similar to that caused by methoxyflurane in patients.⁵⁻⁷

The Fischer 344 rat has been reported to be sensitive to the renal effects of NaF and fluorinated anesthetics, and thus has been widely used to study their associated nephrotoxicities.^{1,5,9,10} Similar renal dysfunction and histologic changes in the proximal tubular cells of Fischer 344 rats have been reported to occur following injection with 0.1 M NaF (.3 ml/100 g b. wt.) for four days, or following exposure to 0.5 per cent methoxyflurane for three hours.^{1,10} However, it has not been determined whether nephrotoxicity induced by NaF or methoxyflurane is primarily related to inhibition of reabsorption in the proximal tubule or to effects in other nephron segments.

In the present study, the tubular site of action of fluoride was examined in Fischer 344 rats using clearance techniques. The renal concentrating and diluting abilities in control rats and in rats pretreated with fluoride were measured to assess the nephron segment primarily affected by fluoride. Pretreatment resulted in diuresis and natriuresis. Free water reabsorption was markedly reduced, while free water excretion remained unchanged. The data suggest that fluoride ions inhibit tubular reabsorption primarily in the ascending limb of Henle's loop.

Methods and Materials

Eighteen technically successful experiments were performed on male Fischer 344 strain rats (Simonsen Labs., Gilroy, California) weighing 250-350 g. Seven rats (b. wt. = 271 ± 23 g) were pretreated for four consecutive days with a daily

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subcutaneous injection (0.2 ml/100 g b. wt.) of 0.2 M NaF. Nine control rats (b. wt. = 302 ± 16 g) received an equal volume of isotonic NaCl. Two additional animals (b. wt. = 283 ± 4 g) were pretreated by anesthetizing them with 0.75 per cent methoxyflurane for three hours four days prior to experiments. The acute experiments were performed one to two days after termination of the injections, or four days after methoxyflurane anesthesia. All rats had free access to food and water until the day of the experiment.

Rats were anesthetized with ethyl-(1-methylpropyl)-malonylthiourea (Inactin), 100 mg/kg b. wt. After tracheostomy, two polyethylene cannulas (PE-10) were placed in the right external jugular vein for infusion, and the right carotid artery was cannulated for blood sampling. A midline incision was made and the left ureter cannulated with polyethylene tubing (PE-50) having a dead-space of approximately 30 μ l. All incision areas were covered with thin Parafilm to reduce fluid loss. Body temperature was maintained at 35–37C. After a suitable prime, all animals received a continuous infusion of a pyrogen-free solution of 2 per cent dextrose with or without the addition of 1 per cent mannitol at a rate of 6 ml/h. Radiolabelled (3 H or 14 C) inulin was included in the infusion solution for measurement of glomerular filtration rate. After the surgical procedure and completion of the priming infusion, one hour was allowed for equilibration. An attempt was made to take five 20–30-minute urine collections from each animal. An arterial blood sample (0.05 ml each) was also taken during each clearance period. Urine was collected into calibrated 1-ml Wintrobe tubes and blood into heparin-treated microhematocrit tubes. A final 1-ml arterial blood sample was collected prior to termination of the experiment.

Duplicate aliquots of all urine and blood samples were measured in constant-bore capillary tubes, transferred to scintillation vials containing 10 ml of Triton counting cocktail, and assayed for radiolabelled inulin using a cooled liquid scintillation spectrometer as described previously.¹¹ The osmolarities of the urine and final blood samples were determined using a direct-reading freezing point-depression osmometer (Advanced Instruments, Needham Heights, Mass.). Sodium and potassium concentrations of aliquots from the same samples were measured using flame photometry. For six animals (four fluoride-treated and two controls), the blood and urinary fluoride concentrations were also determined using a fluoride ion-specific electrode.¹²

The general methods of preparation and handling of samples were similar to those described previously.^{13,14} Clearance values were calculated by formulas found in previous publications.^{15,16} Data are presented as mean \pm one standard error (SE).

Significance was evaluated using Student's t test for unpaired data, linear regression, or analysis of variance F test. $P < .05$ was considered significant.[¶]

Results

Eighteen rats were randomly assigned to control and fluoride-pretreatment groups. Preliminary experiments indicated that rats receiving 0.3 ml/100 g b. wt. of 0.2 M NaF daily became visibly ill and lost weight. Therefore, the dose of NaF in the present study was reduced to 0.2 ml/100 g b. wt. daily and the weight loss was prevented. However, pretreatment prevented normal weight gain, and the mean body weight was about 10 per cent higher at the time of the experiments in control rats than in rats pretreated with fluoride.

Plasma and urinary fluoride concentrations were increased approximately ninefold by NaF pretreatment. Plasma fluoride increased from control levels of 2.7 ± 0.5 to 18.6 ± 7.2 μ mol/l and urinary fluoride rose from 92.0 ± 13.0 to 782.0 ± 186.0 μ mol/l.

The rats pretreated with fluoride or methoxyflurane responded similarly to the fluid load. The data, therefore, were pooled to simplify analysis. Mean urinary excretion data are summarized in table 1. When challenged with an equal fluid load, the rats pretreated with fluoride showed greater increases in urinary flow, glomerular filtration rate, and sodium excretion than did control animals. Urinary osmolarity was lower in the animals treated with fluoride, while potassium excretion was not significantly different in the two groups.

The percentages of the filtered load that were excreted in the final urine for several substances are summarized in table 2. Data expressed in this manner are more indicative of alterations in tubular function compared with overall changes in renal function. Fractional water excretion was nearly 60 per cent higher following pretreatment than in control animals, while fractional sodium excretion was approximately 45 per cent higher. Tubular handling of potassium was similar in the two groups. In pretreated rats, a significantly smaller percentage of the filtered total solute appeared in the urine.

Percentage tubular free water reabsorption was markedly reduced in the pretreated animals, whether analyzed as the mean of all clearance periods in which free water reabsorption occurred, or analyzed as the mean of all clearance periods taken only from animals that did not excrete free

¶ Free water clearance was calculated using the formula:

$$C_{H_2O} = V - C_{os}$$

where V = urine flow and C_{os} = total solute clearance calculated as $C_{os} = V$ (urinary osmolarity/plasma osmolarity). Negative values indicated free water reabsorption ($T^c_{H_2O}$).

TABLE 1. Urinary Data*

	Control	Fluoride	P
Glomerular filtration rate (μ l/min/100 g b.wt.)	814 \pm 31 (48)	1039 \pm 53 (42)	<.001
Urine flow (μ l/min/100 g b.wt.)	10.5 \pm 1.4 (48)	20.1 \pm 1.9 (44)	<.001
Urinary osmolarity (mOsm/l)	1237 \pm 109 (48)	433 \pm 73 (38)	<.001
Serum osmolarity (mOsm/l)	292 \pm 6	292 \pm 4	N.S.†
Na excretion (nEq/min/100 g b.wt.)	111 \pm 7 (48)	206 \pm 17 (44)	<.001
K excretion (nEq/min/100 g b.wt.)	380 \pm 46	409 \pm 38	N.S.

* Values are means \pm SE. Numbers in parentheses indicate numbers of clearance periods.

† N.S. = not significant.

water during the experiment. Percentages of free water excretion were not significantly different in the two groups. It should be noted, however, that only a few positive free water clearance periods were obtained in these studies. This was probably due both to difficulty in completely inhibiting ADH release in anesthetized animals and to the exceptional concentrating ability of the rat.

In figure 1, tubular free water reabsorption is plotted against urinary flow for the control and fluoride-treated rats. The regression lines represent the concentrating abilities of the animals at various urinary flows. The slopes of the regression lines are significantly different ($P < .025$), and indicate that the animals pretreated with fluoride had a renal concentrating defect. That is, they reabsorbed less free water at any given urinary flow than did control animals. The difference was especially marked at low urinary flows, with an apparent upward splay in the control animals, suggesting nephron heterogeneity with respect to loop length and concentrating ability. After fluoride treatment, the data became more homogeneous and linear, suggesting removal of loop function as a source of variability. The effect of fluoride treatment on free water excretion cannot readily be evaluated because only moderate water diuresis was observed in a few periods. However, extrapolation of lines in figure 1 beyond the crossover at 5–6 per cent of water excretion suggest diminished free water excretion in the rats pretreated with fluoride at higher rates of water diuresis.

In figure 2, the urine-to-plasma osmotic ratios are plotted against urine-to-plasma inulin concentration ratios. Osmotic U/P is a measure of urinary concentration and inulin U/P is an index of water reabsorption. The regression lines were calculated from mean data taken from each animal. The slope

of the line for the fluoride-pretreated rats is significantly lower than that of the control line ($P < .025$). The animals treated with fluoride excreted less concentrated urine than controls even when extents of water reabsorption were similar, suggesting reduced osmotic clearance as part of the mechanism of diminished urine concentrating ability. Additionally, rats pretreated with fluoride required more water to eliminate an osmotic load equivalent to that eliminated by the controls. These observations are in agreement with previous reports that fluoride interferes primarily with the renal handling of water.^{2,3}

Discussion

We examined responses of control and fluoride-pretreated Fischer 344 rats to a fluid/osmotic load to determine the site of alteration in renal function induced by fluoride (methoxyflurane). When given the same fluid load, rats pretreated with fluoride showed a greater increase in urinary flow and a greater decrease in urinary osmolarity than did control animals. These changes in renal function are similar to those reported for man, rat, and dog after methoxyflurane anesthesia or NaF treatment, and are consistent with the hypothesis that fluoride ion is the causative agent of nephrotoxicity induced by methoxyflurane.^{5,10}

TABLE 2. Excretion Data*

	Control	Fluoride	P
Water excretion (per cent)	1.27 \pm .15 (48)	2.00 \pm .20 (42)	<.005
Sodium excretion (per cent)	.107 \pm .008 (48)	.155 \pm .015 (42)	<.005
Potassium excretion (per cent)	8.20 \pm .83 (33)	9.20 \pm .73 (30)	N.S.†
Total solute (per cent)	3.57 \pm .17 (48)	2.16 \pm .08 (38)	<.001
Tubular free water reabsorption (per cent)‡	2.66 \pm .21 (43)	0.66 \pm .09 (25)	<.001
Tubular free water reabsorption (per cent)§	2.66 \pm .21 (40)	0.47 \pm .15 (25)	<.001
Free water excretion (per cent)¶	0.82 \pm .39 (5)	1.20 \pm .31 (13)	N.S.
Free water excretion (per cent)**	0.25 \pm .35 (8)	0.83 \pm .32 (13)	N.S.

* Values are means \pm SE. Numbers in parentheses indicate numbers of clearance periods.

† N.S. = not significant.

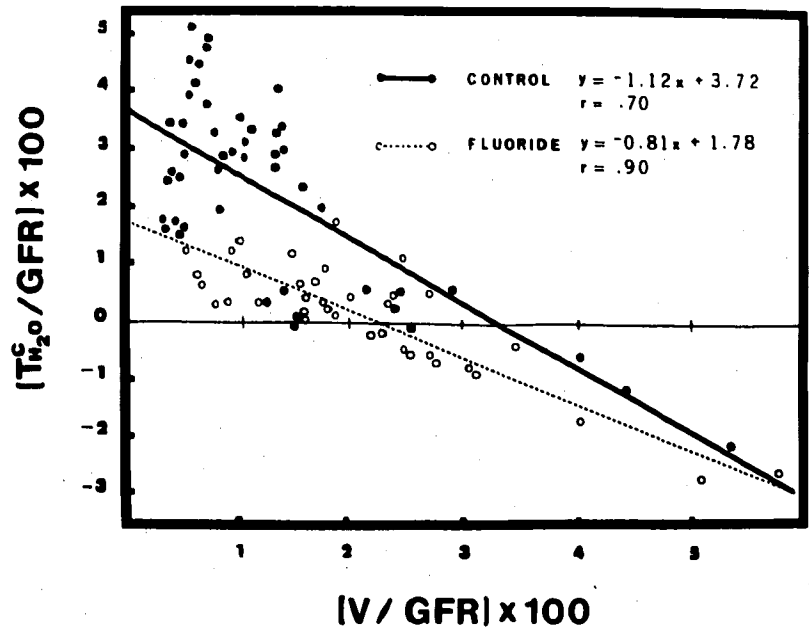
‡ Mean of all negative free water clearance periods.

§ Mean calculated from all clearance periods taken in animals with an overall negative free water clearance.

¶ Mean of all positive free water clearance periods.

** Mean calculated from all clearance periods taken in animals with an overall positive free water clearance.

FIG. 1. Effect of fluoride pretreatment on the ability of the rat kidney to concentrate or dilute the urine in response to a fluid load. Percentage water excretion ($[V/GFR] \times 100$) is plotted against either per cent free water reabsorption (above zero line) or per cent free water excretion (below zero line). Each point represents an individual clearance period.



In the present study, absolute as well as per cent sodium excretion increased significantly after fluoride pretreatment. In the dog⁶ and Wistar rat,⁷ sodium excretion was found to be unaltered by NaF infusion. The variance may be due to species differences or to different experimental conditions.

The observed reduction in tubular free water reabsorption in combination with increased sodium excretion indicates that fluoride (methoxyflurane) inhibited tubular reabsorption primarily in the ascending limb of Henle's loop. Impaired solute reabsorption in the loop is known to diminish the efficiency of the counter-current multiplier and to reduce the cortico-medullary osmotic gradient. Decreased osmotic force for water removal from the collecting duct would lead to antidiuretic hormone (ADH)-insensitive water loss and reduced $T_{H_2O}^C$. In addition to the evidence provided by our data, presented here, the above conclusion is also supported by previous observations that medullary sodium concentration and $T_{H_2O}^C$ were markedly reduced in animals into which large doses of NaF were infused^{6,7} and that diuresis associated with methoxyflurane is unresponsive to ADH.³ Impaired loop reabsorption should also reduce free water generation and excretion. We did not observe this expected change, perhaps due to the limited number of free water clearance periods or to an increased distal delivery secondary to elevated glomerular filtration rate in the fluoride-treated group. As mentioned earlier, extrapolation of the lines in figure 1 suggests diminished C_{H_2O} in the absence of ADH action.

The proximal convoluted tubule has been implicated as the site of fluoride action by histologic

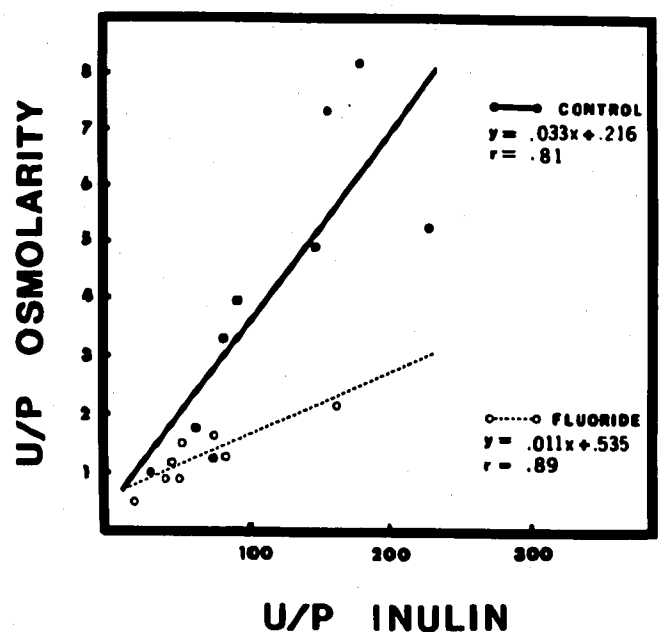


FIG. 2. Relationship between urinary total solute concentration (U/P osmolarity) and tubular water reabsorption (U/P inulin) in control and fluoride-pretreated rats. Each point represents mean data from one rat.

changes observed in the cells of this nephron segment in patients and animals with renal failure induced by methoxyflurane.^{1,3,10} The present studies do not provide direct evidence for inhibition of proximal tubular reabsorption, which would augment both $T_{H_2O}^C$ and C_{H_2O} . However, simultaneous inhibition of proximal and loop reabsorption may have cancelled each other's action on free water clearance and produced the net effect observed in

these experiments. Furthermore, advanced nephrotoxicity may involve several nephron segments.

The collecting duct is also a likely site of fluoride action. A renal concentrating defect could result if the duct became unresponsive to ADH (nephrogenic diabetes insipidus) under the effect of fluoride. Under these conditions, U/P osmolarity should fall below one, sodium excretion and medullary concentration should remain unchanged, and C_{H_2O} should rise. Present and previous observations do not bear out these expectations. However, the collecting duct cannot be excluded as a possible site of action of fluoride. Another possible explanation of the concentrating defect would be a medullary washout induced by fluoride secondary to exaggerated medullary blood flow.

The effect of fluoride appears to depend on its intrarenal concentration. In the present study, plasma fluoride levels were relatively low at the time of the experiments ($20 \mu\text{mol/l}$), while urinary concentrations were high ($> 700 \mu\text{mol/l}$). If the renal handling of fluoride is similar to handling of chloride, fluoride could attain very high levels in the ascending loop. Consistent with this assumption are the observed fluoride concentrations of as much as $5,000 \mu\text{mol/l}$ in renal medullary slices from rats infused with NaF.⁷

The present observations do not provide evidence concerning the mechanism of action of fluoride on renal tubular function. Fluoride, the halogen with the greatest electronegative charge, may have a high affinity for the active chloride pump located in the ascending limb of Henle's loop. By occupying the active site, fluoride may inhibit chloride transport. Alternatively, fluoride, through its known inhibitory action on glycolysis, might reduce the energy supply available for active transport processes that are dependent on anaerobic metabolism in the medullary nephron segments.

In summary, Fischer 344 rats pretreated with NaF or anesthetized with methoxyflurane showed more diuresis and natriuresis than did control animals. Urinary osmolarity was lower in the fluoride-treated group. Free water reabsorption was markedly reduced, while free water excretion was not significantly altered by pretreatment with fluoride. The results suggest that NaF and methoxyflurane alter renal function primarily by inhibiting active chloride transport in the ascending limb of Henle's loop.

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