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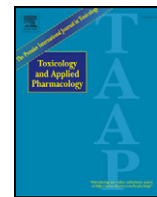
Proximal renal tubular injury in rats sub-chronically exposed to low fluoride concentrations

Mariana C. Cárdenas-González [a], Luz M. Del Razo [a], Jonatan Barrera-Chimal [b], Tania Jacobo-Estrada [a], Esther López-Bayghen [c], Norma A. Bobadilla [b], Olivier Barbier [a],*

[a] Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), México, D. F., México
[b] Unidad de Fisiología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México and Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México, D. F., México
[c] Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), México, D. F., México

ABSTRACT

Fluoride is usually found in groundwater at a very wide range of concentration between 0.5 and 25 ppm. At present, few studies have assessed the renal effects of fluoride at environmentally relevant concentrations. Furthermore, most of these studies have used insensitive and nonspecific biomarkers of kidney injury. The aim of this study was to use early and sensitive biomarkers to evaluate kidney injury after fluoride exposure to environmentally relevant concentrations. Recently weaned male Wistar rats were exposed to low (15 ppm) and high (50 ppm) fluoride concentrations in drinking water for a period of 40 days. At the end of the exposure period, kidney injury biomarkers were measured in urine and renal mRNA expression levels were assessed by real time RT-PCR. Our results showed that the urinary kidney injury molecule (Kim-1), clusterin (Clu), osteopontin (OPN) and heat shock protein 72 excretion rate significantly increased in the group exposed to the high fluoride concentration. Accordingly, fluoride exposure increased renal *Kim-1*, *Clu* and *OPN* mRNA expression levels. Moreover, there was a significant dose-dependent increase in urinary β-2-microglobulin and cystatin-C excretion rate. Additionally, a tendency towards a dose dependent increase of tubular damage in the histopathological light microscopy findings confirmed the preferential impact of fluoride on the tubular structure. All of these changes occurred at early stages in which, the renal function was not altered. In conclusion using early and sensitive biomarkers of kidney injury, we were able to found proximal tubular alterations in rats sub-chronically exposed to fluoride.

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Introduction

Fluoride is ubiquitously present in the environment forming mineral complexes that represent approximately 0.06–0.09% of the earth's crust (Whitford, 1983). Fluoride occurs naturally in many bodies of water due to runoff from the weathering of fluoride-containing rocks and solids and from the leaching of fluoride into groundwater (Edmunds and Smedley, 1996). Hence, the main source of fluoride exposure is drinking water where fluoride concentrations fluctuate between 0.5 and 25 ppm (ATSDR, 2003). The maximum limit for fluoride concentration in drinking water, established by the World Health Organization, is 1.5 ppm (WHO, 2006). This optimal concentration ensures the beneficial effect of fluoride on preventing dental caries. Consequently, in the United States, Spain, Switzerland, Australia, Canada, and the United Kingdom, among others, fluoride is purposely added to the water supplies mostly at around 1 ppm, to promote dental health (Petersen, 2008). Nevertheless, in many areas of the world high fluoride concentrations (>1.5 ppm) occur naturally in ground water. It has been estimated that more than 200 million people, from among 25 countries such as China, India, México and Argentina are affected by endemic fluorosis (WHO, 2006). Chronic fluoride exposure above 1.5 ppm in drinking water has been associated with dental and skeletal fluorosis, decreases in fertility, diminished intellectual capacity and renal impairment (Browne et al., 2005; Chandrajith et al., 2011; Izquierdo-Vega et al., 2008; Nayak et al., 2009; Ortiz-Perez et al., 2003; Rocha-Amador et al., 2007; Xiong et al., 2007).

The disposition of fluoride is characterized by extensive gastrointestinal absorption, which is followed by distribution and association with calcified tissues. Fluoride renal excretion is one of the most important mechanisms for the regulation of fluoride levels in the body. Approximately, 50% of the daily absorbed fluoride is excreted by the kidney. Fluoride is freely filtered through the glomerulus and undergoes a variable degree of proximal tubular

* Corresponding author at: Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Av. Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, Mexico, D.F. 07360, México. Fax: +55 5 747 3395.
E-mail address: obarbier@cinvestav.mx (O. Barbier).

re-absorption, directly related to glomerular ultrafiltrate pH (Whitford, 1994). It has been reported that, the proximal tubule (PT), whose longest portion (proximal convoluted tubule and early part of proximal straight tubule) is localized in the renal cortex, is more susceptible to damage than the glomerulus or any other tubular structure in rat models of acute fluoride nephrotoxicity (Dote et al., 2000; Usuda et al., 1999, 1998). Renal oxidative stress and modification of cellular membrane lipids increased in rats chronically exposed to 30 and 100 ppm of fluoride in drinking water (Guan et al., 2000; Karaoz et al., 2004). However, most of these studies were conducted using high fluoride concentrations or biomarkers that lack the sensitivity and/or specificity to detect early kidney injury before renal dysfunction is established. At present, only a few studies have assessed the renal effects of fluoride at environmentally relevant concentrations. These studies reported changes in protein expression related to alterations in renal metabolism, in the kidney and urine of rats exposed to 5 and 50 ppm of fluoride in drinking water (Kobayashi et al., 2011, 2009). Because the evidence is not conclusive, a better understanding of the early renal effects of fluoride exposure is still needed. Currently, several novel biomarkers are available for the early detection of kidney injury that correlates with histopathological alterations. Further, these biomarkers are easily quantifiable and some of them are nephron segment-specific (Barrera-Chimal and Bobadilla, 2012; Bonventre et al., 2010; Vaidya et al., 2008).

Therefore, the purpose of this study was to evaluate the early kidney injury after exposure to environmentally relevant concentrations of fluoride using early and sensitive biomarkers like kidney injury molecule-1 (Kim-1), clusterin (Clu), osteopontin (OPN), heat shock protein 72 (Hsp72), cystatin-C (Cys-C) and β-2-microglobulin (B2M).

Materials and methods

Animals and treatment. The care and experimental procedures were conducted after approval of the study by the Institutional (Cinvestav-IPN) Animal Care and Use Committee (CICUAL) in accordance with their Guidelines for the Care and Use of Laboratory Animals. Recently weaned male Wistar rats weighing 65 ± 3 g were purchased from Harlan Laboratories (México, D.F.) and were acclimatized for 1 week prior to the commencement of the fluoride exposure. Rats were housed in groups of four per polypropylene cage with sawdust bedding at 20–22 °C room temperature and relative humidity of 40–60% with a 12 h light to dark cycle. Water and food (Lab Diet® 5053, PMI Nutrition International, St. Louis, MO) were freely available in the home cages throughout the experiment.

After the acclimatization rats were randomly divided into 3 groups of 12 animals each group. The fluoride-exposed groups received 15 or 50 ppm of fluoride as sodium fluoride (Sigma Chemical Co., St. Louis, MO) in drinking water for a period of 40 days. The control group was provided with drinking water with 0.5 ppm of fluoride concentration for the same period. Fluoride concentrations were selected based on previous studies that reported that the fluoride concentration in drinking water for rats must be about 4–5 times greater in order to achieve serum fluoride levels comparable to those in humans (Angmar-Mansson and Whitford, 1984). Food, water intake and body weight were carefully monitored three times a week during the fluoride exposure period.

Urine, serum and tissue collection. During the exposure period, urine was collected four times at 10, 20, 30 and 40 days of the study in a non-fasted state. For the first three collections, the animals were placed in metabolic cages, and urine was collected over 6 h. The final urine collection was over 12 h on dry ice. Urine was centrifuged at 3000 g for 10 min (4 °C), and supernatant was aliquoted and stored at −80 °C. After the last urine collection, the rats were euthanized by terminal exsanguination (intracardiac puncture) under pentobarbital sodium anesthesia (60 mg/kg). Blood was centrifuged at 4000 g for

10 min (4 °C) to obtain the serum, which was stored at −80 °C. Through a catheter placed into the abdominal aorta, a renal perfusion was performed using isotonic saline solution (0.9% NaCl). After complete perfusion, both kidneys were excised from each rat and cut transversely into two halves. One half of each kidney was placed in 4% phosphate-buffered formalin for histological analysis. The cortex of the other half was meticulously separated, flash-frozen in liquid nitrogen and stored at −80 °C.

Urinary fluoride concentration and biochemical measurements. The urinary fluoride concentration was assessed every ten days throughout the exposure period in 0.5 ml of urine. Fluoride concentration was determined with a potentiometric method using an ion selective electrode Orion 9609 (Thermo Fisher Scientific Inc.) (Del Razo et al., 1993). The data were normalized against urinary creatinine concentration to correct for variations in urinary dilution.

Urinary and serum creatinine (Cre) concentrations were quantified with a kit-based spectrophotometric assay (Randox Laboratory Ltd. Co. Antrim, UK) and creatinine clearance (ml/min), a measure to estimate the glomerular filtration rate (eGFR), was calculated with the following equation:

$$CreCl = \text{urine flow rate (ml/min)} \\ \times \text{ urine Cre (mg/dl)/serum Cre (mg/dl)}$$

where: CreCl = Creatinine clearance; urine Cre = urinary creatinine concentration; and serum Cre = serum creatinine concentration.

Histological analysis. Formalin-fixed tissue samples were embedded in paraffin, sectioned at 4 μm and stained with periodic acid-Schiff (PAS) reagent. Preparations of three randomly chosen animals per group were analyzed in a blinded fashion. Percentage of injured tubules was determined by counting both tubular injured tubules and total number of tubules per field (magnification ×400). Because fluoride treated rats exhibited tubular flattening, the degree of tubular flattening was evaluated by a morphometric analysis. For this purpose, tubular epithelium thickness was measured in at least 40 different tubules per rat from recorded digital renal cortex microphotographs, using a digital camera incorporated in a Nikon microscope and NIS-Elements D 3.2 software.

Determination of urinary kidney injury biomarkers. Twelve-hour urine samples were used for the determination of urinary kidney injury biomarker excretion rate. Urinary Kim-1, Clu, OPN, B2M and CysC were determined using MILLIPLEX® MAP Rat kidney toxicity panel 1 and panel 2 (Millipore Corp., St. Charles, MO); the manufacturer's instructions were followed. This pre-validated assay uses a microsphere based Luminex® xMAP® technology which combines a sandwich ELISA immobilized on microparticle beads and flow cytometry. Urine samples were thawed approximately 1 h before the assay was performed. A total of 12.5 μl urine sample were used. For panel 1 (Kim-1, Clu and OPN), urine dilution was not necessary, but the urine samples for panel 2 (B2M and CysC) needed a 10-fold dilution. For all measurements, samples were analyzed in duplicate. High and low concentration controls were also included for each plate. The plaque was run on a Luminex 100™ instrument. Urinary Hsp72 protein levels were detected by western blotting as previously described (Barrera-Chimal et al., 2011). Urinary kidney injury biomarker data were expressed as urinary biomarker-excretion rate, regarded as the gold standard corrective method (Tonomura et al., 2013).

Quantitative reverse transcription-PCR. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed using a two-step method. The total RNA was isolated from the renal cortex using TRIzol® reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. RNA concentrations were determined by ultraviolet light absorbance at 260 nm. Complementary DNA

(cDNA) was generated from 2 µg total RNA using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI). PCR was performed with the MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas, Glen Burnie, MA). Duplicate samples were subjected to quantitative PCR (qPCR) using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qPCR profile consisted of an initial denaturation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The primer sequences for Kim-1, Clu and OPN were previously described by Rached et al. (2008). Gene expression changes relative to controls were determined using the $2^{-\Delta\Delta Ct}$ method. Samples were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression. The data are presented as the mean fold change in mRNA expression levels.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 5.0 Software (GraphPad Software, Inc., San Diego, CA). The Shapiro–Wilk test and a normal probability plot were used to test for the normality of the data. Data that meet the criteria necessary for the use of a standard parametric test were analyzed by one-way ANOVA, followed by a post hoc Tukey's test to determine differences among the groups. Those data that did not meet the standard parametric test criteria were analyzed using the non-parametric Kruskal–Wallis test and Dunn's post hoc test for multiple comparisons. For the trend-test, we used the likelihood ratio test statistic with 1° of freedom. Spearman rank correlations (r_s) was calculated to evaluate the relationship between the urinary fluoride concentrations and urinary biomarker excretion rate. Values significantly different from the control are indicated as $^*p < 0.05$.

Results

Food, water intake and body weight gain were not modified by sub-chronic fluoride exposure

There was no significant difference in food and water intake between the control and fluoride-exposed groups during 40 days of fluoride exposure. In consequence, no significant differences were observed in the body weight gain of fluoride-exposed groups (data not shown).

Sub-chronic fluoride exposure in drinking water increased urinary fluoride concentrations

To monitor the levels of fluoride during the exposure period, urinary fluoride concentration was used as exposure biomarker. Fig. 1 shows the average urinary fluoride concentration during 40 days of fluoride exposure. The basal urinary fluoride concentration was 2.36 ± 0.24 mg fluoride/g creatinine (mean ± SEM). As expected, fluoride exposure induced a significant and dose-related increment in urinary fluoride concentration throughout the study. Accordingly, at the first urinary fluoride concentration time point, the difference between control and the 50 ppm fluoride-exposed group was 9.6-fold, whereas the difference between control and the 15 ppm fluoride-exposed group was 5.4-fold. In addition, the difference between the 15 ppm and 50 ppm fluoride-exposed groups was 1.8-fold. The magnitude of these differences was maintained during 40 days of fluoride exposure. At the end of the exposure, the urinary fluoride concentrations were 1.75 ± 0.20, 11.45 ± 1.47 and 24.61 ± 2.55 mg fluoride/g creatinine for control, the 15 ppm and 50 ppm fluoride-exposed groups, respectively.

eGFR was not modified by sub-chronic fluoride exposure

In order to assess the renal function with standard biomarkers, we measure the serum creatinine levels and the creatinine clearance
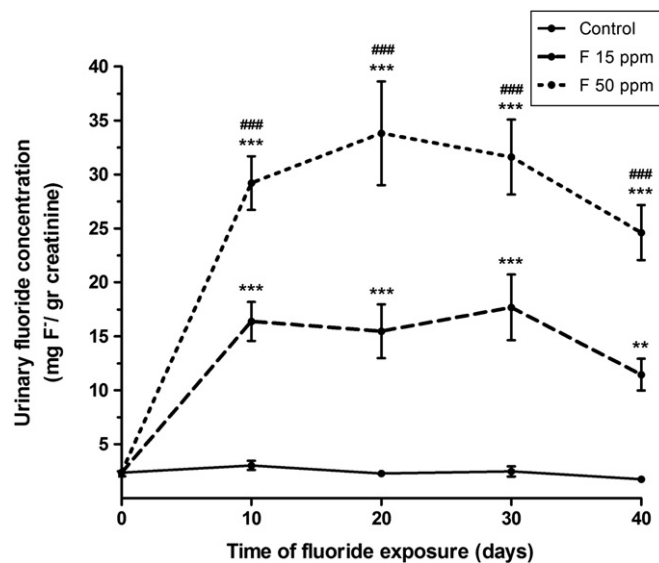

Fig. 1. Urinary fluoride concentration in male Wistar rats exposed to low (15 ppm) and high (50 ppm) fluoride concentrations for 40 days. The data are presented as the mean ± SEM. An * denotes statistically significant differences in fluoride excretion values relative to the control, and a # denotes significant differences in fluoride excretion values between low and high fluoride-exposed groups. Statistical analysis was performed by one-way ANOVA and Tukey's post hoc test. Statistically significant changes are indicated by $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

to estimate the glomerular filtrations rate (eGFR). Fig. 2a and b shows the effect of sub-chronic fluoride exposure on serum creatinine levels and eGFR values, respectively. Although, there was a small dose-dependent increase in the serum creatinine levels and decrease in the eGFR in the fluoride-exposed groups (15 ppm and 50 ppm) compared with control group, the differences were not statistically significant.

Sub-chronic fluoride exposure induced tubular injury

Sub-chronic fluoride exposure induced tubular injury characterized by tubular flattening, loss of proximal tubule brush border, cell detachment and loss of the tubular epithelium continuity (Fig. 3b and c). Tubular flattening was the main structural alteration in rats exposed to 15 ppm of fluoride (Fig. 3b). In contrast, exposure to 50 ppm of fluoride induced a greater injury: tubular flattening and tubular cell detachment (Fig. 3c). These findings were confirmed by the quantitative analysis of the percentage of injured tubules and the measure of tubular epithelial thickness. There was a tendency towards a dose-dependent increase of the percentage of injured tubules in the fluoride-exposed groups (15 ppm and 50 ppm) compared with the control group; this was not statistically significant (Fig. 3d) (trend $p = 0.043$). Fluoride-exposed groups exhibited tubular flattening that was evidenced by the reduction in the tubular thickness; this reduction was statistically significant in rats exposed to 50 ppm of fluoride (Fig. 3e).

Sub-chronic fluoride exposure increased the urinary kidney injury biomarker excretion rate

The urinary Kim-1, Clu, OPN, Hsp72, B2M and CysC excretion rate was increased after sub-chronic fluoride exposure (Fig. 4). In the 50 ppm fluoride-exposed group, urinary Kim-1 and Hsp72 excretion rate was significantly elevated by 3.2- and 5.5-fold, respectively (Fig. 4a and d). Clu and OPN showed a modest increase by 2.3- and 2.5-fold respectively; these differences were statistically significant in the 50 ppm fluoride-exposed group (Figs. 4b and c). No significant
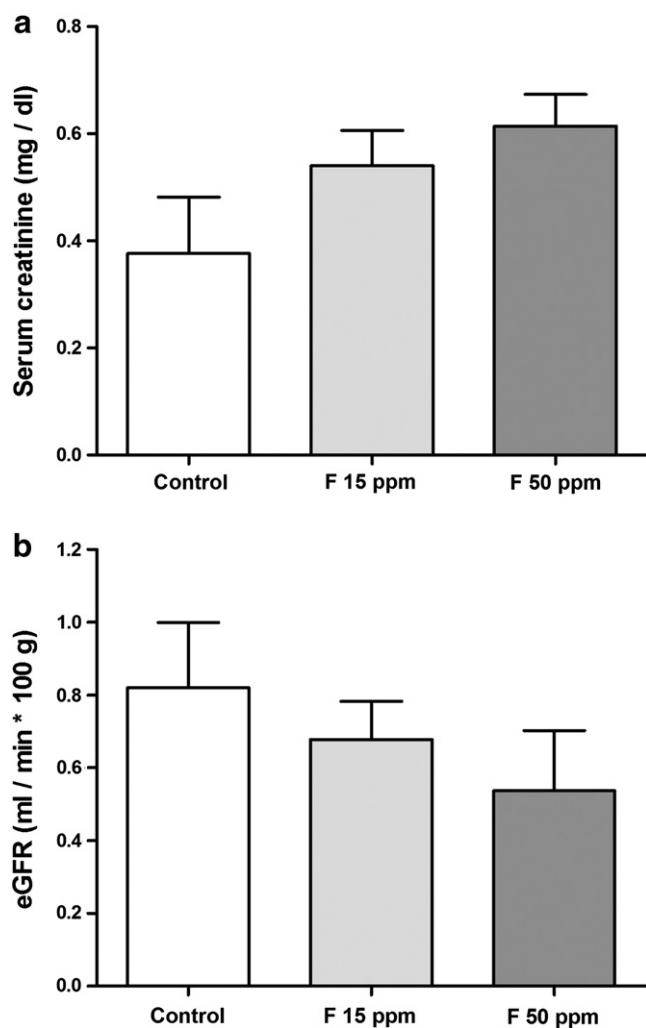
Fig. 2. (a) Serum creatinine levels and (b) estimated glomerular filtration rate (eGFR) measured by creatinine clearance in male Wistar rats exposed to low (15 ppm) and high (50 ppm) fluoride concentrations for 40 days. The data are presented as the mean ± SEM.

increase in urinary Kim-1, Clu, OPN and Hsp72 excretion rate was observed in the 15 ppm fluoride-exposed group. Significant increased urinary B2M (3.2-fold) and CysC (2.7-fold) excretion rate occurred even at 15 ppm of fluoride exposure and was elevated even more at 50 ppm of fluoride exposure (4.7- and 4.5-fold, respectively). Furthermore, a significant positive correlation was observed between the urinary fluoride concentrations and urinary Kim-1, Clu, OPN, Hsp72, B2M and CysC excretion rate (Table 1).

Increased mRNA expression levels of Kim, Clu and OPN in the renal cortex after sub-chronic fluoride exposure

Consistent with the increased levels of urinary Kim-1, Clu and OPN, qRT-PCR analyses showed gene expression increase of these kidney injury biomarkers in the renal cortex of fluoride-exposed groups. Kim-1 mRNA expression was significantly up-regulated in the 50 ppm fluoride-exposed group (6.9-fold). As found in the urinary protein pattern, exposure to 15 ppm of fluoride did not result in significant changes in Kim-1 mRNA expression (Fig. 5a). No exposure-related effect on the OPN mRNA expression was observed (Fig. 5b). Clu mRNA expression was significantly up-regulated since

the exposure to 15 ppm of fluoride and was maintained even until exposure to 50 ppm of fluoride (Fig. 5c).

Discussion

Our data showed a statistically significant increase in the urinary excretion rate of all kidney injury biomarkers evaluated that was significantly correlated with the urinary fluoride concentrations, in rats exposed to fluoride in drinking water for a period of 40 days. Furthermore, these changes occurred without a significant effect on the eGFR. We evaluated biomarkers of functional and structural PT dysfunction, such as B2M and CysC. Actively secreted proteins that are induced or repressed because of PT injury, including Kim-1, Clu, OPN and Hsp72, were examined as well. Urinary Kim-1, Clu, OPN and Hsp72 excretion rate was significantly increased in the 50 ppm fluoride-exposed group. Moreover, urinary B2M and CysC excretion rate was also significantly increased at even the low fluoride exposure concentration (15 ppm).

The outcome and progression of kidney injury overlap widely with the ability for the kidney to repair itself. Kidney injury and tissue repair are dynamic events in the spectrum of kidney disease progression and regression (El Sabbahy and Vaidya, 2011). During these events, proximal tubular epithelial cells (PTEC) undergo functional and structural adjustments. Each of these adjustments is accompanied by a complex pattern of molecular modifications such as changes in protein expression. Currently, these proteins are used as biomarkers for the early detection of kidney injury (Bonventre et al., 2010; Vaidya et al., 2008). Immediately after injury and during repair/regeneration, PTEC markedly express Kim-1, Clu, OPN and Hsp72, and these proteins can be detected in the urine (Barrera-Chimal et al., 2011; Bonventre, 2009; Ishii et al., 2007; Mueller et al., 2003; Ozer et al., 2010; Xie et al., 2001a). The increased expression of these molecules is a very early PTEC signal of kidney damage. For example, an increase in urinary Kim-1 is a useful biomarker of the early stages of cadmium-induced PT injury (Prozialeck et al., 2009). Similarly, urinary Clu levels, as well as changes in both protein and gene expression, are indicative of early kidney injury (Dieterle et al., 2010; Ishii et al., 2007). Neither of these two biomarkers has been evaluated in fluoride induced nephrotoxicity models. In addition to their role as kidney injury biomarkers, OPN and Hsp72 may act as survival factors after oxidative and ischemic damage (Noiri et al., 1999; Ophascharoensuk et al., 1999; Suzuki et al., 2005; Wang et al., 2011). Both of these biomarkers have been evaluated in fluoride-induced nephrotoxicity models. When the oxidative stress induced by fluoride exceeds the antioxidant capacity of renal cells in young mice, protein denaturation and/or misfolding can occur. This event leads to over-expression of Hsp72, an inducible member of the Hsp70 molecular chaperone family (Bouaziz et al., 2007) while OPN appears to have a protective role against the apoptosis induced by fluoride in isolated rat PTEC (Xu et al., 2006).

In this study Kim-1 and Clu mRNA levels were up-regulated in the renal cortex of rats exposed to fluoride in drinking water. Kim-1 mRNA up-regulation correlated well with the patterns found in urine. These results are in accordance with other studies where increases in urinary Kim-1 closely paralleled Kim-1 mRNA up-regulation (Pérez-Rojas et al., 2007; Rached et al., 2008; Vaidya et al., 2006). Clu up-regulation is related to physiological defense from sub-lethally stressed cells during a period of increased oxidative stress (Viard et al., 1999; Zhou et al., 2010). In fact, Clu gene expression is sensitive to even minute alterations in the cellular oxidative burden (Nath et al., 1994; Schwochau et al., 1998). Here, we found that renal Clu mRNA levels were significantly up-regulated since the exposure to 15 ppm of fluoride and maintained until 50 ppm of fluoride exposure. Chouhan and Flora (2008) reported that fluoride might exert its pro-oxidant effect at a relatively low concentration (10 ppm). Accordingly, it is plausible to assume that fluoride
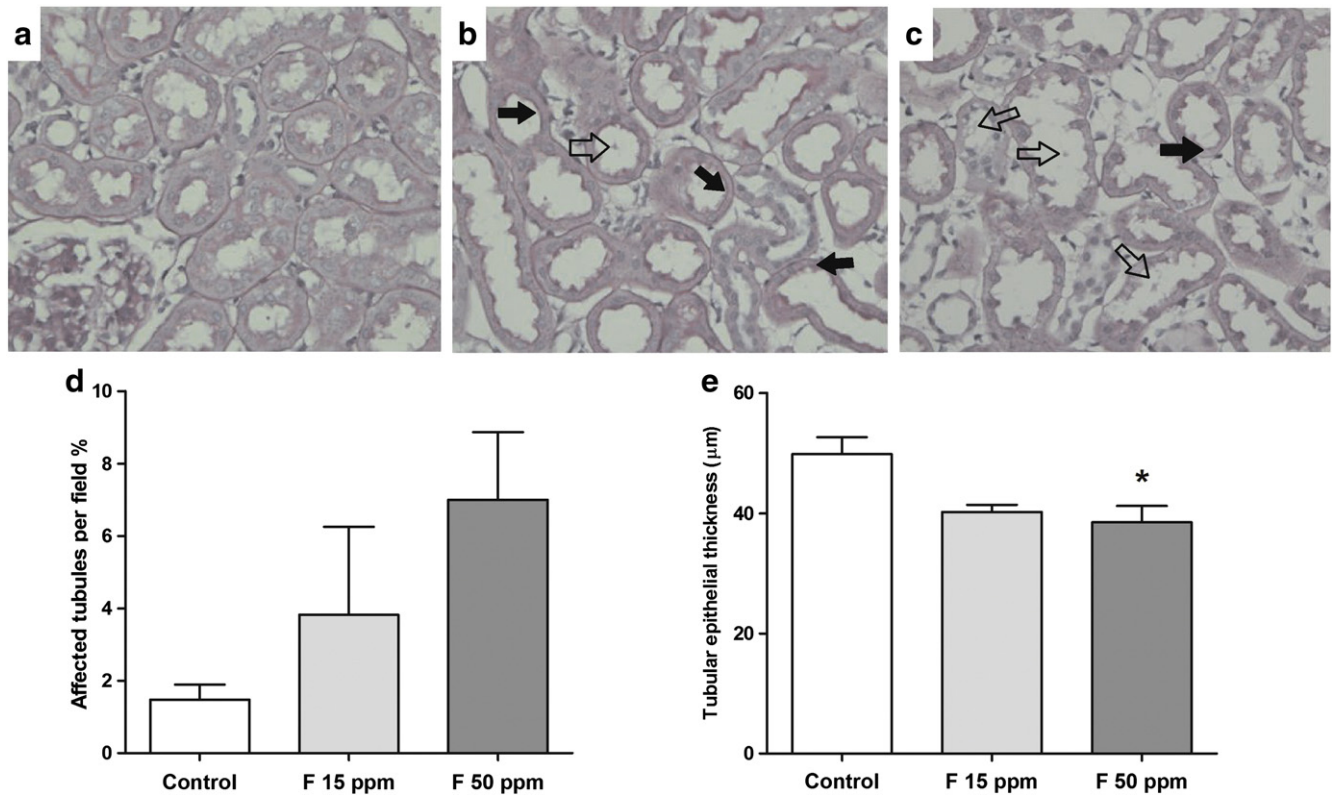
Fig. 3. Light microscopy of kidney sections (4 μm) stained with periodic acid-Schiff (PAS). Representative images of sections from (a) control, (b) 15 ppm and (c) 50 ppm fluoride-exposed groups (400× magnification). Filled arrows indicate tubular flattening, whereas empty arrows point out tubular cell detachment. In the graphs, the data are presented as the mean ± SEM. (d) The percentage of tubules injured per field, and (e) the measure of tubular epithelial thickness in micrometers. Statistically significant changes are indicated by *$p < 0.05$ (one-way ANOVA + Tukey's multiple comparison test); $p < 0.05$, dose-dependent linear trend.

exposure induced renal oxidative burden alterations leading to *Clu* mRNA up-regulation at low as well as high fluoride concentrations. Renal *OPN* mRNA levels showed a tendency to increase similar to that found in protein urine levels, although this change was not statistically significant. It has been reported that OPN that is detected in urine may undergone to extensive post-translational modifications
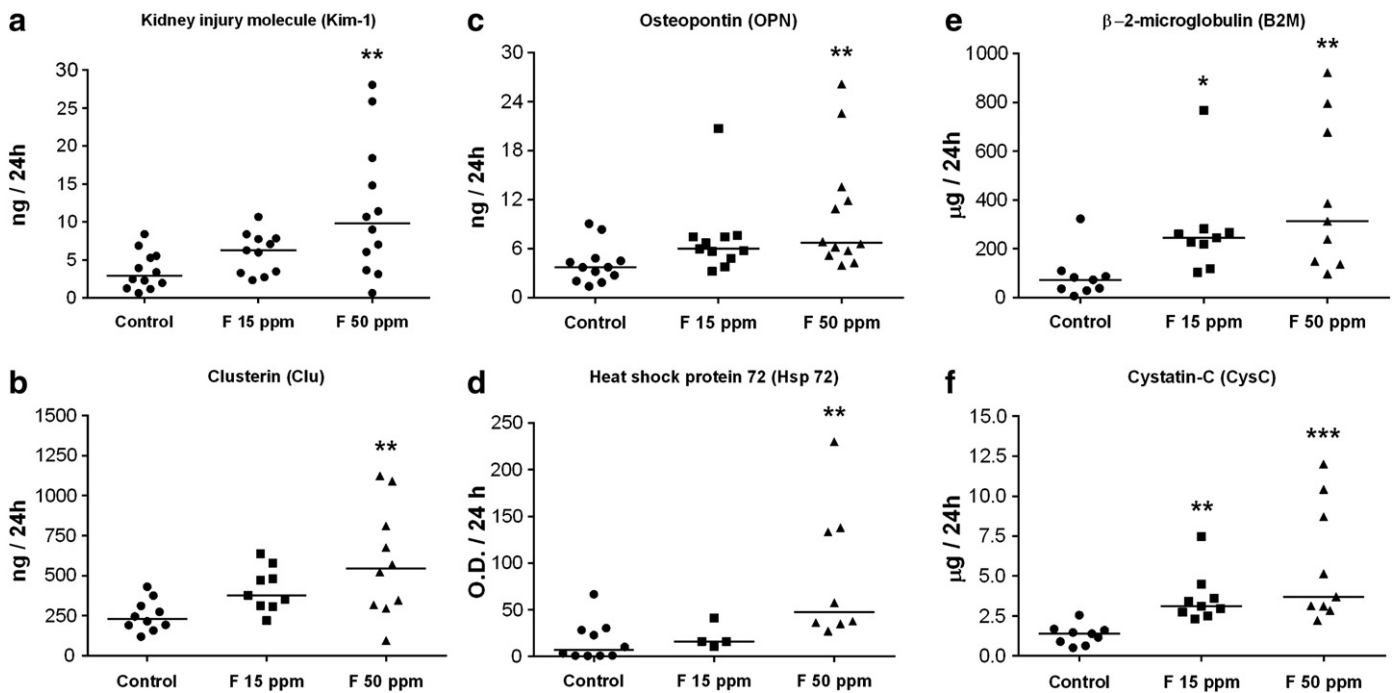

Fig. 4. Increased urinary (a) kidney injury molecule (Kim-1), (b) clusterin (Clu), (c) osteopontin (OPN), (d) heat shock protein 72 (Hsp72), (e) β-2-microglobulin (B2M) and (f) cystatin-C (CysC) excretion rate in male Wistar rats exposed to low (15 ppm) and high (50 ppm) fluoride concentrations for 40 days. The data are presented as the median ± interquartile ranges (n = 4–12). For (a), (b) and (c), statistically significant changes are indicated by *$p < 0.05$ (one-way ANOVA + Tukey's multiple comparison test). For (d), (e) and (f) statistically significant changes are indicated by *$p < 0.05$ (Kruskal–Wallis + Dunn's multiple comparison test).

Table 1

Correlation analysis between urinary fluoride concentration and urinary kidney injury biomarker excretion rate in male Wistar rats exposed to 15 and 50 ppm fluoride concentrations for 40 days.

Urinary excretion rate of the biomarker	Urinary fluoride concentration (mg fluoride/g creatinine)	
	rs value	p
Kim-1	0.4789	0.0048
cbyClu	0.6119	0.0005
OPN	0.6633	<0.0001
Hsp72	0.6158	0.0023
B2M	0.6515	0.0004
CysC	0.7215	<0.0001

Kim-1, kidney injury molecule 1; Clu, clusterin; OPN, osteopontin; Hsp72, heat shock protein 72; B2M β-2-microglobilin and CysC, cystatin-c.
Spearman's correlation coefficient (r_s).

(Kazanecki et al., 2007). Because of these modifications, OPN may not be expected to exhibit similar patterns between mRNA expression and protein levels. Furthermore, OPN secreted in rat urine comes not only from the PT but also from the descending thin limbs of the loop of Henle (DTL) (Xie et al., 2001b). Noteworthy is the fact that, under our experimental conditions OPN mRNA levels were evaluated exclusively from the renal cortex, which mostly contains PTs. Thus, the pattern of both measures may not be comparable.

The increase of renal Kim-1, Clu, OPN and Hsp72 indicates that fluoride was able to induce PT injury while the repair process was in progress. In accordance with these findings, tubular epithelium flattening and tubular cell detachment were the main morphological alterations found in fluoride-exposed groups. Tubular cell detachment may reflect death of tubular cells, which are then sloughed into the lumen. This change is predominant during kidney injury. However, tubular epithelium flattening represents regenerative change, which is more apparent during the recovery phase but not during kidney injury (Racusen and Solez, 1986). This histological findings support our hypothesis that fluoride induced proximal tubular damage with a repair process in progress.

We also observed loss of proximal tubule brush border and patchy loss of tubule cells in the injured tubules. These results suggest the possibility that the reabsorptive capacity of proximal tubule may be also affected by sub-chronic fluoride exposure. To evaluate the possible effect of fluoride exposure on PT structure and function, we assessed urinary B2M and CysC excretion rate. Serum levels of these proteins have been long proposed as glomerular function biomarkers (Dharnidharka et al., 2002; Schardijn and Statius van Eps, 1987). However, increased urinary B2M and CysC levels provide better diagnostic performance for minimal functional and structural PT impairment (Conti et al., 2006; Dieterle et al., 2010; Schaub et al., 2005). Interestingly, urinary B2M and CysC excretion rate performed as the best early kidney injury biomarkers in this study, since they were induced at the low fluoride concentration (15 ppm). These results suggest that fluoride exposure was able to modify the function and/or structure of rat PT. Nonetheless, further investigation will be needed to verify these observations.

In summary, our results revealed that the sub-chronic fluoride exposure at environmentally relevant concentrations induces PT injury. This was clearly demonstrated by the increase of early and sensitive kidney injury biomarkers such as Kim-1, Clu, OPN, Hsp72, B2M and CysC at stages were renal function was not altered. To our knowledge, this is the first time that these early, non-invasive, sensitive and easily detected biomarkers have been used to assess the renal effects of fluoride exposure.

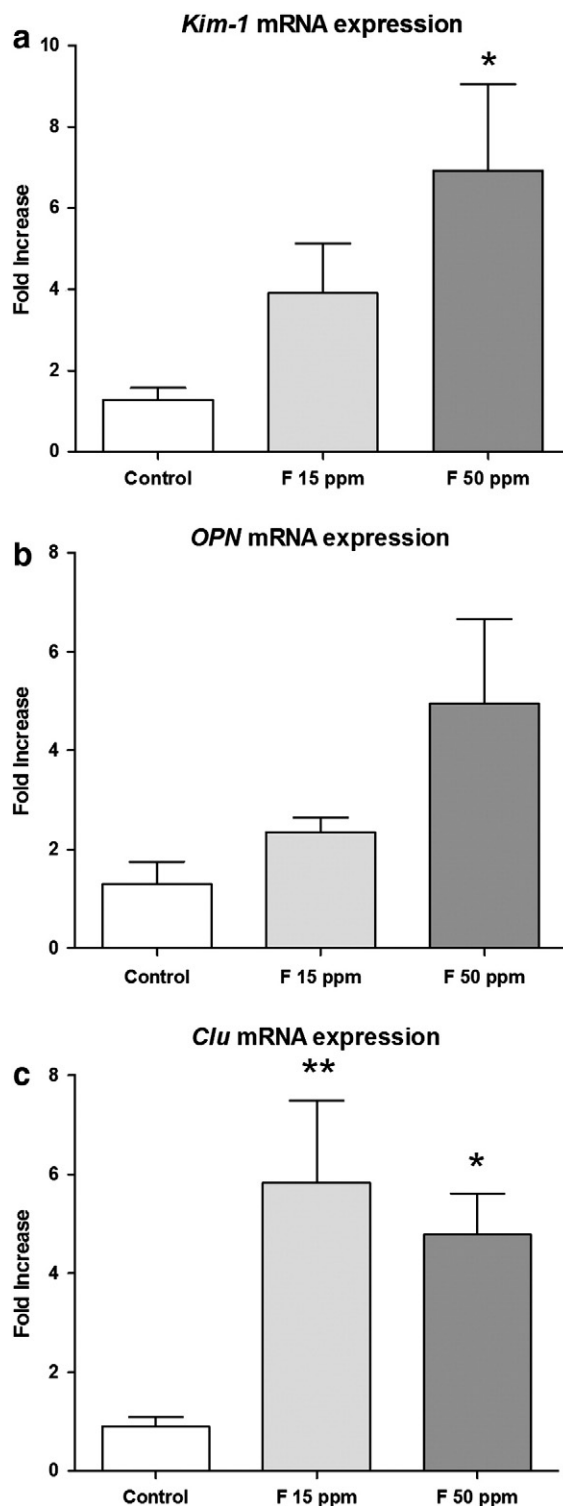## Conflict of interest statement

Authors report that there are none.

Fig. 5. Up-regulation of (a) *kidney injury molecule* (*Kim-1*), (b) *osteopontin* (*OPN*) and (c) *clusterin* (*Clu*) mRNA expression in the renal cortex of male Wistar rats exposed to low (15 ppm) and high (50 ppm) fluoride concentrations for 40 days. The data represent the mean fold change in messenger RNA (mRNA) ± SEM. Statistically significant changes are indicated by *$p < 0.05$ (one-way ANOVA + Tukey's multiple comparison test).

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