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Sub-chronic exposure to fluoride impacts the response to a subsequent nephrotoxic treatment with gentamicin

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ABSTRACT: Fluoride is an important groundwater contaminant, and more than 200 million people are exposed to high fluoride levels in drinking water, the major source of fluoride exposure. Exposure above 2 ppm of fluoride is associated with renal impairment in humans. In rats, moderate levels of fluoride induce kidney injury at early stages in which the glomerular filtration rate (GFR) is not altered. In the present study, we investigated if sub-nephrotoxic stimulus induced by fluoride might impact the response to a subsequent nephrotoxic treatment with gentamicin. Male Wistar rats (~21 days) were exposed to 0, 15 or 50 ppm of fluoride through drinking water during 40 days. Afer that, rats were co-exposed to gentamicin (40 mg kg^{-1} day^{-1}, 7 days). Gentamicin induced a marked decrease in the GFR and an increase in urinary levels as well as the protein and mRNA expression of biomarkers of early kidney injury, such as Kim-1. Interestingly, gentamicin nephrotoxicity was less pronounced in groups previously exposed to fluoride than in the group only treated with gentamicin. Fluoride induced *Hsp72*, a cytoprotective molecule, which might have improved the response against gentamicin. Moreover, fluoride decreased the expression of megalin, a molecule necessary for internalization of gentamicin into the proximal tubule, potentially reducing gentamicin accumulation. The present results suggest that fluoride reduced gentamicin-induced nephrotoxicity by inducing a compensatory response carried out by Hsp72 and by decreasing gentamicin accumulation. These findings should not be interpreted to suggest that fluoride is a protective agent as megalin deficiency could lead to serious adverse effects on the kidney physiology. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: fluoride; gentamicin; kidney injury; Kim-1; megalin; Hsp72

Introduction

Fluoride is naturally present in mineral complexes of many bodies of water (Whitford, 1983). Fluoride ions are released from these fluoride-containing minerals into the groundwater, which is the main source of fluoride exposure (ATSDR, 2003). The World Health Organization has established 1.5 ppm as the maximum limit for fluoride concentration in drinking water, a level considered beneficial for its cariostatic effects (WHO, 2006). Nevertheless, it has been estimated that more than 200 million people from 25 countries, including China, India, Mexico and Argentina, are exposed to high fluoride concentrations (>1.5 ppm) through drinking water (WHO, 2006). Chronic fluoride exposure above 2 ppm has been associated with renal impairment (Xiong *et al*., 2007). Given that renal excretion is the primary pathway for fluoride elimination, the kidney is, therefore, one of the main target organs for fluoride toxicity (Whitford, 1994). In the kidney, fluoride induces oxidative stress and peroxidation of the cell membrane lipids (Guan *et al*., 2000; Karaoz *et al*., 2004). Furthermore, experimental animal studies have shown that the proximal tubule, which is primarily localized in the renal cortex, is the segment of the nephron that is most susceptible to damage by fluoride exposure (Usuda *et al*., 1998; Dote *et al*., 2000). In a previous study carried out in rats, we have demonstrated that exposure to 15 and 50 ppm of fluoride induced a sub-nephrotoxic effect only detected using very sensitive and specific biomarkers of kidney injury. Neither serum creatinine (SCr) nor estimated glomerular filtration rate (eGFR) levels were modified after fluoride exposure. However, urinary levels of kidney injury molecule-1 (Kim-1), clusterin (Clu), osteopotin (OPN), heat shock protein 72 (Hsp72), β-2-microglobulin (B2M) and cystatin-C (CysC) were significantly increased after fluoride exposure. Moreover, while fluoride-induced tubular damage a repair process was in progress (Cárdenas-González *et al*., 2013).

The cumulative nehprotoxicity induced by a sub-toxic stimulus has an effect on the response to a subsequent treatment with a potentially damaging dose with the same or a related stressor

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agent (Calabrese, 2004). This event poses special importance in clinical and environmental scenarios. People exposed chronically to fluoride, with no signs of renal dysfunction, could present a modified response to treatment with potential nephrotoxicants. Gentamicin, one of the most prescribed antibiotics, has a nephrotoxic potential when it is administered for prolonged periods or at an inadequate dosage (IOM-US, 2003; Lopez-Novoa *et al.*, 2011). However, despite the fact that fluoride and gentamicin both pose a significant risk to the development of nephrotoxicity and have a high probability of co-exposure, there is currently no data available showing their potential cumulative nephrotoxicity.

In the present study, we tested the hypothesis that fluoride exposure might impact the response to a subsequent nephrotoxic treatment with gentamicin. For this purpose, traditional and novel kidney injury biomarkers were measured in male rats exposed to 15 or 50 ppm of fluoride for 40 days and subsequently co-exposed to fluoride and a nephrotoxic dose of gentamicin (40 mg kg^{-1} day^{-1}) for 7 days.

Materials and Methods

Animals

The experiments were performed in recently weaned male Wistar rats (~21 days old) purchased from Harlan Laboratories (Distrito Federal, Mexico) weighing 71 ± 9 g. The rats were group-housed in a polypropylene cage with sawdust bedding, at a controlled temperature between 20 and 22 °C and a 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, USA) were freely available in the home cages throughout the experiment. Food, water intake, and body weight were monitored three times a week during the fluoride exposure period and daily during the gentamicin administration. The care and experimental procedures were conducted after approval of the study by the Institutional Animal Care and Use Committee (CICUAL, Cinvestav-IPN) in accordance with their Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Experimental Design

After 1 week of acclimatization, the rats were randomly divided into four groups of six animals each: two fluoride-exposed groups and two groups without fluoride added to the drinking water. The number of animals used was selected based on our previous study Cárdenas-González *et al.* (2013). In that study, we were working with different numbers of animals per group (between 4 and 12), and we found that six was a reasonable number in order to get statistical significance. The fluoride-exposed groups received 15 or 50 ppm of fluoride as sodium fluoride (Sigma-Aldrich, St. Louis, MO, USA) in the drinking water for a period of 40 days. These levels might be considered as environmentally relevant fluoride concentrations as it has been shown that the exposure levels of fluoride must be 4-5 times higher in rats in order to achieve serum fluoride levels comparable to those in humans (Angmar-Månsson and Whitford, 1984). The groups to which supplemental fluoride was not administered were provided with drinking water with a concentration of 0.5 ppm of fluoride for the same period. After 40 days, one of the non-exposed groups and both fluoride-exposed groups were administered with gentamicin 40 mg kg^{-1} (bw) daily by a subcutaneous injection for 7 consecutive days (Garamicin®; Schering-Plough Corp. Kenilworth, NJ, USA). During the gentamicin treatment, fluoride exposure continued. The dosage of gentamicin was selected based on previous studies and considering the inter-lineage and gender sensitivity, as a threshold dose that induces mild acute kidney injury in male Wistar rats (Quiros *et al.*, 2010; Harpur *et al.*, 2011). Urine samples were collected for a period of 12 h, beginning 24 h after the last administration of gentamicin. Finally, after 12 h of urine collection, the blood and both kidneys were obtained from the rats (Fig. 1).

The experimental groups were named as follows:

F0ppm, Control group; rats without fluoride added to drinking water and non-treated with gentamicin.

F0ppm + GM, Gentamicin group; rats that did not have fluoride added to the drinking water but were administered gentamicin (40 mg kg^{-1} day^{-1}) for 7 days.

F15ppm + GM, Co-exposed group 1; rats exposed to 15 ppm of fluoride for 40 days and then co-exposed to fluoride and gentamicin (40 mg kg^{-1} day^{-1}) for 7 days.

F50ppm + GM, Co-exposed group 2; rats exposed to 50 ppm of fluoride for 40 days and then co-exposed to fluoride and gentamicin (40 mg kg^{-1} day^{-1}) for 7 days.

Urine, Serum and Tissue Collection

For urine collection, animals were placed in metabolic cages, and the samples were collected on dry ice for a period of 12 h. Food and water were supplied *ad libitum* during urine collection. The urine was centrifuged at 3000 *g* for 10 min (4 °C), and the supernatant was aliquoted and stored at −80 °C. For tissue collection, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg kg^{-1}) and placed on a homoeothermic table. A catheter was then placed into the abdominal aorta, and animals were euthanized by terminal exsanguination (intracardiac puncture). Immediately after, the right kidney was removed, and the cortex was carefully removed, flash-frozen in liquid nitrogen and stored at −80 °C for molecular analysis. The left kidney was perfused using isotonic saline solution (0.9% NaCl) and cut transversely into two halves. One-half was placed in phosphate-buffered 4% formalin for light microscopic analysis. The remaining half was immediately immersed for 2 min in 2-methylbutane (Sigma-Aldrich), which was previously cooled in liquid nitrogen, and stored in liquid nitrogen for immunofluorescence assays.

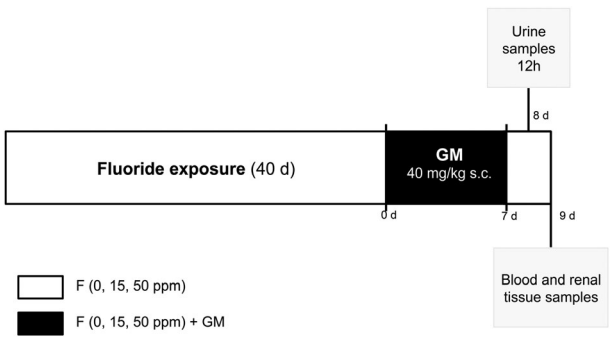

Figure 1. Experimental protocol. Scheme of the experimental protocol for fluoride (F) exposure and co-exposure to F and gentamicin (F+GM). The animals were exposed to fluoride (0, 15 and 50 ppm) via drinking water for 40 days (d). After this period, the animals were co-exposed to fluoride and gentamicin, administered by subcutaneous injection for 7 consecutive days (40 mg kg^{-1} of body weight). Urine samples were collected 24 h after the last gentamicin administration, and blood and renal tissue samples were collected after 48 h.

Urinary Fluoride Concentration and Biochemical Measurements

Measurements of the urinary fluoride concentration, SCr, and urinary creatinine, and the determination of the eGFR were performed as previously described by Cárdenas-González *et al.* (2013).

Histological Analysis

The formalin-fixed tissue samples were embedded in paraffin, sectioned in 5–8 μm slides and stained with hematoxylin and eosin (H&E). Samples from four randomly chosen animals per group were analyzed in a blinded fashion. Digital photographs were taken using a camera connected to an Olympus BX51 (Tokyo, Japan) light microscope. Histological analysis was performed in triplicated using at least three different animals per group.

Immunofluorescence

Kidney sections (6–8 μm) were cut using a Leica CM 1510 cryostat (Wetzlar, Germany) and mounted on poly-L-lysine-coated slides (Sigma-Aldrich), which were held at –70 °C. The sections were fixed for 10 min in methanol at –20 °C, hydrated with PBS for 5 min, and subsequently incubated for 5 min at room temperature in 0.2% PBS-Triton X-100. After that, the tissue sections were washed with PBS and incubated with 0.5% IgG-free albumin (Research Organics, Cleveland, OH, USA) for 1 h at room temperature to reduce non-specific labeling. To differentiate the Kim-1 and caspase-3 locations in the proximal tubule, double labeling was performed using a monoclonal mouse anti-dipeptidyl-peptidase IV (DPP) antibody (1:500; AbD Serotec, Bio-Rad Laboratories, Inc. Hercules, CA, USA) to label the brush border at the luminal region. The frozen sections were incubated overnight at 4 °C with one of the following antibodies: polyclonal goat anti-Kim-1 (1:500; R&D Systems Inc., Minneapolis, MN, USA), rabbit anti-caspase-3 (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), goat anti-megalin (1:100; Santa Cruz Biotechnology Inc), or mouse anti-vimentin (1:100; Invitrogen Life Technologies, Thermo Fisher Scientific Inc. Wilmington, DE, USA). Afterwards, the slides were washed and incubated for 2 h at room temperature with the appropriate secondary antibody: Alexa Fluor® 488 donkey anti-goat, Alexa Fluor® 488 donkey anti-rabbit, Alexa Fluor® 594 donkey anti-goat, or Alexa Fluor® 594 donkey anti-mouse (1:500; Invitrogen Life Technologies, Thermo Fisher Scientific Inc.). After washing, the nuclei were stained with DAPI (Sigma-Aldrich) for 10 min at room temperature. The immunofluorescent signals were evaluated using a confocal inverted microscope (TCS-SP2; Leica, Heidelberg, Germany). The immunofluorescence assays were performed in triplicated using samples from three different animals per group. Non-specific labeling was assessed by the exclusion of the primary antibodies.

Determination of Urinary Kidney Injury Biomarkers

The concentrations of urinary Kim-1, Clu, OPN, B2M and CysC were measured using the MILLIPLEX® MAP Rat kidney toxicity panel 1 and panel 2 (Millipore Corp., Billerica, MA, USA) as previously described (Cárdenas-González *et al.*, 2013). A total of 12.5 μl of the urine sample was used. For panel 1 (Kim-1, Clu and OPN), dilution of the urine was not necessary, but for panel 2 (B2M and CysC), the urine samples were diluted 10-fold. For all measurements, the samples were analyzed in duplicate. The plates were run on a Luminex® instrument. The levels of urinary heat shock protein 72 (Hsp72) were detected by western blotting as previously described (Barrera-Chimal *et al.*, 2011). The urinary kidney injury biomarker data were expressed as the urinary biomarker excretion rates.

Quantitative Reverse Transcription-PCR

Total RNA was isolated from the renal cortex using TRIzol® reagent (Invitrogen Life Technologies, Thermo Fisher Scientific Inc.). Complementary DNA (cDNA) was generated from 2 μg of the total RNA using the ImProm-II™ reverse Transcription System (Promega, Madison, WI, USA). The concentration and purity of the RNA and cDNA were measured by spectrophotometry using a NanoDrop 2000 instrument (Thermo Fisher Scientific). Real-time PCR (qRT-PCR) was performed to analyze the mRNA expression levels of the kidney injury biomarkers (Kim-1, Clu and OPN) and the apoptosis-related molecules, B-cell lymphoma 2 (Bcl2) and bcl-2-like protein 4 (Bax). The PCR was performed using the MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas; Thermo Fisher Scientific Inc.) in the StepOnePlus™ Real-time PCR System (Applied Biosystems®, Invitrogen Life Technologies, Thermo Fisher Scientific Inc.). 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, listed in 5′ to 3′ direction, for *GAPDH* were ACCACAGTCCATGCCATCAC (forward) and TGCCAGTGAGCTTCCCGTT (reverse). The primers used for *Kim-1*, *Clu*, *OPN*, *Hsp-72*, *Bax* and *Bcl2* were previously described (Yang *et al.*, 2002; Rached *et al.*, 2008; Barrera-Chimal *et al.*, 2011). Gene expression changes relative to the control were determined using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene.

Determination of Urinary Gentamicin Levels by the Enzyme-Linked Immunosorbent Assay (ELISA)

The urinary gentamicin concentration was determined using the competitive enzyme immunoassay commercially available MaxSignal® Gentamicin ELISA Test Kit (Bio Scientific Corp,. Austin, TX, USA) with a detection limit of 10 ppb; the manufacturer's instructions were followed. Absorbance was measured using the Infinite® 200 PRO microplate reader (Tecan Trading AG, Männedorf, Switzerland) at 450 nm.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5.0 Software (GraphPad Software, Inc., La Jolla, CA, USA). The Shapiro–Wilk test and a normal probability plot were used to test for the normality of the data. Data that met 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 the differences among the groups. Differences between means from the two groups were analyzed by Student's *t*-test. 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. Differences were considered statistically significant with $P < 0.05$.

RESULTS

General Toxicity Evaluation and Fluoride Exposure Assessment

A significant decrease in body weight gain was observed in all groups treated with gentamicin compared with the F0ppm group.

This was related to a reduction in food intake during the 7 days of gentamicin treatment (Supplementary information Table 1). However, neither gentamicin nor its co-exposure to fluoride caused overt signs of toxicity or death. In order to monitor fluoride exposure, we analyzed urinary concentrations of fluoride. As expected, fluoride exposure induced a significant dose-related increase in the urinary fluoride concentration. Accordingly, urinary fluoride concentrations in the F15ppm + GM and F50ppm + GM groups were significantly increased compared with the F0ppm group (5.8- and 9-fold, respectively) and the F0ppm + GM group (14.6- and 22.5-fold, respectively). The difference in urinary fluoride levels between the co-exposed F15ppm + GM and F50ppm + GM groups (1.5-fold) was also significant (Supplementary information Table 2).

Gentamicin-Induced Nephrotoxicity was Less Pronounced than in Groups Previously Exposed to Fluoride

Gentamicin treatment led to a significant amount of nephrotoxicity, as assessed by traditional biomarkers of renal function (Fig. 2), and renal tissue integrity (Fig. 2A). SCr levels increased more than twofold after gentamicin treatment when compared with those of the F0ppm group (Fig. 2B). However, when rats previously exposed to fluoride were co-exposed to gentamicin, levels of SCr were similar to basal values. Gentamicin treatment also induced a marked decrease in eGFR values in all gentamicin-treated groups compared with the F0ppm group (Fig. 2B). However, the effect of gentamicin treatment on eGFR was less pronounced in the groups previously exposed to fluoride. Specifically, the F50ppm + GM group had significantly higher eGFR values than the F0ppm + GM group. Representative H&E stains obtained from rat kidneys 48 h after the last gentamicin administration are shown in Fig. 3. No morphological alterations were found in the kidneys from the F0ppm group (Fig. 3A). Kidney sections from groups treated with gentamicin (Fig. 3B, C and D) displayed a decrease of Bowman's space and multilobed glomeruli (filled arrows) as well as foci of inflammation containing cellular infiltration (yellow arrows). The hyaline material in tubular lumen (white arrows), loss of tubular basement membrane and tubular cell lysis were also observed (yellow asterisks). There were no visible histological differences between the F0ppm + GM group and the co-exposed F15ppm + GM and F50ppm + GM groups.

Increase of Novel Biomarkers of Kidney Injury by Gentamicin was Less Pronounced n Groups Previously Exposed to Fluoride

Levels of urinary Kim-1, Clu, OPN, Hsp72, B2M and CysC showed a significant increase after gentamicin treatment (Fig. 4), which was greater than the increase induced by the exposure to 15 or 50 ppm of fluoride alone reported in our previous study (Cárdenas-González *et al.*, 2013). For instance, gentamicin induced 44-, 38- and 29-fold increase in Kim-1 levels in the groups F0ppm + GM, F15ppm + GM and F50ppm + GM, respectively, compared with the F0ppm group (Fig. 4A). However, in the co-exposed groups, the increase in the levels of all kidney injury biomarkers was less pronounced than in the F0ppm + GM group. B2M and CysC were the biomarkers that exhibited the most dramatic reduction of the gentamicin-induced effect. In the F50ppm + GM group, B2M and CysC levels were reduced by 80% and 90% respectively, relative to the gentamicin-induced effect (Fig. 4E and F). This reduction in the gentamicin-induced effects was also observed for Clu, OPN and Hsp72 (Fig. 4B, C and D). Sensitive and specific biomarkers of kidney injury indicate that gentamicin-induced nephrotoxicity was less pronounced in rats with previous exposure to fluoride

Reduction in Gentamicin-Induced Kim-1 and Vimentin Expression in Renal Tissue of Groups Previously Exposed to Fluoride

To explore the tissue expression of the biomarkers previously evaluated in urine, we assessed the renal expression of Kim-1, which is one of the most sensitive and specific kidney injury biomarkers (Bonventre, 2009). In addition, vimentin expression was evaluated, as an indirect marker of the repair process, and to identify the differentiation status of the renal epithelial cells (Kusaba *et al.*, 2014). Gentamicin treatment induced a marked increase in the expression of Kim-1 in the F0ppm + GM group (Fig. 5B, green fluorescence). Kim-1 immunoreactivity co-localized with DPP, confirming its exclusive localization in the proximal tubule (Supplementary information Fig. 1). Moreover, Kim-1 immunoreactivity co-localized with vimentin (red fluorescence) in the proximal tubule of the F0ppm + GM group (Fig. 5J, white arrow). However, in the co-exposed F15ppm + GM and F50ppm + GM groups we observed a clear fluoride-dose-dependent reduction of gentamicin-induced expression of Kim-1 (Fig. 5C and D).

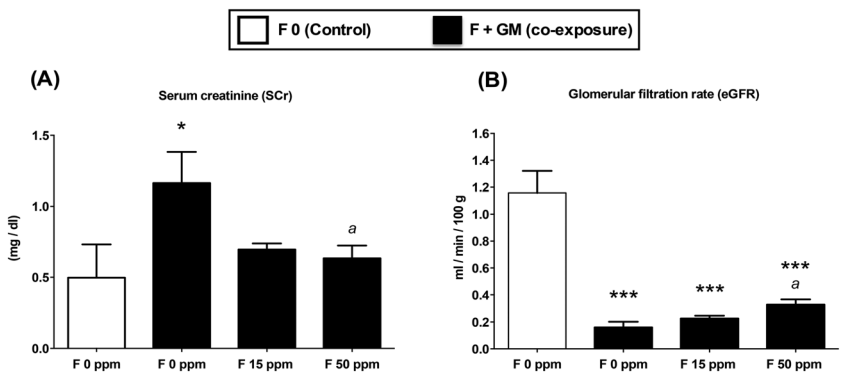

Figure 2. Effect of gentamicin treatment (GM; 40 mg kg^{-1}, 7 days) on (A) serum creatinine concentrations (SCr) and (B) the estimated glomerular filtration rate (eGFR) in rats previously exposed to fluoride for 40 days. The data are presented as the mean ± standard error of the mean (SEM), $n = 6$. Statistically significant changes indicated for $P < 0.05$ (one-way ANOVA + Tukey's multiple comparison test). Asterisks indicate statistically significant differences relative to the F0ppm group (control) (*$P < 0.05$, ***$P < 0.001$). An ^{a} denotes significant differences among the groups treated with gentamicin: F0ppm + GM, F15ppm + GM and F50ppm + GM ($^{a}P < 0.05$).

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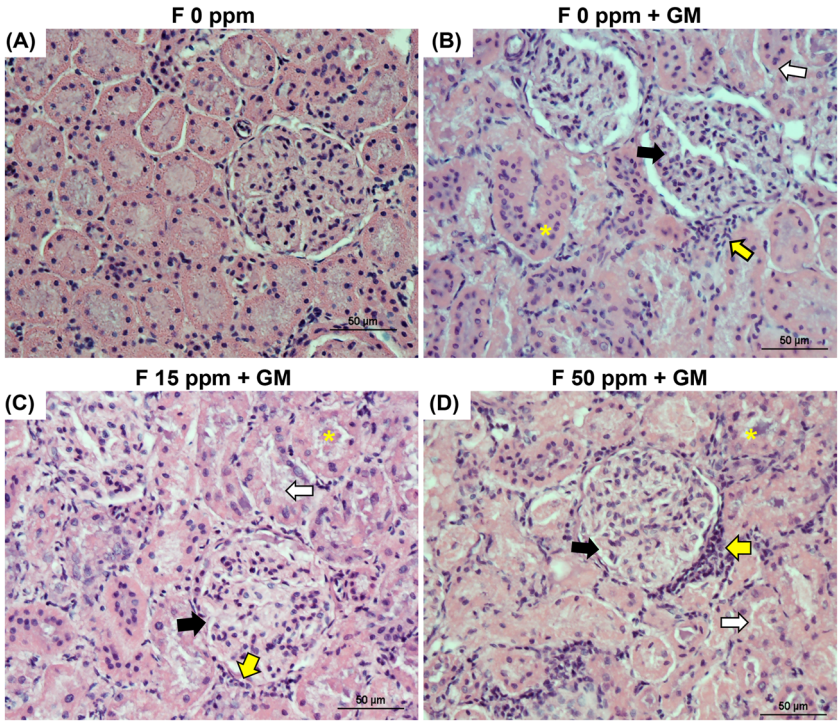

Figure 3. Morphopathological evaluation of rat kidney sections (8 µm) stained with hematoxylin and eosin (H&E). Representative photographs (400×) of renal slices from (A) the F0ppm group (control) and the groups co-exposed to fluoride and gentamicin (GM; 40 mg kg^{-1} day^{-1}, 7 days): (B) F0ppm + GM, (C) F15ppm + GM and (D) F50ppm + GM, respectively. Filled arrows indicate decrements in Bowman's space and/or multilobed glomeruli; yellow arrows indicate cellular infiltration; white arrows indicate hyaline material and yellow asterisks indicate cell lysis.

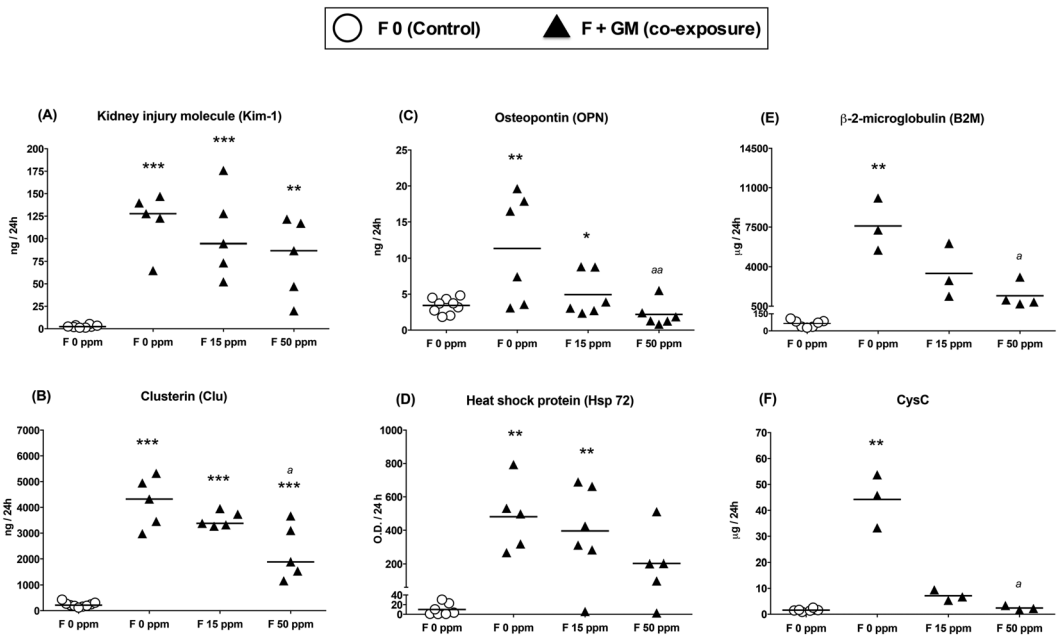

Figure 4. Effect of gentamicin treatment (GM; 40 mg kg^{-1} day^{-1}, 7 days) on urinary (A), kidney injury molecule (Kim-1), (B) clusterin (Clu), (C) osteopontin (OPN), (D) heat shock protein 72 (Hsp72), (E) β-2-microglobulin (B2M) and (F) cystatin-C (CysC) excretion rates in rats previously exposed to fluoride during 40 days. The data are presented as the median ± interquartile ranges ($n = 3$–9). For (A), (B), (C) and (D), statistically significant changes are indicated for $P < 0.05$ by one-way ANOVA + Tukey's multiple comparison test whereas statistically significant changes of (E) and (F) are indicated for $P < 0.05$ by Kruskal–Wallis + Dunn's multiple comparison test. Asterisks indicate statistically significant differences relative to the F0ppm group (control) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). An ^{a} denotes significant differences among the groups treated with gentamicin: F0ppm + GM, F15ppm + GM and F50ppm + GM ($^{a}P < 0.05$, $^{aa}P < 0.01$).

A reduced expression of vimentin and co-localization with Kim-1 was also observed in the co-exposed groups (Fig. 5G, H, K and L). These results were consistent with those found in urine and suggested that co-exposed groups had less damage, and as a consequence less repair than the group treated only with gentamicin.

Changes in the Gentamicin-Induced mRNA Expression Levels of Kim, Clu, OPN and Hsp72 in the Renal Cortex of Groups Previously Exposed to Fluoride

To further explore these findings, mRNA levels of *Kim-1*, *Clu*, *OPN* and *Hsp72* were evaluated in the renal cortex. Consistently, *Kim-1* mRNA expression was significantly induced more than 4700-fold in the renal cortex of the F0ppm + GM group. This upregulation was markedly reduced in the co-exposed groups. For instance, the increase in *Kim-1* mRNA expression in the F15ppm + GM group was 1240-fold and 410-fold for F50ppm + GM group. This represented a reduction of approximately 70% and 90%, respectively, relative to the gentamicin-induced effect. The gentamicin-induced upregulation of *Clu* and *OPN* mRNA was similarly reduced in the co-exposed groups (Fig. 6B and C). However, in contrast with our finding on the protein levels, the gentamicin treatment did not cause significant changes in the expression of *Hsp72* mRNA in the F0ppm + GM and F15ppm + GM groups (6.5- and 2.7-fold, respectively). Interestingly, the *Hsp72* mRNA expression in the F50ppm + GM group was notably upregulated 33.6-fold (Fig. 6D).

Previous Exposure to Fluoride Decreased the Apoptosis Induced by Gentamicin Treatment

In some nephropathies, an increase in the Bax to Bcl2 ratio at both mRNA and protein levels is closely correlated with an increase in caspase-3 activity and thus with apoptosis (Yang *et al.*, 2002). In the renal cortex of the F0ppm + GM group, the ratio of *Bax* to *Bcl2* mRNAs shifted in favor of Bax. However, in the co-exposed F15ppm + GM and F50ppm + GM groups, the Bax to Bcl2 ratio remained close to the control value (Fig. 7A). Accordingly, caspase-3 (green fluorescence) was markedly expressed in the proximal tubule, as indicated by co-localization with DPP (red fluorescence) in the F0ppm + GM group (Fig. 7B b and j), but in the co-exposed groups, gentamicin-induced caspase-3 expression was

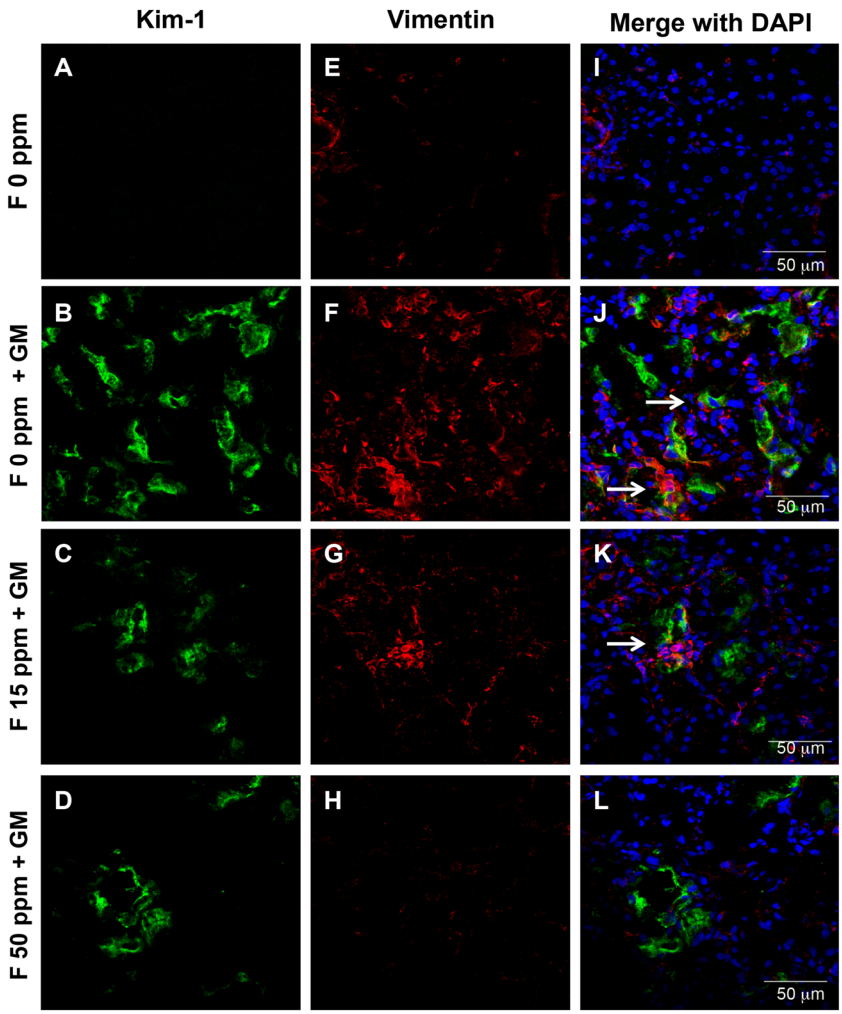

Figure 5. Effect of gentamicin treatment (GM; 40 mg kg^{-1} day^{-1}, 7 days) on the expression of kidney injury molecule-1 (Kim-1) protein (green fluorescence) in kidney sections from rats previously exposed to fluoride during 40 days. Representative photographs were taken using 63 × objectives (scale bar 50 μm) from (A) the F0ppm group (control) and the groups co-exposed to fluoride and gentamicin: (B) F0ppm + GM, (C) F15ppm + GM and (D) F50ppm + GM. Immunofluorescent staining of Kim-1 co-localize with vimentin, a dedifferentiation marker (red fluorescence), in the same tubule (white arrows). The nuclei were stained with DAPI (blue).

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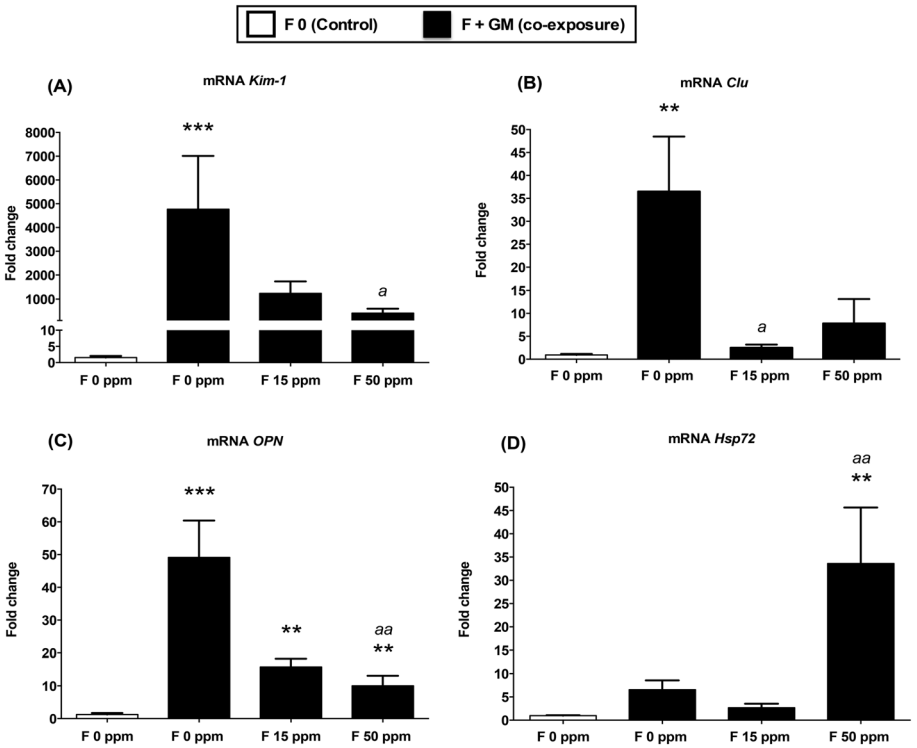

Figure 6. Effect of gentamicin treatment (GM; 40 mg kg^{-1} day^{-1}, 7 days) on the mRNA expression of (A) the kidney injury molecule (*Kim-1*), (B) clusterin (*Clu*), (C) osteopontin (*OPN*) and (D) heat shock protein 72 (*Hsp72*) in the renal cortex of rats previously exposed to fluoride during 40 days. The data are presented as the mean fold change in messenger RNA (mRNA) ± standard error of the mean (SEM), and significant fold changes are presented relative to the F0ppm (control), ($n = 6$). Each gene expression was normalized to the GAPDH expression before calculations of fold change. Asterisks indicate statistically significant differences relative to the F0ppm group (control) (one-way ANOVA + Tukey's multiple comparison test, $^{**}P < 0.01$, $^{***}P < 0.001$). An ^{a} denotes significant differences among the groups treated with gentamicin: F0ppm + GM, F15ppm + GM and F50ppm + GM (one-way ANOVA + Tukey's multiple comparison test, $^{a}P < 0.05$, $^{aa}P < 0.01$).

decreased (Fig. 7B c and d). This finding demonstrates that apoptosis induced by gentamicin treatment was reduced in those groups previously exposed to fluoride.

Expression of Megalin, Direct Indicator of Cellular Capacity for Uptake Gentamicin, Suggest that Fluoride Exposure Reduced Gentamicin Internalization

Gentamicin nephrotoxicity is related to its accumulation primarily in the proximal tubule, where gentamicin is internalized by endocytosis through a megalin-associated endocytic complex (Lopez-Novoa *et al*., 2011). Hence, expression of megalin acts as an indirect indicator of gentamicin uptake by the proximal tubule. We, therefore, examined whether exposure to fluoride prior to the gentamicin treatment could alter the megalin expression, and consequently the internalization of the aminoglycoside into the proximal tubule. For this purpose, the expression of megalin was determined qualitatively by immunofluorescence staining in the proximal tubule. In the F0ppm + GM group, gentamicin markedly induced the expression of megalin in the apical surface and the cytoplasm of proximal tubule epithelial cells (red fluorescence) (Fig. 8A b). Nevertheless, there was a visible decrease in the expression of megalin in the co-exposed groups (Fig. 8 A c and d). Consistent with this observation, in the co-exposed groups the urinary excretion of gentamicin was increased as a function of the previous exposure to fluoride. Thus the fluoride-dependent decrease of megalin expression in co-exposed groups reduced gentamicin internalization into the proximal tubule, potentially contributing to the reduction of gentamicin nephrotoxicity in the F15ppm + GM and F50ppm + GM groups.

Discussion

The goal of the present study was to test if a sub-nephrotoxic stimulus induced by fluoride might impact the response to a subsequent nephrotoxic treatment with gentamicin in rats. In an attempt to mimic a common situation where people exposed chronically to fluoride, with no signs of renal dysfunction, could present a modified response to treatment with potential nephrotoxicants. In the present study, we found that the subchronic exposure to fluoride influences the response to gentamicin-induced nephrotoxicity in rats.

The administration of 40 mg kg^{-1} day^{-1} gentamicin for 7 days caused kidney injury that was characterized by diminished renal function and an increase in sensitive and specific biomarkers of kidney injury. Our data are supported by previous studies in rats treated with different doses of gentamicin, which demonstrated the high sensitivity and specificity of Kim-1, Clu, OPN, CysC and B2M as markers of gentamicin-induced kidney injury (Hoffmann *et al*, 2010; Ozer *et al*., 2010). It has also been reported that the increases of urinary Kim-1 and Clu levels correlate with increased gene and protein expression after gentamicin treatment (Sieber *et al*., 2009). Herein, we found that gentamicin induced increments in both urinary and mRNA levels of not only Kim-1 and Clu but also OPN. Moreover, the pattern observed in urine, and mRNA levels of Kim-1 agreed with the expression pattern of protein in the

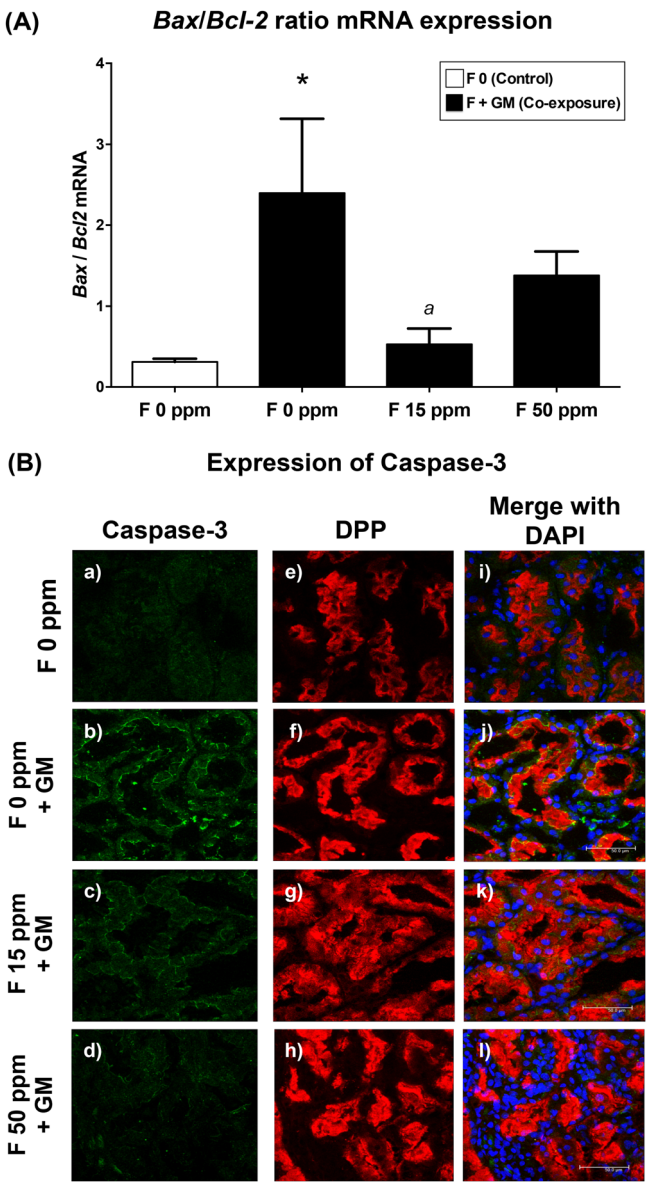

Figure 7. The induction of apoptosis after gentamicin treatment (GM; 40 mg kg–1 day^{-1}, 7 days) is decreased in groups previously exposed to 15 or 50 ppm of fluoride during 40 days. (A) The ratio of *Bax* to *Bcl2* messenger RNA (mRNA) expression in the renal cortex and (B) the expression of Caspase-3 protein (green fluorescence) in kidney sections from rats previously exposed to fluoride for 40 days. In the graph, data are expressed as the ratio of the mean fold change of *Bax* and *Bcl2* ± the standard error of the mean (SEM). *Bax* and *Bcl2* expression levels were normalized to GAPDH expression and the fold change calculation was made relatively to the F0ppm group (control) (one-way ANOVA + Tukey's multiple comparison test, $^{*}P < 0.05$). An ^{a} denotes significant differences among the groups treated with gentamicin: F0ppm + GM, F15ppm + GM and F50ppm + GM (one-way ANOVA + Tukey's multiple comparison test, $^{a}P < 0.05$). The images show representative photographs taken using 63 × objectives from (a) the F0ppm group (control) and the groups co-exposed to fluoride and gentamicin: (b) F0ppm + GM, (c) F15ppm + GM and (d) F50ppm + GM. The nuclei were stained with DAPI (blue).

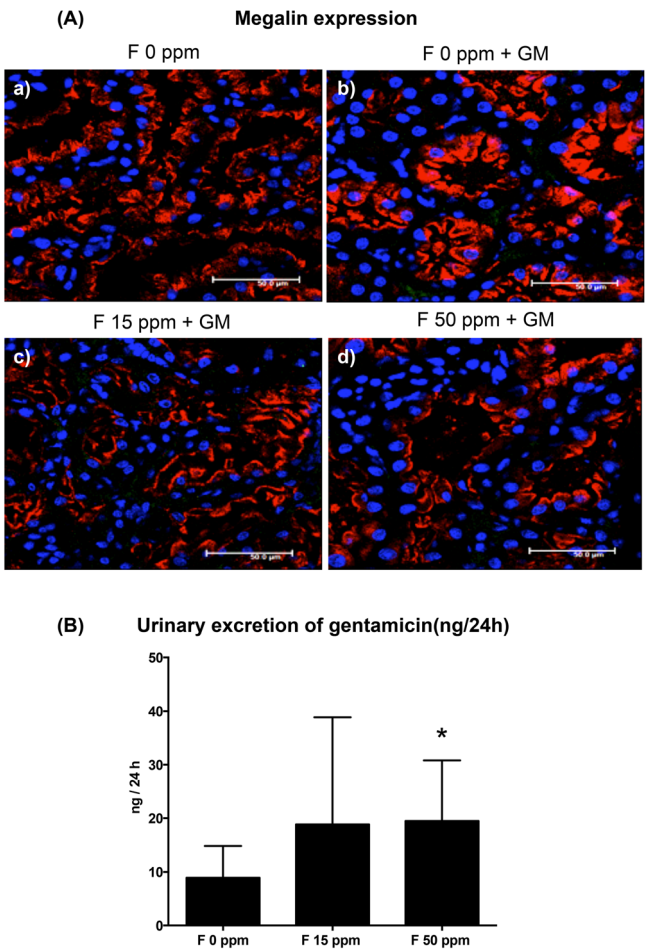

Figure 8. Effect of the co-exposure to fluoride (F) and gentamicin (GM; 40 mg kg^{-1} day^{-1}, 7 days) on (A) the expression of megalin protein (red fluorescence) in kidney sections and (B) the urinary excretion of gentamicin, 24 h after the last administration of gentamicin, in rats previously exposed to fluoride for 40 days. The images show representative photographs taken using 63× objectives from: (A, a) the F0ppm group (control) and the groups co-exposed to fluoride and gentamicin: (A, b, c and d) F0ppm + GM, F15ppm + GM and F50ppm + GM, respectively. The nuclei were stained with DAPI (blue). (B) In the graph, the data of the urinary excretion of gentamicin are expressed as the mean ± standard error of the mean (SEM), $n = 6$. An * denotes significant differences among F0ppm + GM and F50ppm + GM (Student's t-test, $^{*}P < 0.05$).

proximal tubule. However, the gentamicin-induced nephrotoxicity, assessed by traditional and novel biomarkers, was less pronounced in those groups previously exposed to 15 and 50 ppm of fluoride for 40 days.

To the best of our knowledge, the effect of co-exposure to fluoride and gentamicin has never been described before. There are other studies that have investigated the effects of a sub-nephrotoxic stimulus on the response to a subsequent nephrotoxic challenge. Chronic exposure to uranium, at a sub-nephrotoxic level, increased the renal damage caused by the administration of 50 mg kg^{-1} gentamicin for 7 days (Vicente-Vicente *et al.*, 2013). However, a lower dose but longer exposure period of uranium did not enhance the renal sensitivity to different doses of gentamicin (5, 25, 100 and 150 mg kg–1) (Rouas *et al.*, 2011; Poisson *et al.*, 2014). Conversely, exposure to arsenic or cobalt reduced acute kidney injury induced by an ischemia-reperfusion challenge (Yang *et al.*, 2001; Matsumoto *et al.*, 2003). Likewise, the administration of 40 mg kg^{-1} gentamicin for 3 days reduced the nephrotoxic effect caused by subsequent 10-day treatment with the same dose of gentamicin (Pessoa *et al.*, 2011).

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In the course of this study, we investigated several molecular mechanisms that might be possible explanations for the reduction in gentamicin toxicity. Hsp-72, an inducible isoform of Hsp70 subfamily, is one of the major compensatory responses induced by stressful stimuli whose cytoprotective function is largely explained by its anti-apoptotic effect (Emami *et al*., 1991; Fan *et al*., 2003). Apoptosis plays an important role in gentamicin-induced nephrotoxicity. Cytosolic gentamicin activates caspase-3, an executioner caspase, through the mitochondrial pathway and also in an independent fashion. Gentamicin also triggers the translocation of pro-apoptotic molecules, such as cytochrome c, apoptosis-inducing factor and Bax. Together, these events result in cellular death apoptosis closely related to the gentamicin dose (Quiros *et al*., 2011). In the kidney, Hsp-72 protects against toxic and ischemia-induced tubular cell apoptosis, either by preventing caspase activation, thereby inhibiting the translocation of Bax (a pro-apoptotic molecule) and the activation of NF-κB, or by restoring the function of Bcl2 (an anti-apoptotic molecule) (Komatsuda *et al*., 1999; Meldrum *et al*., 2003; Lanneau *et al*., 2007). While tissue levels of Hsp-72 are associated with renoprotection, increased levels of this protein in the urine have been associated with loss of tubular integrity (Mueller *et al*., 2003; Barrera-Chimal *et al*., 2011). Here, we found increased level of urinary Hsp-72 after the gentamicin treatment, which was less intense in the groups previously exposed to fluoride, indicating that the extent of tubular injury induced by gentamicin was reduced in these groups. Interestingly, *Hsp-72* mRNA was markedly expressed in the renal cortex of rats previously exposed to 50 ppm of fluoride for 40 days. In this context, previous exposure to fluoride altered the Bax/Bcl2 ratio towards an anti-apoptotic direction in the renal cortex of rats treated with gentamicin. Furthermore, the expression of the active form of caspase-3 was clearly reduced in the proximal tubules of the groups exposed to fluoride before the gentamicin regimen. Therefore, we suggest that fluoride exposure induced a compensatory response carried out by the expression of *Hsp72* that contributed to decreasing the nephrotoxic effect of gentamicin in rats previously exposed to fluoride.

We have previously reported that fluoride exposure induced proximal tubular epithelial cells dedifferentiated after 40 days of exposure (Cárdenas-González *et al*., 2013). While these cells might be less susceptible to damage, it also implies the loss of the differentiated phenotype of epithelial cells (Bonventre, 2002). Megalin is a hallmark of apical differentiation in the proximal tubular epithelial cells (Christensen *et al*., 2012). Physiologically, megalin has a crucial role in the normal tubular reabsorption of essential substances that otherwise would be lost in the urine. Low-molecular-weight proteins, carrier proteins, lipoproteins, vitamins, trace elements, enzymes, hormones and growth factors have been described as ligands of megalin (Christensen *et al*., 2012). However, exogenous molecules, such as aminoglycosides, can also be ligands of megalin. Gentamicin is distinctively accumulated in the proximal tubule owing to megalin, the primary route for gentamicin internalization (Nagai *et al*., 2001; Nagai and Takano, 2004). In the present study, we found that the expression of megalin was markedly decreased in the groups previously exposed to fluoride. Accordingly, the urinary excretion of gentamicin was increased in the co-exposed groups, suggesting that gentamicin internalization was being reduced. In this regard, we have demonstrated that fluoride exposure for 40 days led to an increased urinary excretion of B2M and CysC (Cárdenas-González *et al*., 2013), proteins totally reabsorbed by the proximal tubule in a megalin-dependent pathway. Therefore, these findings strongly suggest that fluoride exposure reduced not only the uptake of B2M and CysC, but also gentamicin internalization into the proximal tubule, and, as a result, decreasing the gentamicin–nephrotoxic effect rats previously exposed to fluoride. In the future, it will be important to investigate the effects that the diminished expression of megalin, induced by fluoride, might have on the nephrotoxic effect of other toxicants megalin-mediated transport.

In addition, there are other molecules that might be involved in the less pronounced nephrotoxic effect in rats previously exposed to fluoride. Reactive oxygen species (ROS) act as second messengers that activate intracellular signaling pathways and serve as regulators of metabolism, proliferation and survival processes in acute kidney injury models (Ravati *et al*., 2000; Li and Jackson, 2002). Similarly, nitric oxide (NO) down-regulates the inflammatory response diminishing neutrophil recruitment to reduce tissue damage (Park *et al*., 2003). ROS and NO are molecules importantly implicated in the mechanism of fluoride toxicity (Chouhan and Flora, 2008; Barbier *et al*., 2010). The activation of Nrf-2, a transcription factor of genes implicated in cellular survival and the antioxidant response, ameliorates the renal oxidative stress and the subsequent renal inflammation and apoptosis induced by exposure to high levels of fluoride (Thangapandiyan and Miltonprabu, 2014). Additionally, the participation of other Hsps such as Hsp25, 27, 70 and 90 cannot be discarded. After kidney injury, these proteins refold denatured proteins, limit detrimental peptide interactions, favor the translocation of proteins to the correct location and degrade irreparably damaged proteins (Aufricht *et al*., 1998; Van Why and Siegel, 1998; Bidmon *et al*., 2002). Further studies should be performed to identify other molecules involved and unravel a possible molecular mechanism of the sub-nephrotoxic effect induced by fluoride.

In summary, our data suggests that the less pronounced gentamicin-induced nephrotoxicity effect shown in rats previously exposed to 15 and 50 ppm of fluoride might be due to two possible mechanisms. First, fluoride exposure might have induced a compensatory response by up-regulation of the cyto-protective HSPs, in this case, Hsp72, enabling a better and more efficient response to the challenge with gentamicin. Second, exposure to fluoride decreased the expression of megalin, a key molecule for the gentamicin internalization into the proximal tubule, potentially reducing gentamicin accumulation and thereby its nephrotoxicity.

Nevertheless, it is important to emphasize that this finding should not be interpreted to suggest that fluoride is a protective agent. Megalin-mediated endocytosis is one of the principal pathways for the recycling of bioactive ions and molecules such as calcium, iron, 25-hydroxy (OH) vitamin D_3, vitamin B12 and clusterin (Christensen *et al*., 2012). Megalin-deficient rodents and humans display low-molecular-weight proteinuria, disturbed calcium homeostasis and decreased bone mineralization (Leheste *et al*., 2003; Storm *et al*., 2013). At present, we conclude that under our experimental conditions, exposure to fluoride reduced gentamicin-induced nephrotoxicity by inducing a compensatory response carried out by Hsp72 and by decreasing the internalization of gentamicin into the proximal tubule.

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