Toxicology and Applied Pharmacology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

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

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ARTICLE INFO

Article history:
Received 23 May 2013
Revised 26 July 2013
Accepted 29 July 2013
Available online xxxx

Keywords:
Fluoride
Sub-chronic exposure
Kidney injury
Early biomarker
Urinary biomarkers
Kim-1

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

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0041-008X/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.taap.2013.07.026

Please cite this article as: Cárdenas-González, M.C., et al., Proximal renal tubular injury in rats sub-chronically exposed to low fluoride concentrations, Toxicol. Appl. Pharmacol. (2013), http://dx.doi.org/10.1016/j.taap.2013.07.026

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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:

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

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

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