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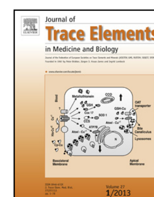

TOXICOLOGY

The decreased expression of mitofusin-1 and increased fission-1 together with alterations in mitochondrial morphology in the kidney of rats with chronic fluorosis may involve elevated oxidative stress

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ABSTRACT

This study was designed to characterize changes in the expression of mitofusin-1 (Mfn1) and fission-1 (Fis1), as well as in mitochondrial morphology in the kidney of rats subjected to chronic fluorosis and to elucidate whether any mitochondrial injury observed is associated with increased oxidative stress. Sixty Sprague-Dawley (SD) rats were divided randomly into 3 groups of 20 each, i.e., the untreated control group (natural drinking water containing <0.5 mg fluoride/L), the low-fluoride group (drinking water supplemented with 10 mg fluoride/L, prepared with NaF) and the high-fluoride group (50 mg fluoride/L), and treated for 6 months. Thereafter, renal expression of Mfn1 and Fis1 at both the protein and mRNA levels was determined by immunohistochemistry and real-time PCR, respectively. In addition, the malondiadehyde (MDA) was quantitated by the thiobarbituric acid procedure and the total antioxidative capability (T-AOC) by a colorimetric method. The morphology of renal mitochondria was observed under the transmission electron microscope. In the renal tissues of rats with chronic fluorosis, expression of both Mfn1 protein and mRNA was clearly reduced, whereas that of Fis1 was elevated. The level of MDA was increased and the T-AOC lowered. Swollen or fragmented mitochondria in renal cells were observed under the electronic microscope. These findings indicate that chronic fluorosis can lead to the abnormal mitochondrial dynamics and changed morphology in the rat kidney, which in mechanism might be induced by a high level of oxidative stress in the disease.

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Introduction

In addition to its well-known effects on skeleton and teeth, excessive accumulation of fluoride has been proposed to cause a vast array of symptoms and pathological changes in many other tissues and organs. Numerous investigations have shown that the underlying mechanism(s) may involve elevated levels of free radicals and attenuated antioxidant defenses, i.e., a high level of oxidative stress [1–4].

The kidney represents the major route for elimination of fluorine from the body and is thus susceptible to fluorosis. Renal dysfunction and abnormal metabolism, as well as histopathological changes have been observed in experimental animals exposed to excessive fluoride [5–8]. A comparative proteomic study of the kidney of puffer fish exposed to high amount of fluoride revealed 32 proteins whose expression was significantly altered [9]. PDZK-1, a protein involved in regulating renal tubular re-absorption was down-modulated in mice with fluorosis [10]. With regards to humans who living in endemic fluorosis area, fluoride level over 2.0 mg/L in drinking water can damage liver and kidney functions in children to an extent that is correlated with dental fluorosis [8]. Furthermore, in another fluoridated area children with renal disease exhibited more severe dental fluorosis than those without such disease [11].

Mitochondria are the cellular center for aerobic energy metabolism and a preferential target of various environmental toxicants. In mammals, cell survival is totally dependent on mitochondrial function [12]. Mitochondria are dynamic organelles,

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constantly undergoing fission and fusion processes that determine their morphology, size, distribution and function [13]. The mitochondrial respiratory chain is the most important source of oxygen free radicals. If overproduced superoxides are not neutralized by endogenous antioxidants or enzymes, they will cause oxidative injury and dysfunction of mitochondria, leading to cell death and other lesions.

Chronic fluorosis has been observed to cause serious damage to mitochondria [14,15]. There are indications that in cultured cells fluoride-induced apoptosis may involve loss of the mitochondrial membrane potential, thereby releasing cytochrome C into the cytosol and leading to apoptotic cell death [16]. Recently, we found that chronic fluorosis disrupts abnormal mitochondrial dynamics in a manner that might lead to a high level of oxidative stress [17].

In the present investigation, we have evaluated the level of oxidative stress together with the expression of mitofusin-1 (Mfn1) and fission-1 (Fis1), both at the protein and mRNA levels, in the kidney of the rats exposed to chronic fluorosis to characterize the pathophysiological mechanism(s) of mitochondrial damage.

Materials and methods

Materials

Rabbit polyclonal antibodies directed toward Mfn1 (Beijing Biosynthesis Biotechnology Co., Ltd., China), kits for measuring malondialdehyde (MDA) and total antioxidant capacity (T-AOC) (Nanjing Jiancheng Bioengineering Institute, China), peroxidase-conjugated polymer carrying antibodies toward rabbit and mouse immunoglobulins (the ChemMateTM Envision TM/HRP-Rabbit/Mouse kit, Gene Technology Co., Ltd., China), Trizol reagents (Invitrogen, USA), QPCR SYBR Green Mix (Infinigen Biotechnology Inc., USA) and all other chemicals (Sigma-Aldrich, USA) were purchased from the sources indicated.

Experimental animals

Thirty SD (Sprague-Dawley) rats (weighing 90–120 g) were purchased from the Experimental Animal Center in Guizhou, China, and ethical permission for the experiment was obtained from the regional ethical committee for animal studies in Guizhou. The humidity ranged from 30 to 55% and temperature remained between 22 and 25 °C. The rats were acclimatized for one week in a housing facility before treatment. The rats were randomly divided into 3 groups with 10 animals in each (half males and half females), e.g., control group (drinking water containing less than 0.5 ppm of fluoride), low-fluoride exposed group (drinking water containing 10 ppm fluoride, prepared with NaF) and high-fluoride exposed group (drinking water containing 50 ppm fluoride). During the study, the drinking water and food were administered to the animals ad libitum. The rats were examined after feeding with NaF for 6 months. At the end of the experiment, dental fluorosis, the level of fluoride in the urine, and body weight were determined.

Detection of the levels of Mfn1 and Fis1 protein by immunohistochemistry

Following fixation, renal tissue from animals in each group was sliced into 4 μm-thick consecutive sections and immunohistochemical analysis performed in accordance with the avidin-biotin peroxidase complex protocol. In brief, the sections were first deparaffinized and then incubated with antibodies directed toward Mfn1 (diluted 1:200), or Fis1 (1:400) at 4 °C overnight. After washing with phosphate-buffered saline (PBS), these sections were incubated with a peroxidase-conjugated polymer carrying antibodies toward rabbit and mouse immunoglobulins. The reactions were visualized with the ChemMateTM DAB$^+$ Chromogen in this same kit. Thereafter, the sections were counterstained with hematoxylin, dehydrated and mounted. As negative controls to assure the antibody specificity, sections were incubated with non-immune antisera. Photomicrographs were obtained using an Olympus microscope. The optical densities of every random visual field were analyzed with a Biomias 2000 Image Analysis System (Institute of Image and Graphics, Sichuan University).

Table 1
Sequences of the primers employed for amplification of Mfn and Fis1 mRNA by quantitative real-time PCR.

mRNA	Sequences (5′–3′)	Length of product (bp)	Annealing temperature (°C)
Mfn1	CCATCACTGCGATCTTCGGCCA CAGCGAGCTTGTTTCTGTAGCCCT	147	60
Fis1	GTAGGGTTACATGGATGCCCAGAGA GGCAAAAGCTCCTCCAGCAG	181	62

Measurement of Mfn1 and Fis1 mRNA by Real-time quantitative PCR

Total RNA was extracted from the renal tissue using the Trizol method. The A260/280 ratio was found to be in the range of 1.8–2.0. Real-time quantitative PCR was carried out using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) in accordance with the manufacture's protocol and analyzed with GeneAmp7300 SDS software. The primers (Table 1) were designed on the basis of the corresponding complete cDNA sequences deposited in GenBank (accession numbers: NM_138976.1 for Mfn1 and NM_001105919.1 for Fis1). The conditions for Real-time PCR were as follows: 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and annealing for 30 s (60 °C for Mfn1 and 62 °C for Fis1), and at 70 °C for 30 s. Both RT-PCR and melting curve analysis were routinely performed after amplification to confirm the specificity of this PCR procedure. To obtain the relative quantitative values for gene expression, the housekeeping gene β-actin was used as an internal control.

Determination of the levels of MDA and T-AOC

After weighing the tissue samples accurately, 10% homogenates were prepared by adding 9 ml normal saline for every g of tissue. The supernatant obtained by centrifugation was used to determine protein with the BCA protein kit. Subsequent steps were performed in accordance with the instructions to the MDA and T-AOC kits. In brief, the level of MDA, one of the products of lipid peroxidation, was quantitated employing the thiobarbituric acid-reactive substance assay (TBARS); whereas, T-AOC was determined by chemical colorimetry and calculated by the following formula: T-AOC (U/mg protein) = OD (control sample)/0.01/30 [4].

Electron microscopy

For electron microscopy, pieces (each approximately 1 mm^3 in volume) of renal cortices were fixed with 3% glutaraldehyde for 2 days at room temperature (RT), washed with 100 mM PBS, post-fixed in osmium tetroxide for 2 hrs at RT, and followed by pre-staining in acetate-barbitone for 10 min. Thereafter, the samples were dehydrated in acetone and paraffinized in Epon 812. Ultrathin sections were isolated on nickel fitters, stained with 2% uranyl acetate for 10 min and then with Reynold's lead citrate for 5 min, and finally examined with a transmission electron microscope (Hitachi-7650, Japan) at 60 kV.

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