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Role of endoplasmic reticulum stress-induced apoptosis in rat thyroid toxicity caused by excess fluoride and/or iodide

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ABSTRACT

Excess fluoride and iodide coexist in drinking water in many regions, but few studies have investigated the single or interactive effects on thyroid *in vivo*. In our study, Wistar rats were exposed to excess fluoride and/or iodide through drinking water for 2 or 8 months. The structure and function of the thyroid, cells apoptosis and the expression of inositol-requiring enzyme 1 (IRE1) pathway-related factors were analyzed. Results demonstrated that excess fluoride and/or iodide could change thyroid follicular morphology and alter thyroid hormone levels in rats. After 8 months treatment, both single and co-exposure of the two microelements could raise the thyroid cells apoptosis. However, the expressions of IRE1-related factors were only increased in fluoride-alone and the combined groups. In conclusion, thyroid structure and thyroid function were both affected by excess fluoride and/or iodide. IRE1-induced apoptosis were involved in this cytotoxic process caused by fluoride or the combination of two microelements.

1. Introduction

Fluorine is an essential trace element in humans and plays a major role in maintaining the normal structure and physiological function of bones and teeth. The human body ingests fluoride mainly through drinking water, in addition to food, industrial pollutants, and drugs (Zhang et al., 2013). The effect of fluoride on the human body follows a U-shaped trend. Low fluoride intake can cause symptoms such as dental caries, while high fluoride intake can induce acute and chronic fluorosis. It can not only result in symptoms such as dental and skeletal fluorosis but also damage the brain, liver, kidney, and other soft tissues, leading to systemic damage (Jha et al., 2011; Jiang et al., 2014; Xiong et al., 2007). The thyroid is an important endocrine organ with a high capacity for fluoride accumulation. Animal experiments have shown that excess fluoride can disrupt thyroid structure and affect thyroid hormone synthesis and secretion (Basha et al., 2011; Bouaziz et al., 2005). However, the existing results are inconsistent, and the specific effect has not been studied.

Excess fluoride has complex effects and mechanisms in thyroid damage. Fluoride can disrupt the redox system in the body, leading to an increase in free radicals and lipid peroxidation products. Additionally, fluoride can reduce functional enzyme systems such as thyroid peroxidase, Na^+, K^+-ATPase, and thyroglobulin hydrolase, causing damage of thyroid structure and function (Agalakova and Guseva, 2008; Marinovich et al., 1997). Moreover, an important mechanism by which fluoride damages the body is inducing apoptosis. Apoptosis is involved in fluoride-induced damage in the nervous, reproductive, and phrenological systems, among other systems (Liu et al., 2011; Yang et al., 2011; Zhang et al., 2013). However, little research has investigated whether fluoride damages the thyroid by inducing apoptosis. In a previous study, we demonstrated that fluoride can induce *in vitro* apoptosis in thyroid cells (Liu et al., 2014b,c), but these results have not been validated *in vivo*. Moreover, the effect of fluoride in thyroid damage is often affected by iodine. An epidemiological survey has found that both fluoride and iodide are excessive in water resources in some regions, such as the Bohai Rim Region of China (Hong et al., 2008) Thus, many resi-

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dents are exposed to the coexisting environment of excess fluoride and iodide.

Iodine and fluorine are both halogens. The thyroid is a target organ of excess iodide, and excess iodide can also induce apoptosis of thyroid cells *in vitro*. Therefore, when excess iodide and fluoride act on the thyroid concurrently, there may exist interactions, and this interactive environment may cause dual damage to the body. We demonstrated excess fluoride and iodide can induce apoptosis in thyroid cells *in vitro* (Liu et al., 2014c). No study has reported whether excess fluoride can affect thyroid structure and function *in vivo*, nor whether apoptosis is involved in thyroid damage induced by a combination of fluoride and iodide in rats.

Endoplasmic reticulum (ER) stress is a novel apoptotic pathway discovered in recent years. ER stress activates the unfolded protein response (UPR) to protect cells from damage and restore normal cell function. When the damage exceeds the recovery capacity of cells, the ER stress signal will turn from pro-survival to pro-apoptosis to quickly remove the excessively damaged cells and maintain a stable internal environment (Kim et al., 2008). Inositol-requiring enzyme 1 (IRE1) is the only anti-stress pathway that can activate the following three apoptotic pathways: C/EBP homologous protein (CHOP), c-jun N-terminal kinase (JNK), and caspase-12 pathway (Gu et al., 2004). *In vitro* study has verified that ER stress is involved in apoptosis of thyroid cells induced by excess fluoride and its combination with excess iodide (Liu et al., 2014a). This suggests that ER stress may be an important reason for thyroid cytotoxicity in rats induced by a combination of fluoride and iodide.

In the present study, we exposed Wistar rats to ambient concentrations of excess iodide and/or fluoride through drinking water and then assessed the effects of excess iodide, excess fluoride, and their combination on thyroid structure and function in rats. We measured the expression of IRE1-related factors, including glucose-regulated protein 78 (GRP78), IRE1, spliced X-box-bindingprotein-1 (sXBP-1), and C/EBP homologous protein (CHOP), to reveal the role of IRE1 pathway-induced apoptosis in the thyroid cytotoxicity caused by fluoride and/or iodide.

2. Materials and methods

2.1. Reagents and instruments

The major reagents included sodium fluoride (analytical reagent, Sinopharm Chemical Reagent, Shanghai, China); potassium iodate (analytical reagent, Sinopharm Chemical Reagent); thyroid hormone radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing, China); terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) apoptosis detection kit (Roche, Switzerland); rabbit anti-rat polyclonal antibodies against GRP78, IRE1, and CHOP (Santa Cruz, USA); TRIzol (Invitrogen, USA); RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA); DreamTap Green PCR Master Mix (Thermo Fisher, USA); and primers (Tsingke, Beijing, China).

2.2. Establishment of the animal model

One-month-old Wistar rats (80 males and 80 females, weighing 110–130 g each) were purchased from the Experimental Animal Research Center of Hubei Province (SCXK2008-0005). The animals were reared in an animal feeding room at 22 °C–24 °C and 40–60% humidity. Male and female rats were fed in separate cages. After 1 week of adaptive feeding (normal diet and water), animals were randomly assigned into eight groups to receive tap water (control; potassium: below detection limit; fluorine: 0.344 mg/L), excess iodide (1.685 mg/L KIO_3), excess fluoride (5, 10, or 20 mg/L NaF), or fluoride plus iodide (5, 10, or 20 mg/L NaF + 1.685 mg/L KIO_3). Each group had 20 animals, including 10 males and 10 females. All the rats had free access to water and food. Exposure was through drinking water. Changes in body weight were monitored weekly. At the end of exposure for 2 or 8 months, 10 rats (five males and five females) were randomly selected from each group and sacrificed after weighing.

2.3. Specimen collection and processing

Before the rats were sacrificed, urine specimens were collected to measure urinary fluoride and iodide. Blood specimens were collected. Sera were separated by centrifugation at 1000*g*/min for 15 min and then stored in a freezer at −20 °C prior to thyroid hormone analysis. The thyroid was immediately isolated and washed with normal saline. The thyroid was dried with filter paper and then weighed to calculate the organ coefficient (thyroid weight/body mass × 100%). Then, one side of the thyroid was fixed in 4% paraformaldehyde for histological studies and the other side immediately frozen in liquid nitrogen at −70 °C for reverse transcription-polymerase chain reaction (RT-PCR) experiments.

2.4. Measurement of urinary fluoride and iodide

Urinary fluoride was measured by ion selective electrode assay (WS/T89-1996) (Wan et al., 1996; Zhang et al., 2015). Urinary iodide was determined by arsenic-cerium catalytic spectrophotometry (WS/T107-2006) (Wu et al., 2012; Yan et al., 2006).

2.5. Morphological observation of thyroid tissue

The fixed thyroid was dehydrated, paraffin-embedded, and sliced into 5-μm-thick sections. After hematoxylin and eosin staining, the section specimens were examined under an optical microscope to observe the morphological changes in thyroid tissue.

2.6. Radioimmunoassay of thyroid hormones

Serum triiodothyronine (T_3), free triiodothyronine (FT_3), thyroxine (T_4), free thyroxine (FT_4), and thyroid stimulating hormone (TSH) levels were measured by radioimmunoassay (Wu et al., 2012; Zhang et al., 2015).

2.7. TUNEL assay of apoptotic cells in thyroid tissue

Apoptotic cells in thyroid tissue were detected using a TUNEL apoptosis detection kit following the manufacturer's instructions. Under an optical microscope, normal nuclei appeared dark blue, while apoptotic nuclei displayed karyopyknosis in a brown color and appeared circular in a crescent or irregular shape. For each section specimen, five fields of view (400×) were randomly selected and the results were photographed with the tissue filling the entire field of view. The percentage of TUNEL-positive cells (positive cell count/number of total cell count) was obtained from each image using Image-Pro Plus 6.0 (Media Cybernetics Inc, Bethesda, Maryland, USA).

2.8. RT-PCR assay of IRE1 pathway-associated gene expression

Total RNA was extracted from thawed thyroid tissue using the TRIzol reagent. The RNA concentration and purity were determined. Total mRNA was reverse-transcribed to synthesize cDNA according to the instructions of the RevertAid First Strand cDNA Synthesis Kit. Template cDNA was added into the corresponding reactions for PCR amplification. The following primers were utilized: GRP78-forward, 5′-ATC AAC CCA GAT GAG GCT GTA GCA-3′; reverse, 5′-AGA CCT TGA TTG TTA CGG TGG GCT-3′ (product length = 235 bp;