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Altered miRNAs expression profiling in sperm of mice induced by fluoride

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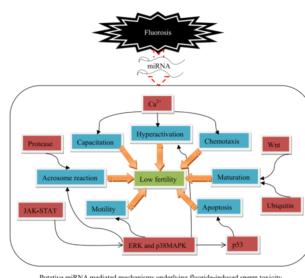
HIGHLIGHTS

- Fluoride altered the sperm miRNAs profiling of mice.
- 31 differentially expressed known miRNAs were identified in fluoride groups.
- Some sperm miRNAs can be as potential biomarkers for fluoride reproductive toxicity.

GRAPHICAL ABSTRACT



Putative miRNA mediated mechanisms underlying fluoride-induced sperm toxicity

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ABSTRACT

The reproductive toxicity of fluoride has become a major concern in the world. Fluoride can decrease the abilities of sperm capacitation, hyperactivation, chemotaxis, acrosome reaction and fertilization, but the studies on the responses of sperm small noncoding RNAs (sncRNAs), especially miRNAs, to fluoride exposure are lacking. miRNAs are demonstrated to influence sperm quality and male fertility by regulating gene expression at post-transcriptional levels or translational repression. The objective of this study is to analyze miRNA profiling in sperm of mice administrated with 25 and 100 mg L^{-1} sodium fluoride (NaF) for 60 d using high-throughput sequencing technology. Along with reduced sperm concentration, survival, motility, and mitochondrial membrane potential, 31 differentially expressed known miRNAs were identified in fluoride groups, compared with the control group. 671 predicted target genes against the 16 altered miRNAs were mainly involved in protease inhibitor activity, apoptosis, ubiquitin mediated proteolysis, and signaling pathways of calcium, JAK-STAT, MAPK, p53, Wnt, which were proved to be directly related to sperm quality. These findings suggested that the altered sperm miRNAs could be potential biomarkers for fluoride reproductive toxicity.

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1. Introduction

Fluoride exists widely in rocks, soil, water, food, coals, toothpastes and others (Doull et al., 2006). Given that fluoride can

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prevent dental caries effectively at low dose, it is added to the water supply in many countries (McDonagh et al., 2000). However, due to such a narrow margin of safety, fluoride often exhibits its detrimental effects on tooth and bone, as well as nonskeletal systems such as liver, kidney, immune system, central nervous system, and reproductive system (Doull et al., 2006; Jagtap et al., 2012; Perumal et al., 2013).

Epidemiological investigation found the apparent connection between the decreasing total fertility rate and the increasing fluoride concentrations (Freni, 1994). Sperm, as the product of spermatogenesis, are highly specialized cells, which functions in transporting and delivering the male genetic information to the descendant on fertilization. It has been reported that excess fluoride could result in the changes in sperm morphology (Chinoy and Narayana, 1994), capacitation (Dvoráková-Hortová et al., 2008), hyperactivation (Sun et al., 2010), chemotaxis (Lu et al., 2014), acrosome reaction (Izquierdo-Vega et al., 2008) and fertilizing ability (Elbetieha et al., 2000; Izquierdo-Vega et al., 2008). In our previous study, microarray analysis was utilized to characterize the effects of fluoride on the gene expression profiling in mice sperm, which provided several important biological clues for further investigations (Sun et al., 2011). Indeed, apart from paternal genome, mammalian sperm are carriers of epigenome in the forms of DNA methylation, histone modifications and noncoding RNAs (ncRNAs) (Yuan et al., 2015).

Importantly, sperm can carry a complex population of coding RNAs and ncRNAs into oocyte at fertilization, which influences fertilization, embryo development, the phenotype of the offspring and possibly future generations (Krawetz et al., 2011; Jodar et al., 2013). And small noncoding RNAs (sncRNAs), which include microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA), have been shown to mainly function as epigenetic factors in controlling gene expression at post-transcriptional levels (He et al., 2009). The miRNA family, as the best characterized of the sncRNAs, has been identified in mammalian sperm and seminal plasma, and been proved to associate with male subfertility (Krawetz et al., 2011; Kotaja, 2014).

miRNAs are a class of short (20–23 nucleotides) single-stranded sncRNAs, which mainly function in post-transcriptionally regulating gene expressions by their interaction with the 3′ untranslated region (UTR) or translational repression (Ambros, 2004; Bartel, 2009). Altered miRNA expression profiles in patients with subfertility suggested some miRNAs can emerge as potential biomarkers for the diagnosis and classification of male fertility (Abu-Halima et al., 2013, 2014). Hence it is possible that fluoride could induce changes in the miRNA profiles of sperm, resulting in altered phenotypes that could be transmitted to offspring. The purpose of this study therefore was to determine the sperm miRNA profiles of normal and fluoride-treated mice by deep sequencing, and to find the potential biomarkers and pathways affected by fluoride.

2. Materials and methods

2.1. Animal treatment

90 male ICR mice weighting about 20–25 g were purchased from the Academy of Military Medical Sciences (Beijing, China). After an acclimatization period of one week, the mice were randomly divided into three groups of 30 animals each, including the control group and two sodium fluoride (NaF) treated groups. Control mice were provided with distilled water, and animals in fluoride groups were given distilled water containing 25 or 100 mg L^{-1} NaF, respectively, based on our previous study (Sun et al., 2016) and the LD_{50} value of 54.4 mg fluoride ion/kg body weight of male mice (Pillai et al., 1987). All mice were supplied with a standard diet and water *ad libitum*, and were housed with controlled temperature (22–25 °C), 12/12-h light/dark cycle, satisfactory ventilation and hygienic conditions. This study was approved by the Ethics Committee of Shanxi Agricultural University (Taigu, China).

2.2. Incisors observation and sperm preparation

At the day 60 of exposure to NaF, mice were sacrificed by cervical dislocation and the incisors were collected and photographed to estimate the status of dental fluorosis. Sperm from the cauda epididymis and vas deferens were collected and dispersed into 1 ml Biggers, Whiuen and Whiuingham (BWW) medium (Genmed Scientific Inc, USA) at 37 °C for further experiments. Partial samples were used for routine sperm analysis and mitochondrial membrane potential (MMP) detection, and others were frozen in liquid nitrogen immediately and stored at −80 °C until RNA extraction.

2.3. Routine sperm analysis and detection of high potential sperm

Aliquots of sperm suspensions were prepared for determination of sperm quality by the methods of Huang et al. (2007) and Sun et al. (2010). Briefly, 10 μl suspension was placed on red blood cell count plate to count sperm concentration. For sperm survival, the same volume was mixed with 10 μl eosin, and the mixture was loaded on the glass slide to count the number of dead and live sperm under the microscope. Another 10 μl suspension was loaded on a 20 μm deep observation chamber at 37 °C, and sperm motility was observed using the Olympus BX51 microscope equipped with CCD DP70 video camera (Olympus Optical, Tokyo, Japan). At least 200 sperm of every sample were calculated for sperm survival and motility. For high potential sperm, JC-1 mitochondrial membrane potential assay kits (Beyotime, Jiangsu, China) and FACS Calibur flow cytometry (Becton Dickinson, Franklin, NJ) were applied to determine the MMP of sperm according to the manufacturer's instructions.

2.4. RNA extraction and small RNA sequencing

The sperm of five mice were pooled as one sample and washed twice in PBS, and then resuspended in the somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in distilled H_2O), in order to produce essentially pure sperm (Ostermeier et al., 2002). Sperm total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of total RNA was determined by gel electrophoresis and Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies Inc., DE, USA). The equal amount of RNA from three samples in each group were pooled for further small RNA sequencing.

In brief, total RNA was purified by electrophoretic separation with denaturing polyacrylamide gel electrophoresis. Then small RNA corresponding to the 18–30 nucleotide band was excised and recovered. After the 3′- and 5′- adapters were ligated to the small RNA, the small RNA-adapter molecules were reverse transcribed for cDNA synthesis. cDNA was amplified as templates with PCR cycles to generate cDNA libraries which were submitted to Illumina Hiseq 2000 (Illumina, San Diego, USA) at Beijing Genomics Institute (Shenzhen, Guangdong, China). The raw data were processed to remove contaminant reads and the length distribution analysis of clean reads was summarized. Small RNA tags were mapped to genome by SOAP software to analyze their expression and distribution on the genome, and were aligned and annotated by miRBase, GenBank and Rfam databases.

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