Ecotoxicology and Environmental Safety 153 (2018) 127–134

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Analysis of digital gene expression profiling in the gonad of male silkworms *(Bombyx mori)* under fluoride stress

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ARTICLE INFO

Keywords:
Fluoride
Bombyx mori
Differential gene expression
Testes
Toxicity

ABSTRACT

Fluorine is an essential element, but excessive fluoride can cause serious effects on the respiratory, digestive, and reproductive systems. Fluorine has been suggested to cause reproductive toxicity in vertebrates, but its potential to reproductively affect invertebrates remains unknown. In the present study, the lepidopteran model insect *Bombyx mori* was used to assess the reproductive toxicity of NaF. The underlying molecular mechanisms were explored by RNA sequencing, and we investigated the testes transcriptomic profile of *B. mori* treated with NaF via a digital gene expression (DGE) analysis. Among 520 candidate genes, 297 and 223 were identified as significantly upregulated or downregulated, respectively. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were carried out on all genes to determine their biological functions and associated processes. The results indicated that numerous differentially expressed genes are involved in the stress response, detoxification, antibacterial, transport, oxidative phosphorylation, and ribosome. The reliability of the data was confirmed by a quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The changed Glutathione S-transferase (GST) activity and glutathione (GSH) content in the NaF-treated groups were increased and reduced respectively. This study reveals that using RNA-sequencing for the transcriptome profiling of *B. mori* testes can lead to better comprehension of the male reproductive toxicity effects of NaF. Furthermore, we expect that these results will aid future molecular studies on the reproductive toxicity of NaF in other species.

1. Introduction

Fluorine is a ubiquitous halogen element that exists naturally in the environment, although it only exists in combination with other elements as fluoride compounds (Camargo, 2003). Fluorides have been applied widely in numerous fields, and they have been used as refrigerants, pharmaceutical preparations, dentifrices, surfactants, phosphate fertilizers, and pesticides, as well as in coal power plants, glass and brickmaking industries, and semiconductor manufacturing (Dietrich, 1993; Camargo, 2003; Weinstein and Davison, 2004; Ozsvath, 2009).

However, fluoride that accumulates in the environment is harmful to animals, plants, and humans. According to estimates, approximately 200 million people in 25 countries drink water with a fluoride ion concentration over 1.5 mg/L (the security constraint set by the World Health Organization). Developing countries, such as India and China, are the worst affected (Ayoob and Gupta, 2006). Excessive fluoride has been shown to be toxic to the liver, nephridium, cerebrum, thyroidea, and testes (Barbier et al., 2010). It is noteworthy that the male reproductive toxicity of fluoride has been followed with interest, especially with regard to vertebrates, such as rats, mice, and rabbits (Chinoy and Sequeira, 1989; Susheela and Kumar, 1991; Gupta et al., 2007). Many epidemiological investigations and animal experiments suggest that increasing the level of fluoride in the environment can affect male reproductive function (Long et al., 2009; Dey and Giri, 2016), leading to reduced sperm counts and motility (Pushpalatha et al., 2005; Kim et al., 2015; Sun et al., 2016), structural changes in spermatozoa and functional incapacitation (Kumar and Susheela, 1993; Sun et al., 2014), decreased testosterone concentrations (Ismail et al., 2014; Dey and Giri, 2016), abnormal changes of epididymis and accessory genital glands (Kumar and Susheela, 1995), and lowered genitality (Elbetieha et al., 2000; Kim et al., 2015). However, the underlying mechanisms of the male reproductive toxicity of fluoride are still not fully elucidated.

Insects are the most diverse species on Earth, and they are in close contact with the natural environment. The silkworm *Bombyx mori* is a vital lepidopteran model insect and has been used widely as a model

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https://doi.org/10.1016/j.ecoenv.2018.01.028
Received 11 August 2017; Received in revised form 9 January 2018; Accepted 11 January 2018

organism in cell biology, physiology, medicine, and toxicology (Hamamoto et al., 2009; Matsumoto et al., 2011; Tansil et al., 2011; Yuan et al., 2013). Silkworms have several experimental advantages, including bio-safety, low cost, the availability of rich genetic resources, and the ability to control experimental conditions and perform experiments quickly and easily. Additionally, they reproduce sexually, are easy to culture and identify gender, and they are not subject to animal ethics regulations. Furthermore, complete genome sequences are available. Therefore, we used the silkworm to study the reproductive toxicity of fluoride to males.

Recently, next-generation sequencing technologies have provided a valid approach for high-throughput sequence determinations to increase our comprehension of the degree in complicity of the eukaryotic transcriptome (Metzker, 2010; Ekblom and Galindo, 2011). Digital gene expression (DGE) is a tag-based RNA sequencing (RNA-seq) technology, which can determine gene expression levels relative to other expression profiles (Qin et al., 2011; Ye et al., 2011). Deep sequencing provides validity, comparability, and profusion of expression profiling data, and could aid synergistic, relative, and compositive genomics research (Hoen et al., 2008).

Our previous study showed that fluoride can damage the gonads of silkworm via oxidative stress, which lead to superoxide and H_2O_2 levels were significant increased, and the histological changes included that abnormal morphology, enlarged cell vacuoles and relative less reproductive cells (Tang et al., 2016). To better understand the testes-damaging molecular toxicological mechanism of NaF, we used RNA-seq to measure whole-genome transcriptional responses in *B. mori* testes exposed to NaF. A DGE analysis was first carried out to reveal and characterize previously undiscovered NaF-induced molecular events. These results will supply significant gene sequence information for toxicological studies of insects. The response of *B. mori* testes to NaF exposure at the transcriptome level is expected to ameliorate our current understanding of the molecular effects associated with NaF-induced male reproductive toxicity.

2. Materials and methods

2.1. Silkworm strains and NaF exposure

Silkworm strain Xian 2 is sex-limited marking strain in which female larvae have markings but males are without. The strains were sourced from our silkworm genetics and breeding laboratory. One- to four-instar silkworm larvae were fed mulberry (*Morus*) leaves four times a day in a 12-h light/dark period at 25 ± 1 °C.

NaF with a purity of approximately 99% was obtained from the Kelon Chemical Reagent Factory (Chengdu, China), and a 1000 mg/L mother liquor of deionized water was prepared before the experiment. The mulberry leaves were soaked in a 200 mg/L NaF solution for 15 min and then dried naturally. The actual fluoride content in the mulberry leaves was 208.42 mg/kg.

Fifth-instar silkworm larvae were divided randomly into two experimental groups with three replicates. Silkworm larvae of the same size were chosen from each group and fed mulberry leaves steeped in either distilled water or the 200 mg/L NaF solution from the first day of the fifth instar to the sixth day of the fifth instar. After fluoride exposure, the silkworms showed severe toxic symptoms, such as uneven growth, weight loss, decreased movement, and dark spots on the epidermis (Fig. 1). The larvae that were fed leaves steeped in distilled water were assigned to the control groups, whereas those fed NaF-treated leaves were the experimental groups.

2.2. Sampling and total RNA extraction

Five male silkworms were selected randomly from each group as the larvae reached the sixth day of the fifth instar. Testes of silkworms in the experimental and control groups were dissected in an ice bath, frozen in liquid nitrogen, and stored in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at −80 °C. Three replicates per group.

Fig. 1. Morphological features of silkworms after fluoride exposure. The left side represents the control group, and the right side represents the NaF-treated group. The red arrow refers to a fluoride-induced abdominal spot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

RNA extraction and sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Total RNA was extracted using Trizol reagent (Invitrogen). RNA purity and concentration was measured using a NanoPhotometer® spectrophotometer (Implen, Westlake Village, CA, USA). RNA integrity was analyzed with 1% agarose gels.

2.3. cDNA library preparation and DGE sequencing

In brief, poly-dT oligo on magnetic beads were used to cleanse mRNA from total RNA samples (3 μg), and then fragmented at a high temperature. cDNA was synthesized through random oligonucleotides and SuperScript II (Invitrogen). Second-strand cDNA was synthesized by DNA polymerase I, and the RNA template was digested using RNase H. Ends were repaired and a 3′ single nucleotide of adenine was added. Fragment sizes of 200 bp were used for PCR. The quality and quantity of the sample library were detected with an Agilent 2100 Bioanalyzer and ABI Step One Plus qPCR System, respectively. Library products were further sequenced with Illumina HiSeq™4000.

2.4. Mapping of DGE reads

Sequence tag preprocessing was implemented based on an antecedently described protocol with some revision (Mortazavi et al., 2008). Raw reads were cleaned by eliminating reads with adaptors, low quality (> 50%) or a high-proportion of unidentified bases (> 10%). Clean data were mapped to the silkworm genome database (http://silkworm.genomics.org.cn/) using Bowtie v2.2.3 and restricted by a maximum of two nucleotide mismatches.

2.5. Quantification and differential expression analysis

Gene expression was computed by using the RPKM (reads per kilobase of transcriptome per million mapped reads) method, with HTSeq software (union model). Differential expression analysis was implemented by the DESeq R package (1.18.0) (Anders and Huber, 2010). The resulting *P*-values were adjusted using the Benjamini and