Original Article

Toxicity of Sodium Fluoride to *Caenorhabditis elegans*[*]

LI Qiao[1], ZHANG Shu Hua[1,#], YU Yuan Hua[1], WANG Li Ping[2], GUAN Shu Wen[2], and LI Peng Fei[2]

1.Life Science and Technology Institute, Changchun University of Science and Technology, Changchun 130022, Jilin, China; 2.College of Life Science, Jilin University, Changchun 130021, Jilin, China

Abstract

Objective To investigate the toxic effect of sodium fluoride (NaF) on the nematode *Caenorhabditis elegans (C. elegans).*

Methods Adult *C. elegans* were exposed to different concentrations of NaF (0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L) for 24 h. To assess the physiological effects of NaF, the brood size, life span, head thrashes, and body bend frequency were examined. Reactive oxygen species (ROS) and cell apoptosis were detected as parameters of biochemical response. The gene expressions were determined by real-time polymerase chain reaction (PCR) to assess the molecular-level response.

Results At the physiological level, the brood size of *C. elegans* exposed to 0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L concentrations of NaF were reduced by 6%, 26%, and 28% respectively in comparison with the control group. The maximum life spans of *C. elegans* exposed to 0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L concentrations of NaF were reduced by 3 days and 5 days, respectively. Head thrashes and body bend frequency both decreased with increasing concentrations of NaF. At the biochemical level, the production of ROS and the incidence of cell apoptosis increased with increasing concentrations of NaF(P<0.05). At the molecular level, different concentrations of NaF exposure raised the expression of stress-related genes, such as *hsp16.1, sod-3, ctl-2, dhs-28, gst-1,* and *cep-1*.

Conclusion NaF exposure could induce multiple biological toxicities to *C. elegans* in a concentration-dependent manner. These toxicities may be relevant to the oxidative stress induced by increased ROS production and accumulation in *C. elegans*.

Key words: Sodium fluoride; *Caenorhabditis elegans*; Toxicity; Real-time PCR

Biomed Environ Sci, 2012; 25(2):216-223 doi:10.3967/0895-3988.2012.02.014 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

INTRODUCTION

Fluorine is a microelement that is essential to humans and animals, but its safe amount for consumption is very small. It has been reported that too much or too little fluorine can induce a number of diseases[1]. Since fluoride was added into tap water in U.S. and Canada to prevent tooth decay in 1945, fluoride has been widely used in water. In addition, the industrial discharge of fluorine has induced environmental pollution. These factors have led to acute human exposure to fluorine. According to the statistics, approximately 25 countries have reported frequent cases of fluorosis. Among these countries, fluorosis is most serious and widespread in China and India[2]. Fluorosis has been paid increasing attention in recent years. The standard concentration of fluoride added into drinking water is 0.7-1.2 mg/L according to the public health service, and an average of 1 mg of fluorine is consumed per day[3]. As reported, approximately 99% of the total body fluoride is

[*]This research was supported by Jilin Provincial Science and Technology Development Project in China, 20100407.

[#]Correspondence should be addressed to ZHANG Shu Hua. E-mail: zhangsh1018@126.com

Biographical note of the first author: LI Qiao, female, born in 1986, master, majoring in environmental pollutants research.

Received: March 9, 2011; Accepted: June 20, 2011

retained in bones and teeth, and the remainder is distributed in highly vascularized soft tissues[4-5]. *In vivo*, fluoride can cross the cell membrane and be distributed in various soft tissues, such as the nervous system, reproductive system, liver, kidneys, skin, and erythrocytes[6-8]. Increased lipid peroxidation and disturbed antioxidant defense systems have been found in the brain, erythrocytes and liver of rats exposed to fluoride[9]. Therefore, studies on the toxic effect of fluorides at different levels are urgent and necessary.

The nematode *Caenorhabditis elegans* is an excellent model organism. *C.elegans* is a small, soil-dwelling nematode with a simple body formed by 959 somatic cells in adult hermaphrodites and 1 031 somatic cells in adult males. Under laboratory conditions, *C. elegans* develops from a unicellular embryo to a fertile adult in 3-5 days. The life cycle of *C. elegans* consists of an embryonic stage and four larval stages (L1-L4)[10]. It is the most thoroughly studied and most completely understood metazoan in terms of molecular and classical genetics, development, behavior, and anatomy. Today, *C. elegans* is widely used in biological studies because of its short lifespan, cellular simplicity, genetic manipulability, and easy cultivation[11-13]. To assess the toxicity of NaF, *C. elegans* were exposed to three concentrations of NaF (0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L) for 24 h. *C. elegans* were investigated and analyzed at the physiological, biochemical, and molecular levels .

MATERIALS AND METHODS

Chemicals and Organisms

Three concentrations of NaF (pure analytical-grade, Sigma-Aldrich Chemical, USA) were used in this study (0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L). These solutions were prepared with M9 medium (2.2 mmol/L KH_2PO_4, 4.2 mmol/L Na_2HPO_4, 8.55 mmol/L NaCl, and 1 mmol/L $MgSO_4$). The wild-type *C. elegans* strain N2 was used and maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli strain OP50*, at 20 °C[14].

Sample Preparation

Young adults (L4 stage) from a synchronized culture (three days after hatching) were exposed to the three concentrations of NaF for 24 h. The control groups were cultivated in M9 medium for 24 h. Three parallel experiments were conducted for all the test types.

Brood Size and Life Span

The methods were performed as previously described[15]. The brood size was assayed by placing a single tested nematode into an individual well of tissue culture plates. The nematode was transferred to a new well every 1.5 days until the nematode finished laying eggs. The progeny were counted the day following the transfer, and the test process lasted about 10 days. The brood size was the summation of eggs laid by the tested nematodes. For the brood size test, at least 10 replicates were performed for each experiment. Life span was assayed by placing 50 nematodes onto a single NGM plate with *OP50* after the nematodes had been exposed to NaF for 24 h. The time of eggs brooding was recorded as t=0. The nematodes were transferred every 2 days to fresh plates during the brood period. The number of survivors was recorded every day. Nematodes that failed to respond to repeated touch stimulation were considered dead. The period of this test was about 27 days. To focus on aging, worms that had become desiccated on the side of the plate after crawling off, or that displayed extruded internal organs or that died because of hatching progeny inside the uterus (matricidal death) were excluded from the analysis. The results of the survival assays were analyzed by GraphPad Prism 5.

Head Thrashes and Body Bend Frequency

The methods were performed as previously described[16]. A thrash was defined as a change in the direction of bending at mid body. For the head thrash assay, the nematodes were washed with M9 medium after they had been exposed to NaF for 24 h. Each nematode was transferred to 60 µL of M9 medium on top of agar. After a 1-min recovery period, the head thrashes were counted for 1 min. A body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the y axis, assuming that the nematode was traveling along the x axis. To assay the body bends, *C. elegans* was placed onto a new plate and scored for the number of body bends at an interval of 20 s. For both head thrashes and body bend frequency tests, 15 nematodes were examined per treatment.

Quantification of Reactive Oxygen Species (ROS)

The quantification of ROS was performed as previously described[17]. To assay ROS, the nematodes that had been exposed to the chemical for 24 h were washed with ddH_2O twice and with M9

medium once. The nematodes were then pendulated in M9 medium mixed with dichlorofluorescein (DCF) at 20 °C for 1 h. ROS was detected using a fluorescence microplate reader (ELX800, Biotek, America). The excitation wave was 528/20 nm, and the absorption wave was 485/20 nm. All of the above procedures were performed away from light.

Cell Apoptosis Assay

The cell apoptosis assay was performed as previously described[18]. Acridine orange (AO) was used to observe cell apoptosis induced by NaF. After exposure to NaF for 24 h, the nematodes were pendulated in M9 medium mixed with AO at 20 °C for 2 h. The concentration of AO was 2.5×10^{-2} g/mL. Then, the nematodes were allowed to recover for 10 min on the top of agar and were mounted in 6×10^{-2} g/mL levamisole onto agar pads on microscope slides. The nematodes were examined using a fluorescence inverted microscope (TE-2000E, Nikon, Japan) to determine cell apoptosis. The excitation wave was 515 nm, and the absorption wave was 488 nm. 15 nematodes were observed per treatment.

Gene Expression Assay by Real-time PCR

For the gene expression assay, we first chose stress-related genes based on previous studies, such as glyceraldehyde 3-phosphate dehydrogenase (*gpd-1*) (as the parameter gene), heat shock protein (*hsp16.1*), superoxide dismutase (*sod-3*), catalase (*ctl-2*), glutathione s-transferases (*gst-1*), deHydrog-enasesb short-chain (*dhs-28*), and *p53*-like protein (*cep-1*). We designed these primers on the basis of the sequences retrieved from *C. elegans* database by Primer Premier 5.0 software (Table 1). After exposure to NaF for 24 h, 100 nematodes were collected for each concentration to isolate the total RNA using an EZ-10 Spin Column Total RNA Isolation Kit (BBI, Canada). Next, the two-step reverse transcription-polymerase chain reaction (RT-PCR) method was used to acquire the cDNA using the RNA PCR Kit (TaKaRa, Japan). The real-time PCR was conducted using the SYBR PCR kit (TaKaRa, Japan) and the 7500 Fast Real-time PCR System (ABI, America). The reaction mixture(25 μL) consisted of 17.25 μL RNase-Free water, 2.5 μL 10×buffer, 2 μL dNTP, 0.25 μL Taq DNA polymerase, 1 μL DNA template, and 0.5 μL of each primer. The real-time PCR proceeded as follows: an initial activation of DNA polymerase at 95 °C for 10 s one cycle followed by 45 cycles of primer annealing and extension at 95 °C for 15 s and 60 °C for 1 min.

Table 1. The Primers for Real-time PCR

Name	Primers (5'-3')
gpd-1	Sense: AGGGAATCCTCGCCTACACTG
	Antisense: ATTCATTGTCGTACCAAGAGACGA
hsp16.1	Sense: CTTTACCACTATTTCCGTCCAGC
	Antisense : GGCTTGAACTGCGAGACATTG
sod-3	Sense: CGAGCTCGAACCTGTAATCAGCCATG
	Antisense: GTTGCTGATTGTCATAAGCCATTGC
ctl-2	Sense: TTCGCTGAGGTTGAACAATCCG
	Antisense: GTTGCTGATTGTCATAAGCCATTGC
gst-1	Sense: GATGATCTTCGGCCAGGTTC
	Antisense: TGTCTCGTTGGAGCCATTGA
dhs-28	Sense: TGGGATCTTATCTTCAAGGTCCA
	Antisense: TGGG ACCAGGGTATTTGCC
cep-1	Sense: ACGCTCACTCGTCGACTGCT
	Antisense: ACACTGAATCGTGCCCTGCT

Data Analysis

All data in this report were expressed as means±SD, which were analyzed by Origin 6.0 software and GraphPad Prism 5.0 software. The statistical differences between the control group and the experimental groups were determined with the aid of the parametric *t*-test. A probability level of 0.05 was considered statistically significant.

RESULTS

Toxic Effects of NaF on the Reproduction of C. elegans

In this report, the brood sizes of all three experimental groups were smaller than those of the control group. As shown in Figure 1, the brood size of the nematodes exposed to 0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L concentrations of NaF were reduced by 6% ($P<0.05$), 26% ($P<0.01$), and 28% ($P<0.01$) , respectively compared with the control group. The 0.38 mmol/L and 3.8 mmol/L concentrations of NaF exposure induced a more severe effect on the brood size compared with the control group. This also indicated that the reproductive toxicity of NaF was concentration- dependent.

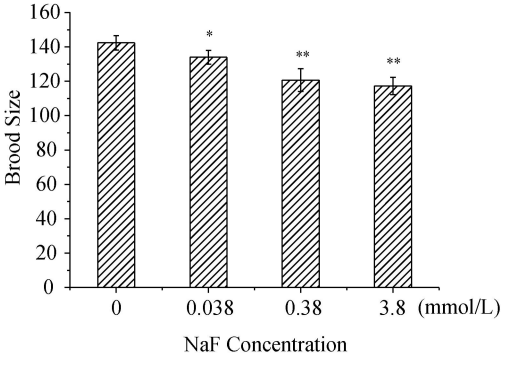

Figure 1. Brood sizes are reduced dramatically in *C. elegans* exposed to NaF. Bars represent means±SD. $^{*}P<0.05$; $^{**}P<0.01$.

Life Span Defects Caused by NaF Toxicity

When the nematodes were exposed to 0.038 mmol/L, 0.38 mmol/L, and 3.8 mmol/L concentrations of NaF, the maximum life spans were reduced by three days, three days and five days compared with the control group, respectively (Figure 2a). Moreover, the mean life span of the nematodes exposed to 3.8 mmol/L concentration of NaF were remarkably decreased compared with the control group (P<0.05) (Figure 2b). However, no significant differences were found among the 0.038 mmol/L and 0.38 mmol/L concentrations groups and the control group. Accordingly, exposure to high concentration (3.8 mmol/L) of NaF could cause more severe life span reduction. The low concentrations (0.038 mmol/L and 0.38 mmol/L) in the NaF exposure groups exhibited no apparent changes compared with the control group.

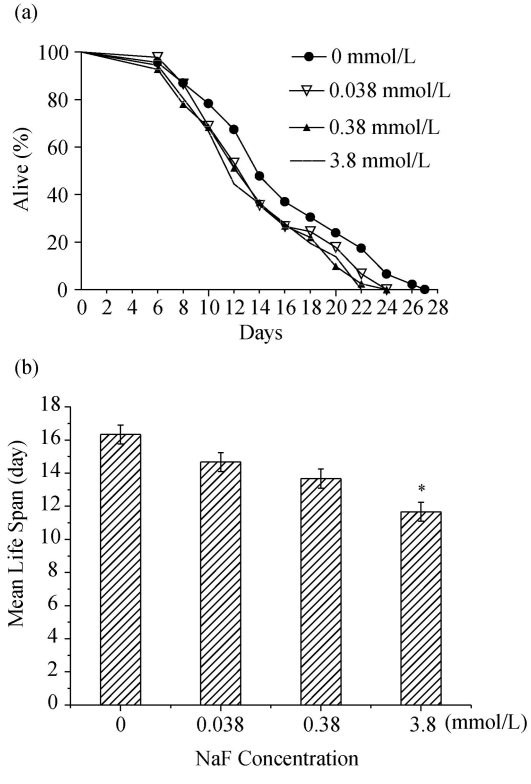

Figure 2. (a) Life spans of *C. elegans* exposed to different concentrations of NaF. (b) Mean life spans of *C. elegans* exposed to different concentrations of NaF. Bars represent means±SD. $^{*}P$<0.05.

Locomotion Behavior Assay

Head thrashes and body bends were used to assay the locomotion behavior of *C. elegans*[19-20]. As shown in Figure 3, the frequency of the head thrashes and body bends both decreased dramatically after exposure to NaF, even at the low concentration of 0.038 mmol/L (P<0.05). More severe phenotypes were observed for the 0.38 mmol/L and 3.8 mmol/L concentrations of NaF (P<0.01). The results indicated that the toxic effect of NaF on the locomotion behavior of *C. elegans* was concentration- dependent.

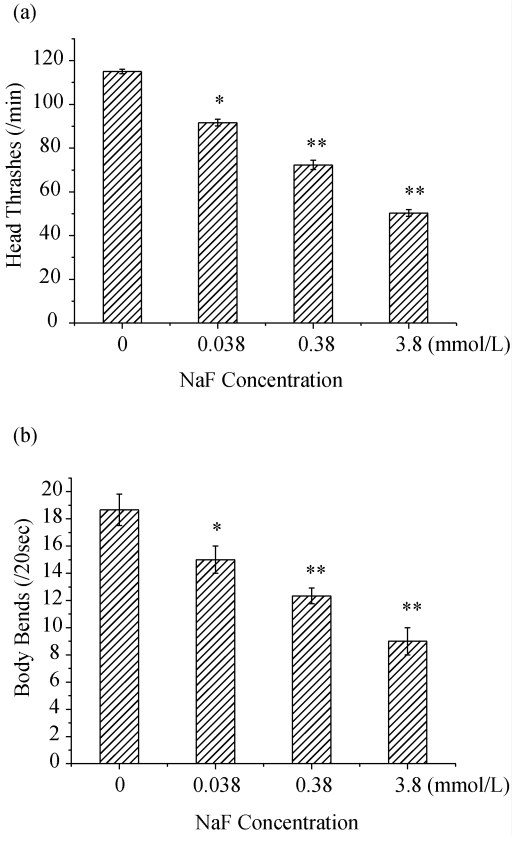

Figure 3. NaF exposure causes locomotion behavior defects in *C. elegans*. (a) Head thrashes of *C. elegans* exposed to NaF show a concentration-dependent decrease. (b) Body bend frequency of *C. elegans* exposed to NaF show a concentration-dependent decrease. Bars represent means±SD. $^{*}P$<0.05; $^{**}P$<0.01.

ROS Assay

ROS is the main in vivo free radical in humans and animals. An ROS assay can help us understand the toxic effects of NaF on *C. elegans* at the biochemical level. As shown in Figure 4, ROS increased rapidly with the increased concentration of NaF (P<0.05). This relationship indicated that the generation of ROS in the tested nematodes was concentration-dependent. This result is consistent

with a report showing increased free radical in the liver of rats exposed to NaF[21].

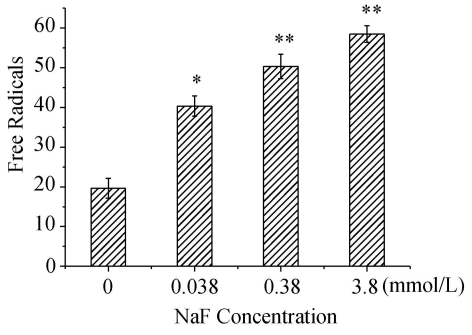

Figure 4. NaF exposure effects on reactive oxygen species. Bars represent means±SD. $^{*}P$ <0.05; $^{**}P$<0.01.

Cell Apoptosis Assay

Acridine orange (AO) was used to determine cell apoptosis. We found that the apoptotic cells were yellow or orange under the fluorescence inverted microscope because of DNA cleavage. The normal cells were green. As shown in Figure 5, increasing numbers of apoptotic cells were found in *C. elegans* with increased NaF concentration. Apoptotic cells were spread over nearly the entire body of *C. elegans* exposed to 3.8 mmol/L concentration of NaF.

Gene Expression Assay

We determined the gene expression of *hsp16.1, sod-3, ctl-2, dhs-28, gst-1,* and *cep-1*. As shown in Figure 6, the expression of g*st-1, dhs-28, hsp16.1, sod-3,* and *cep-1*all increased compared with the control group. The expression of *ctl-2* decreased in the nematodes exposed to the 0.038 mmol/L concentration of NaF compared with the control group but increased in the nematodes exposed to the 0.38 mmol/L and 3.8 mmol/L concentrations of NaF. These results indicated that the expression of stress- related genes changed after NaF exposure. Taken together, the altered expression of stress-related genes at the molecular level suggested that severe oxidative stress was induced by NaF exposure in *C. elegans.*

DISCUSSION

In this study, we investigated the toxicity of NaF to *C. elegans* at the physiological, biochemical and molecular levels. By assaying the three levels and the relationships among them, we were able to explore the toxicity of NaF in a comprehensive and intensive way.

Brood size reflects the reproductive capacity of *C. elegans*. Thus, brood size can help us assess the toxicity of NaF to the reproductive system of *C. elegans*. The brood sizes of all three experimental groups were reduced compared with the control group. This result indicates that NaF exposure could induce reproductive toxicity to *C. elegans*. Moreover, the toxicity was concentration-dependent.

Life span is an important parameter for assessing the toxicity of NaF. *C. elegans* provides an excellent model system for the study of aging because of its relatively short life span. As a life span indicator, the maximum and mean life span of *C. elegans* after NaF exposure were reduced in all three experimental groups compared with the control group. This result reveals that NaF exposure causes reduced life span of *C. elegans* in a concentration-dependent manner.

Fluorine and its compounds can accumulate in the brain, inducing overoxidation in the brain tissue of animals. Recent reports have confirmed the neurotoxicity of NaF to organisms[22]. It has been reported that NaF exposure can induce damage to the nervous system of rats[23-24]. The behavioral phenotypes for both the head thrashes and body bend frequency were remarkably decreased in the NaF exposure groups compared with the control group. This result demonstrates that NaF can induce neurotoxicity to *C. elegans.*

Reactive oxygen species (ROS) are mainly produced in mitochondria during respiration by the incomplete reduction of oxygen and as a side-product of cellular reactions[25]. Environmental stressors, such as radiation, drugs, metal ions, ultraviolet (UV) radiation, and heat, can generate free radicals, including ROS, thereby causing damage to tissues, cells and nucleic acids[26]. Numerous researches have considered that the mechanism of fluorosis is mainly determined by the metabolic disorder of free radicals[27]. Increased free radical generation have been proposed as mediators of the toxic effects of fluoride on soft tissues[28]. ROS in *C. elegans* increased along with an increasing concentration of NaF, which indicates that fluorosis may be related to the oxidative damage induced by increased ROS in *C. elegans.*

Apoptosis results from the action of a genetically encoded suicide program that leads to a series of characteristic morphological and biochemical changes. Fluorine is a protoplasmic poison that can accumulate in all tissues because it can penetrate

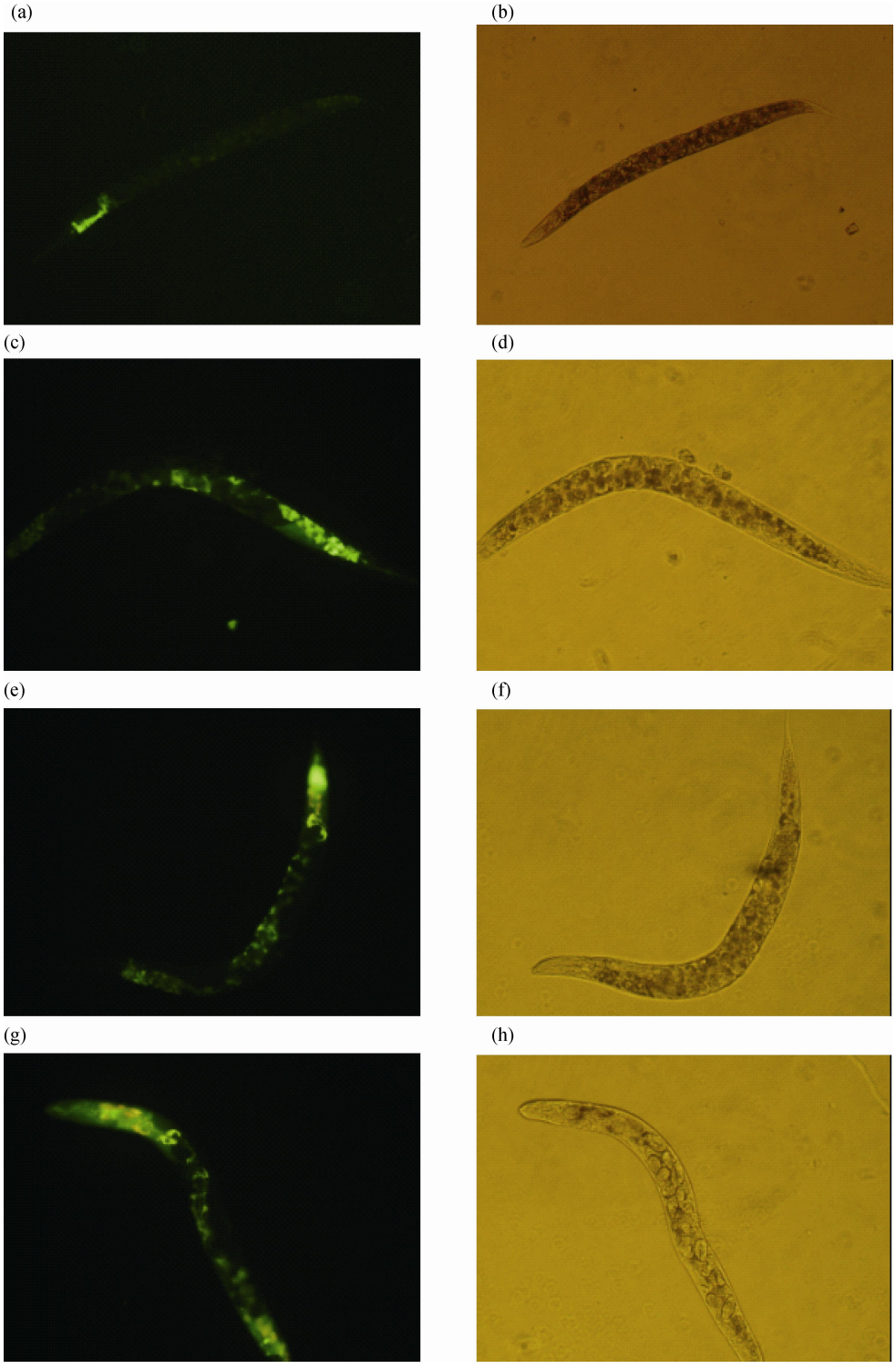

Figure 5. (a) Apoptosis of the control group under dark field. (b) Apoptosis of the control group under bright field. (c) Apoptosis of *C. elegans* exposed to 0.038 mmol/L concentration of NaF under dark field. (d) Apoptosis of *C. elegans* exposed to 0.038 mmol/L concentration of NaF under bright field. (e) Apoptosis of *C. elegans* exposed to 0.38 mmol/L concentration of NaF under dark field. (f) Apoptosis of *C. elegans* exposed to 0.38 mmol/L concentration of NaF under bright field. (g) Apoptosis of *C. elegans* exposed to 3.8 mmol/L concentration of NaF under dark field. (h) Apoptosis of *C. elegans* exposed to 3.8 mmol/L concentration of NaF under bright field.

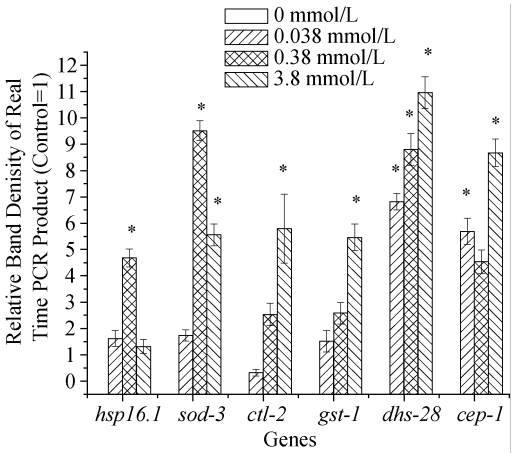

Figure 6. NaF exposure effect on stress-related gene expression. Bars represent means±SD. *P<0.05.

the cell wall of almost any tissue and combine with the protoplasm[29-30]. The cytotoxicity of fluorine is mainly exhibited in two ways: the inhibition of cell growth and the inducing of cell apoptosis. Thus, it is an effective way to study the mechanism of fluorosis by assessing the toxic effect of NaF on cells. In this study, the toxic effect of NaF induced far more apoptosis in *C. elegans* compared with the control group. The result is consistent with the report that apoptosis could be induced by the generation of ROS in organisms[31].

Many environmental stresses result in an increased generation of active oxygen species in animals and the changes of related gene expression. In this study, the expression of some oxidative stress-related genes and that of apoptosis were tested. Temperature elevation or a variety of chemical agents would induce the heat shock response in both eukaryotic and prokaryotic systems, resulting in the induced synthesis of a group of heat shock polypeptides (HSPs)[32]. *Sod-3* and *ctl-2* are reported to play an important role in the oxidative stress response[33]. *Dhs-28* has the function of an oxidoreductase. Glutathione S-transferases (GSTs) performs functions ranging from catalyzing the detoxification of electrophilic compounds to protecting against peroxidative damage[34]. *Cep-1* is *a p53*-like protein gene that is concerned with immunity and apoptosis induced by DNA damage[35-36]. The results of these genes expression were consistent with those of the above ROS assay and apoptosis assay. These facts also indicate that cells respond to oxidative stress by inducing the expression of stress-related genes, by repairing stress-related damage or by inactivating ROS[37].

The findings of the present study demonstrate that NaF could cause multiple biological defects in a concentration-dependent manner at physiological, biochemical and molecular levels. The main injury induced in *C. elegans* by NaF was oxidative damage caused by increased ROS production and its accumulation in these organisms. Therefore, understanding of the multiple biological toxicities of fluorine to *C.elegans* may help us to know the potential toxicity of fluorine to human, and may also provide new clues to reveal the mechanism of fluorine toxicity and lay the foundation for prevention and treatment of fluorosis. Above all, the present study can provide a sound scientific basis for the safe use of fluoride in medical and health care services.

ACKNOWLEDMENTS

The nematode strain used in this study was provided by the *Caenorhabditis* Genetics Center, USA. We are grateful to Prof. FAN (College of Medicine, MUSC, Charleston, SC, USA) for critical and valuable advice on the manuscript revision.

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