FLUORIDE-INDUCED THYROID CELL APOPTOSIS

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SUMMARY: In addition to causing skeletal and dental fluorosis, fluoride (F) in drinking water may damage other organs including the thyroid. The objective of this study was to explore the toxicity of F on immortalized human normal thyroid cells (Nthy-ori 3-1) exposed to 0, 0.1, 1, and 3 mmol/L of sodium fluoride (NaF) *in vitro*. After 24 hours incubation, measurements were made of cell viability and of the lactate dehydrogenase (LDH) leakage rate by MTT and LDH assay. The reactive oxygen species (ROS) level, the constituent ratio of the cell cycle, and the apoptosis rate were measured by flow cytometry. Cell viability was decreased ($p<0.01$) in the 1 and 3 mmol/L F-treated groups (76.64±9.13% and 64.04±6.32% respectively) compared to the control group (100.00±0.00%). The LDH leakage rate and the ROS level were increased ($p<0.01$) in the 3 mmol/L F-treated group (48.66±7.15% and 29993.50±1786.86 FI respectively) compared to the control group (35.24±3.02%) and 13021.33±1067.55 FI). The % of G0/G1 phase cells was decreased ($p<0.01$) in the 1 mmol/L F-treated group (40.76±5.65%) compared to the control group (60.09±1.76)% but the % of S phase cells (54.05±4.59%) was increased ($p<0.01$) compared to the control group (32.59±2.43%). The % of apoptotic cells was increased ($p<0.01$) in the 3 mmol/L F-treated group (20.09±3.22%) compared to the control group (9.64±3.44%). The p53 mRNA expression was not changed in the F-treated groups ($p>0.05$) but in the 3 mmol/L F-treated groups the Bax mRNA expression increased ($p<0.05$) and the Bcl-2 mRNA expression decreased ($p<0.05$) compared to the control group. Thus F in Nthy-ori 3-1 cells may decrease cell viability, increase the LDH leakage rate and the ROS level, block growth in the S phase, and induce apoptosis.

Key words: Apoptosis; Bax; Bcl-2; Cell cycle; Fluoride; Oxidative stress; p53; Thyroid.

INTRODUCTION

Endemic fluorosis is a biogeochemical disease which seriously impairs human health. A high intake of fluoride (F) may damage not only skeletal tissues but also soft tissues.[1,2] Previous epidemiological studies have demonstrated that high-levels of F may damage the structure of the thyroid and disturb thyroid hormone secretion homeostasis.[3] Although apoptosis has been demonstrated to play an important role in toxicity of F to a variety of organs,[4,5] its importance in F-induced thyroid toxicity is not known. p53-induced apoptosis is one of the vital apoptotic pathways in mammals. p53, the most extensively studied tumor suppressor, plays a role in promoting cell-cycle arrest, DNA repair when cells are damaged, and, when cell damage is serious, in inducing apoptosis in order to prevent the damaged cells changing into tumor cells.[6] p53 can activate the transcription of genes that encode apoptotic effectors, such as Bax and it can repress the transcription of anti-apoptotic genes such as Bcl-2 to induce apoptosis.[7] In the

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present study, we used immortalized human normal thyroid cells (Nthy-ori 3-1 cells) to explore the effects of various doses of F on the thyroid on cell viability, lactate dehydrogenase (LDH) leakage rate, reactive oxygen species level (ROS), cell-cycle alteration, apoptosis, and the mRNA expression levels of p53, Bax and Bcl-2 in order to further understand the mechanism and nature of F-induced thyroid cytotoxicity.

MATERIALS AND METHODS

Materials: Microplate Reader (Bio-Rad, Type 680, USA), LSR II Fluorescence-activated cell sorting (FACS) flow cytometer (Beeton Diekinson, USA), MCO-15AC CO_2 incubator (Sanyo, Japan), fetal bovine serum (FBS, Gibco, USA), Rosewell Park Memorial Institute (RMPI) 1640 medium (Hyclone, USA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Biomol, USA), propidium iodide (PI, Sigma, USA), Triton X-100 (Fisher, USA), reactive oxygen species (ROS) detection kit (Beyotime Institute of Biotechnology, China), Revert Aid First Strand cDNA Synthesis Kit and Dream Taq Green PCR master mix (Fermentas, Lithuania). NaF (Guaranteed reagent, China Pharmaceutical Group, Shanghai Chemical Reagent Company, China).

Cell culture and treatment: Cells of the simian virus (SV)-40 transformed human thyroid epithelial cell line Nthy-ori 3-1 (HPACC) were cultured in the RMPI medium with 10% FBS at 37ºC in a humid atmosphere with 5% CO_2. Exponentially growing cells (data from MTT assay) were treated with 0 (control), 0.1, 1, and 3 mmol/L NaF solution respectively. After 24hr exposure, cells were kept for further assays.

MTT assay: The cells were cultured in 96-well plates. After 24hr NaF solution exposure, 200 μL medium and 20 μL MTT solution (5 mg/mL) were re-added. After another 4 hr at 37ºC incubator in the dark, 150 μL DMSO per well was added, after discarding the culture medium, and the culture plate was shaken for 10 min. The optical density (OD) of each well was measured at 490 nm with a microplate reader. The results were calculated by the formula:

$$S = \frac{(OD_{\text{treated well}} - OD_{\text{blank}})}{(OD_{\text{control well}} - OD_{\text{blank}})} \times 100\%.$$

LDH leakage rate assay: The supernatant (S) and cells (C) were collected respectively after 24hr incubation with NaF solution. Supernatant was centrifuged at 702.5 g for 10 min. An equal volume of PBS with 1% Triton X-100 was used to extract the remaining LDH from the cells.[8,9] The OD value was measured at 440 nm by the microplate reader. The results were calculated by the formula:

$$\text{LDH leakage rate\%} = \frac{(OD_s - OD_{\text{blank}})}{(OD_s - OD_{s\ \text{blank}} + OD_c - OD_{c\ \text{blank}})} \times 100\%.$$

s = supernatant, c = cells

ROS level assay: According to the instructions for the ROS assay kit, cells were collected after 24 hr incubation with NaF solution, washed twice with PBS, incubated with 250 μL DCFH-DA (10 μM) for 20 min at 37ºC in the dark, and then washed three times with PBS to remove the residual DCFH-DA. DCF fluorescence intensity (FI) was measured via flow cytometry (excitation 488 nm; emission 525 nm).

Cell cycle and apoptosis assay: Approximately 1.0×10^6 cells from each group were collected after 24 hr incubation with NaF solution, washed twice with PBS, and fixed at 4ºC for 24 hr with cold 75% ethanol, which was prechilled at –20ºC. The cells were then washed with PBS again. Thereafter, cells were re-suspended in 100 μL PC buffer (1.611% $Na_2HPO_4{\cdot}12H_2O$, 0.074% sodium citrate, pH=7.8) for 30 min at room temperature, and then washed with PBS. 300 μL RNase A (50 μg/mL) was added for incubation away from light for 30 min at 37ºC, then 33.3 μL 10×PI solution (500 μg/mL) containing 1% Triton X-100 were added for incubation away from light for 30 min at 4ºC. The percentages of cells in the different phases of the cell cycle were measured with flow cytometry. The cell cycle and apoptotic rate were analyzed by the ModFit LT software and the CellQuest software respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis: Total RNA was extracted with Trizol reagent. First-strand cDNA was synthesized from 3 μg of total RNA with the Revert Aid First Strand cDNA Synthesis Kit. PCR was performed in a total reaction with green PCR master mix. The reaction conditions were as follows: 95ºC preheat for 1 min 50 sec, followed by 40 cycles of 95ºC for 30 sec, 59.3ºC for 30 sec, 72ºC for 1 min, and then 72ºC for 7 min. β-actin was used as an internal control. PCR products were separated on a 2% agarose gel, stained with goldview, scanned by the Tanon Gis-1000UV trans-illuminator and quantifed by Quantity One 4.6.2 software. The PCR primer pairs are as follows: p53 (NM_000546.5, 172bp): forward, CAT AGT GTG GTG GTG CCC TAT GAG, reverse, CAA AGC TGT TCC GTC CCA GTA GA; Bax (NM_138761.3, 143bp): forward, GCG AGT GTC TCA AGC GCA TC, reverse, CCA GTT GAA GTT GCC GTC AGA A; Bcl-2 (NM_000633.2, 143bp): forward, TCG CCC TGT GGA TGA CTG AG, reverse, CAG AGT CTT CAG AGA CAG CCA GGA; β-actin (NM_001101.3, 186bp): forward, TGG CAC CCA GCA CAA TGA A, reverse, CTA AGT CAT AGT CCG CCT AGA AGC A.

Statistical analysis: All results are represented as mean±SD. Data were analyzed by one-way ANOVA followed by Student-Newman-Keuls using SPSS (11.5) software. A p value < 0.05 was considered as a significant difference.

RESULTS

The cell viability in the 1 and 3 mmol/L F-treated groups was significantly lower than that in the control and the 0.1 mmol/L F-treated groups. There was no statistically significant difference between the 0.1 mmol/L F-treated group and the

control group. The cell viability in the 3 mmol/L F-treated group was significantly lower than in the 1 mmol/L F-treated group (Table 1).

Table 1. Effect of fluoride on cell viability, LDH leakage rate, and ROS level in Nthy-ori 3-1 cells (Values are mean±SD)

Concentration (mmol/L)	Cell viability (%, n=8)	LDH leakage rate (%, n=4)	ROS level (Fluorescence intensity, n=3)
0	100.00±0.00[a]	35.24±3.02[e]	13021.33±1067.55[i]
0.1	99.42±6.43[b]	34.07±4.09[f]	12335.67±1271.21[j]
1	76.64±9.13[c]	36.76±2.87[g]	12163.50±3892.62[k]
3	64.04±6.32[d]	48.66±7.15[h]	29993.50±1786.86[l]
F	58.042	8.541	40.908
p	0.000	0.003	0.000

Note: [c] compared to [a], q=10.009, p<0.01; [c] compared to [b], q=8.165, p<0.01; [d] compared to a, q=16.643, p<0.01; [d] compared to [b], q=13.368, p<0.01; [d] compared to [c], q=5.400, p<0.01; [h] compared to [e], q=5.815, p<0.01; [h] compared to [f], q=6.326, p<0.01; [h] compared to [g], q=5.159, p<0.01; [l] compared to [i], q=14.742, p<0.01; [l] compared to [j], q=13.719, p<0.01; [l] compared to [k], q=13.853, p<0.01.

The LDH leakage rate in the 3 mmol/L group was significantly higher than that in the control, 0.1, and 1 mmol/L groups. There were no statistically significant differences among the other groups (Table 1).

The ROS level in the 3 mmol/L F-treated group was remarkably higher than that in the other groups (Table 1). There were no statistically significant differences among the other groups.

Compared to the other groups, the G_0/G_1 phase cells of the 1 mmol/L group were decreased and the percentage of cells in S phase increased (Table 2).

Table 2. Effect of fluoride on cell cycle in Nthy-ori 3-1 cells (Values are mean±SD, %, n=3)

Concentration (mmol/L)	G0/G1	S	G2/M
0	60.09±1.76[a]	32.59±2.43[e]	7.15±1.13
0.1	57.43±1.87[b]	39.15±1.57[f]	3.41±0.33
1	40.76±5.65[c]	54.05±4.59[g]	5.20±2.68
3	61.02±2.49[d]	31.76±7.38[h]	7.22±5.06
F	24.223	15.209	1.158
p	0.000	0.001	0.384

Note: [c] compared to [a], q=10.014, p<0.01; [c] compared to [b], q=8.640, p<0.01; [c] compared to [d], q=10.496, p<0.01; [g] compared to [e], q=8.110, p<0.01; [g] compared to [f], q=5.632, p<0.01; [g] compared to [h], q=8.425, p<0.01.

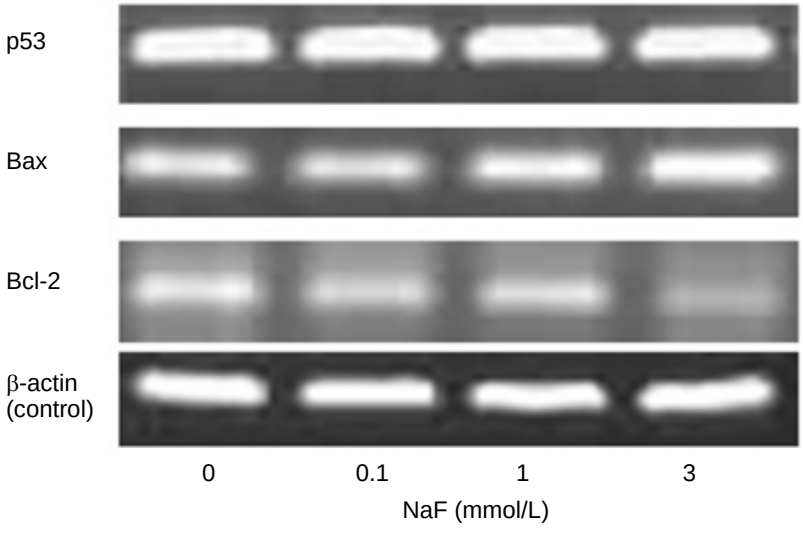
The percentage of apoptotic cells in the 3 mmol/L F-treated group was higher than that in the other groups (Table 3). There were no significant differences among other groups.

Table 3. Effect of fluoride on apoptosis in Nthy-ori 3-1 cells (Values are mean±SD, %, n=3)

Concentration (mmol/L)	Apoptosis
0	9.64±3.44[a]
0.1	10.80±2.41[b]
1	11.41±2.44[c]
3	20.09±3.22[d]
F	32.180
p	0.000

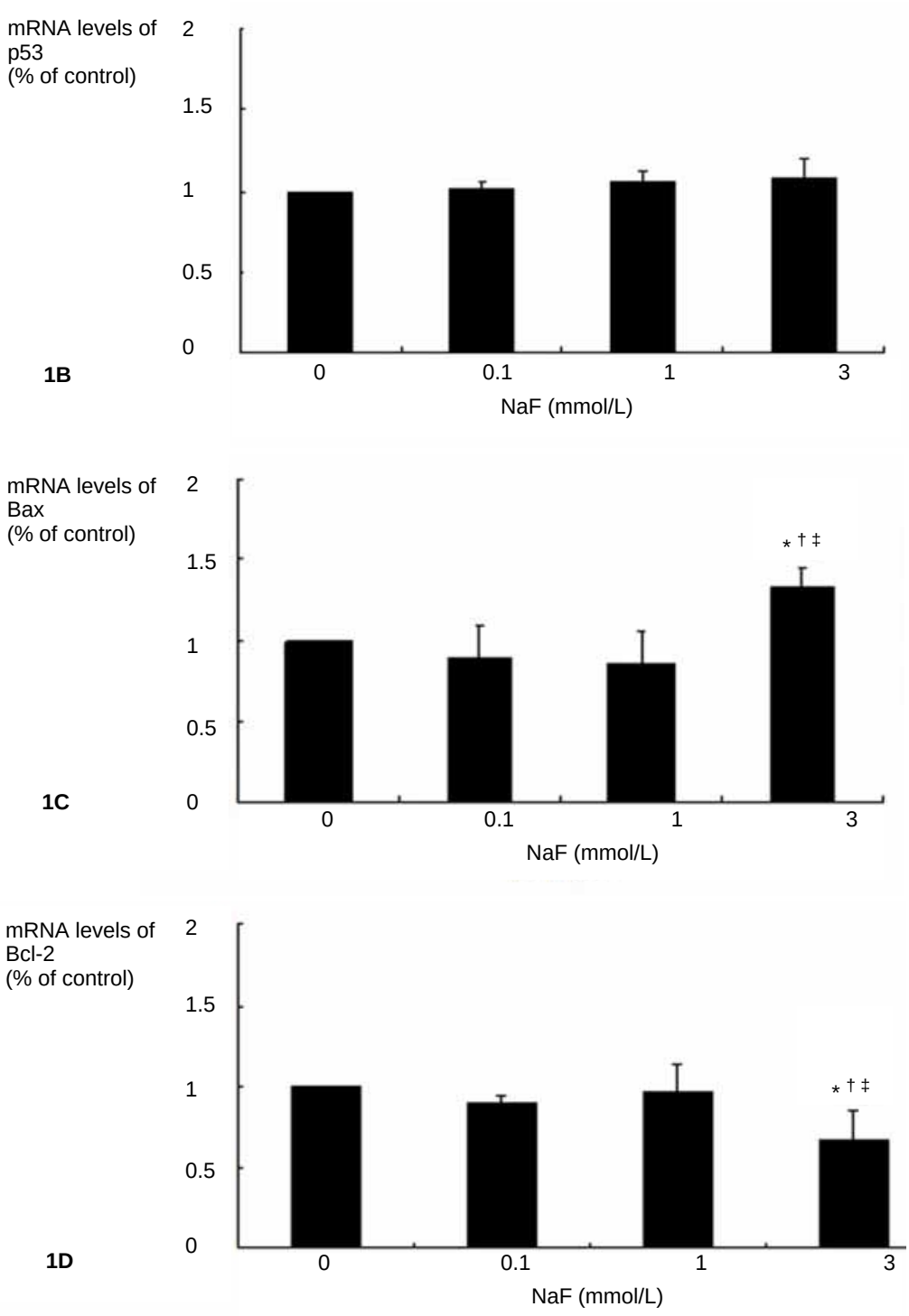
Note: [d] compared to [a], q=12.386, p<0.01; [d] compared to [b], q=11.011, p<0.01; [d] compared to [c], q=10.065, p<0.01.

There were no significant differences in the mRNA expression of p53 among the control and the F-treated groups (Figures 1A and 1B). However, the mRNA expression of Bax in the 3 mmol/L F-treated group was markedly increased compared to the control, 0.1, and 1 mmol/L F-treated groups (Figures 1A and 1C). The mRNA expression of Bcl-2 in the 3mmol/L F-treated group was significantly decreased compared to the control, 0.1, and 1 mmol/L F-treated groups (Figures 1A and 1D).



1A

Figure 1A. The effects, using RT-PCR, on Nthy-ori 3-1 cells of fluoride and iodide, on mRNA expression levels of p53, Bax, and Bcl-2.



Figures 1B, 1C, and 1D. The effects, using RT-PCR, on Nthy-ori 3-1 cells of fluoride and iodide, on mRNA expression levels of p53 (Figure 1B), Bax (Figure 1C), and Bcl-2 (Figure 1d). *p<0.05 as compared to the control group; †p<0.05 as compared to the 0.1 mmol/L NaF group; ‡p<0.05 as compared to the 1 mmol/L NaF group.

DISCUSSION

Many epidemiological investigations and animal experiments have found that F can affect thyroid function.[3,10] The results of this study indicated that thyroid cell viability decreased and LDH leakage rate increased with an increasing NaF concentration. This demonstrated that F can damage thyroid cells. Recently a few studies have suggested that F can induce apoptosis of many types of cell, such as the osteoblastic cell,[11]indicating that apoptosis may be involved in F-toxicity. In the present study, we found that F could induce apoptosis of human thyroid cells and a correlation was present between the impairment caused by F and F-induced apoptosis.

ROS such as molecules, ions, and free radicals with oxidative capacity are continually produced by aerobic organisms during metabolic processes.[12] An elevated level of intracellular ROS can serve as a signal of oxidative stress and activate the apoptosis signaling pathway.[13] Previous experiments have shown that F can cause oxidative stress in young porcine thyroid cells.[14] However, no reports were found on whether F could cause the same effect on intracellular ROS in human thyroid cells. Our results suggest that high doses of F can significantly elevate the ROS level of human thyroid cells, resulting in oxidative stress and the induction of apoptosis.

Zhang et al. demonstrated that a higher concentration of F could induce cell-cycle arrest at S phase in rat primary hippocampal neurons.[2] Similarly, Liu et al. found that F could arrest long bone cells at S phase and decrease mitotic cells.[15] When arrested at S phase, the cells would repair the damaged DNA, but if the repairing failed, preventing fully recovery, the cells would be induced to apoptosis in order to reduce genomic instability and to decrease the possibilities of mutation and carcinogenesis.[16,17] The present study showed that 1 mmol/L F could block human thyroid cells in S phase and 3 mmol/L F could induce apoptosis in the human thyroid cells without arresting the cells at S phase. This is possibly due to 24 hr exposure to 1mmol/L F causing mild DNA damage which was able to be repaired while the cells were arrested at S phase, while 24 hr exposure to 3 mmol/L F caused severe DNA damage with the cells undergoing irreversible apoptosis.

p53 acts to integrate multiple stress signals into a series of diverse anti-proliferative responses. One of the most important p53 functions is its ability to activate apoptosis.[6] In addition, there are intricate relationships between apoptosis and the Bcl-2 family, which includes anti-apoptotic family members, e.g., Bcl-2, and pro-apoptotic family members, e.g., Bax and Bak.[18] p53 is activated by many stimuli including lead, UV light, and hypoxia. The activated p53 can decrease the expression of Bcl-2 and increase the expression of Bax. It is widely accepted that these three proteins are very important in deciding whether or not cell suicide occurs.[6,19] However, the activation of Bax and Bcl-2 are not affected not only by the p53 pathway but also by other pathways, such as with endoplasmic reticulum (ER) stress.[20] In the present study, F did not change the mRNA expression of p53 of the thyroid cells, suggesting F excess induces apoptosis in thyroid cells through

a p53-independent mechanism involving oxidative stress. Interestingly, the study of Vitale et al[21] found that iodide excess can also induce apoptosis in thyroid cells through a p53-independent pathway. In the present study, the increased apoptosis in the human thyroid cells due to F was a result of the increase in the pro-apoptotic factor Bax and the decrease in the anti-apoptotic factor Bcl-2 and these changes occurred via p53-independent pathways.

In summary, F can damage human thyroid cells, decrease cell viability, and increase LDH leakage rates. There is a strong association between the thyroid cytotoxicity of F and F-induced thyroid cell apoptosis. F can cause oxidative stress to activate the apoptotic pathway and induce apoptosis through intracellular ROS generation. Meanwhile, F can also change the cell cycle distribution and arrest cells at S phase. In addition, the F-induced apoptosis in thyroid cells is activated through a p53-independent mechanism which can change the expression of Bax and Bcl-2. However, the specific mechanism of F-induced apoptosis is complex and requires further exploration.

ACKNOWLEDGEMENTS

This study was sponsored by the National Natural Science Foundation of China (30972555, 81372934) and the Tianjin Key Medical Scientific and Technological Research Project (10KG215).

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