IN VITRO HORMESIS EFFECTS OF SODIUM FLUORIDE ON KIDNEY CELLS OF THREE-DAY-OLD MALE RATS

Qin-qing Tang,[a] Xiao-jing An,[a] Jun Du,[a] Zheng-xiang Zhang,[a] Xiao-jun Zhou[a]

Nanjing, China

SUMMARY: Reaction with methyl thiazolyl tetrazolium (MTT) was used to investigate *in vitro* stimulatory (hormesis) effects of sodium fluoride (NaF) on kidney cells collected from three-day-old Sprague-Dawley male rats. The cell cultures were exposed to incremental concentrations of NaF ranging from of 0 (control) to 160 µmol/L and from 500 to 16,000 µmol/L. The mean optical density (OD) ± SD decreased from the control value of 0.591±0.119 to a minimum of 0.468±0.065 at 20 µmol NaF/L before returning to the control level near 160 µmol/L. At 500 µmol/L the OD was 0.545±0.066, after which it decreased monotonically to 0.387±0.046 at 4000 µmol/L, with cell death being complete at 16,000 µmol/L. These results indicate that 20 µmol/L is the lowest concentration at which a stimulatory (hormesis) effect of NaF is observed in kidney cell cultures of very young rats. In addition, results of flow cytometry and RNA detection confirmed these MTT findings.

Keywords: Flow cytometry; Fluoride and kidney cells; Hematoxylin-eosin staining; Hormesis effects; Kidney cells; Methyl thiazolyl tetrazolium (MTT); Paradoxical concentration effects; Ribonucleic acid.

INTRODUCTION

In recent years, the existence of paradoxical dose-response effects of fluoride (F) has been a topic of interest in this journal.[1-6] For kidney cells, the toxicity of F is well established,[7,8] but whether there are paradoxical concentration effects of F in kidney cells and whether they might include stimulatory (hormesis) effects is not known.[9] In the present *in-vitro* study, we explored the dose-effect relationship between F levels and damage to kidney cells using methyl thiazolyl tetrazolium (MTT), flow cytometry, and RNA extraction.

MTT is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Active mitochondrial dehydrogenases of living cells cause the conversion of the soluble yellow dye to the insoluble purple formazan. Dead cells do not cause this change.[10-11] By optical density (OD) measurements, the level of cell viability can therefore be detected.

MATERIALS AND METHODS

Materials: Four male 3-day-old Sprague-Dawley rats were obtained from the Laboratory Animal Center of Nanjing Jinling Hospital. Dulbecco's Modified Eagle Medium (DMEM), trypsin, and fetal bovine serum (FBS) were supplied by the Hua Mei Biocompany of China. MTT was purchased from Sigma Company, USA. A flow cytometry detecting kit for cell viability was obtained from the Nanjing Kai Ji Biocompany of China.

Cell culture: Secondary kidney cells were obtained from the kidneys of the four 3-day-old male Sprague-Dawley rats. The kidneys were sliced and then digested by 0.25% trypsin-0.02% EDTA for 15 min. The supernatant was discarded after

[a]For Correspondence: Prof Xiao-jun Zhou, Department of Pathology, Nanjing University School of Medicine and Nanjing Jinling Hospital, Nanjing 210002, PR China.
E-mail: zhouxj1@yahoo.com.cn

centrifugation under 1000 r/min for 5 min. Afterward, the cells were adjusted to 10^6/mL and cultured in 25-mL tissue culture flasks plated with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. When the cells had been grown to confluence, 0.25% trypsin was used for the secondary culture. The cell number was adjusted to a density of 1×10^6 cells/mL. (Figure 1).

Fluoride treatment for detection of hormesis: When the cells had again grown to confluence, they were exposed to 0, 5, 10, 20, 40, 80, and 160 µmol NaF/L for 48 hr. The cells were then washed twice in PBS buffer.

Haematoxylin and eosin (HE) staining: HE staining was performed so that the nuclei and cytoplasms could be observed clearly.[12] (Figure 2 and 4)

MTT: The optical density (OD) was read at 555 nm against a background wavelength of 630 nm. The MTT reduction assay by OD was used as a qualitative index of cell viability.[13] Dimethyl sulfoxide (DMSO) was used as the solvent.

Flow cytometry: Flow cytometry was performed to determine cell viability using Annexin V-FITC/PI staining. An analysis of 10,000 cells was conducted for each sample.

Further fluoride treatment: In the results with the above NaF concentrations as described below, the effects of five additional NaF concentrations were studied: 0, 500, 1000, 4000, and 16000 µmol/L. HE staining and MTT testing were performed as before.

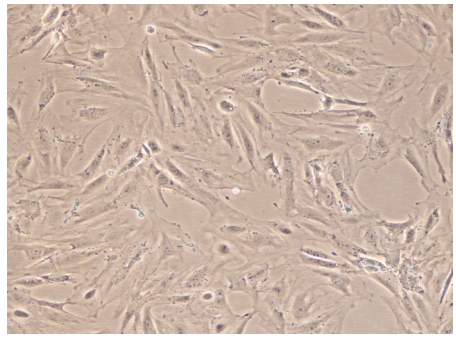
RNA Extraction: Total cellular RNA was extracted using Trizol reagent (1 mL Trizol per $5–10\times10^6$ kidney cells. Finally, gel electrophoresis with 1.5% agarose was used to detect the RNA (Figure 5).

Statistical analysis: Paired-samples T-test was performed to analyze differences in the OD value or viability between the fluorosed group and the control group using SPSS 15.0 Software. Results are expressed as Mean±SD. Differences with $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

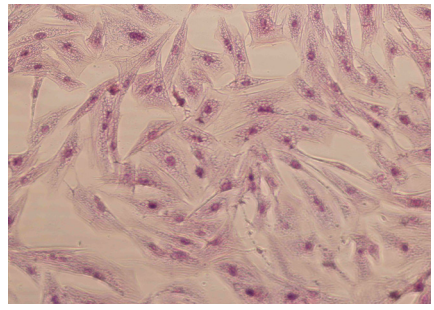
Secondary Cultured Kidney Cells: After culturing for 48 hr, the active cultures adhered to the walls of the flasks, and showed a spindly appearance (Figure 1).

Figure 1. Secondary kidney cells grown to confluence (x100).



Results of HE staining: With HE staining the cell cultures, including those exposed to NaF, showed clear nuclei and membranes (Figure 2).

Figure 2. HE staining of kidney cells, which showed clear nuclei and membranes (x100).



MTT detection: Table 1 and Figure 3 indicate a maximum cell viability at a F concentration of 20 µmol NaF/L (Table 1).

Table 1. Optical density (OD) value of kidney cells (n=7 in each group)

Treatment group	Optical density value							Mean optical density±SD
Control	0.720	0.509	0.465	0.714	0.515	0.496	0.715	0.591±0.119
5 µmol/L	0.600	0.505	0.503	0.456	0.440	0.508	0.574	0.512±0.058 (p = 0.094)
10 µmol/L	0.565	0.443	0.362	0.463	0.435	0.640	0.576	0.498±0.098 (p = 0.089)
20 µmol/L	0.539	0.459	0.437	0.457	0.441	0.375	0.568	0.468±0.065 (p = 0.007)†
40 µmol/L	0.586	0.512	0.474	0.454	0.440	0.467	0.531	0.495±0.051 (p = 0.047)*
80 µmol/L	0.589	0.553	0.516	0.367	0.415	0.507	0.581	0.504±0.084 (p = 0.153)
160 µmol/L	0.745	0.616	0.612	0.598	0.553	0.539	0.708	0.624±0.076 (p = 0.328)

Compared to the control, *p<0.05; †p<0.01.

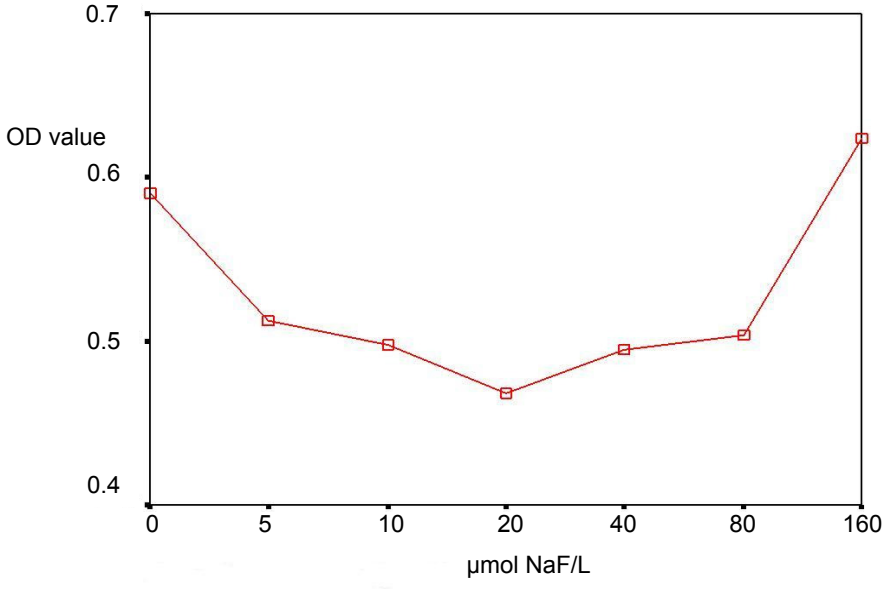


Figure 3. Mean OD detected with MTT from zero to 160 µmol NaF/L.

Flow cytometry: The results of flow cytometry also indicated that the maximum stimulatory effects occurred was at 20 µmol NaF/L (Table 2).

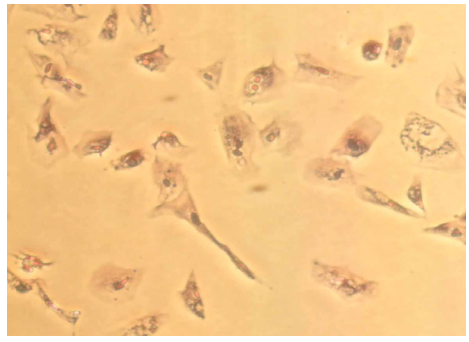
Table 2. Viability of kidney cells (n=3 in each group, mean±SD)

Treatment Group	Viability (%)
Control	65.21±4.27
5 µmol/L	50.59±5.75 (p = 0.068)
10 µmol/L	52.19±8.53 (p = 0.058)
20 µmol/L	41.91±5.47 (p = 0.007)*
40 µmol/L	49.78±3.66 (p = 0.058)
80 µmol/L	52.47±4.30 (p = 0.069)
160 µmol/L	61.03±3.55 (p = 0.453)

Compared to the control, *p<0.05.

Further fluoride treatment: HE staining: After exposure to 16000 µmol NaF/L for 24 hr, most of the cells in the cultures appeared to be dead (Figure 4). Similar results were seen at 4000 µmol NaF/L. However, the cultures exposed to 500 and 1000 µmol NaF/L were similar to the controls.

Figure 4. HE staining of kidney cells exposed to 16,000 µmol/L NaF (x100).



MTT detection (Table 3):

Table 3. Optical density (OD) value of kidney cells (n=7 in each group)

Treatment Group	Optical density value	Mean Optical density±SD
Control	0.671 0.561 0.584 0.623 0.550 0.582 0.497	0.581±0.055
500 µmol/L	0.599 0.524 0.480 0.483 0.517 0.662 0.548	0.545±0.066 (p = 0.270)
1,000 µmol/L	0.433 0.416 0.579 0.441 0.455 0.433 0.571	0.475±0.069 (p = 0.041)*
4,000 µmol/L	0.342 0.358 0.399 0.443 0.451 0.336 0.379	0.387±0.046 (p = 0.001)†
16,000 µmol/L	0.334 0.312 0.274 0.363 0.397 0.225 0.428	0.333±0.070 (p = 0.001)†

Compared to the control, *p<0.05; †p<0.01.

RNA extraction (Table 4 and Figure 5):

Table 4. Average abundance of the extracted RNA (n=3 in each group, mean±SD)

Treatment Group	Average abundance
Control	174.48±2.15
500 µmol/L	150.74±6.47 (p = 0.032)*
1,000 µmol/L	108.74±2.17 (p = 0.001)†
4,000 µmol/L	95.73±3.22 (p = 0.001)†
16,000 µmol/L	83.44±2.25 (p = 0.000)†

Compared to the control, *p<0.05; †p<0.01.

Hormesis or "paradoxical" concentration effects are exhibited when the rate of a reaction or a response at first actually *increases* with low concentrations of a toxic or inhibitory reagent and then *decreases* at still higher concentrations. Alternatively, a paradoxical concentration effect may at first be observed as a

decrease and then an *increase* in the rate of reaction or response with increasing concentrations of the reagent, followed by a further decrease.[1]

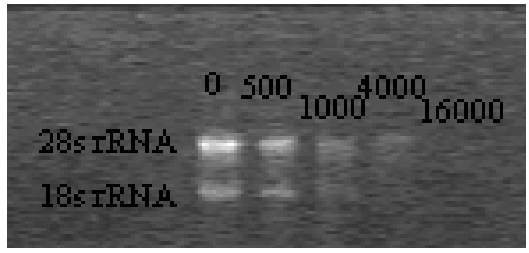


Figure 5. Total RNA extracted from kidney cells. The average abundance of the bands decreased.

In this work, OD measurements by MTT, flow cytometry, and RNA analysis of newborn male rat kidney cell cultures showed that the mean rate of cell growth gradually *increased* at first with increasing F concentration. At ca. 20 µmol F/L, however, the cell growth rate reached a statistically significant maximum ($p<0.05$) before beginning to decrease monotonically at higher F concentrations until cell death began to occur. Thus, we observed a "paradoxical" concentration effect[1-6] of F in which 20 µmol NaF/L is the lowest concentration at which a hormesis or stimulatory effect[9] of NaF occurred on cell growth in kidney cell cultures.

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REFERENCES

1 Burgstahler AW. Paradoxical dose-response effects of fluoride [editorial]. Fluoride 2002;35(3):143-7; Comment on editorial. Fluoride 2002;35(4):230.
2 Jenkins N. Rejoinder [letter]. Fluoride 1997;30:272-3, commenting on Schatz, A. Paradoxical effects: critique of a study. Fluoride 1997;30:131-3.
3 Schatz A. Fluoridation and infant deaths [letter]. Fluoride 1997;30:200.
4 Mitchell EA, Thompson JMD, Borman B, Ford RPK, Dick EA. No association between fluoridation of water and SIDS [letter]. Fluoride 1997;30:199.
5 Schatz A. Schatz: paradoxical effects [letter]. Fluoride 1998;31:245.
6 Schatz A. Schatz: more on paradoxical effects [letter]. Fluoride 1999;32:43-4.
7 Cittanova ML, Lelongt B, Verpont MC, Geniteau-Legendre M, Wahbe F, Prie D, et al. Fluoride ion toxicity in human kidney collecting duct cells. Anesthesiology 1996;84(2):428-35.
8 Yu R, Xia T, Wang A, Chen X. Effects of selenium and zinc on rat renal apoptosis and change of cell cycle induced by fluoride. Biomed Environ Sci 2006;19(6):439-44.
9 Calabrese EJ, Baldwin LA. Scientific foundations of hormesis. Crit Rev Toxicol 2001;31(4,5):351-695.
10 Freimoser FM, Aebi M. The MTT[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetra-zolium Bromide] assay is a fast and reliable method for colorimetric determination of fungal cell. Appl Environ Microbiol 1999;65(8):3727-9.
11 Hanelt M, Gareis M, Kollarczik B. Cytotoxicity of mycotoxins-evaluated by the MTT cell culture assay. Mycopathologi 1994;128(3):167-74.
12 Lillie RD. Histopathologic technic and practical histochemistry. 3rd ed. New York: McGraw-Hill;1965.
13 Aziz DM. Assessment of bovine sperm viability by MTT reduction assay. Anim Reprod Sci 2006;92:1-8.