FLUORIDE-INDUCED CELL CYCLE ARREST, APOPTOSIS, AND DNA DAMAGE IN TM3 MOUSE LEYDIG CELLS

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SUMMARY: This study was designed to evaluate the effect of fluoride (F) on the cell cycle, apoptosis, and DNA damage in TM3 mouse Leydig cells *in vitro* by the methods of flow cytometry (FCM) and single cell gel electrophoresis (SCGE). TM3 mouse Leydig cells were exposed to 0, 200, 400, 600, and 800 µmol NaF/L for 24 hr. By FCM, the number of cells in G_0/G_1 phase were much higher, whereas the cells in S phase, G_2+M phase, and the proliferation index (PI) value were much lower than in the control group. Moreover, the percentage of Leydig cell apoptosis increased with increasing concentration of F compared with the control group. From SCGE, tail length increased with increasing concentration of F, and the effects were dose-dependent. The results show that F can cause cell cycle aberration, induce apoptosis, and DNA damage in cultures of TM3 mouse Leydig cells.

Keywords: Cell cycle aberration; DNA damage; Fluoride and Leydig cells; Leydig cell apoptosis; Single cell gel electrophoresis; TM3 mouse Leydig cells.

INTRODUCTION

Fluoride (F) is an environmental and industrial pollutant with cumulative toxic effects.[1] Previous studies have shown that exposure to F can cause structural changes and disturb the functions of many tissues and cells.[2-14] Leydig cells are the primary cells involved in secreting testosterone, which is a vital steroid hormone for both spermatogenesis and sex characteristics in males.[15] Excessive F can decrease the secretion of testosterone in male rats,[10] and impair spermatogenesis in male mice.[14] Our recent study results showed that oxidative stress plays a crucial role in the toxic effect of F excess on Leydig cells *in vitro*, which is consistent with previous *in vivo* reports.[4,5]

In addition, investigators have shown that high F intakes induced cell cycle arrest,[16,17] apoptosis,[9,16-19] and DNA damage,[17,20,21] in various tissues and cells. However, to our knowledge, there have been no systematic studies on the effects of F on the cell cycle, apoptosis, and DNA damage in Leydig cells. As part of a larger study, the purpose of the present study was to examine the reproductive toxicity of F in TM3 mouse Leydig cells *in vitro* using flow cytometry (FCM) and single cell gel electrophoresis (SCGE).

MATERIALS AND METHODS

Materials: As in our preceding reporting this issue of *Fluoride*, the TM3 mouse Leydig cells line was purchased from the cell bank of the Chinese Academy of Science. The DMEM/F12 culture medium, horse serum, and fetal bovine serum were supplied by Gibco (USA). DNA damage was determined with the reagent kit provided by the Biolab (China). Unless otherwise stated, other reagents and solutions were purchased from Beijing Dingguo Biological Institute.

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Cell culture: The TM3 mouse Leydig cells were cultured in DMEM/F12 medium with 5% horse serum, 2.5% fetal bovine serum, and 1% antibiotics (100 U/mL penicillin; 0.1 mg/mL streptomycin, Invitrogen). The cells were grown at 37ºC in a humidified incubator with 5% CO_2 in the air.

Cell cycle detection: The cells were exposed to 0, 200, 400, 600, and 800 µmol NaF/L for 24 hr after the cells were fixed on the Fisher well flasks. The cells were then trypsinized, washed twice in cold phosphate buffer solution (pH 7.4), suspended in cold phosphate buffer solution at a concentration of 1×10^6 cells/mL and centrifuged (1200×g), to remove the supernatant. The cells were then resuspended in 5 µL 0.25% Tritonx-100 and 5 µL propidium iodide, and incubated for 30 min at 25ºC in the dark. To each tube, 500 µL of PBS was added, and analysis by flow cytometry (Cytomics FC-500, Beckman Coulter) was conducted within 1 hr.

Annexin-V apoptosis detection: The isolated cells were washed twice with cold PBS, and then suspended in 1×binding buffer at a concentration of 1×10^6 cells/mL. Next, 100 µL of the cell mixture was transferred to a 2-mL culture tube and 5 µL of Annexin V-FITC and 5 µL of propidium iodide were added, followed by gentle vortexing, and then incubation for 30 min at 25ºC in the dark. Finally, 400 µL of 1×binding buffer was added to each tube, and analysis by flow cytometry was conducted within 30 min.

Single cell gel electrophoresis: The mono-cellular suspensions were adjusted to a density of 1×10^6 cells/mL for single cell gel electrophoresis (SCGE), which was performed essentially according to the published procedure.[21]

Statistical analysis: Statistical analysis was performed with 17.0 for Windows. All parameters determined in the study are expressed as mean ± SD. A value of $p<0.05$ was considered significant.

RESULTS

Cell cycle detection: According to the data in Table 1 and Figure 1, the percentage of TM3 mouse Leydig cells in G_0/G_1 phase showed a concentration-dependent increase when compared with that of the control.

Table 1. Cell cycle of the TM3 mouse Leydig cells (Values are mean±SD)

NaF (µmol/L)	No. of samples	Phase			
		G_0/G_1	S	G_2+M	PI
0 (control)	5	50.96±2.17	37.99±1.69	11.05±0.33	49.04±1.20
200	5	51.88±1.63	36.89±0.78	11.23±0.64	48.12±0.86
400	5	60.20±2.38*	28.59±0.84*	11.21±0.98	39.81±1.53†
600	5	65.50±0.86*	25.61±2.06*	8.89±1.01†	34.50±2.09*
800	5	71.03±1.20*	13.50±1.55*	15.48±0.46*	28.98±1.21*

Compared with the control group, †$p<0.05$, *$p<0.01$.

$$\text{Proliferating index (PI)} = \frac{S + (G_2+M)}{(G_0/G_1) + S + (G_2+M)} \times 100\%$$

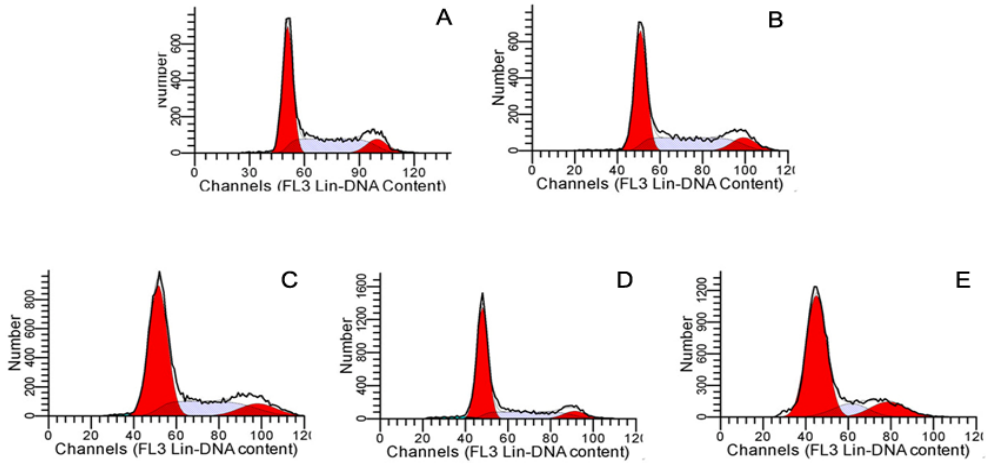

Figure 1. A, B, C, D, and E are the histograms of cell cycle progression of TM3 mouse Leydig cells with 0, 200, 400, 600, and 800 μmol NaF/L treatment for 24 hr, respectively.

Apoptosis detection: As shown in Table 2 and Figure 2, the percentage of TM3 mouse Leydig cells apoptosis was significantly higher, and the effect was dose-dependent compared with the control group.

Table 2. Percentage of F-induced Leydig cell apoptosis (Values are mean±SD)

NaF (μmol/L)	No. of samples	Apoptosis Rate (%)
0 (control)	5	2.2±0.2
200	5	2.4±0.2
400	5	4.0±0.3*
600	5	5.2±0.1*
800	5	6.9±0.4*

Compared with the control group, *p<0.01.

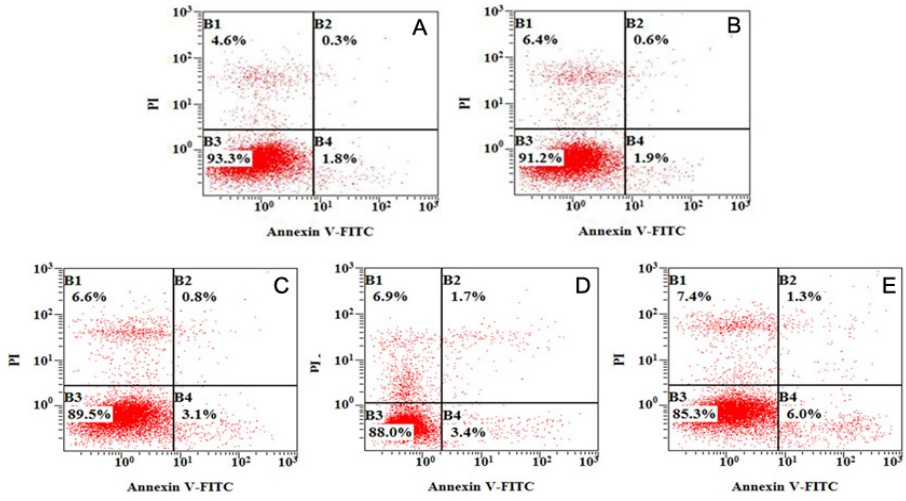

Figure 2. A, B, C, D, and E are the apoptosis rates of TM3 mouse Leydig cells with 0, 200, 400, 600, and 800 μmol NaF/L treatment for 24 hr, respectively.

Single cell gel electrophoresis: As seen in Table 3 and Figure 3, the tail length of the Leydig cells increased considerably in a dose-dependent manner In comparison with the control.

Table 3. Length of comet tail in TM3 mouse Leydig cells induced by F
(Values are mean±SD)

NaF (μmol/L)	No. of samples	No. of cells analyzed	Length of tail (μm)
0 (control)	5	100	3.500±2.646
200	5	100	6.000±3.367†
400	5	100	10.250±7.676*
600	5	100	11.500±3.941*
800	5	100	15.754±6.745*

Compared with the control group, $^{\dagger}p<0.05$, $^{*}p<0.01$.

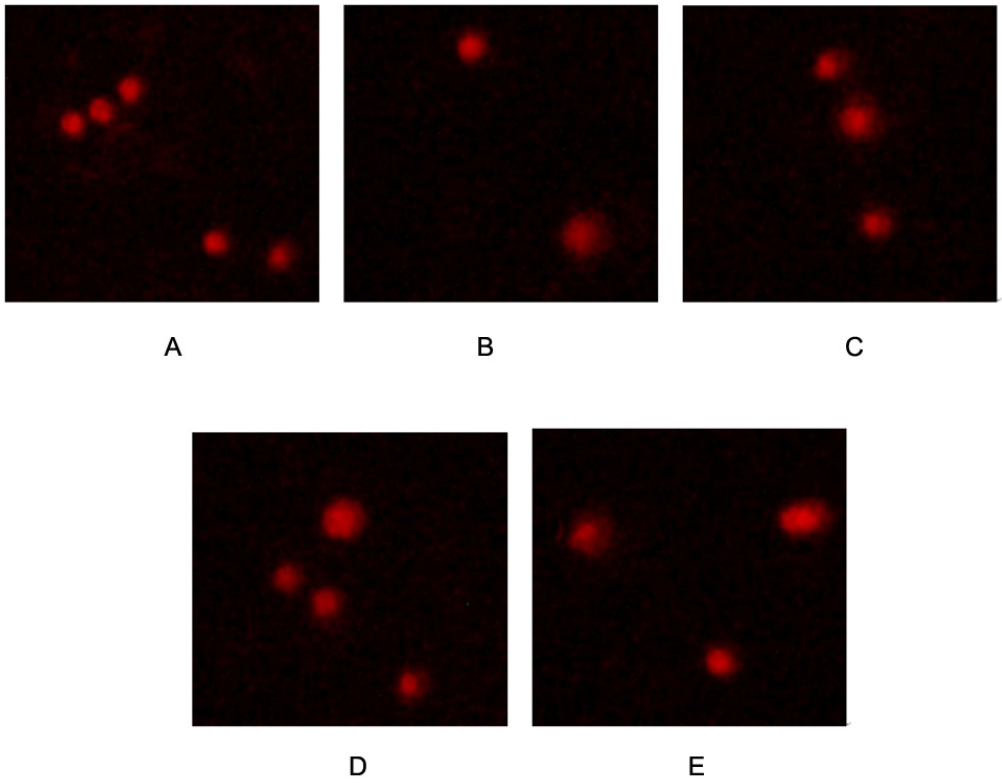

Figure 3. A, B, C, D, and E are single cell gel electrophoresis of nuclei in TM3 mouse Leydig cells with 0, 200, 400, 600 and 800 μmol NaF/L treated for 24 hr respectively.

DISCUSSION

It is well known that the cell cycle is one of the key regulatory mechanisms in cell growth. An active cell cycle includes the G_0/G_1, S, G_2, and M phases.[22] Many cytotoxic and genotoxic agents arrest the cell cycle at different phases and then induce apoptosis.[23,24] Previous studies have demonstrated that fluoride (F) is a cytotoxic agent and can cause G_0/G_1 phase cell cycle arrest in renal cells.[16] Since

Leydig cells are the primary cells that maintain secondary sex characteristics, there is need for data regarding the impact of F on these cells, particularly the cytotoxicity of F on Leydig cells *in vitro*. On the basis of the information derived from the results of the present study, it is suggested that F has the ability to interfere with regulation of the cell cycle in Leydig cells and can induce cell cycle arrest at the G_0/G_1 phase. The decreased cell population in S phase, G_2+M, and PI value showed that the proliferation of Leydig cells was significantly inhibited, which could be the reason why F has been found to decrease the secretory volume of testosterone in rats.[10,25]

The mechanism of cell cycle aberration induced by F appears to be were associated with the following factors:

(1) Excessive DPCs (DNA-protein crosslinks) can physically block the progression of replication and transcription of DNA.[26] Therefore, the enhanced DPCs in TM3 mouse Leydig cells can be seen as responsible for the inhibition and disturbance of cell proliferation cycles.

(2) Cell cycle progression is tightly regulated by various protein regulators. Current evidence suggests that activated ATM and ATR phosphorylate Chk, Brca1, p53, and other downstream checkpoint proteins to carry out cellular responses following genomic stress.[27] A possible explanation for this observation is that a greater percentage of Leydig cells in the G_0/G_1 phase could be due to F affecting G_1/S checkpoint proteins, which would result in G_1 phase arrest. However, this requires further research to identify the mechanisms involved.

As the natural process of programmed cell death, apoptosis is essential for the balance between cell growth and cell death.[28] In our study, flow cytometry demonstrated that apoptosis of Leydig cells following exposure to F showed a marked dose-dependent increase, which is consistent with previous studies on various tissues and cells. The mechanisms of apoptosis induced by F are associated with the following factors:

(1) Our results indicate that excessive F can induce uncontrolled intensified free-radical processes and decrease the activities of some enzymes involved in the antioxidant defense system in TM3 mouse Leydig cells, resulting in oxidative stress, which is known to trigger cellular apoptosis.[29]

(2) It is also possible that a self-protection mechanism was activated in order to eliminate abnormal cells which accumulated in the G_0/G_1 phase.

(3) F induced DNA damage is known to lead to cell apoptosis.[20,21] In our study, SCGE showed that the degree and rate of DNA damage were gradually increased with increasing F in the treatment groups, which may be an important factor in apoptosis. The above changes may therefore be involved in the mechanism of cell apoptosis induced by F.

In conclusion, F has been found to cause G_0/G_1 arrest, induce apoptosis, and DNA damage in TM3 mouse Leydig cells, which will have a negative impact on the reproductive cells.

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