FLUORIDE-INDUCED OXIDATIVE DAMAGE AND DNA-PROTEIN CROSSLINKS IN TM3 MOUSE LEYDIG CELLS

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SUMMARY: This *in vitro* study was conducted to evaluate the effect of fluoride (F) on oxidative damage and DNA-protein crosslinks (DPCs) in TM3 mouse Leydig cells. Sodium fluoride at concentrations of 0, 200, 400, 600, and 800 µmol/L was administered to TM3 mouse Leydig cells for 24 hr. The activities of the antioxidant enzymes glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT), along with the contents of glutathione (GSH) and malondialdehyde (MDA), plus the levels of DPCs were determined. The results showed that F administration significantly altered the levels of all of these factors compared to those of the control. The activities of antioxidant enzymes and GSH contents showed a dose-dependent decrease, the levels of lipid peroxidation and DPCs showed a dose-dependent increase. Combining all the data, the results suggest that F induces the oxidation hyperirritability in cultures of TM3 mouse Leydig cells, resulting in DPCs.

Keywords: Antioxidant enzymes; DNA-protein crosslinks; Fluoride-induced cell damage; Lipid peroxidation; Oxidation hyperirritability; TM3 mouse Leydig cells.

INTRODUCTION

Fluoride (F) is involved in various industrial practices and is a ubiquitous ingredient of most drinking water, dental products, and foodstuffs.[1] Fluorosis can be induced from excessive intake of F over a prolonged period of time.[2,3] Environmental factors have been the major problem on male reproductive function in many regions around the world.[4] In a review of studies on F and male reproductive function, we found exposure of rats to F adversely affects male reproduction through altering sperm quality,[5-7] spermatogenesis,[8] cellular morphology,[6] testis protein levels, metabolic enzyme activities,[9] and the antioxidant defense system.[10,11] However, there appears to have been no systematic *in vitro* studies on the toxicity of F on male reproductive cells.

Generally speaking, the types of DNA damage in cells include single strand breaks, double strand breaks, incomplete excision repair sites, DNA-DNA crosslinks, and DNA-protein crosslinks (DPCs). DNA breaks usually occur fairly rapidly and are usually readily repaired, whereas DPCs develop slowly and are not easily repaired. Excessive DPCs may anchor chromatin and prevent it from self-remolding. DPCs also act as bulky helix-distorting adducts that physically block the progression of replication, transcription, DNA incision, and repair during gene expression.[12,13] Excessive DPCs can be induced by the accumulation of hydroxyl radicals and superoxide anion radicals in cells.[13] Wang et al.[14] found that high F intake can induce oxidative damage to bovine splenic lymphocyte, resulting in DPCs.

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In addition, Leydig cells are known to play an important role in maintaining spermatogenesis and secondary sex characteristics.[15] To our knowledge, there are no systematic *in vitro* reports on the effects of F on oxidative damage and DPCs in Leydig cells. In the present study, we conducted an *in vitro* investigation of oxidative damage and DPCs in TM3 mouse Leydig cells exposed to various concentrations of NaF.

MATERIALS AND METHODS

Materials: 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). Hoechst 33258, calf thymus DNA, and proteinase K were supplied by Sigma (USA). NaF, Tris, SDS (sodium dodecyl sulfate), and KCl were purchased from Shanghai Chemical Reagent (China). All chemicals were of analytical grade.

Cell cultures: The cells were cultured in DMEM/F12 medium with L-glutamine, supplemented with 5% horse serum, 2.5% fetal bovine serum, 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen).

Determination of oxidative damage parameters in the TM3 mouse Leydig cells: The cells were exposed to 0, 200, 400, 600, and 800 μmol NaF/L for 24 hr after the cells grew against the walls of flasks (Fisher). The cells were then trypsinized, washed twice in phosphate buffered saline (PBS) at pH 7.4, and the cell number was adjusted to a density of 1×10^7 cells/mL. Cells were broken up by ultrasound and then immediately centrifuged at 1000×g for 15 min at 4ºC. After the total protein in the supernatant was determined,[16] the activities of GSH-Px, SOD, and CAT, and the contents of GSH and MDA were tested by means of commercially available assay kits purchased from Nanjing Jiancheng Technology Co., Ltd., China.

Determination of the DPCs in TM3 mouse Leydig cells: The formation of DPCs was monitored based on fractionation of the protein-bound and free DNA by sodium dodecyl sulfate/potassium (SDS/K^+) precipitation according to the method by Wang et al.[14] with a few modifications. The steps were:

(1) *Lysis of cells:* the isolated cells were harvested at 1500×g for 4 min, removal the supernatant and resuspension in 0.5-mL of PBS (PH 7.4), lysation of the cells with 0.5 mL of 2% SDS solution, gentle vortexing, and then incubation for 10 min at 65ºC.

(2) *Extraction of cell-free DNA:* One-tenth mL of 20 mmol/L Tris-HCl containing 1.0 mol/L KCl was added, followed by passing the resultant mixture six times through a 1-mL polypropylene pipette tip to favor shearing of DNA for a uniform length. The samples were placed on ice for 5 min and were then collected by centrifugation at 10000×g for 5 min. The supernatants were collected in different labeled tubes. The pellets (containing DPC) were washed three times by resuspending them in 1 mL of washing buffer (0.1 mol/L KCl, 0.1 mmol/L EDTA, and 20 mmol/L Tris-HCl at pH 7.4), followed by heating at 65ºC for l0 min, and

chilling in ice for 5 min. The latter supernatants from each wash were added to the previous one with unbound fractions of DNA.

(3) *Extraction of DNA bounded with protein:* The final pellet was resuspended in l mL proteinase K solution (0.4 mg/mL soluble in a wash buffer) and digested for 3 hr at 50ºC. The tubes were then placed on ice for 5 min and centrifuged at 12000×g for 10 min at 4ºC. The supernatants were collected to determine the levels of crosslinked DNA.

(4) *DPCs quantification:* DNA standards were prepared at concentrations of 100, 200, 500, 1000, 2000, and 5000 ng/mL. Two mL of a standard DNA, free DNA, and crosslinked DNA were mixed with 1 mL of 400 ng/mL of freshly prepared Hoechst 33258. The mixtures were placed in dark for 30 min. Fluorescence intensity was determined by excitation at 350 nm and emission at 450 nm using fluorescence spectrometry (F-4500, Hitachi).

Statistics: All the statistical analyses were performed using SPSS 17.0 and presented as means ± standard deviation of five replicates.

RESULTS

Changes in the activities of GSH-Px, SOD, and CAT in TM3 mouse Leydig cells: According to the data in Table 1, the activities of GSH-Px, SOD, and CAT showed a significant dose-dependent decrease in TM3 mouse Leydig cells.

Table 1. Changes in the activites of GSH-Px, SOD, and CAT in TM3 mouse Leydig cells (Values are mean±SD)

NaF (μmol/L)	No. of samples	GSH-Px activities (U/mg prot)	SOD activities (U/mg prot)	CAT activities (U/mg prot)
0 (control)	5	3.512±0.259	36.732±1.976	19.364±2.017
200	5	3.374±0.251†	35.554±3.252	18.712±3.141
400	5	3.037±0.365*	33.823±2.365	16.756±2.242†
600	5	2.669±0.115*	29.057±1.770†	13.249±1.812*
800	5	1.175±0.203*	16.212±3.201*	9.287±3.230*

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

Changes in the contents of GSH and MDA in TM3 mouse Leydig cells: As seen in Table 2, the GSH contents showed a significant dose-dependent decrease in TM3 mouse Leydig cells. On the other hand, the MDA contents showed a significant dose-dependent increase.

Table 2. Changes in the contents of GSH and MDA in TM3 mouse Leydig cells (Values are mean±SD)

NaF (μmol/L)	No. of samples	GSH contents (U/mg prot)	MDA contents (nmol/mg prot)
0 (control)	5	16.476±3.125	0.098±0.007
200	5	15.845±2.221	0.110±0.011†
400	5	12.256±3.923*	0.191±0.007*
600	5	9.351±2.160*	0.354±0.010*
800	5	6.114±3.125*	0.479±0.037*

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

Changes in the levels of DPCs in TM3 mouse Leydig cells: From the Figure, with DNA of calf thymus as the standard for measuring the spectrophotometric results, the DPCs values in Table 3 showed a significant increase in TM3 mouse Leydig cells. In the regression equation for the Figure, $y = 0.0025x \pm 3.7974$ and $R^2 = 0.9994$, where y are fluorescence values and x are DNA concentrations of calf thymus.

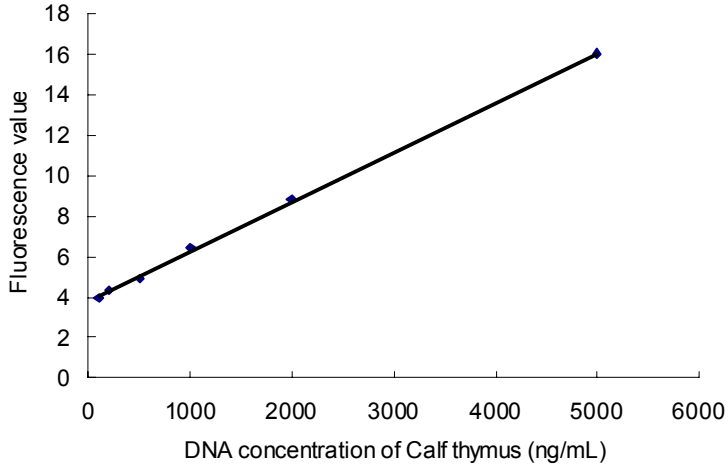

Figure. Standard curve of DNA concentration

Table 3. The level of DPCs in TM3 mouse Leydig cells (Values are mean±SD)

NaF (μmol/L)	No. of samples	DPC module
0 (control)	5	0.097±0.002
200	5	0.103±0.003
400	5	0.147±0.013*
600	5	0.186±0.010*
800	5	0.214±0.009*

Compared with the control group *p<0.01.

DISCUSSION

Fluoride (F) is a well-known environmental and industrial pollutant that is distributed from the plasma to all the tissues and organs of the body.[17] The uncontrolled overproduction of free-radicals induced by F is harmful to tissues and cells.[18-20] F can alter the activities of enzymes involved in free-radical metabolism and impair the antioxidative defense system in liver,[21] kidney,[21,22] brain,[23] embryo,[24] and cecal tonsil.[25] In this connection, 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] However, to date, there have been no studies on the effects of F on the functions of Leydig cells *in vitro*. Here we conducted such an investigation of oxidative damage induced by F to TM3 mouse Leydig cells.

Lipid peroxidation occurs under the attack of free-radicals against membrane lipoproteins via polyunsaturated fatty acids (PUFA) on biological structures, producing MDA as one of the end products.[26] The contents of MDA in tissues and cells thus increase after intensified free-radical processes. Consequently, determination of MDA contents provides a good measure of lipid peroxidation. The present *in vitro* study demonstrated that MDA contents increased in TM3 mouse Leydig cells induced by F in a dose-dependent manner, which is consistent with a previous *in vivo* report.[11] Oxidative stress is a result of disturbed oxidants and antioxidants balance in biological systems.[27] To counter the oxidative stress, the antioxidant defense mechanism operates in the cells. The antioxidant systems include antioxidant enzymes like GSH-Px, SOD, and CAT, together with non-enzymatic antioxidants like GSH. Our results indicate that the balance between the oxidative and antioxidative systems was disturbed during F exposure, probably making the cells more susceptible to biochemical injury. Oxidative stress may be induced by intensified free-radical reactions or by reduced concentration of antioxidants, possibly also by those two processes occurring simultaneously. That is why, in parallel, we have examined both antioxidants levels and the MDA contents.

As noted in the Introduction, DNA-protein crosslinks (DPCs), which are usually induced at a basic level in normal cells, have a connection between DNA and nuclein.[13] Excessive DPCs can be induced by environmental pollutants and carcinogens.[28,29] DPCs may anchor chromatin and prevent DNA from self-remodeling. DPCs can physically block the progression of replication and transcription that lead to the loss of some important genes.[12,13] Excessive DPCs are a pathological phenomena of free-radicals in those cells that could not be eliminated in time to prevent DPCs from occurring.[13] Under normal conditions, free-radicals production and elimination are in a dynamic balance. In our study, F impaired the production of free-radical scavengers, such as GSH-Px, SOD, CAT, and GSH, resulting in the accumulation of free-radicals, which induced DPCs in Leydig cells *in vitro*. However, we recognize there is need for further research to ascertain the definite mechanisms of these events.

In summary, according to the results of the present study and the aforementioned discussion, it is concluded that F can disturb the balance of oxidants and

antioxidants, thereby causing oxidative damage of TM3 mouse Leydig cells *in vitro*, resulting in DPCs.

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