iTRAQ-BASED PROTEOMIC ANALYSIS OF TESTIS FROM MICE EXPOSED TO THE FLUORIDE ION

Zilong Sun,[a,b,*] Chenguang Liu,[a] Sujuan Li,[a] Yuxiang Yu,[a] Ruiyan Niu,[a,b] Jundong Wang[a,b]

Taigu, Shanxi, People's Republic of China

ABSTRACT: Isobaric tags for relative and absolute quantification (iTRAQ)-based comparative proteomics techniques were applied in this study to detect global changes in the protein profiles of testis in male Kunming mice exposed to 0, 25, and 100 mg/L sodium fluoride (NaF) for 60 days. The results showed that 88 proteins were differentially expressed in both the 25 and 100 mg/L NaF groups. Enrichment analysis of GO and KEGG revealed that these altered proteins were involved in seven GO molecular functions, in 34 GO biological processes, and in five KEGG pathways. The down-regulated proteins related to reproductive functions discussed in detail included ubiquitin-conjugating enzyme E2 R1, ubiquitin-40S ribosomal protein S27a, aldehyde dehydrogenase, histone H1.4, serine/threonine-protein phosphatase, and sperm acrosome membrane-associated protein 4. In conclusion, the data suggest that fluoride ion exposure may adversely affect ubiquitination, phosphorylation, retinoic acid synthesis, histone-protamine replacement, and the sperm acrosome membrane. Consequently, fluoride ion exposure may interfere with spermatogenesis, sperm motility, and the acrosome reaction.

Keywords: Fluoride; iTRAQ; Proteomics; Testis.

INTRODUCTION

The fluoride ion (F) is a global contaminant that is traceable in various environmental samples such as water, soil, plants, and volcanic ash. Humans are continuously exposed to F in food, drinking water, dental products, and F-containing dust that can be inhaled. This results in the accumulation of F in many different tissues. [1] It is demonstrated that 12 weeks of high dose exposure can increase the F concentration about twofold in serum, sevenfold in liver and kidney, ninefold in brain, and twelvefold in testis.[2]

Epidemiological data has linked F exposure to male infertility.[3,4] Experimental studies have indicated that F may alter testis morphology, decrease testosterone secretion, reduce sperm quality, and disturb spermatogenesis.[5,6] However, the testis protein profile of animals treated with F has not been studied yet. Therefore, in this study, isobaric tags for relative and absolute quantification (iTRAQ)-based comparative proteomics techniques were applied in order to detect the differently expressed proteins in the testes of mice treated with different doses of F.

MATERIALS AND METHODS

Establishment of animal model: All experiments in this study were approved by the Institutional Animal Care and Committee of Shanxi Agricultural University. Male Kunming mice, aged 8 weeks, were provided by the Experimental Animal Center of Shanxi Medical University. After acclimation to the laboratory

[a]College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, People's Republic of China; [b]Shanxi Key Laboratory of Ecological Animal Science and Environmental Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, People's Republic of China; *For correspondence: Zilong Sun, Shanxi Agricultural University, Taigu, Shanxi, 030801, People's Republic of China; E-mail: sunzilong2000@outlook.com.

conditions for 7 days, the mice were administered 0, 25, or 100 mg NaF/L in drinking water for 60 days, and given free access to food and water. After fasting overnight, mice were sacrificed by cervical dislocation. Testes were quickly removed, snap-frozen in liquid N_2, and stored at –80ºC for proteomic analysis.

iTRAQ labeling and strong cation exchange (SCX) chromatography: The testes, about 100 mg in volume, were extracted with lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM ethylene diamine tetraacetic acid (EDTA) for 5 min. Ten mM dithiothreitol (DTT) was then added. After sonication for 15 min and centrifugation at 25,000 g for 20 min, the supernatant was mixed with 5× volume of chilled acetone, incubated at –20ºC for 2 hr, and centrifuged at 4ºC, 16,000 g, for 20 min. The precipitate was dissolved and centrifuged using the same treatment as in the first two steps. Then the supernatant was incubated with 10 mM DTT at 56ºC for 1 hr to reduce disulfide bonds. After incubation with chilled acetone at –20°C for 2 hr, the solution was centrifuged at 25,000 g for 20 min. The precipitate was mixed with 200 µL 0.5 M triethylammonium bicarbonate (TEAB), sonicated for 15 min, and centrifuged at 25,000 g for 20 min. The supernatant was analysed to detect the protein concentration using the Bradford method. The testis proteins of three mice were pooled together for one sample. Protein samples were loaded on the 12% SDS-PAGE gel followed by a 12 hr trypsin digestion at 37ºC (trypsin:protein = 1:20). Peptides were reconstituted in 0.5 M TEAB and labeled with the isobaric tags (iTRAQ reagent, Applied Biosystems), and incubated at room temperature for 2 hr for further SCX chromatography.

The iTRAQ-labeled peptides were reconstituted with 4 mL buffer A (25 mM NaH_2PO_4 in 25% acetonitrile, pH 2.7) and loaded onto a 4.6×250 mm Ultremex SCX column with a flow rate of 1 mL/min with a gradient of 5% buffer B (25mM NaH_2PO_4, 1 M KCl in 25% acetonitrile, pH 2.7) for 7 min, with 5%–60% buffer B for 20 min, and then with 60%–100% buffer B for 2 min. The system was then maintained at 100% buffer B for 1 min. Elution was monitored by measuring the absorbance at 214 nm.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis: A 10 µL peptide fraction with a concentration of 0.5 µg/µL was loaded onto a 2 cm C18 trap column (Shimadzu, Kyoto, Japan) and eluted at 8 µL/min for 4 min. Then, the 35 min gradient operation was run at 300 nL/min starting from 5% to 35% buffer C (95% acetonitrile, 0.1% formic acid), followed by a 5 min linear gradient to 60%. After 2 min, the linear gradient was increased to 80%, the elution was maintained at 80% for 2 min, and finally it was returned to 5% for 1 min. Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON, Canada) fitted with a Nanospray III source (AB SCIEX, Concord, ON, Canada) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA, USA).

Data and pathway analysis: The raw MS/MS data were inputted into Proteome Discoverer 1.2 software (Thermo Fisher Scientific) to identify the peptides. To reduce the probability of false identification, proteins were identified from

peptides with more than 95% confidence. Proteins with corrected p values <0.05 and fold changes of >1.2 were considered as significant. Gene ontology (GO) term and KEGG pathway enrichment were performed using the GO (http://www.geneontology.org/) and KEGG databases (http://www.genome.jp/kegg/).

RESULTS

Using iTRAQ, a total of 6,563 proteins were identified in this study. Compared with the control group, 511 proteins were altered in the 25 mg/L NaF group, out of which 297 were up-regulated and 214 were down-regulated. In the 100 mg/L NaF group, 201 proteins were up-regulated and 160 were down-regulated proteins. show Eighty-eight proteins were differentially expressed in both the low and high NaF groups (Tables 1A-1G). After enrichment analysis and analysis by the GO and KEGG databases, we found that the proteins commonly altered in both the 25 and 100 mg/L NaF groups were involved in seven GO molecular functions (Table 2), in 34 GO biological (Tables 3A and 3B), and in five KEGG pathways (Table 4).

Table 1A. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
Q3TDU5	Milk fat globule-EGF factor 8 protein, isoform CRA_a	Mfge8	1	21.4	1.894	1.528
O89017	Legumain	Lgmn	5	21.4	1.761	1.41
Q5DTL0	MKIAA4152 protein (fragment)	Enpp5	5	13.2	1.752	1.459
Q61599	Rho GDP-dissociation inhibitor 2	Arhgdib	2	23.5	1.738	1.325
Q3TJZ6	Protein FAM98A	Fam98a	3	11.8	1.694	1.635
Q9D855	Cytochrome b-c1 complex subunit 7	Uqcrb	4	29.7	1.693	1.264
B7ZWC4	Insulin-like growth factor 2 receptor	Igf2r	4	2	1.671	1.478
P40935	Phenylethanolamine N-methyltransferase	Pnmt	5	20.7	1.629	1.328
Q9CRD2	ER membrane protein complex subunit 2	Emc2	4	14.8	1.626	1.384

Table 1B. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
E9Q2H1	E3 ubiquitin-protein ligase UBR5	Ubr5	8	3.7	1.601	1.209
Q496Q2	Glutathione peroxidase	Gpx5	1	39.4	1.591	1.448
Q9Z0S9	Prenylated Rab acceptor protein 1	Rabac1	2	15.7	1.568	1.534
Q571E4	N-acetylgalactosamine-6-sulfatase	Galns	3	6.3	1.562	1.255
Q3TTZ3	Putative uncharacterized protein (fragment)	Myo1b	13	17.4	1.518	1.401
Q4FJZ7	Ada protein	Ada	6	18.2	1.514	1.63
Q8CC70	Protein 9230110C19Rik	9230110C19 Rik	6	24.3	1.508	1.541
Q8BK60	Putative uncharacterized protein	Serpinb1a	8	26.4	1.496	1.997
Q3TC98	Putative uncharacterized protein	Vps39	7	13.6	1.494	1.595
O08797	Protein Serpinb9	Serpinb9	10	29.9	1.473	1.267
Q5DU67	MFLJ00088 protein (fragment)	Ganc	5	8.4	1.461	1.876
Q9JI90	E3 ubiquitin-protein ligase RNF14	Rnf14	3	6.6	1.456	1.288
Q3TIZ0	Putative uncharacterized protein	Tuba1c	3	61.7	1.44	1.925
G3X8X7	Vacuolar protein sorting 16 (yeast)	Vps16	6	10.5	1.424	1.295
Q3SXD3	HD domain-containing protein 2	Hddc2	3	14.6	1.393	1.275

Table 1C. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
P51174	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	Acadl	7	21.4	1.393	1.234
Q8BND5	Sulfhydryl oxidase 1	Qsox1	12	19.3	1.389	1.622
Q3TQD9	Putative uncharacterized protein (fragment)	Sardh	7	8.7	1.384	1.416
Q91W43	Glycine dehydrogenase (decarboxylating), mitochondrial	Gldc	16	24.1	1.377	1.319
Q3UBS0	Putative uncharacterized protein	Apoe	4	16.1	1.351	1.57
P29788	Vitronectin	Vtn	4	9.6	1.35	1.741
Q8BJ64	Choline dehydrogenase, mitochondrial	Chdh	8	22	1.344	1.33
Q9R0H0	Peroxisomal acyl-coenzyme A oxidase 1	Acox1	11	27.5	1.34	1.224
Q3THX5	Putative uncharacterized protein	Mvp	15	25.9	1.336	1.296
D3Z3G5	Sulfotransferase	Sult1a1	3	16.4	1.303	1.729
Q3UMR5	Calcium uniporter protein, mitochondrial	Mcu	6	22.6	1.298	1.336
Q80XN0	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	Bdh1	7	23.9	1.294	1.313
Q3UMY5	Echinoderm microtubule-associated protein-like 4	Eml4	7	9.8	1.277	1.446

Table 1D. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
Q3TZZ7	Extended synaptotagmin-2	Esyt2	7	11.8	1.265	1.307
Q7TMR0	Lysosomal Pro-X carboxypeptidase	Prcp	6	15.9	1.259	1.356
Q64726	Zinc-alpha-2-glycoprotein	Azgp1	9	29.3	1.259	1.249
Q8K0C4	Lanosterol 14-alpha demethylase	Cyp51a1	10	22.9	1.254	1.404
Q9CZS1	Aldehyde dehydrogenase X, mitochondrial	Aldh1b1	12	37.4	1.254	0.738
Q3TIH8	Putative uncharacterized protein	Zpr1	6	17	1.245	1.41
F8WJE0	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	Samhd1	12	27.2	1.242	1.274
Q8BPI1	Protein kintoun	Dnaaf2	8	17.8	1.242	1.237
P32261	Antithrombin-III	Serpinc1	13	35.9	1.221	1.271
Q5BKS2	Protein phosphatase 1B, magnesium dependent, beta isoform	Ppm1b	4	13.9	1.216	1.241
E0CXW2	Uncharacterized aarF domain-containing protein kinase 5	Adck5	2	5.1	1.215	1.486
Q9DCJ9	N-acetylneuraminate lyase	Npl	12	44.7	1.206	1.236
P28665	Murinoglobulin-1	Mug1	16	27	0.826	0.638
D3Z4K0	Protein Ankrd36	Ankrd36	6	5.2	0.803	0.764
Q80Y75	DnaJ homolog subfamily B member 13	Dnajb13	9	42.7	0.789	0.766
Q9CSV6	Vesicle transport protein SFT2C	Sft2d3	1	6.7	0.787	0.77

Table 1E. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
Q3U6M5	Putative uncharacterized protein	Ranbp1	2	10.8	0.784	0.799
Q3UEJ7	Putative uncharacterized protein	Ass1	13	35.7	0.773	0.632
Q8CFI2	Ubiquitin-conjugating enzyme E2 R1	Cdc34	4	17	0.769	0.764
G3UWD6	Glucose-6-phosphate 1-dehydrogenase	G6pd2	8	26.5	0.768	0.723
Q05816	Fatty acid-binding protein, epidermal	Fabp5	4	41.5	0.758	1.227
Q91Z31	Polypyrimidine tract-binding protein 2	Ptbp2	5	19.6	0.752	0.833
Q3TV94	Putative uncharacterized protein	Ssr1	3	11.9	0.752	0.813
P06683	Complement component C9	C9	10	20.6	0.752	0.586
Q9D8E6	60S ribosomal protein L4	Rpl4	4	14.8	0.749	0.674
P54116	Erythrocyte band 7 integral membrane protein	Stom	5	27.1	0.728	0.746
Q91WS0	CDGSH iron-sulfur domain-containing protein 1	Cisd1	3	34.3	0.723	0.761
Q80XK0	Serine/threonine-protein phosphatase (fragment)	Ppp3cc	4	16.4	0.72	0.752
Q810N3	Protein BC049730	BC049730	2	8.9	0.711	0.708
Q5H8C4	Vacuolar protein sorting-associated protein 13A	Vps13a	35	14.6	0.711	0.687
E9Q0C6	Protein Gm14569	Gm14569	22	19.7	0.705	0.762

Table 1F. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
Q922Q9	Chitinase domain-containing protein 1	Chid1	8	29.8	0.703	0.816
P97290	Plasma protease C1 inhibitor	Serping1	6	14.9	0.695	1.49
Q78Y63	Phosducin-like protein 2	Pdcl2	5	28.8	0.694	0.703
P43274	Histone H1.4	Hist1h1e	2	14.6	0.675	0.637
Q3UF30	Putative uncharacterized protein	S100a10	2	17.5	0.659	0.7
Q99K70	Ras-related GTP-binding protein C	Rragc	3	9	0.65	0.805
Q3THJ0	60S ribosomal protein L18a	Rpl18a	3	19.3	0.644	0.692
Q8BFZ3	Beta-actin-like protein 2	Actbl2	2	25	0.641	0.733
A0A087WNP6	Protein CDV3	Cdv3	2	13.2	0.638	0.687
P62983	Ubiquitin-40S ribosomal protein S27a	Rps27a	6	46.2	0.634	0.828
Q61646	Haptoglobin	Hp	10	29.1	0.631	3.444
Q80ZQ0	Sperm acrosome membrane-associated protein 4	Spaca4	1	9.4	0.616	0.473
P23242	Gap junction alpha-1 protein	Gja1	2	7.9	0.615	0.651
B7ZN13	Aldehyde dehydrogenase	Aldh3a1	2	5.3	0.595	0.559
D3Z3B2	V-type proton ATPase 16 kDa proteolipid subunit (Fragment)	Atp6v0c	1	15.5	0.571	0.764
O54962	Barrier-to-autointegration factor	Banf1	2	40.4	0.57	0.732

Table 1G. Differentially expressed proteins from the testis of the mice in the 25 mg/L NaF and 100 mg/L NaF groups compared with the control group

Accession No.	Protein name	Gene name	Unique peptides	Coverage (%)	Fold change in the 25 and 100 mg/L NaF groups compared to the control group	
					Group	
					25 mg/L NaF	100 mg/L NaF
Q9CPT4	UPF0556 protein C19orf10 homolog	D17Wsu104e	3	21.7	0.559	0.771
Q3UFI4	60S ribosomal protein L6	Rpl6	2	8.8	0.474	0.685
B2RUF0	Y box protein 2	Ybx2	5	24.8	0.464	0.685
P17665	Cytochrome c oxidase subunit 7C, mitochondrial	Cox7c	1	14.3	0.427	0.811

Table 2. Gene ontology (GO) molecular functions with enrichment in both the 25 mg/L NaF and 100 mg/L NaF groups compared to the control group

GO term	p value of the NaF groups compared to the control group		Number and ratio of differential proteins (%), with annotation of >3%, of the NaF groups compared to the control group	
	Group		Group	
	25 mg/L NaF	100 mg/L NaF	25 mg/L NaF	100 mg/L NaF
Carboxylic acid binding	0.048	0.037	15 (3.4%)	12 (3.8%)
Endopeptidase inhibitor activity	0.012	0.001	14 (3.2%)	14 (4.4%)
Endopeptidase regulator activity	0.009	0.001	15 (3.4%)	14 (4.4%)
Enzyme inhibitor activity	0.028	0.004	20 (4.6%)	18 (5.7%)
Oxidoreductase activity	0.001	<0.001	54 (12.3%)	44 (14.0%)
Peptidase regulator activity	0.018	0.002	16 (3.6%)	15 (4.8%)
Serine-type endopeptidase inhibitor activity	0.001	<0.001	12 (2.7%)	13 (4.1%)

Table 3A. Gene ontology (GO) biological processes with enrichment in both the 25 mg/L NaF and 100 mg/L NaF groups compared to the control group

GO term	p value of the NaF groups compared to the control group		Number and ratio of differential proteins (%), with annotation of >3%, of the NaF groups compared to the control group	
	Group		Group	
	25 mg/L NaF	100 mg/L NaF	25 mg/L NaF	100 mg/L NaF
Alcohol metabolic process	0.021	0.004	19 (4.3%)	17 (5.4%)
Carboxylic acid catabolic process	0.044	0.011	17 (3.9%)	15 (4.8%)
Catabolic process	<0.001	0.039	118 (27%)	74(23.6%)
Defense response	0.012	0.012	30 (6.9%)	23 (7.3%)
Inflammatory response	0.013	0.027	15 (3.4%)	11 (3.5%)
Macromolecular complex subunit organization	<0.001	0.025	80 (18.3%)	49 (15.7%)
Negative regulation of hydrolase activity	0.044	0.002	17 (3.9%)	17 (5.4%)
Negative regulation of peptidase activity	0.034	0.001	14 (3.2%)	15 (4.8%)
Organic acid catabolic process	0.044	0.011	17 (3.9%)	15 (4.8%)
Organic acid metabolic process	0.029	0.001	54 (12.4%)	47 (15%)
Organic hydroxy compound metabolic process	0.005	0.002	23 (5.3%)	19 (6.1%)
Organic substance catabolic process	<0.001	0.024	111 (25.4%)	71 (22.7%)
Oxidation-reduction process	0.021	0.008	57 (13%)	45 (14.4)
Oxoacid metabolic process	0.032	0.001	53 (12.1%)	46 (14.7%)
Protein complex subunit organization	<0.001	0.013	67 (15.3%)	42 (13.4%)
Regulation of peptidase activity	0.029	0.002	20 (4.6%)	19 (6.1%)
Response to alcohol	0.001	0.017	23 (5.3%)	15 (4.8%)

Table 3B. Gene ontology (GO) biological processes with enrichment in both the 25 mg/L NaF and 100 mg/L NaF groups compared to the control group

GO term	p value of the NaF groups compared to the control group		Number and ratio of differential proteins (%), with annotation of >3%, of the NaF groups compared to the control group	
	Group		Group	
	25 mg/L NaF	100 mg/L NaF	25 mg/L NaF	100 mg/L NaF
Response to corticosteroid stimulus	<0.001	<0.001	22 (5%)	14 (4.5%)
Response to extracellular stimulus	0.018	0.019	22 (5%)	17 (5.4%)
Response to glucocorticoid stimulus	<0.001	<0.001	21 (4.8%)	14 (4.5%)
Response to hormone stimulus	0.006	0.001	44 (10.1%)	37 (11.8%)
Response to lipid	<0.001	<0.001	42 (9.6%)	31 (9.9%)
Response to nutrient	0.003	0.006	17 (3.9%)	13 (4.2%)
Response to nutrient levels	0.022	0.026	21 (4.8%)	16 (5.1%)
Response to organic cyclic compound	0.001	<0.001	42 (9.6%)	36 (11.5%)
Response to organic substance	0.038	0.025	82 (18.8%)	62 (19.8%)
Response to oxygen-containing compound	0.005	0.017	56 (12.8%)	40 (12.8%)
Response to steroid hormone stimulus	<0.001	<0.001	32 (7.3%)	25 (8%)
Single-organism biosynthetic process	0.034	<0.001	26 (5.9%)	26 (8.3%)
Single-organism catabolic process	0.005	0.007	25 (5.7%)	19 (6.1%)
Small molecule biosynthetic process	0.049	<0.001	25 (5.7%)	26 (8.3%)
Small molecule catabolic process	0.005	0.007	25 (5.7%)	19 (6.1%)
Steroid metabolic process	0.015	0.001	15 (3.4%)	15 (4.8%)
Sulfur compound metabolic process	0.030	<0.001	17 (3.9%)	18 (5.8%)

Table 4. KEGG pathways with enrichment in both the 25 mg/L NaF and 100 mg/L NaF groups compared to the control group

KEGG pathway	p value of the NaF groups compared to the control group		Number and ratio of differential proteins (%), with annotation, of the NaF groups compared to the control group	
	Group		Group	
	25 mg/L NaF	100 mg/L NaF	25 mg/L NaF	100 mg/L NaF
Alpha-linolenic acid metabolism	0.025	0.007	4 (0.92%)	4 (1.34%)
Complement and coagulation cascades	0.020	<0.001	11 (2.52%)	13 (4.36%)
Glycine, serine and threonine metabolism	0.005	0.010	8 (1.83%)	6 (2.01%)
Glycosaminoglycan degradation	0.004	0.043	5 (1.15%)	3 (1.01%)
Ribosome	<0.001	0.018	27 (6.19%)	10 (3.36%)

DISCUSSION

Researchers can obtain a global profile of proteins in a certain tissue using proteomics, which can be used to investigate the toxicological effects of environmental contaminants. iTRAQ is a relatively sensitive and reproducible technique that has been widely used in quantitative proteomic studies.[7] In the present study, the differentially expressed proteins, and enriched GO terms and KEGG pathways of testis from mice treated with NaF provided new explanations for testicular toxicity. Here, representative proteins related to reproductive function are discussed in detail.

The ubiquitination of proteins is considered to be an important post-translational modification for modulating protein stability and function.[8] Notably, there is a high rate of ubiquitination in testis.[8] During the maturation of spermatozoa, ubiquitin-mediated protein modification and degradation are required.[9] In this study, ubiquitin-conjugating enzyme E2 R1, which is responsible for catalyzing the activated ubiquitin during the process of ubiquitination, was found to be significantly down-regulated in both the 25mg/L and 100 mg/L NaF groups. In addition, the expressions of E3 ubiquitin-protein ligase UBR5, E3 ubiquitin-protein ligase RNF14, and ubiquitin-40S ribosomal protein S27a were also altered by F exposure. The changes in these proteins suggest that ubiquitination in testis may be disturbed by F, which further influences normal spermatogenesis. The

findings in this study provide the molecular explanation for the earlier observations.

Retinol plays an essential role in the differentiation of spermatogonia.[10] Therefore, a deficiency in retinol can attenuate spermatogenesis. It has also been found that the level of aldehyde dehydrogenase, a retinoic acid synthesizing enzyme, is low in men with infertility.[10] In the current experiment, the protein expression of aldehyde dehydrogenase was down-regulated, suggesting that fluoride may interfere with spermatogenesis by disturbing retinoic acid biosynthesis.

Chromodomain helicase DNA binding protein 5(Chd5) is a master regulator of the histone-to-protamine replacement that is required for spermiogenesis.[11] Chd5-deficient male mice exhibited defective sperm chromatin compaction and infertility, in concert with a low expression of histone H1.4.[11] In the present study, histone H1.4, one of the histone variants, was reduced by treatments of both 25 and 100 mg/L NaF, implying that F-induced male infertility may be associated with disruption of the histone variant exchange.

Serine/threonine-protein phosphatase has been demonstrated to participate in the regulatory mechanism by which sperm gain motility activation.[12] A large number of documents have shown that F ingestion can lead to low sperm motility. In this study, the level of serine/threonine-protein phosphatase was significantly decreased in both the 25mg/L and 100 mg/L NaF groups, providing a new target molecule to explain the F-induced low sperm motility.

Previous literature has revealed that sperm from F-treated mice exhibits a decreased capability of undergoing the acrosome reaction or oocyte fertilization.[13, 14] In this study, Spaca4, a functional protein that is associated with the sperm acrosome membrane, was reduced by the different doses of F. Its down-regulation explains in part the F-induced low sperm acrosome reaction.

Enrichment analysis of GO and KEGG indicated that F-induced differentially expressed proteins were involved in diverse biological processes and pathways, including those concerned with oxidation-reduction, steroid hormone stimulation, steroid metabolism, and ribosomal function. This is supported by the pre-existing knowledge that F can evoke oxidative stress, decrease testosterone secretion, and influence protein metabolism.

CONCLUSIONS

Taken together, the iTRAQ techniques applied in this study led to the discovery of novel differentially expressed proteins in the testes of mice treated with various doses of fluoride. The proteins discussed here are involved in diverse biological processes associated with F-induced reproductive toxicity, suggesting that the fluoride ion may adversely affect ubiquitination, phosphorylation, retinoic acid synthesis, histone-protamine replacement, and the sperm acrosome membrane. Consequently, fluoride ion exposure may interfere with spermatogenesis, sperm motility, and the acrosome reaction.

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DATA AVAILABILITY

Readers who want to access the raw data should contact Z Sun, E-mail: sunzilong2000@outlook.com.

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