Acta Biochim Biophys Sin, 2015, 47(7), 504–515
doi: 10.1093/abbs/gmv045
Advance Access Publication Date: 10 June 2015
Original Article

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Alteration of sperm protein profile induced by cigarette smoking

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Received 21 February 2015; Accepted 12 April 2015

Abstract

Cigarette smoking is associated with lower semen quality, but how cigarette smoking changes the semen quality remains unclear. The aim of this study was to screen the differentially expressed proteins in the sperm of mice with daily exposure to cigarette smoke. The 2D gel electrophoresis (2DE) and mass spectrometry (MS) analyses results showed that the mouse sperm protein profile was altered by cigarette smoking. And 22 of the most abundant proteins that correspond to differentially expressed spots in 2DE gels of the sperm samples were identified. These proteins were classified into different groups based on their functions, such as energy metabolism, reproduction, and structural molecules. Furthermore, the 2DE and MS results of five proteins (Aldoa, ATP5a1, Gpx4, Cs, and Spatc1) were validated by western blot analysis and reverse transcriptase-polymerase chain reaction. Results showed that except Spatc1 the other four proteins showed statistically significant different protein levels between the smoking group and the control group ($P < 0.05$). The expressions of three genes (Aldoa, Gpx4, and Spatc1) were significantly different ($P < 0.05$) at transcription level between the smoking group and the control group. In addition, five proteins (Aldoa, ATP5a1, Spatc1, Cs, and Gpx4) in human sperm samples from 30 male smokers and 30 non-smokers were detected by western blot analysis. Two proteins (Aldoa and Cs) that are associated with energy production were found to be significantly altered, suggesting that these proteins may be potential diagnostic markers for evaluation of smoking risk in sperm. Further study of these proteins may provide insight into the pathogenic mechanisms underlying infertility in smoking persons.

Key words: cigarette smoking, mass spectrometry, protein profile, sperm, male fertility

Introduction

Infertility is a global concern and male infertility accounts for ~50% of human infertility cases [1]. Cigarette smoking can impair fertility [2] and may affect the health of offsprings [3,4].

Previous studies have shown that cigarette smoking can lower sperm density, sperm mobility, and semen volume [5,6]. Low pregnancy rates in infertile couples undergoing *in vitro* fertilization are highly associated with the male partner's smoking habits. Cigarette smoking may damage the chromosome during spermatogenesis,

which is thought to lower pregnancy rates. Rubes *et al.* [7] found that cigarette smoking was associated with increased sperm disomy in teenage men. The abnormal chromosome structure can affect pregnancy and early embryo development [8–10].

Several studies have explored how cigarette smoking affects sperm and semen quality, as well as how cigarette smoking alters the levels of male hormones that are associated with sperm number and motility [11,12], DNA damage in sperm [13], and lower sperm membrane permeability and acrosin activity [14]. High-throughput methods have also been used to analyze the genes that are differentially expressed in sperm from smoking and non-smoking males. However, more studies are needed to provide an in-depth understanding of the sperm proteome and elucidate the roles of sperm proteins in regulating motility, capacitation, acrosome reaction, and fertilization.

This work aims to determine how cigarette smoking affects the sperm protein profile and to identify the molecular mechanism underlying the changes in sperm protein profile.

Materials and Methods

Cigarette smoke exposure

C57BL/6J mice were provided by the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China).

Seven-week-old mice were randomly assigned into the smoking group and control group, with 20 mice in each group. A special device was designed and used to expose the mice in the smoking group to a similar level of exposure as human smokers experience. A 150 W vacuum pump was used to suck and draw smoke into a 30 cm × 30 cm × 30 cm glass box.

First, the mice in the smoking group were placed into the glass box, and the system was closed. The cigarette was lit and the vacuum pump was concurrently turned on to draw cigarette smoke into the box. After the cigarette was depleted, the vacuum pump was turned off and the mice were continuously exposed to the smoke for 1 h. The mice were exposed twice a day with a 1-h interval. The mice in the control group were placed in a box with identical conditions, but the cigarette was not lit. This exposure process was performed for 6 weeks.

Mouse oxygen content analysis

Fifteen male mice were randomly divided into three groups, a smoking group, a recovery group, and a control group, with five mice in each group. Mice in the cigarette-smoking group and recovery group were treated with cigarette smoke for 2 weeks as above and then used for oxygen content analysis, while mice in the control group remained untreated.

The arterial blood of the smoking group mice was collected immediately after the final smoke exposure, while the arterial blood of recovery group mice was collected 2 h after the final smoke exposure. The blood gas of blood samples was analyzed using a GEM premier 3000 blood gas analyzer (Instrumentation Laboratory, Lexington, USA).

Mouse sample collection

Mice were killed by cervical dislocation, and the epididymides were collected. The cauda of the epididymis was carefully isolated, and the spermatozoa were forced out after a nick was cut in the cauda epididymis. The isolated spermatozoa were suspended in human tube fluid (HTF: 5.9 g/l NaCl, 0.35 g/l KCl, 0.049 g/l $MgSO_4{\cdot}7H_2O$, 0.054 g/l KH_2PO_4, 0.57 g/l $CaCl_2$, 2.1 g/l $NaHCO_3$, 0.5 g/l glucose, 3.4 ml/l sodium lactate, 0.037 g/l sodium pyruvate, 0.075 g/l ampicillin, 0.05 g/l streptomycin, 4 g/l bovine serum albumin, and 0.002 g/l phenol red) for the subsequent treatments. The spermatozoa suspended in HTF were purified through swim-up in Earle's balanced salt solution (EBSS: 6.68 g/l NaCl, 0.4 g/l KCl, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 0.14 g/l KH_2PO_4, 0.2 g/l $CaCl_2$, 2.2 g/l $NaHCO_3$, and 0.01 g/l phenol red) containing 10% fetal bovine serum. The purified spermatozoa were precipitated through centrifugation at 1000 *g* and 4°C for 15 min, followed by three times wash with phosphate-buffered saline (PBS). Then, sperm samples were stored at −80°C for the subsequent 2D gel electrophoresis (2DE) and RNA extraction.

Human sample collection

The human study was approved by the Ethics Committee of Shanghai Jiaotong University. Donors signed consent forms before semen samples were obtained. This study included samples from 30 smoking and 30 non-smoking men collected in the Key Laboratory of Contraceptive Drugs and Devices of the National Population and Family Planning Commission of China. The age of the non-smoking group men ranged from 20 to 44 years, with the mean age of 30.1 years. The age of the smoking group men ranged from 28 to 44 years, with the mean age of 36.7 years. The smoking group men smoked for at least 12 years, smoking 20 cigarettes a day. Each sample was centrifuged to remove seminal plasma. After the 10 × loading buffer (RIPA lysis buffer) and normal saline was added, the sample was boiled for 5 min. Then, the samples were stored at −80°C until use for western blot analysis.

Evaluation of sperm motility and progressive motility

The cauda epididymis of the C57BL/6J mice was isolated and placed in EBSS that was pre-balanced at 37°C, with 5% CO_2 and 95% air. The cauda region was pierced using a surgical blade to allow the sperm to disperse into the medium for 10 min. The sperm suspension was transferred to a new micro-centrifuge tube for analysis using a computer-assisted semen analysis system (Hamilton Thorne CASA System, Beverly, USA). Ten microliters of sample was transferred into a 10-μm thick microcell and analyzed according to the operating manual. Progressive motility was defined as the percentage of sperm with an average path velocity ≥25 μm/s. For each sample, four fields with a mean total number of ∼1000 sperm cells were evaluated. For the capacitated spermatozoa analysis, bovine serum albumin was supplemented in the sperm suspension to a final concentration of 3 mg/ml. After 2 h of incubation at 37°C, with 5% CO_2 and 95% air, the motility and progressive motility of the capacitated sperm were tested.

2D electrophoresis

One milliliter of sample buffer [9.5 M urea, 4% CHAPS, 2% Dithiothreitol (DTT), 0.5% immobilized pH gradient (IPG) buffer, and protease inhibitors] was added into two sperm sample, and a homogenate was generated using Dounce tissue grinders. To break up the cells, the homogenate was sonicated at 80 W for 10 s ×5 with 15 s intervals. These steps were performed on ice. The mixture was then centrifuged at 20,000 *g* and 4°C for 45 min. The protein concentration in the supernatant was quantified by Bradford method using Bio-Rad protein assay reagent (Hercules, USA).

For 2D electrophoresis (2DE), 100 and 400 μg of proteins were loaded onto analytical and preparative gels, respectively. The Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences, Buckinghamshire, UK) and pH 3–10 IPG strips (13 cm, nonlinear; Amersham Biosciences) were used for isoelectric focusing (IEF). The IPG strips were rehydrated for 12 h in 250 μl of rehydration buffer with the protein samples. IEF was performed in four steps: 30 V for 12 h, 500 V for

1 h, 1000 V for 1 h, and 8000 V for 8 h. The gel strips were equilibrated for 15 min in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 1% DTT). This step was repeated using an identical buffer but with 4% iodoacetamide instead of 1% DTT. The strips were then used for 2DE after transfer into 12.5% SDS–polyacrylamide gels. Electrophoresis was performed using the Hofer SE 600 system (Amersham Biosciences) at 15 mA per gel for 30 min followed by 30 mA per gel until the bromophenol blue reached the end of the gel. Three replicate experiments were performed for each sample.

Image processing and analysis

Protein spots on the analytical gels were visualized through silver staining. The gels were stained using a modified silver staining method that was compatible with subsequent mass spectrometric analyses. The stained gels were scanned using UMax Powerlook 2110XL (UMax, Taiwan, China), and the image analysis was performed using Imagemaster 2D Platinum 6.0 (Amersham Biosciences). Each paired spot was manually verified to ensure high reproducibility between the normalized spot volumes in the gels produced from the triplicate data. The overlapping ratio was used to calculate the change in protein expression, and the proteins with a 1.5-fold or greater overlap ratio were considered differentially expressed.

MALDI-TOF–MS

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF–MS) was used to determine the protein composition for the protein spots of interest. The tryptic peptide samples were transported to Shanghai GeneCore BioTechnologies Co. Ltd (Shanghai, China) for MALDI-TOF–MS peptide mass fingerprint (PMF) analysis. The dry peptide samples were reconstituted in 2 μl of standard diluent (20:80 ACN:water) and spotted on a 384-well OptiTOF stainless steel plate. The samples were covered with 5 mg/ml cyano-4-hydroxycinnamic acid in 50% ACN and 0.1% trifluoroacetic acid before they were dried. The MS and MS/MS data for the protein identification were collected using a MALDI-TOF–TOF instrument (4800 proteomics analyzer; Applied Biosystems, Foster City, USA). The instrument parameters were established using the 4000 Series Explorer software (Applied Biosystems). The MS spectra were recorded in reflector mode with the mass range of 800–4000 and a 2000 focus mass. The MS experiments included a CalMix5 standard to calibrate the instrument (ABI 4700 Calibration Mixture). For one primary MS spectrum, 25 subspectra with 125 shots per subspectrum were accumulated using a random search pattern. For the MS calibration, the trypsin autolysis peaks ([M + H] + 842.5100 and 2211.1046) were used as internal calibrates. Up to 10 of the most intense ion signals were selected as precursors for MS/MS acquisition, excluding the trypsin autolysis peaks and matrix ion signals. For one primary MS spectrum, 50 subspectra with 50 shots per subspectrum were accumulated using a random search pattern in the MS/MS positive ion mode. The collision energy was 2 kV, the collision gas was air, and the default calibration was used with Glu1-Fibrino-peptide B ([M + H] + 1570.6696), which was spotted onto the Cal 7 positions of the MALDI target. The combined PMF and MS/MS queries were performed using the MASCOT search engine 2.2 (Matrix Science, Ltd, London, UK) embedded in the GPS-Explorer Software 3.6 (Applied Biosystems) on the International Protein Index (IPI) mouse v 3.87 database with the following parameters: 100 ppm mass accuracy, trypsin cleavage, one missed cleavage allowed, carbamidomethylation as a fixed modification, methionine oxidation allowed as variable modification, and 0.4 Da MS/MS fragment tolerance. The GPS-Explorer protein confidence index ≥95% was used for further manual validation.

Western blot analysis

The protein samples were isolated from the sperm, separated on a 10% SDS–polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer (5% skimmed milk powder in PBS) at room temperature for 1 h followed by incubation with the appropriate primary antibody at room temperature for 1 h or at 4°C overnight. Five antibodies for the selected proteins were used: Spatc1 Rabbit PolyAb (ABGENT, San Diego, USA; 1:500), Aldoa Rabbit PolyAb (Proteintech Group, San Diego, USA; 1:1500), Gpx4 Rabbit PolyAb (Proteintech Group; 1:1000), Cs Rabbit PolyAb (Proteintech Group; 1:2000), and ATP5a1 Mouse mAb (Epitomics, California, USA; 1:1000). GAPDH Rabbit mAb (Cell Signal Technology, Massachusetts, USA; 1:1000) was used to detect the internal reference GAPDH. After three times wash with PBS, the membranes were incubated with a secondary antibody (Goat anti Rabbit IgG-HRP, Maibio, Shanghai, China; 1:20,000 or Goat anti mouse IgG-HRP; 1:10,000) at room temperature for 1 h followed by three times wash with Tris-buffered saline with Tween 20. The bound HRP was detected using Millipore Immobilon Western Chemiluminescent HRP substrate (Billerica, USA) according to the manufacturer's instructions, and the final blot was exposed to X-ray film. The bands were equilibrated to the protein concentration in the samples and transformed into values using ImageJ (ImageJ, 1.44p, National Institutes of Health, Bethesda, USA).

Comparative proteomic analysis

The differentially expressed proteins in our datasets were identified and categorized based on their functions. We acquired the identified proteins' Gene-IDs and mRNA sequences in FASTA format from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The gene ontology (GO) annotations for the identified proteins were based on BLAST results from Blast2GO (www.blast2go.de). The pathway networks were determined after the differentially expressed protein list was uploaded to the Kyoto Encyclopedia of Genes and Genomes (KEGG) server (http://www.genome.jp/kegg/tool/map/pathway1.html).

Quantitative real-time polymerase chain reaction

The RNA samples were isolated from the sperm by Trizol reagent (Invitrogen, Carlsbad, USA), followed by an RNase-free DNase I

Table 1. Primers used in real-time PCR

Gene	Forward primer	Reverse primer	Amplicon length (bp)
Gpx4	GGAGCCAGGAAGTAATCAAG	CGCAGCCGTTCTTATCAATG	203
Cs	AGACTGACCCTCGCTATTCC	CAGGACTGTGTAGTAGTTCATC	215
Aldoa	CCCAGGAGGAGTACATCAAG	GGCATGGTTAGAGATGAAGAG	119
Spatc1	CCCACCGAAAGTCTAACAAG	GGACAGGATTCTTCGGTCTA	110
ATP5a1	CTGCCACTCAACAGCTCTTG	GGCTGATAACGTGAGACAAG	123

(TaKaRa, Dalian, China) treatment to remove the contaminating genomic DNA, and the samples were reverse transcribed into cDNA using Fermentas RevertAid reverse transcriptase (Fermentas, Burlington, USA; EP0441). The cDNA concentration was determined using a Spectrumlab 752S UV spectrophotometer and adjusted to ~100 ng/l. Quantitative real-time polymerase chain reaction (PCR) was performed using Bestar real-time PCR mastermix (SybrGreen) (DBI Bioscience, Ludwigshafen, German) and the ABI PRISM 7500 system (Applied Biosystems) according to the manufacturer's protocols. The primers were designed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/) and provided by Invitrogen (Table 1). The transcript levels were normalized to the corresponding *GAPDH* gene transcript levels.

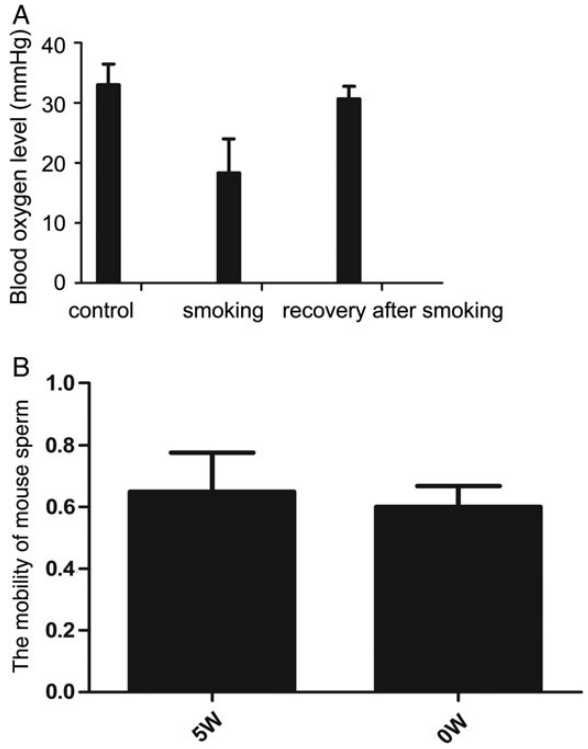

Figure 1. Blood oxygen levels of mice and the mobility of mice sperm (A) A histogram of the blood gas of smoking mouse ($n = 5$). (B) A histogram of the progressive motility of sperm from the control and 5-week treated groups ($n = 3$).

Statistical analysis

Statistical analyses were performed using the Mann–Whitney *U* test. A significant difference is indicated by '*' ($P < 0.05$). An extremely significant difference is indicated by '**' ($P < 0.01$).

Results

Oxygen levels in the smoking mice and the smoking mice sperm

We aimed to verify whether cigarette smoking changes mouse sperm mobility. Thus, sperm motility in cigarette-smoking and non-smoking mice was tested. However, the results showed no statistically significant difference (**Fig. 1**).

Protein profile changes due to cigarette smoke exposure in mouse sperm

To investigate the sperm protein profile changes due to cigarette smoking, soluble proteins from the smoking and control group samples were compared using the 2DE technique. Over 1000 spots were matched from three analytical gels and one preparative gel. The 2DE gel analyses yielded 38 spots with a statistically significant (>2 folds) change between the two groups (**Fig. 2**).

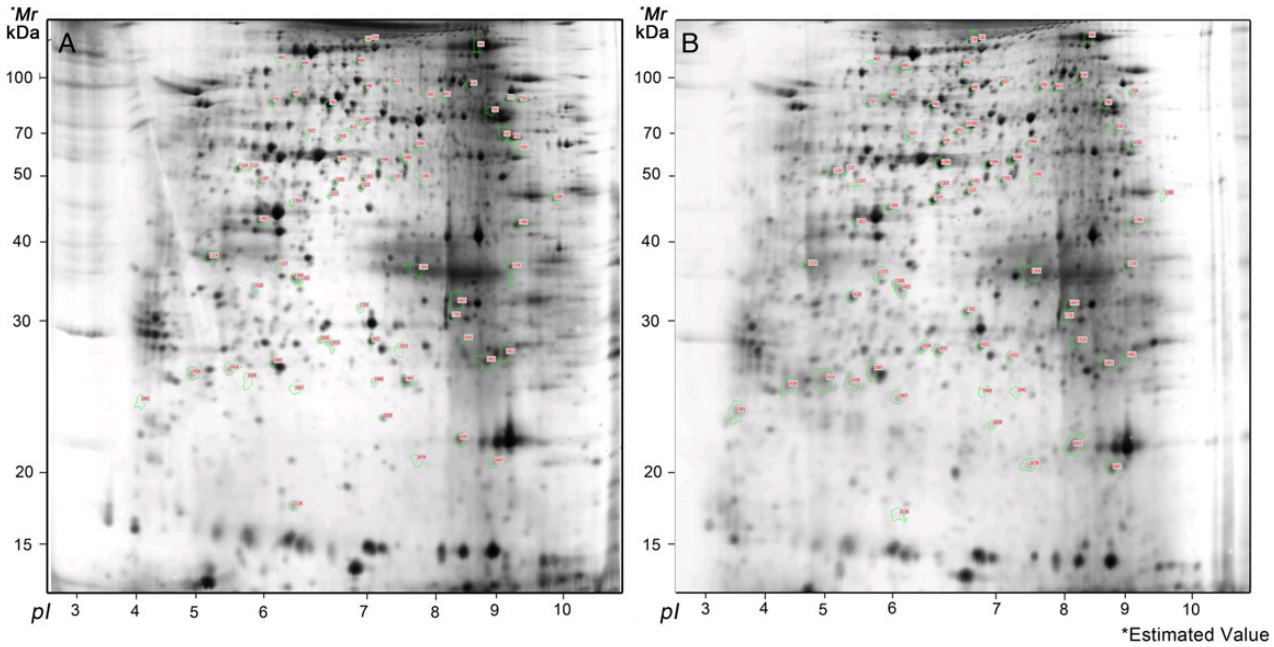

Figure 2. A comparison of the 2DE maps for the total proteins extracted from the mice sperm (A) Proteins extracted from the control group sperm. (B) Proteins extracted from the treated group sperm. The numbered spots were significantly increased or decreased in the treated group compared with the control group. $n = 3$.

Table 2. Proteins with significantly altered expression in the mice sperm samples from the smoking group and control group

No.	Spot name	Accession number	Identified protein name	Unique peptides detected	Sequence coverage (%)[a]	Mascot score	Protein score CI%	Function[b]	Alteration
1	786	IPI00221402	Aldoa (Fructose-bisphosphate aldolase A)	5	49	676	100	Plays a key role in glycolysis and gluconeogenesis. In addition, may also function as scaffolding protein	Up
2	1862	IPI00121288	Ndufb10 (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10)	2	69	246	100	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), that is believed not to be involved in catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone	Down
3	1730	IPI00473667	Tuba3b (Tubulin alpha-3 chain)	9	27	288	100	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain	Down
4	952	IPI00113141	Cs (Citrate synthase, mitochondrial)	6	26	484	100	Acetyl-CoA + H_2O + oxaloacetate = citrate + CoA	Down
5	930	IPI00118594	Pdha2 (Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial)	3	40	203	100	The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA and CO_2, and thereby links the glycolytic pathway to the tricarboxylic cycle	Down
6	1892	IPI00648105	Prdx1 (Peroxiredoxin-1)	0	25	98	100	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system but not from glutaredoxin. May play an important role in eliminating peroxides generated during metabolism. Might participate in the signaling cascades of growth factors and tumor necrosis factor-alpha by regulating the intracellular concentrations of H_2O_2. Regulates GDPD5 function by reducing an intramolecular disulfide bond	Up
7	1288/1853	IPI00857439	ATP5a1 (ATP synthase subunit alpha, mitochondrial)	2/2	21/23	194/85	100/99.994	Mitochondrial membrane ATP synthase (F1F0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F1—containing the extramembraneous catalytic core, and F0—containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F1. Rotation of the central stalk against the surrounding alpha3beta3 subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits. Subunit alpha does not bear the catalytic high-affinity ATP-binding sites	Down

Continued

Table 2. Continued

No.	Spot name	Accession number	Identified protein name	Unique peptides detected	Sequence coverage (%)[a]	Mascot score	Protein score CI%	Function[b]	Alteration
8	1598	IPI00551191	Cyb5d1 (Cytochrome b5 domain-containing protein 1)	3	19	211	100	Belongs to the cytochrome b5 family	Down
9	717	IPI00421017	Spatc1 (Speriolin)	8	35	697	100	Found in a complex with CDC20, CDC27, and TUBG1. Interacts with CDC20	Down
10	2043	IPI00187284	1700065I17Rik (1700065I17Rik)	10	63	519	100	Probably involved in redox regulation of the cell. Regulates the activation of NF-kappa-B in the cytosol by a modulation of I-kappa-B-alpha phosphorylation	Up
11	875	IPI00308885	Hspd1 (60 kDa heat shock protein, mitochondrial)	0	26	72	99.897	Implicated in mitochondrial protein import and macromolecular assembly. May facilitate the correct folding of imported proteins. May also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix	Up
12	1895	IPI00128209	Ak1 (Adenylate kinase isoenzyme 1)	5	59	384	100	Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Also possesses broad nucleoside diphosphate kinase activity. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism	Up
13	693	IPI00331182	Gpd2 (Glycerol-3-phosphate dehydrogenase, mitochondrial)	0	13	70	99.841	sn-glycerol 3-phosphate + a quinone = glycerone phosphate + a quinol	Down
14	1826	IPI00117281	Gpx4 (Phospholipid hydroperoxide glutathione peroxidase, mitochondrial)	6	38	149	100	Protects cells against membrane lipid peroxidation and cell death. Isoform mitochondrial is required for normal sperm development and male fertility. Could play a major role in protecting mammals from the toxicity of ingested lipid hydroperoxides. Essential for embryonic development. Protects from radiation and oxidative damage	Down
15	1030	IPI00323233	Smcp (Sperm mitochondrial-associated cysteine-rich protein)	1	13	62	98.951	Involved in sperm motility. Its absence is associated with genetic background dependent male infertility. Infertility may be due to reduced sperm motility in the female reproductive tract and inability to penetrate the oocyte zona pellucida	Down
16	1564	IPI00230559	Odf1 (Outer dense fiber protein 1)	3	44	448	100	Component of the ODFs of spermatozoa. ODFs are filamentous structures located on the outside of the axoneme in the midpiece and principal piece of the mammalian sperm tail and may help to maintain the passive elastic structures and elastic recoil of the sperm tail	Up
17	1183/486/623/1125/1124/735/1620/1824	IPI00856846	Odf2 (Outer dense fiber protein 2)	6/4/0/3/5/1/2	29/19/17/22/22/11/13	542/254/79/227/412/77/160	100/100/99.977/100/100/99.967/100	Seems to be a major component of sperm tail ODFs	Down

Continued

Table 2. Continued

No.	Spot name	Accession number	Identified protein name	Unique peptides detected	Sequence coverage (%)[a]	Mascot score	Protein score CI%	Function[b]	Alteration
18	912/1306/1981	IPI00169463	Tubb2c (Tubulin beta-4B chain)	0/4/2	16/19/34	67/235/149	99.661/100/100	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain	Up
19	862	IPI00990455	LOC100503183 (Glutathione S-transferase Mu 2)	5	39	321	100	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles	Up
20	1186	IPI00380903	Spata22 (Spermatogenesis-associated protein 22)	0	0	36	0	Probably plays a role in facilitating the assembly of multimeric protein complexes inside the ER and mass spectrometry	Down
21	465	IPI01008515	Nrap (Nebulin-related-anchoring protein)	0	0	54	93.532	May be involved in anchoring the terminal actin filaments in the myofibril to the membrane and in transmitting tension from the myofibrils to the extracellular matrix	Up
22	700	IPI00338762	Sult1c2 (Sulfotransferase 1C2)	0	36	62	98.975	Sulfotransferase that utilizes 3′-phospho-5′-adenylyl sulfate as sulfonate donor to catalyze the sulfate conjugation of drugs, xenobiotic compounds, hormones, and neurotransmitters	Up

[a]The percentage coverage was defined as the ratio (%) of the protein sequence covered by the matched peptides.
[b]The functions are classified according to published literatures and Swiss Prot database (http://www.uniprot.org/).

To identify the proteins with altered expression upon exposure to cigarette smoke, 41 spots were selected for MS analyses, and 22 proteins were successful identified using MS based on the Mascot score and protein score confidence interval (Table 2). Among the 22 proteins identified, 10 proteins were up-regulated, and 12 were down-regulated. The up-regulated proteins included Aldoa, Odf1, Hspd1, Ak1, Tubb2c, Nrap, Prdx1, Sult12c, 1700065I17Rik, and LOC100503183. The down-regulated proteins included ATP5a1, Spata22, Gpd2, Smcp, Gpx4, Cs, Spatc1, Odf2, Ndufb10, Tuba3b, Pdha2, and Cyd5d1. Because one spot may include various proteins, these results were further verified by western blot analysis.

Ontology analysis of the identified proteins

To classify the proteins from the MS experiments, GO annotation was performed; each identified protein was classified based on a GO functional annotation. The combined graphics show that the ontology analysis results were created using Blast2Go V. 2.6.0. Because one protein could be classified with multiple terms, the total counts may exceed 22. These differentially expressed proteins were found to be primarily involved in cellular processes (nine proteins), metabolic processes (eight proteins), developmental processes (six proteins), signaling (six proteins), biological regulation (six proteins), multicellular organismal process (six proteins), and localization (five proteins) (**Fig. 3A**). In terms of 'molecular function', these differentially expressed proteins were enriched in binding (nine proteins) and catalytic activity functions (seven proteins) (**Fig. 3B**).

Pathway analyses for the identified proteins

To better understand the identified proteins' global network functions, we submitted the protein Gene_IDs to KEGG server, and two of the proteins were found to be involved in glycolysis (Aldoa and LOC100503183), which is the metabolic pathway that converts glucose into pyruvate (**Fig. 4A**). Two proteins were involved in the TCA cycle (Pdha2 and Cs), which involves an important series of enzyme-catalyzed chemical reactions in living cells that use oxygen for cellular respiration (**Fig. 4B**).

Western blot analysis of mouse proteins

Five proteins (Aldoa, ATP5a1, Gpx4, Cs, and Spatc1) were selected to validate their 2DE and MS results, by western blot analysis, with GAPDH as an internal reference. These proteins were clearly detected (**Fig. 5A**). Aldoa was up-regulated, whereas ATP5a1, Gpx4, Cs, and Spatc1 were down-regulated. Except Spatc1, the other four proteins examined showed statistically significant different levels between the smoking group and the control group ($P < 0.05$) (**Fig. 5B**). This result is in agreement with the 2DE and MS analysis results.

Real-time PCR results of mouse mRNAs

Real-time PCR was also performed to determine whether the mRNA levels for each identified protein were also altered by cigarette smoking. Down-regulation was clearly seen in all the mRNAs tested (**Fig. 6**), suggesting that the expressions of three genes (*Aldoa*, *Gpx4*, and *Spatc1*) were significantly different ($P < 0.05$) at transcription level between the smoking group and the control group. However, the result of Aloda was inconsistent with its 2DE and MS results

Effect of cigarette smoking in human subjects

Cigarette smoking altered mouse sperm protein profiles, but whether it could produce a similar effect in human sperm is still unknown.

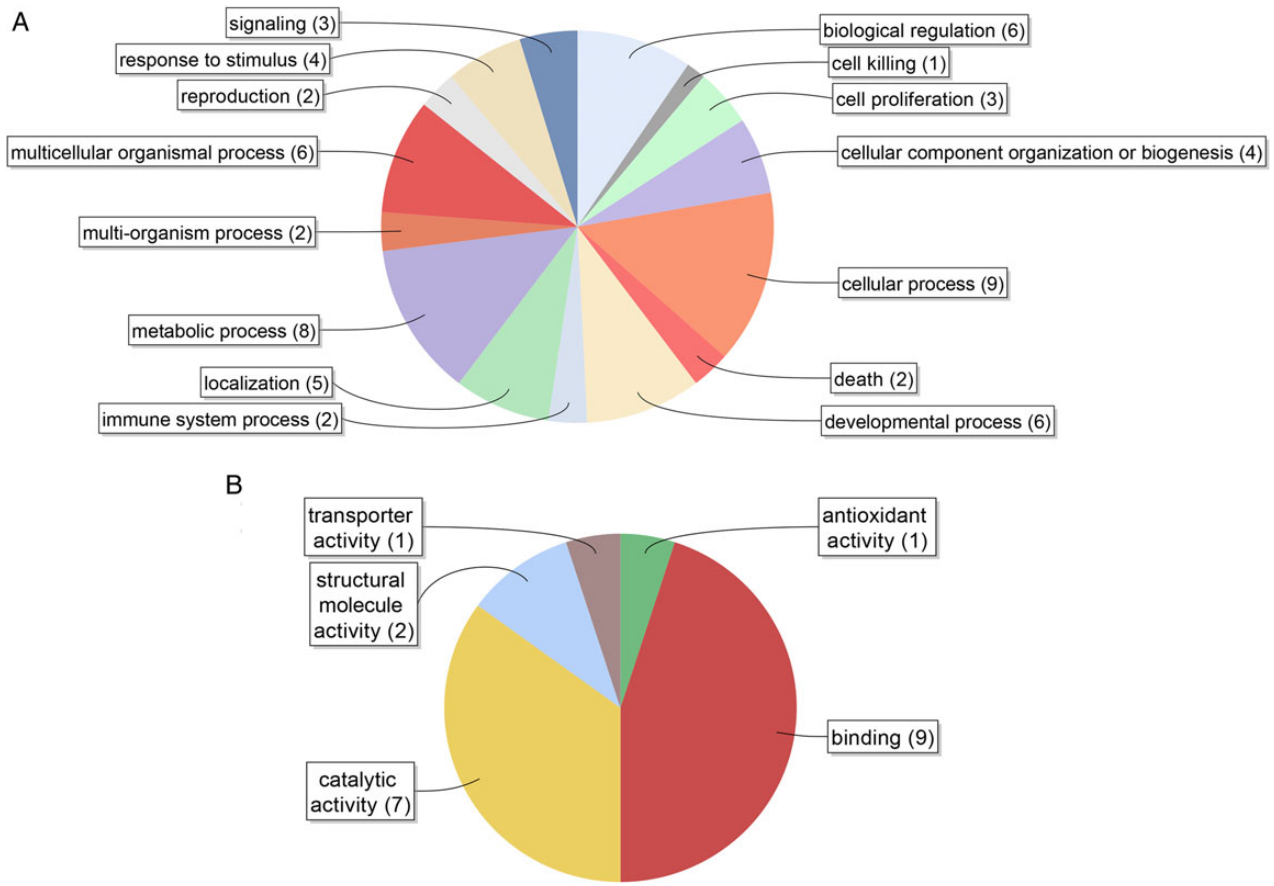

Figure 3. GO distribution of differentially expressed proteins extracted from the treated group and control group sperm (A) A pie chart of differentially expressed proteins that were classified based on major biological process categories. (B) A pie chart of differentially expressed proteins that were classified based on major molecular function categories.

Thus, human samples from 30 male smokers who smoke 20 cigarettes per day and had smoked for at least 12 years with the mean age 36.7 years, as well as samples from 30 non-smokers with the mean age 30.1 years were collected. These five proteins were also clearly detected by western blot analysis (**Fig. 7A**). All protein levels were lower in samples from the smoking group when compared with the control group. However, only the Aldoa and Cs levels showed a statistically significant difference between the smoking group and the control group ($P < 0.05$) (**Fig. 7B**).

Discussion

The 2DE results demonstrated that cigarette smoke exposure can alter the mouse sperm protein profile. Twenty-two altered proteins were identified using MS analysis. Five proteins (Aldoa, ATP5a1, Gpx4, Cs, and Spatc1) were selected to validate their 2DE and MS results. The western blot analysis results showed a trend similar to the 2DE and MS analyses. Real-time PCR was performed to determine whether there are any changes in the transcription level of the genes for each identified protein. The real-time PCR results were not consistent with the western blot results, suggesting that these genes might have post-transcriptional regulation. And this post-transcriptional regulation could change the expression level of each identified protein. The real-time PCR result may not really reflect the expression level of each identified protein. So western blot analysis was used to validate the 2DE and MS results, and results indicated that the 2DE and MS results were reliable.

These proteins were clustered into groups based on their functions and were enrichment in certain groups. Through pathway analyses, we demonstrated that the identified proteins were primarily grouped into the group of energy metabolism function.

Smoking may induce oxidative stress. After cigarette smoke exposure, Prdx1 expression is altered. Prdx (peroxiredoxin) is a multifunctional redox protein with thioredoxin-dependent peroxidase activity. One study has shown that if mice lack the Prdx1, they produce more cellular ROS, which suggests that Prdx1 expression is an essential antioxidant in sperm [15]. The up-regulated Prdx1 expression may indicate an oxidative stress response.

GPx4 is an intracellular antioxidant enzyme that directly reduces peroxidized phospholipids. GPx4 is strongly expressed in testis and spermatozoa mitochondria. Imai *et al.* [16] found that spermatocyte-specific GPx4 knock-out male mice were infertile despite normal plug formation after mating, and they displayed significantly lower spermatozoa levels. The down-regulated Gpx4 expression might also cause sperm to produce more ROS and indicate an oxidative stress response.

The GO analysis showed that certain altered proteins were related to structural molecule activity; these proteins included Odf2, Tuba3b, and Spatc1, which showed down-regulated expression in smoking mice. This result suggests reduced cell growth. Odf2 is a major protein

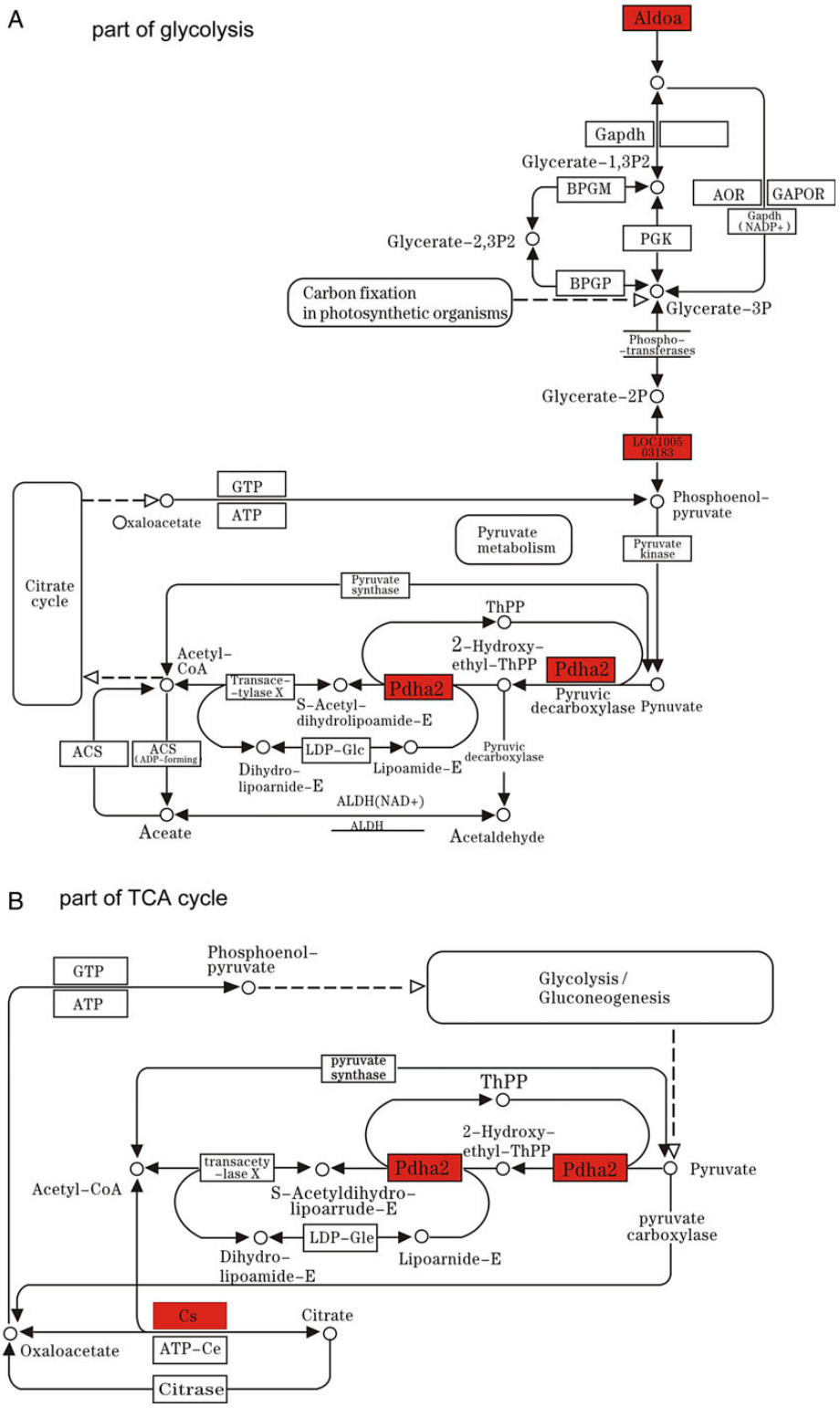

Figure 4. Pathways involved in this study (A) 'Glycolysis' pathway map modified from the KEGG pathway map. The red events indicate the location of the proteins identified herein. (B) 'TCA cycle' pathway map modified from the KEGG pathway map. The red events indicate the location of the proteins identified herein.

in the outer dense fibers (ODFs) of the sperm tail and is involved in sperm motility. The reduced protein expression indicates impaired sperm motility. Spatc1 (Speriolin) is a novel centrosomal protein in the connecting piece region of mouse and human sperm that is transmitted to the mouse zygote and can be detected throughout the first mitotic division [17]. Although the exact function of Spatc1 in sperm is not known, it is very important because the down-regulated expression of Spatc1 reduces the sperm fertility.

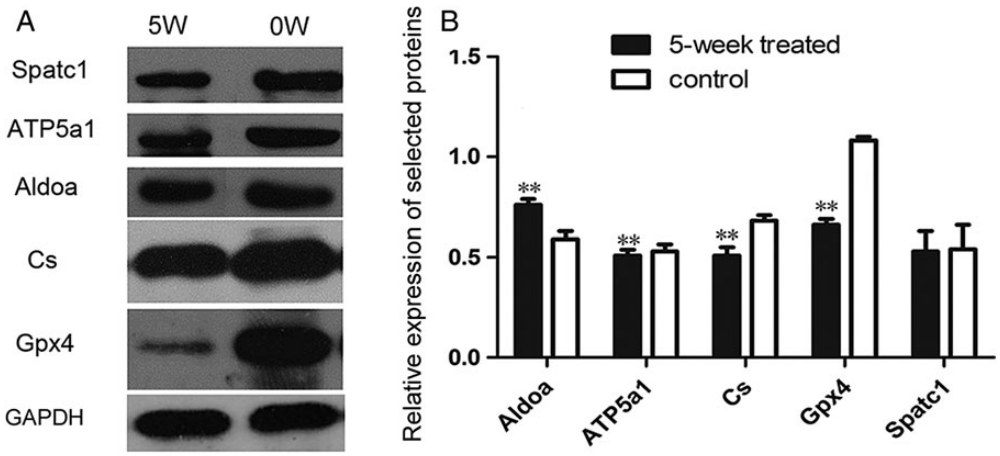

Figure 5. Western blot analysis of selected protein in mice sperm samples from the control group and smoking group (A) Western blots of samples from the control group ($n = 3$) and 5-week treated group ($n = 3$). (B) A histogram of the western blot analysis for the control group and 5-week treated group. The bars represent the mean ± SD from three individual experiments ($n = 3$). $^{**}P < 0.01$.

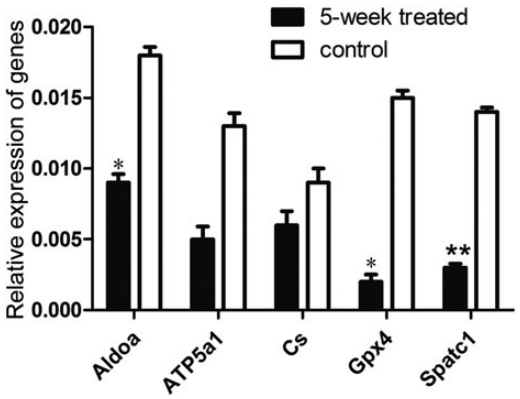

Figure 6. Real-time PCR analysis of the differentially expressed protein transcripts The quantity of each transcript is shown as a 'relative quantity', which represents the ratio of a specific transcript to an internal reference. $^{*}P < 0.05$ and $^{**}P < 0.01$, $n = 3$.

Based on the GO analysis, two proteins, pyruvate dehydrogenase α2 (Pdha2) and glycerol-3-phosphate dehydrogenase 2 (Gpd2), are related to reproduction. They were down-regulated by smoke exposure. Pdha2 is a component of the pyruvate dehydrogenase complex, which is expressed in post-meiotic spermatogenic cells. One study has shown that Pdha2 exhibits capacitation-associated protein tyrosine phosphorylation; thus, Pdha2 may be associated with the capacitation process [18], but the Pdha2 function during capacitation is still unclear. The MALDI-TOF–MS results showed that Pdha2 expression was down-regulated by cigarette smoke exposure, which indicated that cigarette smoking is harmful to sperm capacitation. Gpd2 is only phosphorylated in capacitated hamster spermatozoa and is non-canonically localized in the acrosome and principal piece in human, mouse, rat, and hamster spermatozoa. However, it is localized in the somatic cell mitochondria. This non-canonical localization may imply a role for Gpd2 in acrosome reaction and hyperactivation. In addition, the enzymatic activity of Gpd2 during capacitation is positively correlated with hyperactivation and the acrosome reaction, which indicates

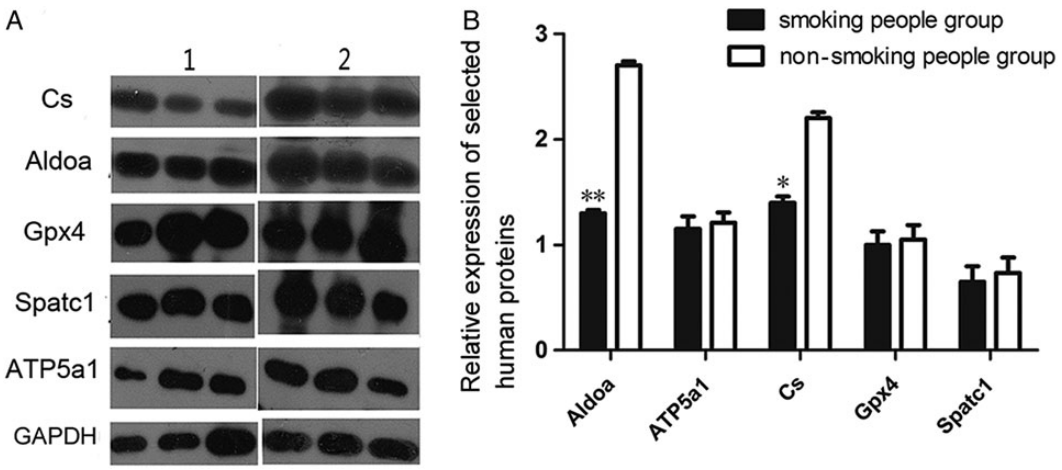

Figure 7. Western blot analysis of selected protein in human sperm samples from the control group and smoking group (A) Western blots for the sample human non-smoking and smoking groups ($n = 3$). (B) A histogram of the western blot results for the human non-smoking and smoking groups. The bars represent the mean ± SD from three individual experiments ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$.

that Gpd2 may be required for sperm capacitation [19]. The altered expression of such proteins may indicate that sperm reproductive function is disturbed.

Ak1 is associated with sperm outer microtubular doublets and ODFs. It is one part of the mouse sperm flagellum accessory structures. This localization would allow the interconversion of ATP and ADP between the fibrous sheath, where ATP is produced by glycolysis and the axonemal dynein ATPases Ak1, which is in the flagellar accessory structures, provides a buffering mechanism for the adenylate energy charge for sperm motility [20]. The up-regulated Ak1 expression also indicates a response to the low oxygen partial pressure and glycolysis.

Atp5a1 is an ATP synthase subunit, and the reduced expression level of this protein will disrupt energy generation. In addition to the inhibited aerobic respiration, the reduced Atp5a expression will reduce the ATP supply, which impairs sperm function and motility because they require adequate energy. In this study, ATP5a1 expression was found to be down-regulated the sperm of smoke-exposed mice.

Smcp is a cysteine- and proline-rich structural protein that is closely associated with the sperm mitochondria keratinous capsules in the mitochondrial sheath surrounding the ODFs and axoneme. One study showed that Smcp-deficient spermatozoa bind the oocyte, but the number of fertilized eggs is reduced by more than 3 folds relative to the wild-type control [21]. The result herein showed that Smcp expression was down-regulated. In addition to inhibiting aerobic respiration, lower Smcp expression may cause mitochondrial dysfunction and a reduced ATP supply, which impairs sperm motility because such functions require adequate energy.

After exposure to cigarette smoke, Aldoa, which is an important protein for glycolysis, was up-regulated in mouse sperm. Aldoa is a glycolysis enzyme that promotes reversible FBP cleavage to triose phosphate, G-3-P and DHAP. Aloda is in the mouse sperm flagellum accessory structures [22,23]. The flagellum is an important organ for sperm mobility. Thus, the energy pathway can affect sperm mobility in flagellum. Previous studies showed that spermatocytes and spermatids prefer oxidative phosphorylation for ATP production [22]. However, the oxygen partial pressure was found to be very low in this study. We speculated that sperm may switch to glycolysis for ATP production. Thus, the up-regulated Aldoa expression indicates a response to such conditions.

Cs expression was also altered by cigarette smoking in mouse sperm. Cs is the first and rate-limiting enzyme in the tricarboxylic acid cycle and plays a key role in regulating energy production for mitochondrial respiration. Cs is a reliable mitochondrial volume marker [24], and several reports have speculated that mitochondrial dysfunction may be a factor for infertility [25–27]. Certain evidence suggested that mitochondria might play a key role in spermatozoa motility energy maintenance, which is a major male fertility determinant [28]. Furthermore, one study suggested that spermatozoa motility largely depends on the sperm midpiece mitochondrial volume [24]. Thus, the down-regulated Cs expression can alter mitochondrial volume and spermatozoa motility.

Five proteins were also detected in human sperm and results showed a trend similar to that in the mouse sperm. Of the five proteins we detected in human samples, only Aldoa and Cs showed a statistically significant difference between the smoking group and the control group. These two proteins are also related to energy metabolism.

However, the expression of Aldoa was down-regulated in the human sperm from smoking men, which was different from the results in mouse sperm. We speculate that different conditions yielded the different results. We measured the oxygen content when the mouse was treated with cigarette smoke exposure, and the results showed that the PO2 was lower. However, the human smoking group did not have low oxygen partial pressure. The different PO2 may have yielded the different Aldoa expression in sperm. Wang *et al.* [29] found that the Aloda levels were lower in sperm from asthenozoospermia patients, which suggests that Aldoa is important for human sperm quality.

The Cs expression was also found to be down-regulated by cigarette smoking. As discussed above, Cs is important for sperm mobility. The change in Cs expression will alter the mitochondrial volume and spermatozoa motility.

In summary, we show that daily cigarette smoke exposure alters the mouse sperm protein expression profile. Many of the affected proteins have functions related to catalytic activity and associated with energy production in certain pathways. Altered expression of such proteins may lower the ATP supply and impair sperm function and motility. We also checked five proteins in human samples, and found that two proteins (Aldoa and Cs) were significantly altered, which are also associated with energy production. The results in human sample are still preliminary and should be confirmed by larger sample study. Nevertheless, these proteins may be potential diagnostic markers, and further studies on these proteins may help better understand the pathogenic mechanisms underlying infertility in smokers.

Funding

This work was supported by the grants from Shanghai Jiao Tong University Medical Engineering Joint Project (No. YG2012 MS56), the National Natural Science Foundation of China (No. J1210047) and the Specialized Research Fund for the Doctoral Program of Higher Education of China (No. 20130073110061).

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