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Identification of differentially expressed proteins in fresh and frozen–thawed boar spermatozoa by iTRAQ-coupled 2D LC–MS/MS

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Abstract

Cryodamage is a major problem in semen cryopreservation, causing changes in the levels of proteins that influence the function and motility of spermatozoa. In this study, protein samples prepared from fresh and frozen–thawed boar spermatozoa were compared using the isobaric tags for relative and absolute quantification (iTRAQ) labeling technique coupled to 2D LC–MS/MS analysis. A total of 41 differentially expressed proteins were identified and quantified, including 35 proteins that were present at higher levels and six proteins that were present at lower levels in frozen–thawed spermatozoa by at least a mean of 1.79-fold ($P<0.05$). On classifying into ten distinct categories using bioinformatic analysis, most of the 41 differentially expressed proteins were found to be closely relevant to sperm premature capacitation, adhesions, energy supply, and sperm–oocyte binding and fusion. The expression of four of these proteins, SOD1, TPI1, ODF2, and AKAP3, was verified by western blot analysis. We propose that alterations in these identified proteins affect the quality of cryopreserved semen and ultimately lower its fertilizing capacity. This is the first study to compare protein levels in fresh and frozen–thawed spermatozoa using the iTRAQ technology. Our preliminary results provide an overview of the molecular mechanisms of cryodamage in frozen–thawed spermatozoa and theoretical guidance to improve the cryopreservation of boar semen.
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Introduction

Freezing semen has become an indispensable technique for breeding livestock in the cattle industry (Pons-Rejraji *et al*. 2009). However, farrowing rates decrease by 20–30% and litter size drops by two to three piglets when using cryopreserved boar semen. Because of the sublethal damage, ∼40–50% of the sperm do not survive cryopreservation, and even when using similar numbers of motile sperm, fertility is still lower after thawing compared with that of sperm in fresh semen (Watson 2000). As a result, cryopreserved semen is not routinely used in the pig industry (Bailey *et al*. 2008). To improve cryopreservation technology for boar semen, many studies have focused on understanding the mechanism underlying the cryodamage. It has been shown that the most evident damages are to the plasma membrane, acrosome, mitochondrial sheath (mid-piece), and axonema after freezing and thawing (Cerolini *et al*. 2001), and studies on the proteins of cryopreserved sperm have revealed profound implications for fertility and embryo development (Oliva *et al*. 2009). Cryopreservation changes the functional state of many proteins, such as enzymes related to sperm metabolism (Huang *et al*. 1999), proteins related to capacitation and acrosome reaction (Tabuchi *et al*. 2008), proteins related to membrane and structure (Desrosiers *et al*. 2006), and proteins related to apoptosis (Jeong *et al*. 2009). All these variations influence the structural integrity, biological processes, and function of sperm, ultimately reducing their fertilization capacity.

To explore the mechanism of cryodamage, two-dimensional gel electrophoresis has been used to detect changes in proteins in sperm, and many differentially expressed proteins have been found in human sperm (Zhang & Xiong 2013), sea bass sperm (Zilli *et al*. 2005),

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and sheep sperm (Li *et al*. 2011) after cryopreservation. However, differentially expressed proteins have not been identified in these studies. The proteome profiles of 'good' and 'poor' boar sperm after freezing have also been compared using LC–MS/MS analysis, which indicated that boar spermatozoa contain large amounts of proteins whose susceptibility to cryopreservation and implications for sperm function are yet to be characterized (Feugang *et al*. 2011). In current proteomics research, the method used for the quantification of proteins has developed into a combination of isobaric tags for relative and absolute quantification (iTRAQ) and LC–MS/MS (Niu *et al*. 2009). The multiplexing capability allows different protein samples to be simultaneously quantified with a control standard sample in the same run (Tannu & Hemby 2006). In the present study, the iTRAQ-coupled 2D LC–MS/MS approach was utilized for the first time to examine changes in the proteins of fresh and frozen–thawed sperm on a global scale to understand the primary mechanism and process of cryodamage in spermatozoa. Overall, we aimed to provide a foundation for the optimization and improvement of cryopreservation technology.

Materials and Methods

Collection and pre-treatment of semen

In this study, animal care and sample collection procedures were approved and conducted under established standards of the Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China. Semen samples were randomly and normally collected from six healthy Yorkshire stud boars (age: 2–3 years, farrowing rate: 80–90%, and litter size: 11–13) from the Beijing HotBoar Swine AI Service Centre. The sperm-rich fraction of each ejaculate was manually collected using the gloved-hand method. After collection, the semen samples were immediately filtered through a 100 μm semen filter paper to remove gelatinous material and diluted at a ratio of 1:1 (v:v) at room temperature. The diluted semen samples were transported to our laboratory at 25–30°C in <1 h for quality assessment and subsequent analysis.

Sperm quality analyses were carried out using microscopy to ensure the quality of ejaculates (motility $>80\%$ and deformation ratio $<15\%$). The semen samples of each animal were divided into two fractions, one of which was pooled with equal numbers of sperm from all animals as the fresh semen sample pool and the other fraction was used for the cryopreservation analysis. The semen samples were cryopreserved using Pursel's method (Pursel & Johnson 1975) and modified by Purdy's method (Purdy 2008). After storage of samples for at least 10 h in liquid nitrogen, 0.25 ml of frozen semen straws of each animal was thawed for 20 s in 37°C water. Quality assessment of frozen semen was carried out to ensure that the motility in each straw was more than 30%, and then the validated semen samples were pooled with an equal number of sperm for each animal.

Protein extraction

Before protein extraction, the fresh and frozen semen sample pools were purified by centrifugation through a Percoll density gradient (Naaby-Hansen *et al*. 1997). After washing three times with PBS, the sperm pellets were resuspended in PBS at the same sperm concentration as before protein extraction. From 100 μl of each of the resuspended fresh and frozen semen sample pools, total proteins were extracted using the cold-acetone method. Each of the samples was incubated at −20°C for 2 h after addition of 20 ml of 10% trichloroacetic acid in acetone and then centrifuged at 35 000 ***g*** for 15 min at 4°C. The supernatant was discarded without disturbing the white pellet. To reduce acidity, the pellet was washed with 20 ml acetone and centrifuged again at 35 000 ***g*** for 15 min at 4°C. The wash step with acetone was repeated three times. Each precipitate was dried in a vacuum concentrator for 5 min. The dried pellet (50 mg) was lysed with 1 ml protein extraction reagent (8 M urea, 4% (w/v) CHAPS, and 20 mM HEPES (pH 8.5)), and then 10 μl of 1 mM phenylmethylsulphonyl fluoride, 10 μl of 2 mM EDTA, and 10 μl of 10 mM dithiothreitol were added. After dissolving for 1 h, the solution was centrifuged at 40 000 ***g*** for 15 min at 4°C to remove non-soluble impurities.

iTRAQ labeling

Cysteines in all the denatured protein samples were blocked as described in the iTRAQ protocol (Applied Biosystems). Proteins were precipitated using the cold-acetone method again and then dissolved in 50% TEAB (Sigma) with 1% SDS. The protein content of the samples was determined using the bicinchoninic acid protein assay, and the samples were stored at −80°C for future use. Each protein sample (100 μg) was then digested with 5 μg sequencing grade modified trypsin (Promega) at 37°C for 36 h. The digested samples were dried in a centrifugal vacuum concentrator. The protein pellets were dissolved in 30 μl of 50% TEAB with 70 μl isopropanol and labeled with iTRAQ reagents according to the protocol of 8Plex iTRAQ labeling kit (Applied Biosystems). The iTRAQ-labeled peptides were analyzed using MALDI–TOF/TOF–MS/MS to ensure complete digestion. During labeling, iTRAQ tags 117 and 119 were added to the digested protein samples of the fresh and frozen–thawed spermatozoa respectively. The iTRAQ-labeled samples were then pooled and subjected to strong cation exchange (SCX) fractionation.

SCX fractionation

The labeled samples were fractionated using a HPLC system (Shimadzu, Kyoto, Japan) connected to an SCX column (Luna 5u column, 4.6 mm I.D.×250 mm, 5 μm, 100 Å; Phenomenex, Torrence, CA, USA). The retained peptides were eluted using buffer A (10 mM KH_2PO_4 in 25% ACN, pH 3.0) and buffer B (2 M KCl and 10 mM KH_2PO_4 in 25% ACN, pH 3.0), and fractions were collected in 1.5 ml microfuge tubes. Flow rate was set at 1 ml/min for 61 min. The following gradient was applied: 30 min with 100% buffer A; increasing to 30–31 min with 5% buffer B; increasing to 31–46 min with 30% buffer B; increasing to 46–51 min with 50% buffer B, maintained for 4 min; decreasing to

55–60 min with 0% buffer B and maintained for 1 min to remove the excess reagent. All solutions used were freshly prepared and filtered through a 0.22 μm filtration membrane. Fraction collection started 31 min following injection and continued to be carried out every 1 min to obtain a total of 38 fractions. For high-salt concentration fractions, an additional step was used to desalt fractions using a Strata-X 33u Polymeric Reversed Phase Column (Phenomenex). The resulting fractions were dried in a vacuum concentrator, and each fraction was redissolved in 0.1% formic acid solution before reversed-phase nLC–tandem mass spectrometry.

Reversed-phase nanoliquid chromatography–tandem MS (LC–MS/MS)

We equalized the amount of peptides of each fraction before injection into the NanoLC System. For analysis using MALDI–TOF/TOF, the SCX peptide fractions were pooled to obtain 17 fractions. From each fraction, a 10 μl portion was injected twice using the Proxeon Easy NanoLC System (Odense, Denmark). Peptides were separated on a C18 analytical reversed-phase column from Agela (75 μm I.D.×100 mm, 300 Å, 5 μm particle size, Wilmington, DE, USA) at a flow rate of 300 nl/min solvent (solution A: 5% acetonitrile and 0.1% formic acid; solution B: 95% acetonitrile and 0.1% formic acid) in 120 min.

A linear LC gradient profile was used to elute peptides from the column. The gradient started with 5% solution B. After equilibration in 5% solution B, a multi-slope gradient started 10 min after the injection signal as follows: 45% solution B at 80 min, 80% solution B at 85 min, maintained for 15 min, and 5% solution B at 105 min and held for 15 min.

The fractions were analyzed using a hybrid quadrupole/time-of-flight MS (MicroTOF-Q II, Bruker Daltonics, Bremen, Germany) with a nanoelectrospray ion source. The data were collected and analyzed using Data Analysis Software (Bruker Daltonics). The MS/MS scans from 50 to 2000 *m/z* were recorded. Nitrogen was used as the collision gas. The ionization tip voltage and interface temperature were set at 1250 V and 150°C respectively.

Statistical analysis

All mass spectrum data were collected using a Bruker Daltonics micrOTOF control and processed using Bruker Daltonics Data Analysis Software. The database of *Sus scrofa* (wild boar) (containing 36 605 sequences, updated on 24 February 2011) was downloaded from the National Center for Biotechnology Information (NCBI) and Mascot search engine version 2.3.01 was used for identification and quantification. Parameters were set as follows: specifying trypsin as the digestion enzyme, cysteine carbamidomethylation as fixed modification, and iTRAQ 8Plex on N-terminal residue, iTRAQ 8Plex on tyrosine (Y), iTRAQ 8Plex on lysine (K), glutamine as pyroglutamic acid, and oxidation on methionine (M) as variable modifications. The tolerance settings for peptide identification in Mascot searches were 0.05 Da for MS and 0.05 Da for MS/MS. MS/MS spectra were utilized if the Mascot peptide ion score was above identity threshold and a significance threshold of *P* value was less than 0.05, which were required for protein identification, and 'require bold red' was set 'on'. The relative quantification of proteins was achieved by dividing the peak intensity of 117 by 119 iTRAQ reporter groups, and then the Medium Method was used for statistical normalization. Protein quantification was carried out based on a unique peptide. A cutoff of 1.75-fold regulation was chosen, and proteins with quantification ratios of 1.75-fold for low (<0.57) or high (>1.75) relative protein levels were considered as differentially regulated. Although a low cutoff of 1.75 entails a risk of reporting false-positive hits particularly when comparing pooled samples, the possibility of iTRAQ ratio compression due to co-eluting unregulated peptides of similar *m/z* requires a low cutoff value to avoid missing possibly regulated candidates (Bantscheff *et al*. 2008). An automatic decoy database search was also carried out.

Western blot analysis

From the differentially expressed proteins, we randomly selected the proteins triosephosphate isomerase 1 (TPI1), superoxide dismutase (Cu-Zn) (SOD1), outer dense fiber 2 (ODF2), and A kinase anchoring protein 3 (AKAP3) for western blot analysis to validate their expression levels in fresh and frozen–thawed spermatozoa. Samples containing 40 μg total proteins were separated by 12% SDS–PAGE. After transferring onto PVDF membranes by electroblotting, the proteins were then probed with primary antibodies: AKAP3 polyclonal antibody (T3217, Epitomics, Burlingame, CA, USA) at 1:100 dilution, SOD1 polyclonal antibody (PAB14492, Abnova, Walnut, CA, USA) at 1:5000 dilution, TPI1 polyclonal antibody (AV48144, Sigma) at 1:1000 dilution, ODF2 polyclonal antibody (SAB2501725, Sigma) at 1:200 dilution, and β-actin MAB (SC4970, Cell Signaling Technology, Beverly, MA, USA) at 1:1000 dilution respectively at 4°C overnight. The membranes were then rinsed with TBST (CW004, CWbio, Beijing, China) and incubated with a fluorescently labeled secondary antibody diluted 1:10 000 in TBS with 5% milk for 1 h in the dark. After rinsing the membranes in TBST again, the protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Western blot analysis was carried out using each boar sample (six biological repeats), and Student's *t*-test was used (Fig. 1B). Western blot data were normalized using β-actin (internal control).

Results

Identification and quantification of differentially expressed proteins

In this study, the labeling efficiency with iTRAQ reagents was 93.9%. The peptide mass was searched against the *S. scrofa* database, and the false discovery rate for peptide–spectrum matches determined by a Mascot 'decoy' search was 3.45%. Based on the selection criteria described in the 'Materials and Methods' section, 41 proteins were found to be differentially expressed after cryopreservation using iTRAQ-coupled 2D LC–MS/MS, among which 27 and 14 proteins were quantified based on two and one unique peptides

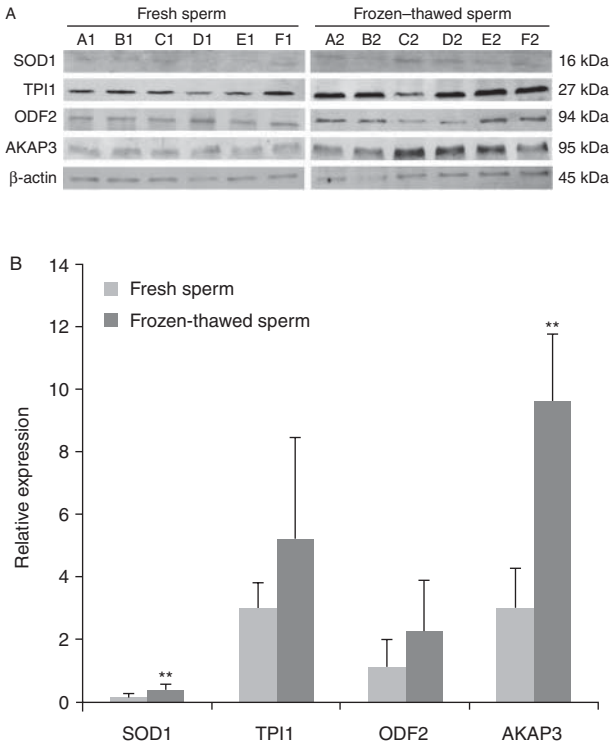

Figure 1 (A) Western blot analysis of AKAP3, SOD1, TPI1, and ODF2 proteins in fresh spermatozoa (A1, B1, C1, D1, E1, and F1) and frozen–thawed boar spermatozoa (A2, B2, C2, D2, E2, and F2). β-actin was used as the loading control. (B) Quantification of protein levels in fresh and frozen–thawed spermatozoa based on western blot analysis. The bar graph represents data of AKAP3, SOD1, TPI1, and ODF2 from six biological repeats respectively. $^{**}P<0.01$. The expression of AKAP3, SOD1, TPI1, and ODF2 was upregulated in frozen–thawed semen compared with that in fresh samples.

respectively. A total of 35 proteins were expressed at higher levels (Table 1) in frozen–thawed boar spermatozoa, whereas six proteins were expressed at lower levels (Table 2), and the levels of three proteins mainly expressed in epididymis were reduced during the freeze–thaw process (Table 2). The average fold differences in the relative amount ranged between 1.79 and 13.18. The version numbers were obtained from the NCBI for all the proteins (Tables 1 and 2).

The differentially expressed proteins were classified into ten distinct categories according to their functions (Fig. 2). MS proteomics data have been deposited in the Peptideatlas database. The raw MS data are publicly available for download from PeptideAtlas (http://www.peptideatlas.org/PASS/PASS00208) (Supplementary Table 1, see section on supplementary data given at the end of this article).

Western blot analysis of AKAP3, SOD1, TPI1, and ODF2

Western blot analysis was carried out to validate the levels of differentially expressed proteins. We randomly selected the proteins SOD1, TPI1, ODF2, and AKAP3 from the differentially expressed proteins identified by LC/MS analysis. Changes in protein levels determined by western blot analysis shown in Fig. 1A were generally consistent with the variations recorded in the LC–MS/MS analysis. Quantification of the proteins SOD1, TPI1, ODF2, and AKAP3 based on western blot analysis is shown in Fig. 1B. The levels of AKAP3, SOD1, TPI1, and ODF2 were increased in frozen–thawed boar spermatozoa compared with those in the fresh counterpart samples.

Discussion

In this study, some differentially expressed proteins were identified to be closely related to sperm premature capacitation, adhesions, energy supply, and sperm–oocyte binding and fusion. To our knowledge, this study is the first to demonstrate the presence of differentially expressed proteins in fresh and frozen–thawed boar sperm using iTRAQ-coupled 2D LC–MS/MS. This study could provide basic information for understanding the primary mechanism and process of cryodamage. A previous study has shown that, compared with the 'slow' cooling rate method, premature capacitation or other damages to the sperm are decreased on using the 'rapid' cooling rate method (Kumar *et al*. 2003). Our study indicated that we could optimize the cooling rates or dilution components, e.g. by adding some adhesions to prevent the sperm from premature capacitation and adding some new highly efficient energy material to ensure the energy requirement of sperm and also to improve the quality of frozen sperm.

Spermadhesins

Spermadhesins have been found in porcine seminal plasma (Haase *et al*. 2005), which are mainly secreted by seminal vesicles and highly expressed in the tail of the epididymis. Their binding to the acrosomal zone of the sperm head plays an important role in capacitation, as well as in sperm–oocyte recognition and binding. The differential proteins identified in this study, AWN1 and PSPC1 (PSP1), belong to the spermadhesin family. AWN1 adheres to the zona pellucida through carbohydrate recognition (Sanz *et al*. 1992), and PSPC1 is a heterodimer of glycosylated sperm adhesion proteins (Assreuy *et al*. 2003). Previous research on the protein–carbohydrate interactions of PSPC1/PSP2–oligosaccharide complexes has indicated that the spermadhesin molecules can play an important role in the sperm–oocyte binding event (Topfer-Petersen 1999). The reduction of these spermadhesins in frozen–thawed spermatozoa would promote premature capacitation, affect sperm–oocyte binding, and ultimately lower fertility.

Table 1 Proteins exhibiting increased expression levels in frozen spermatozoa.

Version number	Protein name	MW (kDa)	pI	Peptide matches	Number of unique peptides[a]	Protein score	Sequence coverage (%)	Protein ratio[b]	Log2 protein ratio
ACD02421.1	Cardiac muscle ATP synthase H+-transporting mitochondrial F1 complex α-subunit 1 (ATP5A1)	59 764.75	9.21	2	1	225.08	22.42	2.64	1.40
ABD92704.1	Triosephosphate isomerase 1 (TPI1)	26 862.90	6.53	3	3	140.82	37.90	3.94	1.96
P04178.2	RecName: Full=Superoxide dismutase (Cu-Zn) (SOD1)	16 052.97	6.03	4	4	126.84	22.22	3.54	1.83
XP_001926739.2	PREDICTED: ras-related protein Rab-2A (RAB2A)	23 674.82	6.08	4	3	82.60	20.75	3.65	1.87
1AN1_E	Chain E, leech-derived tryptase inhibitor trypsin complex	24 141.65	8.26	16	16	233.08	26.01	3.22	1.68
ACU43591.1	Zona pellucida-binding protein 2 transcript variant 1 (ZPBP2)	38 011.84	8.53	4	4	106.86	15.03	2.90	1.54
XP_003127098.2	PREDICTED: glyceraldehyde-3-phosphate dehydrogenase, testis-specific-like (LOC100517097)	40 550.83	8.54	5	5	151.10	14.82	2.63	1.40
ADJ53353.1	Outer dense fiber 2 (ODF2)	94 001.21	8.18	36	36	666.27	28.77	2.57	1.36
AAA31131.1	Acrosin precursor (EC 3.4.21.10)	46 042.62	9.66	10	10	273.38	25.30	2.95	1.56
NP_001231868.1	PREDICTED: glutathione S-transferase Mu 5-like (LOC10051423)	27 200.57	6.83	21	20	442.11	73.33	2.37	1.24
XP_001929699.1	PREDICTED: uncharacterized protein C1orf56-like (LOC100155103)	37 371.72	9.03	15	14	393.98	28.74	2.09	1.06
XP_003135135.2	PREDICTED: LOW QUALITY PROTEIN: a-kinase anchor protein 4-like (*Sus scrofa*) (LOC100513773)	96 530.12	6.40	61	61	1167.82	44.84	2.00	1.00
ADC38901.1	A-kinase (PRKA) anchor protein 3 (AKAP3)	94 841.16	6.03	6	5	224.86	10.30	1.93	0.95
CAA72379.1	pP47 protein	46 721.68	6.15	9	9	305.70	34.47	2.00	1.00
XP_003127397.1	PREDICTED: L-amino acid oxidase-like (IL4I1)	68 124.37	9.54	2	2	141.71	15.63	2.12	1.09
XP_003122400.2	PREDICTED: tubulin-β chain-like (LOC100516352)	63 867.08	5.51	20	20	375.69	19.72	2.28	1.19
ABL73884.1	Angiotensin I-converting enzyme (ACE)	151 246.00	6.23	3	2	128.53	8.33	1.96	0.97
XP_003125420.1	PREDICTED: apolipoprotein B-100-like (APOB)	102 434.00	5.50	14	1	39.00	4.00	10.25	3.36
XP_003130394.1	PREDICTED: sulfhydryl oxidase 1-like isoform 1 (QSOX1)	63 166.00	9.16	4	1	33.00	4.96	10.15	3.34
AAC48718.1	90 kDa heat shock protein (HSP90AA1)	85 121.00	4.93	13	1	39.00	6.68	3.74	1.90
AAV65756.1	Mitochondrial associated cysteine-rich protein (SMCP)	12 495.00	8.51	2	1	69.00	12.50	2.09	1.06
CAA34557.1	Unnamed protein product	12 085.00	12.05	4	2	92.00	18.48	13.18	3.72
ADE28531.1	Sperm equatorial segment protein 1 (SPESP1)	39 896.00	5.32	2	1	38.00	6.53	1.97	0.98
XP_003124619.2	PREDICTED: pancreatic secretory granule membrane major glycoprotein GP2 (GP2)	59 412.00	5.02	4	1	47.00	4.73	2.53	1.34
ADE28547.1	Sperm acrosome associated 1 (SPACA1)	32 846.00	4.44	4	1	74.00	14.92	4.07	2.02
BAI47603.1	Chaperonin-containing TCP1, subunit 7 (η) (CCT7)	59 949.00	6.74	8	1	44.00	10.68	3.51	1.81
ADC38871.1	Rhophilin-associated protein 1 (ROPN1)	24 108.00	5.18	5	1	46.00	21.23	2.73	1.45

Table 1 *Continued.*

Version number	Protein name	MW (kDa)	pI	Peptide matches	Number of unique peptides[a]	Protein score	Sequence coverage (%)	Protein ratio[b]	Log2 protein ratio
ABD77251.1	Mitochondrial ATP synthase, H+-transporting F1 complex β-subunit (ATP5B)	47 060.00	4.99	14	1	38.00	14.87	3.18	1.67
AAR88362.1	Phosphoglycerate kinase 2 (PGK2)	45 266.00	8.31	29	8	213.00	17.99	1.79	0.84
XP_003126012.3	PREDICTED: polycystic kidney disease and receptor for egg jelly-related protein-like (PKDREJ)	247 713.00	8.98	19	1	95.00	2.57	8.02	3.00
AAB21327.2	Phospholipid hydroperoxide glutathione peroxidase (PHGPx)	18 016.00	7.81	9	1	52.00	34.84	3.03	1.60
BAA04497.1	Zona pellucida-binding protein (ZPBP) precursor	40 454.00	9.24	16	2	70.00	20.57	3.75	1.91
XP_001929621.1	PREDICTED: tubulin α-3 chain (TUBA3D)	50 068.46	4.91	15	5	538.59	43.02	2.33	1.22
P02550.1	RecName: Full=Tubulin α-1A chain; AltName: Full=α-tubulin 1; AltName: Full=Tubulin α-1 chain (TUBA1A)	50 044.52	5.01	12	1	375.69	37.00	2.47	1.31
XP_003126645.3	PREDICTED: tubulin α-8 chain-like (LOC100518253)	49 959.55	4.98	9	4	369.30	17.00	2.13	1.09

MW, molecular weight.
[a]The number of unique peptides used for protein quantification. [b]Protein ratio is the iTRAQ reporter ion ratio. Only proteins quantified by iTRAQ with 1.75-fold change for high relative protein levels (>1.75) was considered as differentially regulated.

Anti-oxidative stress proteins

Mild peroxidation appears to promote the capacitation of spermatozoa. However, when exposed to oxidative burst, a large number of free radicals and reactive oxygen species (ROS) can cause polypeptide chains in the sperm to become fractured, polymerized, and cross-linked and consequently result in protein function changes, premature capacitation, and senescence (Aitken 1997, Stadtman & Levine 2003, Kankofer *et al*. 2005). The differential proteins, glutathione S-transferase (GST) Mu 5-like and SOD1, identified in our study belong to the cytosolic GST family and the Cu-Zn superoxide dismutase family respectively, which play important roles in the metabolism of toxic compounds and prevent cells from being attacked by acute toxicity and oxidant compounds. Phospholipid hydroperoxide glutathione peroxidase (PHGPx) belongs to the family of glutathione peroxidases, which was viewed as a kind of protectant against hydroperoxides (Tramer *et al*. 2002). We presumed that manipulation including centrifugation, dilution, and cooling may increase the levels of intracellular ROS and free radicals during the process

Table 2 Proteins exhibiting decreased expression levels in frozen spermatozoa.

Version number	Protein name	MW (kDa)	pI	Peptide matches	Number of unique peptides[a]	Protein score	Sequence coverage (%)	Protein ratio[b]	Log2 protein ratio
XP_003124797.1	PREDICTED: zymogen granule protein 16 homolog B-like (LOC100520553)	19 102.64	5.87	27	26	119.23	21.30	2.04	−1.02
AAD00096.1	16 kDa secretory protein	16 676.34	6.39	5	4	153.52	36.91	2.78	−1.46
AAB21636.1	Zona pellucida-binding protein, AWN1=C12 fragment (swine, sperm, peptide partial, 22 aa)	2174	4.14	10	9	473	68.18	2.00	−1.01
AAB50711.1	PSP94-like protein	12 865	8.29	10	2	79	26.13	3.45	−1.78
AAB58956.1	Porcine inhibitor of carbonic anhydrase precursor (pICA)	79 636	5.88	5	1	70	9.09	1.89	−0.93
AAC48399.1	PSPI	14 719	8.33	408	38	4954	66.17	4.55	−2.17

MW, molecular weight.
[a]The number of unique peptides used for protein quantification. [b]Protein ratio is the iTRAQ reporter ion ratio. Only proteins quantified by iTRAQ with 1.75-fold change for low relative protein levels (<0.57) were considered as differentially regulated.

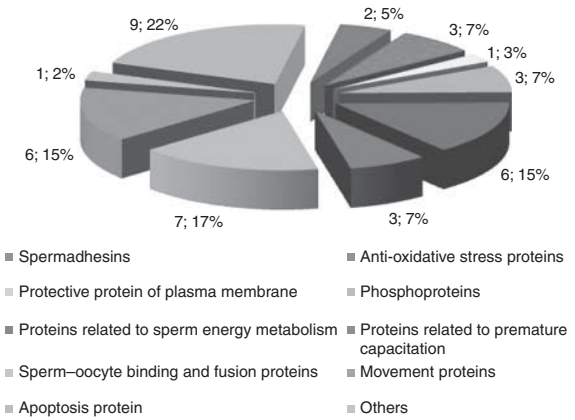

Figure 2 Classification of 41 differentially expressed proteins in fresh and frozen spermatozoa. The functional categorization of proteins was based on their annotations in the database.

of semen freezing and thawing (Li *et al*. 2010), and the increase in the levels of these proteins in frozen–thawed spermatozoa may be a protective response of boar sperm to cold stimulation and oxidation stress to prevent damage by freezing and toxic substances in the cryopreservation medium, such as glycerol.

Protective proteins of plasma membrane

Boar sperm is particularly sensitive to cold shock due to the low cholesterol:phospholipid ratio and the lower distribution of cholesterol in the inner membrane versus the outer membrane (Johnson *et al*. 2000). With a decrease in temperature, the resulting massive efflux and loss of membrane cholesterol combined with HCO_3- influence membrane fluidity, capacitation, and acrosomal reaction (Kadirvel *et al*. 2009). The 16 kDa secretory protein, secreted by the epithelial cells of the epididymis, can specifically bind to free cholesterol to regulate the cholesterol content of sperm membrane (Okamura *et al*. 1999). Its lower levels in frozen–thawed sperm may reduce membrane cholesterol levels, which may lower the stability of the sperm membrane. The 16 kDa secretory protein level could be used to evaluate sperm membrane lesions.

Phosphoproteins

With a decrease in temperature, larger numbers of ions flow into the sperm and activate soluble adenylyl cyclase (sAC) and protein kinase A (PKA); in turn, the residues of serine, threonine, and tyrosine are phosphorylated, resulting in a state of sperm hyperactive motility and premature capacitation (Jha & Shivaji 2002). With the development of capacitation, the proportion of tyrosine phosphorylated proteins in sperm increases, gradually extending from the sperm head to the tail (Nagdas *et al*. 2005). AKAP4 (Turner *et al*. 1998) and AKAP3 (Vijayaraghavan *et al*. 1999) can bind specifically to cAMP-dependent PKA and catalyze the phosphorylation of target proteins to regulate cellular responses. PKA can be targeted to the sperm tail by interaction with tyrosine phosphorylated AKAP3 during capacitation and sperm motility can be improved by enhancing the interaction between AKAP3 and PKA (Luconi *et al*. 2004). The AKAP3 binding to the protein rhophilin-associated protein 1 (ROPN1), containing the RII dimerization/docking (R2D2) domain, is regulated by PKA phosphorylation (Fiedler *et al*. 2008). The increase in the levels of ROPN1, AKAP4, and AKAP3 in frozen–thawed sperm suggests that these three proteins may play an important role in premature capacitation.

Sperm energy metabolism

TPI1 can catalyze the conversion of dihydroxyacetone phosphate into glyceraldehyde-3-phosphate, and glyceraldehyde-3-phosphate dehydrogenase can catalyze the synthesis of 1,3-bisphosphoglycerate containing energy-rich phosphate bonds from the oxidative dehydrogenation of glyceraldehyde-3-phosphate, while phosphoglycerate kinase 2 can catalyze the conversion of 1,3-bisphosphoglycerate and ADP into 3-phosphoglycerate and ATP. The increase in the levels of these glycolytic enzymes in frozen–thawed sperm may be a cell stress reaction to low temperature or a response to increased demand for energy in the hyperactivated state of premature capacitation in spermatozoa. Cardiac muscle ATP synthase H+-transporting mitochondrial F1 complex α-subunit 1, also called ATP synthase α-subunit, is a mitochondrial ATP synthase subunit coded by ATP5A1. Mitochondrial ATP synthase takes part in oxidative phosphorylation and catalyzes ATP synthesis using transmembrane proton motive force (Boyer 1997, 2002). Hence, both the glycolysis pathway and the tricarboxylic acid cycle are active in sperm to meet the energy demand of a high activity state induced by freezing. If insemination cannot be performed at the right time, the sperm would become 'exhausted' and fertilization efficiency is greatly lowered. The 90 kDa heat shock protein (HSP90AA1 (HSP90)), a member of the heat shock protein family, exerts its essential ATP-dependent chaperone action on more than 300 client proteins involved in cell growth, differentiation, and survival. The higher levels of HSP90AA1 in frozen–thawed spermatozoa in our study may be a cell stress response to low temperature. However, other studies have shown that the levels of HSP90AA1 in spermatozoa are decreased substantially after cryopreservation (Huang *et al*. 1999, Cao *et al*. 2003*a*, 2003*b*). The relationship between cryopreservation and higher HSP90AA1 levels in boar sperm should be studied further. In addition, mitochondrial ATP synthase, H+-transporting F1 complex β-subunit (ATP5B) was also present at higher levels in frozen–thawed spermatozoa.

Interestingly, we also detected its high expression in Y sperm in our previous study (Chen *et al*. 2012).

Proteins related to premature capacitation

A previous study has shown that spontaneous capacitation, acrosomal reaction, and premature capacitation of frozen–thawed spermatozoa are inhibited by ACE inhibitors (Foresta *et al*. 1991), which indicates that ACE may also be an important factor for premature capacitation in frozen–thawed sperm. The levels of ACE on the surface of spermatozoa have been shown to be inversely proportional to their fertilization capacity (Kohn *et al*. 1998, Shibahara *et al*. 2001). Therefore, the increase in ACE levels in frozen–thawed spermatozoa may reasonably explain the problem of low fertility rate. Protein pP47, located in the plasma membrane of boar sperm, has a potential function in membrane remodeling and zona pellucida binding. This protein has been detected in the acrosome membrane of *in vitro* capacitated sperm, testicular sperm, and epididymal spermatozoa and highest expression levels have been found in the capacitated sperm (Ensslin *et al*. 1998). In addition, polycystic kidney disease and receptor for egg jelly-related protein-like (PKDREJ) is also localized in the plasma membrane of the acrosomal region, which controls acrosomal exocytosis through the process of capacitation (Sutton *et al*. 2008). Therefore, we deduced that the increase in the levels of pP47 and PKDREJ may be related to premature capacitation in frozen–thawed semen as well.

Sperm–oocyte binding and fusion proteins

Seven of the regulated proteins that we detected are involved in the penetration of zona pellucida and sperm–oocyte fusion. Acrosin is a typical serine proteinase with trypsin-like specificity and is localized in the acrosomal matrix in its precursor form, proacrosin (Hardy *et al*. 1991). The active enzyme is involved in the lysis of the zona pellucida, thus facilitating the penetration of the sperm through the innermost glycoprotein layers of the ovum (Glogowski *et al*. 1998). Zona pellucida-binding protein 2 transcript variant 1 is also localized in the acrosomal matrix, implicated in the initial binding of the sperm acrosome to zona pellucida and upregulated significantly during sexual maturity (Song *et al*. 2010). The increase in the levels of these proteins observed in our study should be studied further. Sperm equatorial segment protein 1 (SPESP1) and sperm acrosome associated 1 (SPACA1) are acrosome membrane proteins. Studies have shown that *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ mouse sperm have a lower egg-fusing ability compared with the WT sperm (Fujihara *et al*. 2010), and both the binding and fusion of hamster sperm with hamster eggs have been found to be significantly inhibited by the antiserum against SPACA1 (Hao *et al*. 2002). The research on apolipoprotein B-100-like is analogous to that on SPESP1; heterozygous male knockout mice are infertile and it has been found that sperm cannot penetrate the zona pellucida (Huang *et al*. 1996). The increase in the levels of these three proteins in frozen–thawed spermatozoa and their function should be studied further. In addition, some other proteins related to sperm–oocyte binding and fusion, such as chaperonin-containing TCP1, subunit 7 (η) (CCT7) and zona pellucida-binding protein (ZPBP (SP38)) precursor, were also present at higher levels after cryopreservation.

Movement proteins

α-tubulin and β-tubulin are the basic units of the axoneme microtubule, which is surrounded by longitudinal ODFs (Turner *et al*. 2001). The ODF2 protein is one of the chief constituents of ODFs (Petersen *et al*. 1999). By forming the basic structure of the motor organ, ODFs give strong support to the sperm tail. By electron microscopy (EM), Hu (2009) observed that after cryopreservation, ODFs are increased in number or thickened and the microtubules are separated and arranged in a disorderly manner; moreover, the number of axonemes is increased or they are morphologically abnormal. The increased levels of ODF2 and β-tubulin in our study are consistent with Hu's EM observations. The content of the two proteins changed after cryopreservation, which apparently destroyed the characteristic '9+9+2' structure of the sperm tail and impaired the motor ability of sperm. In addition, mitochondrial associated cysteine-rich protein, localized in the capsule associated with the mitochondrial outer membranes, is thought to function in the organization and stabilization of the helical structure of the sperm's mitochondrial sheath and can also enhance sperm motility (Hawthorne *et al*. 2006). The increase in its levels may reduce the structure stability of frozen–thawed sperm and may also be associated with sperm premature capacitation.

Apoptosis protein

Premature apoptosis occurs and semen quality declines after sperm freezing and thawing (Jeong *et al*. 2009). Therefore, the freezing solution for boar sperm has been optimized by screening anti-apoptosis reagents to reduce the apoptotic rate of cryopreserved spermatozoa. L-amino acid oxidase is closely associated with sperm apoptosis, and its activity can be detected after damage or death (Shannon & Curson 1972, 1982). The increase in the levels of L-amino acid oxidase in frozen–thawed semen may be closely related to apoptosis in cryopreserved sperm. It may also directly cause cell senescence or death by increasing the permeability of the sperm

membrane. Therefore, L-amino acid oxidase activity could be used as a means to detect the level of damage to spermatozoa.

Conclusion

In summary, the results of this study provide the first overview of alterations in the proteome of fresh and frozen–thawed boar sperm and emphasize the fact that the cause of cryodamage is multifactorial. Many of the altered proteins were mainly related to premature capacitation in different stages, and some proteins were closely related to sperm–oocyte binding and fusion, movement, and apoptosis. Collectively, these changes offer explanations for mechanisms responsible for the cryodamage of sperm, which provides theoretical guidance for further development of an efficient cryopreservation technology for semen of boar and other species sensitive to cold shock.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0313.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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