Proteomic Analysis of Testicular Ischemia-Reperfusion Injury in Rats

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ABSTRACT. Testicular torsion is a urological emergency that leads to serious testicular damage and male infertility. We performed this study to identify specific proteins that are differentially expressed in response to testicular torsion and detorsion-induced ischemia-reperfusion (I-R) injury. Adult male rats were divided into two groups: a sham-operated group and a testicular I-R group. Testicular torsion was induced by rotating the left testis 720° in a clockwise direction for 1 hr, and then, detorsion was performed for 24 hr. After this testicular tissues were collected, protein analysis was performed using two-dimensional gel electrophoresis and Western blot analyses. Testicular I-R injury resulted in serious histopathologic damage to the germinal cells in the seminiferous tubules and increased the number of TUNEL-positive cells in testicular tissue. Specific protein spots with a greater than 2.5-fold change in intensity between the sham-operated and testicular I-R groups were identified by mass spectrometry. Among these proteins, levels of peroxiredoxin 6, thioredoxin, heterogeneous nuclear ribonucleoproteins, ubiquitin carboxyl terminal hydrolase isozyme L5 and zinc finger AN1-type domain 3 were decreased in the testicular I-R group compared to the sham-operated group. Moreover, Western blot analysis clearly showed the decrease of these proteins in the testicular I-R group. These proteins have spermatogenesis and anti-oxidative functions. These findings suggest that testicular I-R results in cell death due to altered expression of several proteins with spermatogenesis and anti-oxidation functions.
KEY WORDS: ischemia-reperfusion injury, proteomics, rat, testicular torsion-detorsion.

Testicular torsion is a urologic emergency that mainly affects newborns, children, adolescents and young adults [7, 11]. The most common type of testicular torsion is prenatal testicular torsion, which happens prenatally or within one month of life [8, 32]. Testicular torsion causes testis dysfunction, including male infertility. Infertility results from a serious defect in spermatogenesis and affects about 5% of human males. Testicular injury caused by spermatic cord torsion causes edema and testicular ischemia [2, 4, 6]. Torsion reduces the oxygen supply to the testes, and reperfusion leads to the formation of nitrogen and reactive oxygen species (ROS) [2, 15]. Highly toxic metabolites of oxygen induce the overproduction of ROS and the activation of oxidizing enzymes, consequently leading to cytoskeletal, cell membrane and mitochondrial damages [30]. However, antioxidant agents can eliminate the ROS by functioning as free radical scavengers [3]. The mechanisms of testicular I-R are unclear. We hypothesized that various proteins may contribute to the process of testicular I-R. However, little information is known about the changed proteins expression during testicular I-R. Thus, we identified proteins that were differentially regulated in response to testicular I-R injury.

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MATERIALS AND METHODS

Experimental animals: Male Sprague-Dawley rats (230–250 g, 10 weeks, n=20) were purchased from Samtako Co. (Animal Breeding Center, Osan, Korea) and were randomly divided into 2 groups, sham-operated group and testicular ischemia-reperfusion (I-R) group (n=10 per group). Rats were used for the morphological study (n=5 per group) and the molecular biological study (n=5 per group). Animals were maintained under controlled temperature (25°C) and lighting (14:10 light/dark cycle) and were allowed free access to water and food. All animal experiments were carried out in accordance with the guidelines that were approved by the ethics committee concerning animal research at Gyeongsang National University.

Testicular ischemia-reperfusion: Testicular ischemia and reperfusion injury was carried out as previously described method [37]. Rats were anesthetized with sodium pentobarbital (100 mg/kg) and were kept in a supine position. The left testis was exposed through a left-sided longitudinal incision and rotated 720° in a clockwise direction, and this torsion position was maintained by fixing the testis to the scrotum with 4–0 silk suture [37]. The incision was sutured and was reopened after 1 hr of torsion. The testis was counter-rotated to its natural position, and the testicular tissues were removed after 24 hr. In the sham-operated group, the left testis was brought out by a left-sided longitudinal incision, and then, a 4–0 silk suture was placed through the tunica albuginea. After the left testis was replaced into the scrotum, the incision was closed. The sham-operated group was constituted to investigate the effect of surgical stress on spermatogenesis. The testis was frozen in liquid nitrogen and stored at −70°C

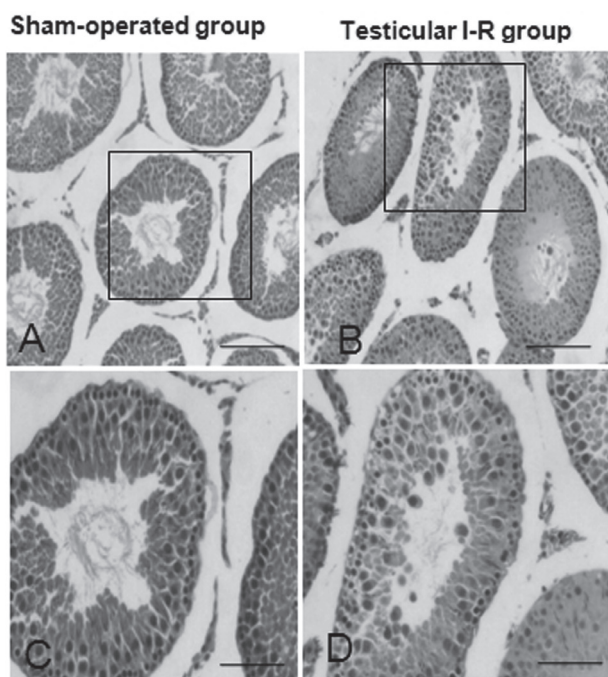**Sham-operated group**   **Testicular I-R group**

Fig. 1.   Histopathological photos of the testis tissue in sham-operated and testicular I-R groups. Sham-operated animals have a normal testicular architecture and regular seminiferous tubular morphology (A and C). In testicular I-R group, testis appears a markedly morphological change with severe distortion of tubules and non-cohesive germinal cells (B and D). C and D photos indicate the magnification of square in A and B. Hematoxylin and Eosin stain. Scale bar: A and B, 100 μm; C and D, 50 μm.

until use for proteomic and Western blot analyses.

Histological analysis: Testis tissues were fixed in 4% neutral buffered paraformaldehyde, embedded with paraffin and cut into 4 μm thick slices. The sections were deparaffinized in xylene and rehydrated in gradient ethanol from 100% to 70%. The sections were stained using hematoxylin and eosin solution. The morphological changes of testis tissues were observed using light microscopy.

TUNEL histochemistry: Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) histochemistry was carried out using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambridge, MA, U.S.A.). Briefly, paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol and washed with PBS. The sections were subjected to proteinase K digestion (20 μg/ml) for 20 min and blocked with 0.3% hydrogen peroxide in methyl alcohol for 10 min. The sections were washed in PBS and incubated in equilibration buffer for 30 min, and then, TdT labeling reaction mixture was applied to each specimen and incubated at 37°C for 1 hr. The reaction was stopped with stop solution for 5 min, and the section was incubated with blocking buffer for 10 min. The sections were labeled with digoxigenin peroxidase and visualized with diaminobenzidine (DAB) substrate. The sections were counterstained with hematoxylin, dehydrated

in graded alcohol, cleared and coverslipped with permount. To quantitate the incidence of apoptosis, the seminiferous tubules containing three or more apoptotic cells by TUNEL stain were calculated [18]. The apoptosis percentage was calculated by the ratio of the positive seminiferous tubules of apoptosis to the total number of seminiferous tubules in cross sections.

Two-dimensional gel electrophoresis and silver staining: Testis tissues were homogenized on ice in lysis buffer [8 M urea, 4% CHAPS, ampholytes and 40 mM Tris-HCl (pH 7.2)] and centrifuged at 16,000 g for 20 min at 4°C. The samples were kept at −70°C until use. The total protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Protein samples (50 μg) were applied to the immobilized pH gradients gel strips (17 cm, pH 4–7, pH 6–9, Bio-Rad) with sample buffer (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer and bromophenol blue). Isoelectric focusing (IEF) was performed using the Protean IEF Cell (Bio-Rad). After rehydration for 13 hr at 20°C, IEF was carried out three steps at 20°C: 250 V (15 min), 10,000 V (3 hr) and then 10,000 to 50,000 V. For the second dimension analysis, strips were equilibrated in equilibration buffer [6 M urea, 1% dithiothreitol (DTT), 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8) and bromophenol blue] for 10 min and in the same buffer containing 2.5% iodoacetamide for 10 min. Separation was performed on 7.5–17.5% gradient gels followed by electrophoresis in a Protein-II XI electrophoresis equipment (Bio-Rad) at 10°C. Current conditions were 5 mA/gel for 2 hr and 10 mA/gel for 10 hr. The gels were fixed in solution (50% methanol and 12% acetic acid) for 2 hr, washed with 50% ethanol for 20 min and incubated in 0.02% sodium thiosulfate for 1 min. After washing with distilled water, the gels were reacted with silver stain solution for 20 min and developed in a solution, and the reactions were stopped by stop solution (1% acetic acid).

Image analysis and protein identification: The silver stained gels were scanned using Agfar ARCUS 1200™ (Agfar-Gevaert, Mortsel, Belgium). The scanned gel images were used to measure differentially expressed proteins between groups using PDQuest software (Bio-rad). The selected spots were cut from gels, distained using 50% acetonitile solution and dried for 20 min using a vacuum centrifuge. The gel particles were incubated with reduction solution (10 mM DTT in 0.1 M NH$_4$HCO$_3$) at 56°C for 45 min and alkylation solution (55 mM iodoacetamide in 0.1 M NH$_4$HCO$_3$) for 30 min. The gel particles were washed with 0.1 M NH$_4$HCO$_3$ for 15 min, and the same volume of acetonitrile was added. And then, the gel spots were followed by incubation with trypsin-containing digestion buffer. Matrix solution was made using ACHC solution (α-cyano-4-hydroxycinnamic acid in acetone) and nitrocellulose solution (nitrocellulose in acetone and isopropanol) at a ratio of one to four. After the preparation of matrix solution, calibrants (angiotensin and neurotensin) were added. The samples were dissolved in the matrix solution by pipetting, loaded on a MALDI plate, dried completely and then washed by 0.1% trifluoroacetic acid. MALDI TOF MS was carried out to using Voyager-
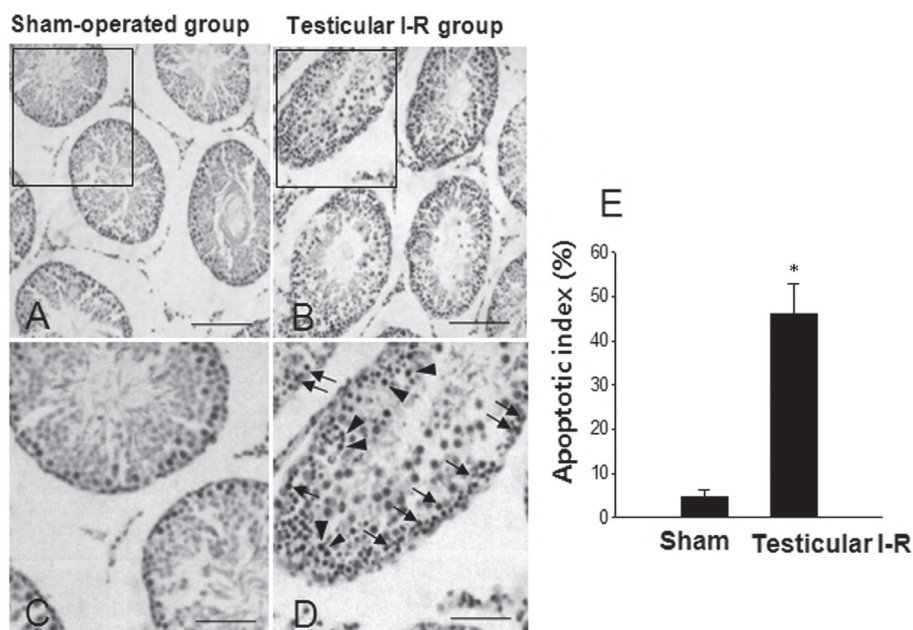
Fig. 2.   Representative photos of TUNEL staining in testis of sham-operated and testicular I-R animals. Positive cells of TUNEL staining were increased in testicular I-R group. The positive cells of TUNEL staining were detected in spermatogonia (arrows) and spermatocytes (arrowheads). C and D photos indicate the magnification of square in A and B. Scale bar: A and B, 100 μm; C and D, 50 μm. E: Quantitative analysis of apoptosis in testis. The number of seminiferous tubules containing three or more apoptotic cells was calculated by TUNEL stain. The apoptosis percentage was calculated as the ratio of apoptosis-positive seminiferous tubules to the total number of seminiferous tubules. Data (n=5) are represented as mean ± SEM. * $P<0.05$ (vs. Sham).

DETM STR biospectrometry work station (Applied Biosystem, Forster city, CA, U.S.A.). Results of MALDI TOF were analyzed by MS-FIT and ProFound (http://prospector. ucsf.edu/ucsfhtml13.4/msfit.htm and http://129.85.19.192/ profound_bin/WebProFound.exe). Sequence database was used SWISS PROT and NCBI.

Western blot analysis: Total protein (30 μg) was applied to each lane on to 10% SDS-polyacrylamide gels. Electrophoresis and immunoblotting were performed and the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with anti-zinc finger AN1-type domain 3 (Sigma), anti-heterogeneous nuclear ribonucleoproteins, anti-ubiquitin carboxyl terminal hydrolase isozyme L5, anti-peroxiredoxin-6, anti-thioredoxin and actin antibody (diluted 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), as the primary antibody. And, the membrane was incubated with horseradish peroxidase-conjugated-rabbit IgG or mouse IgG, as secondary antibody (diluted 1:5,000, Pierce, Rockford, IL, U.S.A.), and signals were detected by ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) according to the manufacturer's protocol.

RNA isolation and reverse transcription-PCR amplification: RNA was extracted from testis tissue using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). First strand cDNA synthesis was performed with use of 1 μg of total RNA and Superscript III reverse transcriptase (Invitrogen) following the manufacturer's protocol. The PCR amplifications were performed with the following primers; ubiquitin carboxyl terminal hydrolase isozyme L5, 5′-TTTTCTTTTCAAGTGGCAGCC-3′ and 5′-GATAGCCTGAGTGGCACAAGC-3′, heterogenous nuclear ribonucleoproteins, 5′-TCCTCTCCTGCTAAGCTT-3′ and 5′-CGGGGATCCACCGTCATGTCTAAG-3′, zinc finger AN1-type domain 3, 5′-AGGGCCTAAGAAGGGTTCAT-3′ and 5′-CTGCCTAGCGTTTACCACTG-3′, peroxiredoxin-6, 5′-AGCGTCACCACTGCCGCCATG-3′ and 5′-GTACTGGATGTGCAGATGCAGCC-3′, thioredoxin, 5′-TTCTTTCATTCCCTCTGTG-3′ and 5′-TCCGTAATAGTG-GCTTCG-3′, and actin, 5′-GGGTCAGAAGGACTCCTACG −3′ and 5′-GGTCTCAAACATGATCTGGG −3′. The amplification PCR program consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles from 94°C for 30 sec, annealing at 54°C for 30 sec and an extension at 72°C for 1 min and a final extension for 10 min at 72°C. PCR products were run on 1% agarose gel and visualized under UV light.

Data analysis: All data are expressed as mean ± SEM. The intensity analysis of protein spots was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, U.S.A.). The results in each group were compared by Student's *t*-test. The difference for comparison was considered significant at $P<0.05$.
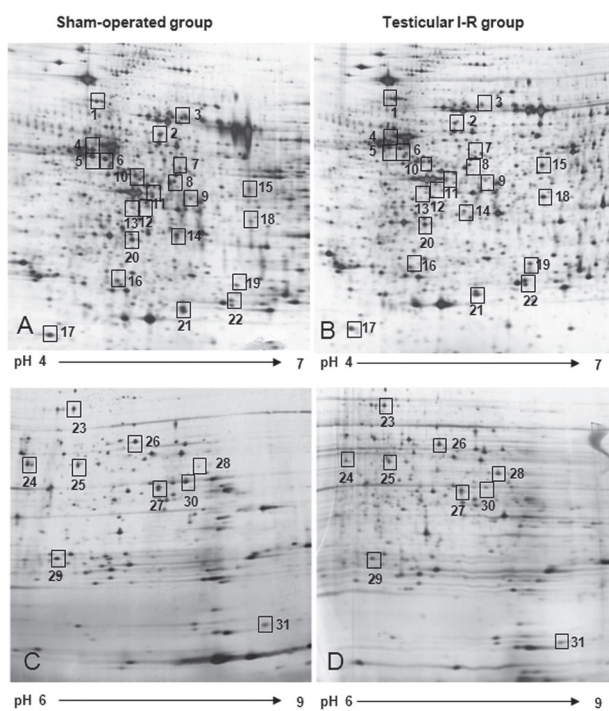
Fig. 3. Two-dimensional SDS-PAGE analysis of proteins in the testis from sham-operated (A and C) and testicular I-R (B and D) groups. Isoelectric focusing was performed at pH 4–7 and pH 6–9 using IPG strips, followed by second-dimensional separation on 7.5–17.5% gradient SDS gels stained with silver. Squares indicate the protein spots that were differentially expressed between sham-operated and testicular I-R groups.

RESULTS

Testes from sham-operated animals had a normal testicular architecture and seminiferous tubular morphology with normal spermatogenesis, including primary and secondary spermatocytes, spematids and spermatozoa (Fig. 1A and 1C). However, in testicular I-R animals, marked morphological changes were evident with severe distortion of tubules. Some tubules contained a few primary and secondary spermatocytes, while other tubules had non-cohesive germinal cells with pyknotic nuclei and extensive disorganization (Fig. 1B and 1D). TUNEL histochemical staining was performed to evaluate apoptotic cell death. The number of TUNEL-positive cells was significantly higher in testicular I-R animals than sham-operated animals (Fig. 2A–2D). TUNEL-positive cells were specially observed in spermatogonia and spermatocyte. However, a few seminiferous tubules in sham-operated animals had TUNEL-positive cells. The apoptotic index was $4.3 \pm 1.3\%$ and $43.8 \pm 6.8\%$ in sham-operated and testicular I-R animals, respectively (Fig. 2E).

Figure 3 shows the two-dimensional electrophoresis maps in the pH ranges of 4–7 and 6–9 for testes proteins from sham-operated and testicular I-R injured animals. Approximately 900 protein spots were present in the pH 4–7 map and

200 protein spots in the pH 6–9 map. We detected thirty-one protein spots with more than a 2.5-fold change in intensity between sham-operated and testicular I-R injured animals. Among the identified proteins, twenty-seven proteins were identified by MALDI-TOF analysis with protein sequence coverage of 10–69% (Table 1). However, four proteins were not identified by MALDI-TOF analysis and were named as unknown proteins. Among the identified proteins, levels of ubiquitin carboxyl terminal hydrolase isozyme L5, zinc finger AN1-type domain 3, heterogeneous nuclear ribonucleoproteins, peroxiredoxin 6 (Prdx-6) and thioredoxin (Trx) were decreased in testicular I-R injury animals compared to sham-operated animals. In contrast, levels of Rab GDP dissociation inhibitor beta, guanidioacetate N-methyltransferase, proteasome subunit beta type-4, hydroxymethylglutaryl CoA synthase and one unknown protein were increased in testicular I-R injury animals relative to sham-operated animals

Western blot analysis demonstrated that ubiquitin carboxyl terminal hydrolase isozyme L5, zinc finger AN1-type domain 3 and heterogeneous nuclear ribonucleoproteins levels were significantly decreased in testicular I-R injury animals compared to sham-operated animals (Fig. 4). Protein levels are presented as the ratio of the intensity of the protein to that of actin. Ubiquitin carboxyl terminal hydrolase isozyme L5 levels were 0.85 ± 0.02 and 0.65 ± 0.03 in sham-operated and testicular I-R animals, respectively (Fig. 4A). Heterogeneous nuclear ribonucleoproteins levels were 0.81 ± 0.03 and 0.66 ± 0.04 in sham-operated and testicular I-R animals (Fig. 4B). Zinc finger AN1-type domain 3 levels were 0.77 ± 0.04 and 0.63 ± 0.03 in sham-operated and testicular I-R animals (Fig. 4C). Moreover, peroxiredoxin-6 and thioredoxin levels were significantly decreased in testicular I-R injury animals compared to sham-operated animals. Peroxiredoxin-6 levels were 0.78 ± 0.02 and 0.53 ± 0.02 in sham-operated and testicular I-R animals, respectively (Fig. 4D). Thioredoxin levels were 0.82 ± 0.02 and 0.47 ± 0.03 in sham-operated and testicular I-R animals (Fig. 4E).

RT-PCR analysis clearly demonstrated that ubiquitin carboxyl terminal hydrolase isozyme L5, zinc finger AN1-type domain 3, heterogeneous nuclear ribonucleoproteins, peroxiredoxin-6 and thioredoxin levels were significantly decreased in testicular I-R injury animals compared to sham-operated animals. The intensity of RT-PCR product was normalized to that of actin product. Ubiquitin carboxyl terminal hydrolase isozyme L5 levels were 0.78 ± 0.03 and 0.61 ± 0.02 in sham-operated and testicular I-R animals, respectively (Fig. 5A). Heterogeneous nuclear ribonucleoproteins levels were 0.89 ± 0.02 and 0.62 ± 0.03 in sham-operated and testicular I-R animals (Fig. 5B). Zinc finger AN1-type domain 3 levels were 0.62 ± 0.03 and 0.37 ± 0.02 in sham-operated and testicular I-R animals (Fig. 5C). Peroxiredoxin-6 levels were 0.82 ± 0.04 and 0.45 ± 0.02 in sham-operated and testicular I-R animals, respectively (Fig. 5D). Thioredoxin levels were 0.75 ± 0.03 and 0.50 ± 0.02 in sham-operated and testicular I-R animals (Fig. 5E).

Table 1. List of identified proteins that were significantly differentially expressed between sham-operated and testicular I/R groups

Spot No.	Protein name	Accession No.	MW (Da)	pI	Mass match	Sequence coverage (%)	Ratio I-R/Sham
1	Zing finger AN1-type domain 3 (ZFAND3)	Q5U2M7	25066	6.45	6/97	31	0.32 ± 0.02*
2	cAMP-dependent protein kinase inhibitor beta (PKI β)	P27775	7581	5.01	4/102	69	0.31 ± 0.03*
3	Metallothionein-2 (MT2)	P04355	6140	8.23	2/101	29	0.29 ± 0.02*
4	Ubiquitin carboxyl terminal hydrolase isozyme L5 (Uch-L5)	Q9WUP7	37593	5.24	5/111	31	0.34 ± 0.03*
5	Alpha-1-antiproteinase: antitrysin	P17475	46107	5.70	11/97	32	0.32 ± 0.02*
6	Alpha-1-antiproteinase	P17475	46107	5.70	7/104	28	0.26 ± 0.03*
7	Ras-related protein Rab-18	P35293	23021	5.24	3/139	10	0.34 ± 0.02*
8	Guanine deaminaseos	Q9WTT6	50984	5.56	13/140	32	0.35 ± 0.03*
9	Ras-related protein Rab-1A	Q6NYB7	22663	5.93	5/120	26	0.34 ± 0.02*
10	Uncharacterized protein C10	Q5X146	29097	5.47	9/116	39	0.35 ± 0.03*
11	Serum albumin precursor	P07724	6848	5.75	8/120	15	0.31 ± 0.02*
12	Protein S100-A1	P35467	10553	4.37	2/116	26	0.33 ± 0.04*
13	Killer cell lectin-like receptor subfamily B	A4KWA1	24756	6.51	5/104	34	0.32 ± 0.03*
14	Heat shock protein 90-alpha	P07901	29645	4.98	7/131	20	0.34 ± 0.02*
15	Rab GDP dissociation inhibitor beta	P50399	50505	5.93	6/101	20	2.52 ± 0.04*
16	Heterogeneous nuclear ribonucleoproteins (HnRNPs)	Q9Z204	34364	4.92	11/134	27	0.33 ± 0.04*
17	Diablo homolog, mitochondrial	Q9JIQ3	26813	6.15	4/125	17	0.34 ± 0.04*
18	Unknown						2.83 ± 0.04*
19	Guanidinoacetate N-methyltransferase	P10868	26390	5.69	5/124	33	2.51 ± 0.02*
20	Unknown						0.31 ± 0.03*
21	Perodoxiredoxin-6 (Prdx-6)	O35244	24803	5.64	8/135	34	0.32 ± 0.04*
22	Proteasome subunit beta type-4	P34067	29178	6.45	7/131	24	2.78 ± 0.04*
23	Protein FAM136A	Q9CR98	15664	7.56	8/107	38	0.32 ± 0.04*
24	G protein-regulated inducer of neurite outgrowth 1	Q3UNH4	95437	8.14	16/95	12	0.24 ± 0.03*
25	Aspartylaminopeptidase	Q9Z2W0	52522	6.82	8/109	17	0.33 ± 0.04*
26	Pyruvate Kinase isozymes M1 / M2	P11980	57781	6.63	7/99	14	0.31 ± 0.02*
27	Homeobox protein	P52651	23120	6.86	10/115	29	0.32 ± 0.04*
28	Hydroxymethylglutaryl CoA synthase	P54869	56786	8.65	7/123	15	3.52 ± 0.03*
29	Unknown						0.27 ± 0.03*
30	Unknown						0.21 ± 0.03*
31	Thioredoxin (Trx)	P97615	18220	7.74	4/105	18	0.34 ± 0.03*

Proteins name and accession number are listed according to the SWISS-PROT database. MW, molecular weight; pI, isoelectrical point. Ratio is described as spots intensity of testicular I/R group to spots intensity of sham-operated group. Data (n=5) are represented as mean ± SEM. *$P<0.05$ (vs. Sham).

DISCUSSION

This study clearly demonstrates that testicular I-R injury leads to serious histopathologic damage of the testis, including distortion of the seminiferous tubules and separation of germinal cells, as well as an increase in the number of apoptotic cells. Testicular I-R injury leads to the generation of ROS, and excessive ROS generation overcomes endogenous free radical scavenger's capacity. The accumulation of toxic oxide causes apoptosis in germ cells [14, 20]. Moreover, it has been revealed that apoptotic cells were observed in the spermatogonia and other spermatogenic cells in testicular I-R [21, 34]. We confirmed that apoptotic cells were specially observed in spermatogonia and spermatocytes. Moreover, using a proteomics approach, we identified thirty-one differentially-expressed proteins following testicular I-R injury. Among these proteins, we focused on specific proteins that have spermatogenesis and anti-oxidative functions in the discussion.

Ubiquitin thioesterase is a ubiquitin carboxyl terminal hydrolase (Uch). Ubiquitin plays a critical role in various cellular processes including cell differentiation, cell protection under oxidative stress, signal transduction and apoptosis [31]. Uch isoenzymes (Uch-L) mainly affect spermatogenesis in the testis. Among Uch-L, Uch-L1 and Uch-L4 mRNAs are expressed in spermatogonia, while Uch-L3 and Uch-L5 mRNAs are expressed in spermatids and spermatocytes [22]. Moreover, Uch-L1 has been shown to be down regulated in germ cells during testicular ischemia and reperfusion, and Uch-L1 deficiency results in infertility [33]. A decrease in Uch-L1 expression leads to a decline in ubiquitination, and thus, ubiquitination level decreases [33]. However, over-
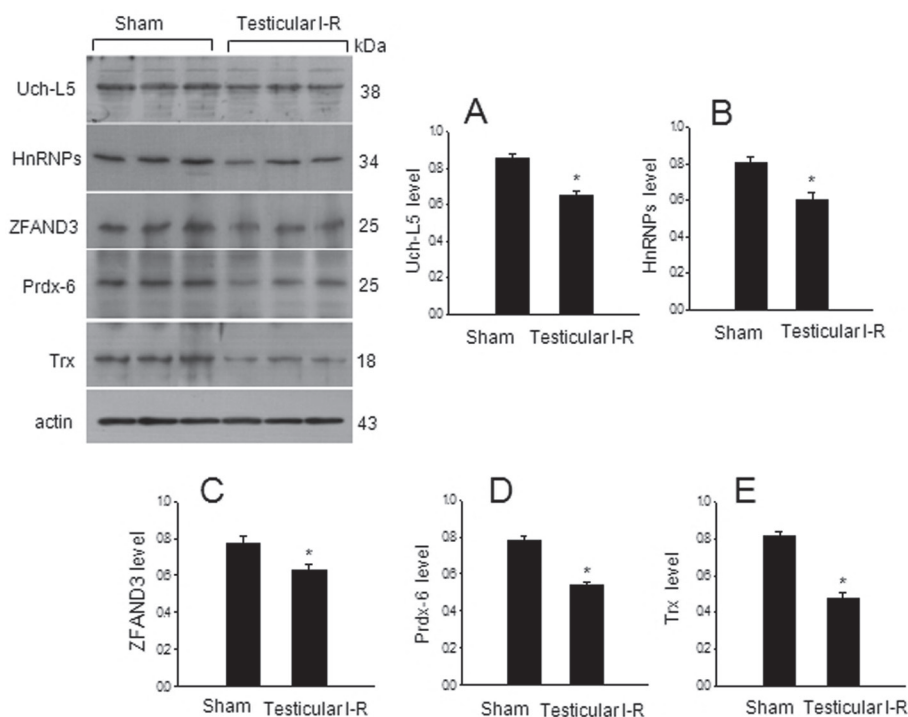
Fig. 4.   Western blot analysis of ubiquitin carboxyl terminal hydrolase isozyme L5 (Uch-L5), hetero-
geneous nuclear ribonucleoproteins (HnRNPs), zinc finger AN1-type domain 3 (ZFAND3), peroxire-
doxin 6 (Prx-6) and thioredoxin (Trx) in the testis from sham-operated and testicular I-R groups. Each
lane represents an individual experimental animal. These images are representative photos of Western
blot. The levels of proteins were normalized by actin. Data (n=5) are represented as mean ± SEM.
* $P<0.05$. (vs. Sham).

expression of the UCH-L1 also induces germ cell apoptosis
and inhibits spermatogenesis, leading to male sterility [35].
Thus, the proper quantity of UCH-L1 expression in testis
is very important for normal spermatogenesis. This study
showed the decrease of Uch-L5 in testicular I-R injury. The
decrease of Uch-L5 can contribute to spermatogenesis dys-
function. We therefore speculate that a decrease in expres-
sion of Uch-L5 mediates testicular I-R-induced apoptotic
cell death and defects in spermatogenesis

Zinc finger AN1-type domain 3 (ZFAND3), also known
as testis-expressed sequence 27 (Tex27), was originally de-
tected in mouse testis [24]. ZFAND3 is present in post-mei-
otic cells during spermatogenesis [9]. Moreover, ZFAND3
mRNA is expressed primarily in spermatids in the testis and
oocytes in the ovary [29]. It is accepted that ZFAND3 is a
useful marker of spermatogenesis, because ZFAND3 has a
critical physiological function related to germ cell matura-
tion [29]. Thus, the regulation of ZFAND3 expression in
the testis is critical for spermatogenesis, and a decrease in
ZFAND3 expression may lead to the inactivation of sper-
matogenesis [9]. We found that the level of ZFAND3 was
decreased in the testis as a result of testicular I-R injury. The
decrease of ZFAND3 expression declines spermatogenic
ability. Thus, our data demonstrated that the testicular I-R
injury leads to a decrease of ZFAND3 and continuously

results in a serious defect in spermatogenesis.

Heterogeneous nuclear ribonucleoproteins (HnRNPs) are
family proteins which have common structural domains.
HnRNPs play important roles in DNA repair, telomere
biogenesis, cell signaling during gene transcription and
translation [5]. Moreover, HnRNPs have multiple roles in
tumor development, including angiogenesis and cell inva-
sion [5]. Among HnRNP family proteins, HnRNP G-T is a
germ cell-specific nuclear protein that is expressed mostly
in pachytene spermatocytes [38]. The presence of HnRNP
G-T is important for normal germ cell development [13].
HnRNP-L acts as a key regulator of spermatogenic cell
apoptosis and growth [23]. Knockout of the HnRNP-L gene
leads to inhibition of proliferation and an increase in apopto-
sis of spermatogenic cells [23]. We found that testicular I-R
injury resulted in increased apoptotic cell death in germ cell
and serious testis tissue damage. In particular, we observed a
significant decrease in HnRNPs in response to testicular I-R
injury, which may explain the increase in apoptosis of germ
cells and resultant dysfunction of spermatogenesis.

The peroxiredoxin (Prdx) family proteins are involved
in cell differentiation, proliferation and gene expression.
Among these family proteins, Prdx-6 is also known as an-
tioxidant protein 2. Prdx-6 protects liver tissue against mi-
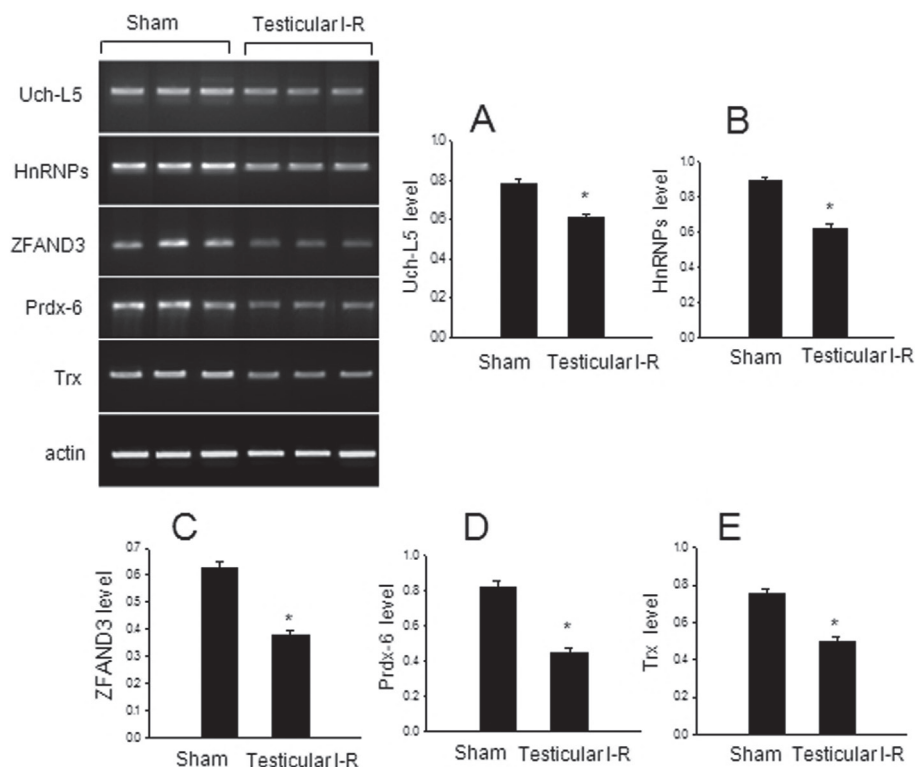tochondrial dysfunction during hepatic ischemia-reperfusion

Fig. 5. Reverse transcription-PCR analysis of ubiquitin carboxyl terminal hydrolase isozyme L5 (Uch-L5), heterogeneous nuclear ribonucleoproteins (HnRNPs), zinc finger AN1-type domain 3 (ZFAND3), peroxiredoxin 6 (Prx-6) and thioredoxin (Trx) in the testis from sham-operated and testicular I-R groups. Each lane represents an individual experimental animal. These images are representative photos of RT-PCR. The band intensity of RT-PCR product was normalized to that of actin product. Data (n=5) are represented as mean ± SEM. * P<0.05. (vs. Sham).

and contributes to the mitochondrial trafficking [12]. Prdx6-knockout mice were more susceptible to injury, increased tissue damage in liver and heart injury [12, 26]. Prdx-6 is highly expressed in epithelial cells and the Sertoli cells of the testis [14]. Prdx-6 protects Leydig cells against oxidative stress [39]. Moreover, over-expression of Prdx-6 results in resistance to cytotoxicity induced by chemical materials and promotes cell proliferation [10, 16]. We showed that testicular I-R injury induces a decrease in Prdx-6 levels. Moreover, Western blot analysis and RT-PCR analysis clearly demonstrated that Prdx-6 levels are markedly decreased in rats with testicular I-R injury, which would decrease anti-oxidant activity in the tissues of the testis, resulting in testicular damage.

Thioredoxin (Trx) is a small redox protein that suppresses apoptosis and protects cells against oxidative stress. Trx contributes to several cellular processes, including redox signaling and oxidative stress responses [36, 38]. Redox regulation is an essential step in the normal spermatogenesis process. Thus, oxidative stress is one of the major causative factors of male infertility [1]. Moreover, sperm-specific Trx is expressed in spermatozoa and in developing testicular germ cells [28]. The sperm redox system plays a key role in protecting spermatozoa from ROS until fertilization [27]. Thus,

a decrease in Trx expression indicates a decline in anti-oxidative ability and spermatogenesis. We found the decrease of Trx expression in testicular ischemic injury. The decrease of Trx expression indicates a decline in anti-oxidative ability and spermatogenesis. A previous study demonstrated that the expression of thioredoxin-1 and thioredoxin-2 was significantly decreased in cerebrums of rats with ischemia and reperfusion injury [19]. During ischemia and reperfusion injury, excessive radical production is produced and leads to protein oxidation and DNA damage [17]. Thioredoxin may reduce the free radical production and remove oxygen free radicals. In this study, we identified the decrease of Trx in testicular I-R injury using a proteomics. We also confirm this decrease using Western blot analysis and RT-PCR analysis. Our results indicate that the testicular I-R injury-induced reduction in Trx leads to testicular cell death.

In the present study, we obtained these results at 24 hr after testicular I-R injury. However, the expression of apoptosis-related proteins is correlated with the time of reperfusion after testicular I-R [25]. Thus, we purpose the fact that several new proteins can be identified according to time course after testicular I-R injury. In conclusion, this study showed that levels of peroxiredoxin 6, thioredoxin, heterogeneous nuclear ribonucleoproteins, ubiquitin carboxyl

terminal hydrolase isozyme L5 and zinc finger AN1-type domain 3 proteins decreased significantly in response to testicular I-R injury. These proteins have anti-oxidative and spermatogenesis functions. Thus, these findings suggest the fact that testicular I-R injury causes testicular damage due to changes in the expression of several proteins.

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