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学位論文

Genetic and histopathological analysis of spermatogenesis after short-term testicular torsion in rats

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Genetic and histopathological analysis of spermatogenesis after short-term testicular torsion in rats

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BACKGROUND: Patients with testicular torsion (TT) may exhibit impaired spermatogenesis from reperfusion injury after detorsion surgery. Alteration in the expressions of spermatogenesis-related genes induced by TT have not been fully elucidated.
METHODS: Eight-week-old Sprague-Dawley rats were grouped as follows: group 1 (sham-operated), group 2 (TT without reperfusion) and group 3 (TT with reperfusion). TT was induced by rotating the left testis 720° for 1 h. Testicular reperfusion proceeded for 24 h. Histopathological examination, oxidative stress biomarker measurements, RNA sequencing and RT-PCR were performed.
RESULTS: Testicular ischemia/reperfusion injury induced marked histopathological changes. Germ cell apoptosis was significantly increased in group 3 compared with group 1 and 2 (mean apoptotic index: 26.22 vs. 0.64 and 0.56; $p = 0.024$, and $p = 0.024$, respectively). Johnsen score in group 3 was smaller than that in group 1 and 2 (mean: 8.81 vs 9.45 and 9.47 points/tubule; $p = 0.001$, $p < 0.001$, respectively). Testicular ischemia/reperfusion injury significantly upregulated the expression of genes associated with apoptosis and antioxidant enzymes and significantly downregulated the expression of genes associated with spermatogenesis.
CONCLUSION: One hour of TT followed by reperfusion injury caused histopathological testicular damage. The relatively high Johnsen score indicated spermatogenesis was maintained. Genes associated with spermatogenesis were downregulated in the TT rat model.

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IMPACT:

- How ischemia/reperfusion injury in testicular torsion (TT) affects the expressions of genes associated with spermatogenesis has not been fully elucidated.
- This is the first study to report comprehensive gene expression profiles using next generation sequencing for an animal model of TT.
- Our results revealed that ischemia/reperfusion injury downregulated the expression of genes associated with spermatogenesis and sperm function in addition to histopathological damage, even though the duration of ischemia was short.

INTRODUCTION

Testicular torsion (TT) is a urological emergency and occurs most commonly during puberty.[1,2] Torsion is caused by twisting of the spermatic cord, which restricts blood flow to the testis. Immediate surgical detorsion is required to preserve the testis from ischemia-induced necrosis, but the restoration of blood supply to the untwisted testis paradoxically induces reperfusion injury.[3] Recent studies reported that ischemia and reperfusion after surgical detorsion both affect the testis.[4,5]

Ischemia/reperfusion (I/R) injury causes the production of reactive oxygen species (ROS) and free radicals, followed by oxidative stress in tissues.[6,7] Oxidative stress is the imbalance between ROS production and the antioxidant system.[8] Previous studies showed that oxidative stress in the testis induces lipid peroxidation and germ cell apoptosis.[9–11] Malondialdehyde (MDA), an end product of lipid peroxidation, is one of the indicators of ROS production.[12,13] Superoxide dismutase (SOD) is an antioxidant enzyme that scavenges superoxide anion, a type of ROS.[6,14] Studies have shown that ROS produced during testicular I/R injury are associated with germ cell apoptosis.[15–17]

Testicular I/R injury in patients leads to unfavorable prognoses, such as testicular atrophy and subsequent impaired spermatogenesis. The abnormal spermatogenesis after TT results in decreased semen qualities, including sperm quantity and quality.[18] Therefore, even though the untwisted testis exhibits an improved color and appears preserved, spermatogenesis may be impaired by subsequent reperfusion injury. Several previous reports have demonstrated the impairment of spermatogenesis after TT in human patients and experimental animal models.[3,19] A limited number of studies have reported alterations in the

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expressions of genes associated with spermatogenesis after TT.[20,21] However, the effect of TT on the expression of genes regulating spermatogenesis has not been fully elucidated.

Spermatogenesis includes many processes, such as meiosis, acrosome biogenesis and flagellum formation.[22] Comprehensive analysis of the expressions of genes involved in each of these processes by RT-PCR may be technically challenging. The effects of I/R injury on brain and kidney function in animal models have previously been investigated using next generation sequencing (NGS).[23,24] To date, NGS for testicular I/R injury has not been reported.

In this study, we performed RNA sequencing (RNA-seq) using NGS in a rat model of TT to investigate the expression profile of genes associated with spermatogenesis in the testis subjected to I/R injury caused by TT. We also performed histopathological examination, apoptosis evaluation and measurement of oxidative stress biomarkers. This is the first exploratory study to investigate the effect of testicular I/R injury on spermatogenesis in a rat model of TT using RNA-seq.

MATERIALS AND METHODS

Animals

Eight-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were used in this study. Rats were housed in plastic cages in a temperature-controlled room under a 12 h light/dark cycle, with *ad libitum* access to food and water. All animal experiments were conducted in accordance with Japanese laws and the "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006) and were approved by the Animal Care and Use Committee for Kagawa University (approval number: 2018-18690).

Study design

The study groups are shown in Fig. S1. Twenty-five rats were divided into three groups: group 1 (sham-operated group, $n = 10$); group 2 (TT without reperfusion group, $n = 5$); and group 3 (TT with reperfusion group, $n = 10$). In groups 1 and 3, five rats were used for histopathological examination and the other five rats were used for biochemical examination. Three of the five rats subjected to biochemical examination were also used for gene expression profiling. All five rats in group 2 were used for histopathological examination only.

Surgical procedures

Before surgical procedures, rats were anesthetized by sevoflurane inhalation (7% for induction, 2–3% for maintenance) followed by subcutaneous injection of buprenorphine hydrochloride (0.01 mg/kg; Otsuka Pharmaceutical, Tokyo, Japan) as an analgesic.

Group 1. Rats in group 1 underwent a sham operation. The skin of the surgical area was shaved and a scrotal incision was made. The tunica vaginalis was opened and the left testis was exteriorized. The testis was immediately returned to the scrotum and fixed with a 4-0 nylon suture, the wound was then closed with a 3-0 nylon suture. The rats were maintained under sevoflurane anesthesia for 1 h before euthanasia.

Group 2. Group 2 was subjected to TT without reperfusion. The left testis was rotated 720° counterclockwise and fixed with a 4-0 nylon suture. The wound was closed with a 3-0 nylon suture, and the torsion of the testis was maintained for 1 h before euthanasia.

Group 3. Rats in group 3 underwent TT in the same manner as rats in group 2. After 1 h of ischemia, the scrotal skin was reopened and the testis was detorted. Reperfusion of the left testis was allowed for 24 h, and the rats were then euthanized.

Rats were euthanized by an overdose of sevoflurane followed by transcardial perfusion with saline.

Histopathological examination

Transcardial perfusion with 10% neutral-buffered formalin was performed immediately after euthanasia. The testes were removed and a small incision was made in the tunica albuginea; the testes were then immersed in 10% neutral-buffered formalin. The testes were dehydrated in ethanol and embedded in paraffin. The embedded tissues were sectioned at 5 μm thickness and stained with hematoxylin and eosin. Histopathological evaluation of the seminiferous tubules was performed and four parameters were examined: sloughing of germ cells; necrosis of germ cells; vacuolation in the seminiferous tubule; and spermatogenesis. Histopathological images were captured using a DP73 digital microimaging camera attached to a BX53 microscope (Olympus Corporation, Tokyo, Japan). Parameters were examined in 100 circular seminiferous tubule sections per testis, as described below. Oblique or oval sectioned tubules were not examined.

Sloughing of germ cells. To assess germ cell sloughing, we evaluated the presence of immature germ cells shedding into the lumen of the seminiferous tubule as described in a previous study.[4] Quantitative data were obtained by calculating the percentage of sloughing-positive tubules.

Necrosis of germ cells. We evaluated the necrosis of the germinal epithelium by calculating the necrosis score of the seminiferous tubules as described in a previous study.[25] We evaluated the extent of necrosis in the tubule and scored each tubule using the following criteria:0–25% = mild (1 point), 25–75% = moderate (2 points), and 75–100% = severe (3 points). The mean necrosis score of 100 tubule sections per testis was calculated and statistically analyzed.

Vacuolation of seminiferous tubules. Vacuolation of the seminiferous tubules was scored in the same manner as necrosis in the germinal epithelium. The mean score was calculated from 100 tubule sections per testis.

Evaluation of spermatogenesis. We used the Johnsen score[26] to evaluate spermatogenesis in the seminiferous tubules: 10: complete spermatogenesis and normally organized tubule; 9: many spermatozoa present but the germinal epithelium is disorganized; 8: only a few spermatozoa present; 7: no spermatozoa but numerous spermatids present; 6: only a few spermatids present; 5: no spermatozoa or spermatids but numerous spermatocytes present; 4: only a few spermatocyte present; 3: only spermatogonia present; 2: no germ cells but only Sertoli cells present; and 1: no germ cells and no Sertoli cells present. The mean Johnsen score of 100 tubule sections per testis was calculated and statistically analyzed.

Apoptosis evaluation

To evaluate germ cell apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed with an In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. For each testis, 100 circular tubule sections were examined as described above for the hematoxylin and eosin–stained sections. The apoptotic index was calculated as the mean number of TUNEL-positive cells per tubule.

RNA sequencing

Library preparation and RNA-seq. After euthanasia, part of the left testis was removed and homogenized with Buffer RLT included in the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was extracted using the kit in accordance with the manufacturer's instructions. The quality and quantification of RNA samples were evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Messenger RNA (mRNA) was isolated from total RNA samples using a NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA). RNA libraries were prepared using NEBNext Ultra II RNA Library Prep with Sample Purification Beads (New England Biolabs). Briefly, isolated mRNA samples were fragmented, and random primers were hybridized to the fragmented mRNA. First strand complementary DNAs (cDNAs) were synthesized from fragmented and primer-hybridized mRNAs, followed by second strand cDNA synthesis. Double-stranded cDNAs were then end-repaired to create blunt ends, followed by adapter ligation and PCR enrichment using NEBNext Multiplex Oligos for Illumina (New England Biolabs). The adapters for RNA-seq were ligated to the double-stranded cDNA, and hairpin loop-shaped adapters were opened by Uracil-Specific Excision Reagent (USER) enzyme. The adapter-ligated cDNA samples were enriched for RNA-seq by PCR. RNA-seq was performed by AZENTA Japan (Tokyo, Japan) using Illumina HiSeq (Illumina, San Diego, CA).

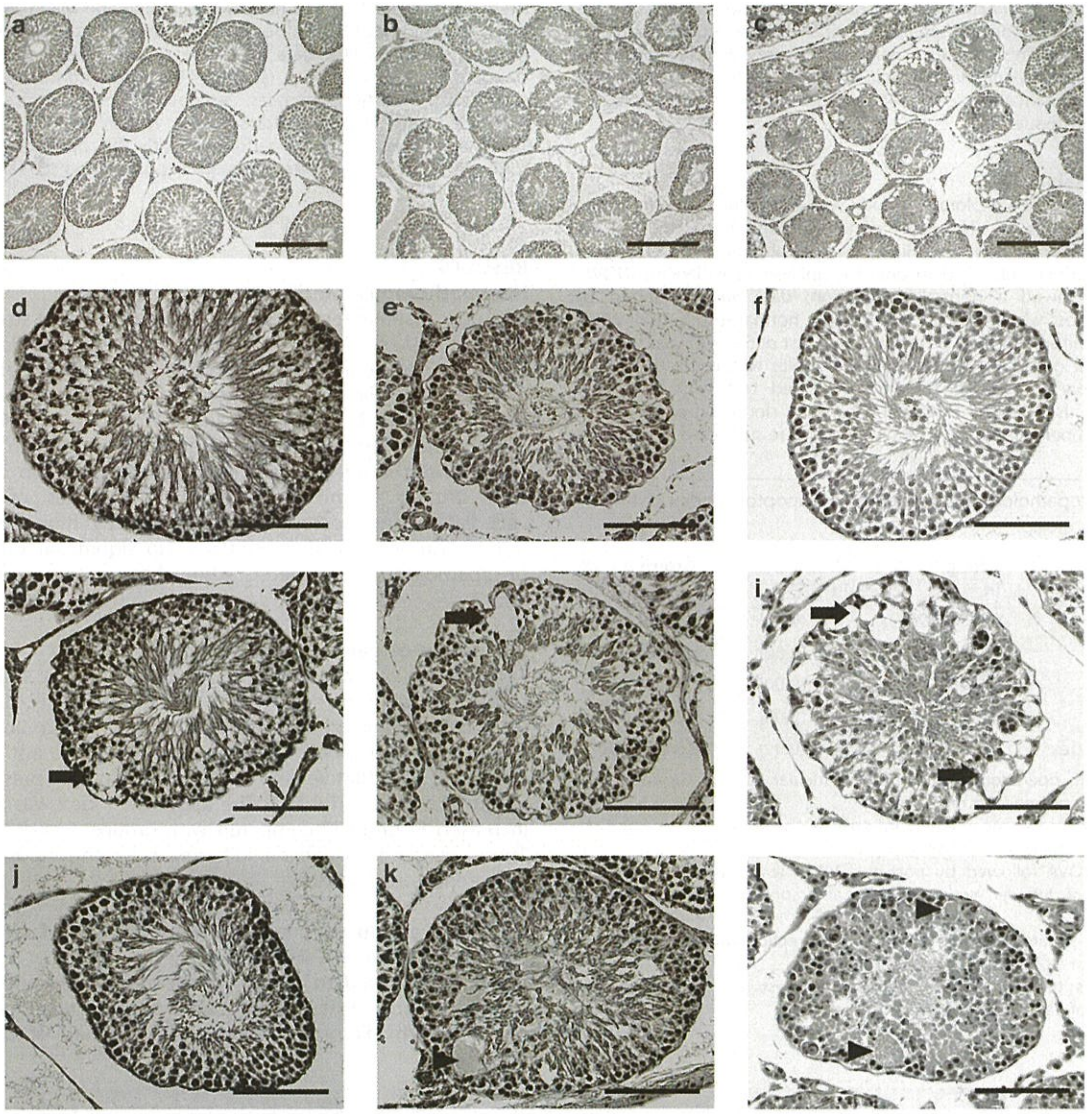

Fig. 1 Photomicrographs of testicular tissues stained with hematoxylin and eosin. Group 1. **a** Many normal seminiferous tubules are observed. **d** A tubule with sloughing of immature germ cells into the lumen. **g** A vacuole is very rarely present in the germinal epithelium (arrow). **j** A normal seminiferous tubule with complete spermatogenesis is seen. Group 2. **b** Tubular disorders are rarely present. **e** Germ cell sloughing is rarely seen in the lumen. **h** A vacuole is rarely observed in the germinal epithelium (arrow). **k** Necrotic tissues are rarely present in a seminiferous tubule (arrowhead). Group 3. **c** Marked tubular damage is frequently present, as shown in (**f, i, l**). **f** Germ cell sloughing is frequently seen in the lumen. **i** Numerous vacuoles are frequently present in the germinal epithelium of seminiferous tubules (arrows). **l** Necrosis (arrowheads) and impairment of tubular organization and spermatogenesis are occasionally seen. Scale bars: 300 μm (**a–c**) and 100 μm (**d–l**). Group 1: sham operation, group 2: 1 h testicular torsion, group 3: 1 h testicular torsion then 24 h reperfusion.

Analysis of RNA-seq data. The raw RNA-seq files underwent quality control using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) followed by trimming of adapters and low-quality data using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Single-end reads were then aligned to the genome assembly of *Rattus norvegicus* (Rnor_6.0) using Bowtie 2.[27] Read count data were uploaded to the integrated Differential Expression and Pathway analysis (iDEP) platform (http://bioinformatics.sdstate.edu/idep94/) and bioinformatics analysis was performed as described in a previously published manual.[28] Using iDEP, the pre-processed data were subjected to K-means clustering with four clusters, followed by enrichment analysis using Gene Ontology.

RT-PCR

Gene expression was evaluated by real-time RT-PCR using a One Step TB Green® PrimeScript™ RT-PCR Kit II (Takara Bio, Shiga, Japan) and ViiA™ Real-Time PCR System (Thermo Fisher Scientific). The expression levels of 19 genes associated with apoptosis, antioxidant enzymes and sperm were analyzed: *Ace, Bax, Bbs4, Bcl2, Casp3, Casp8, Casp9, Fas, Fasl, Gopc, Gpx1, Ho1, Hspa2, Prdx1, Prdx4, Shcbp1l, Sod1, Sod2* and *Tex14*. Thermal cycling protocols for PCR amplification were as follows: 42 °C for 5 min; 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 59 °C for 15 s and 72 °C for 15 s. Relative gene expression levels were calculated using ΔΔCt methods; *Gapdh* was used as an internal control. Primers used for RT-PCR are listed in Table S1.

Biochemical analysis

Concentrations of MDA, a marker of lipid peroxidation, and the activity of SOD, an antioxidant enzyme, were measured in homogenized testicular tissue samples. MDA levels were measured by a thiobarbituric acid reactive substance assay using a malondialdehyde assay kit (Japan Institute for the Control of Aging, Shizuoka, Japan), following the manufacturer's instructions. Absorbance was read at 532 nm using a SH-9000Lab microplate reader (Corona Electric, Ibaraki, Japan). MDA levels were expressed as

nmol/g wet tissue. SOD activities were quantified using a SOD Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. This kit uses a colorimetric assay with formazan dye produced from water-soluble tetrazolium salt when it is reduced with superoxide. Absorbance was read at 450 nm. SOD activities were expressed as units/mg wet tissue.

Statistical analysis

The sample size of this exploratory study was determined by referencing the previous studies.[29–31] Normality of the continuous parameters was analyzed with Shapiro–Wilk test. Histopathological data, except for germ cell sloughing, were subjected to one-way analysis of variance (ANOVA) when data followed a normal distribution; data were analyzed by Kruskal–Wallis test when they did not follow a normal distribution. When there was overall significance, post-hoc Tukey test or Steel–Dwass test was performed after one-way ANOVA or Kruskal–Wallis test, respectively, as a multiple comparison test. Pearson's chi-squared test with Bonferroni correction was used for the statistical analysis of sloughing of germ cells (quantified as a percentage). Biochemical data were subjected to Student's *t*-test when they followed a normal distribution and Mann–Whitney *U* test when they did not follow a normal distribution. Student's *t*-test was used for analysis of RT-PCR data.[29,32–34] Data are presented as mean and standard error of the mean (SEM). $P < 0.05$ were considered statistically significant. Statistical analyses, except for Steel–Dwass test, were performed with SPSS statistics software version 26 (IBM, Armonk, NY). The Steel–Dwass test was performed with the open-source statistical software EZR (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html).[35]

Table 1. Histopathological parameters and apoptotic index of the testis in each group.

Parameters	Group 1 ($n = 5$)	Group 2 ($n = 5$)	Group 3 ($n = 5$)
Johnsen score[a]	9.45 (0.07)	9.47 (0.09)	8.81 (0.11)*†
Sloughing[b]	3.00 (1.76)	3.20 (0.80)	67.20 (3.23)*†
Vacuolation[c]	0.00 (0.00)	0.01 (0.00)	0.86 (0.19)*†
Necrosis[a]	0.00 (0.00)	0.01 (0.00)	1.08 (0.23)*†
Apoptotic index[a]	0.64 (0.08)	0.56 (0.09)	26.22 (5.68)*†

Group 1: sham operation, Group 2: 1 h testicular torsion, Group 3: 1 h testicular torsion then 24 h reperfusion.
Data are presented as mean (standard error of the mean).
*$P < 0.05$ vs group 1, †$P < 0.05$ vs group 2.
[a]One-way ANOVA followed by post-hoc Tukey test was performed for comparisons of Johnsen score, necrosis and apoptotic index between groups.
[b]Pearson's chi-squared test with Bonferroni correction was performed for comparisons of sloughing between groups.
[c]Kruskal–Wallis test followed by Steel–Dwass test was performed for comparisons of vacuolation between groups.

RESULTS

Histopathological findings

In group 1, seminiferous tubules exhibited normal structures and spermatogenesis (Fig. 1a). In group 3 (Fig. 1c), tubules exhibited abnormal histological findings (vacuolation, germ cell sloughing and necrosis) (Fig. 1f, i, l), whereas few of these features were found in groups 1 (Fig. 1d, g, j) and 2 (Fig. 1b, e, h, k). Quantitative data of the histopathological parameters are shown in Table 1. The Johnsen score was significantly lower in group 3 compared with groups 1 and 2 ($P < 0.05$). The scores of sloughing, vacuolation and necrosis were significantly higher in group 3 than in groups 1 and 2 ($P < 0.05$). No significant differences in histopathological parameters were observed between groups 1 and 2.

Evaluation of germ cell apoptosis

Figure 2 shows histopathological images of TUNEL staining of the testis samples from each group. While few TUNEL-positive cells were detected in groups 1 (Fig. 2a, d) and 2 (Fig. 2b, e), a large number of TUNEL-positive germ cells were present in group 3 (Fig. 2c, f). Quantitative data of TUNEL staining are presented as an apoptotic index in Table 1. The apoptotic index was significantly increased in group 3 compared with groups 1 and 2 ($P < 0.001$). There was no significant difference in the apoptotic index between groups 1 and 2.

Gene expression profiling and identification of differentially expressed genes (DEGs)

RNA-seq was used to assess the differences in gene expression between groups 1 and 3. RNA-seq data were analyzed using the iDEP platform. Figure 3a shows the heatmap of the k-means

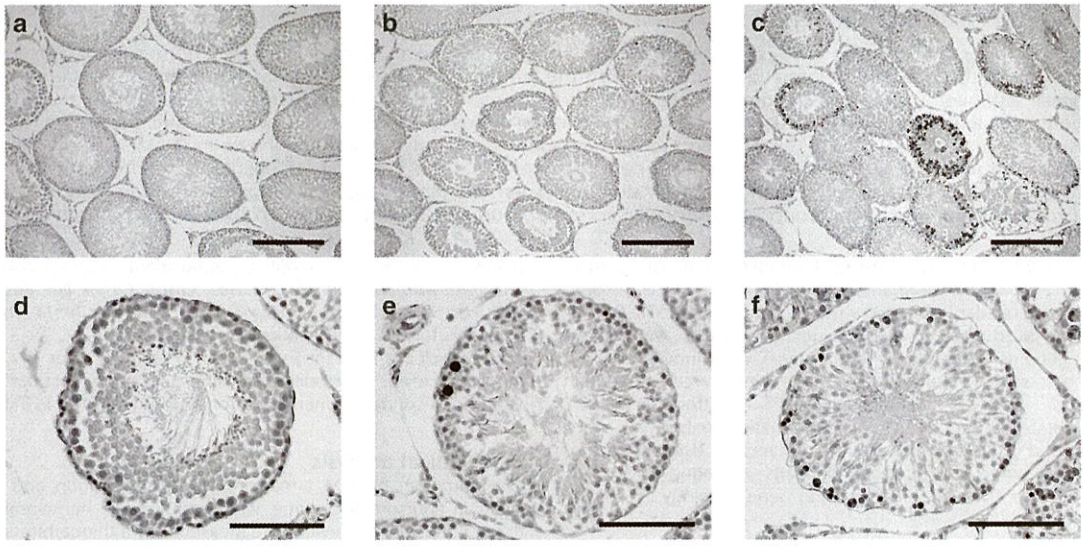

Fig. 2 TUNEL staining of testicular tissues. Group 1. **a** Almost all tubules show negative staining for TUNEL. **d** No apoptosis is seen. Group 2. **b** Almost all germ cells in tubules show negative staining for TUNEL. **e** A few TUNEL-positive germ cells are seen. Group 3. **c** Many tubules with TUNEL-positive germ cells are seen. **f** Many TUNEL-positive germ cells are frequently seen. Scale bars: 300 μm (**a**–**c**) and 100 μm (**d**–**f**). Group 1: sham operation, group 2: 1 h testicular torsion, group 3: 1 h testicular torsion then 24 h reperfusion.

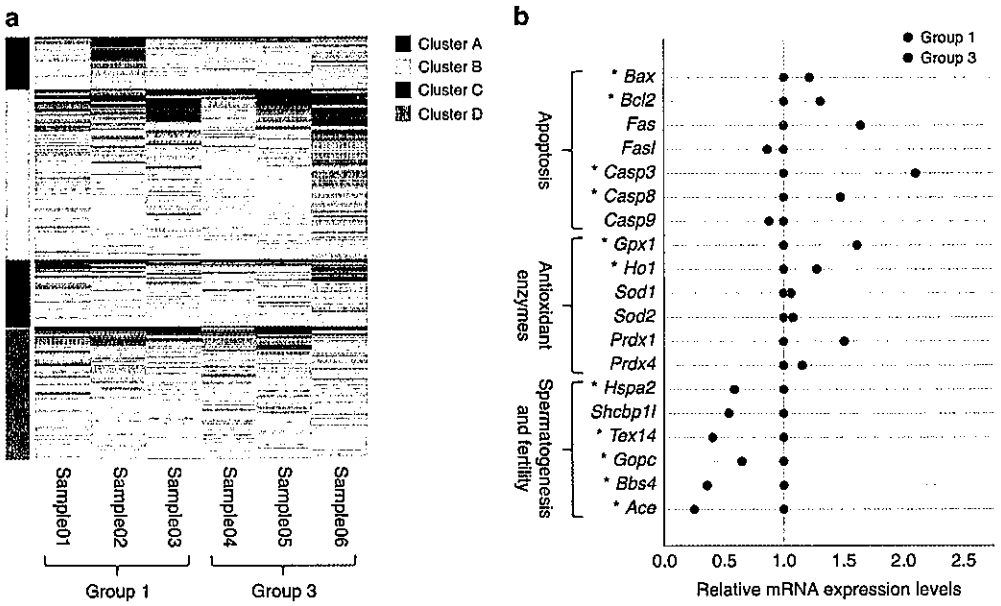

Fig. 3 Gene expression profiling of testis tissue. a Heatmap analysis of RNA-sequencing data from groups 1 and 3. Upregulated and downregulated genes are shown in red and blue, respectively. In clusters B (yellow) and D (green), there are differences in the expression levels of genes between groups 1 and 3. Clustering method: K-means clustering (min. level = 15, most variable genes to include = 2000, clusters = 4). **b** Gene expression analysis by RT-PCR. Testicular RNA expression levels were calculated relative to levels in the sham group. *$P < 0.05$ vs group 1. Group 1: sham operation, group 2: 1 h testicular torsion, group 3: 1 h testicular torsion then 24 h reperfusion.

clustering under the following conditions: Min. level = 15; Most variable genes to include = 2000; Clusters = 4. Genes in cluster B were upregulated in group 3 compared with group 1, while genes in cluster D were downregulated in group 3 compared with group 1. The details of k-means clustering are shown in Table S2. Enrichment analysis was then performed to identify pathways in each cluster (Table S3). Cluster B included pathways associated with the oxidation-reduction process, apoptotic process, and cell death. Cluster D included pathways associated with male gamete generation and sperm function. The analysis identified 69 DEGs, including 55 upregulated genes and 14 downregulated genes, in group 3 compared with group 1 (false discovery rate = 0.1, minimum fold-change = 2) (Table S4).

Quantification of gene expression by RT-PCR

We next examined the expression levels of genes associated with enriched pathways identified by RNA-seq, particularly genes associated with apoptosis, antioxidant enzymes and sperm, using RT-PCR. The RT-PCR results are presented in Table 2, and dot plots of RT-PCR data are shown in Fig. 3b. Among the genes associated with apoptosis, *Bax*, *Bcl2*, *Casp3* and *Casp8* were significantly upregulated in group 3 compared with group 1 ($P < 0.05$). The expression levels of *Casp9*, *Fas*, and *Fasl* showed no significant difference between the two groups. Among the genes associated with antioxidant enzymes, the expression of glutathione peroxidase 1 (*Gpx1*) and heme oxygenase 1 (*Ho1*) were significantly upregulated in group 3 compared with group 1 ($P < 0.05$). The expression levels of genes encoding superoxide dismutase (*Sod1*, *Sod2*) and peroxiredoxin (*Prdx1*, *Prdx4*) showed no significant differences between groups 1 and 3. Among the genes associated with sperm, the expression of *Ace*, *Bbs4*, *Gopc*, *Hspa2*, and *Tex14* were significantly downregulated in group 3 compared to group 1 ($P < 0.05$). There was no significant difference in the expression level of *Shcbp1l* between the two groups.

Biochemical findings

Figure 4 shows the results of MDA levels and SOD activity. Testicular MDA levels were 227.7 (15.8) and 267.0 (10.4) nmol/gram wet tissue in groups 1 and 3, respectively. Testicular SOD activities were 55.2 (7.7) and 60.1 (5.9) units/mg wet tissue in groups 1 and 3, respectively. There were no significant differences in testicular MDA levels and SOD activity between groups 1 and 3 ($P > 0.05$).

DISCUSSION

In the present study, we investigated expression alterations of genes, including genes related to spermatogenesis, after TT. Using a rat model of TT, we performed gene expression profiling together with the histopathological and biochemical evaluation of testicular damage caused by I/R injury.

We used a short-term TT rat model to elucidate how I/R injury after surgical detorsion affects spermatogenesis. We aimed to recreate the condition of postoperative testicular I/R injury in human patients who undergo immediate surgical detorsion and ipsilateral orchidopexy. Therefore, we needed to avoid excessive testicular ischemia, which restricts intraoperative testicular salvage. Our results showed that 1 h of 720° torsion without reperfusion did not induce significant apoptosis and necrosis in the testes compared with sham operated control testes. This finding indicates that the testis is viable under this condition of ischemia. A previous study reported that germ cell apoptosis increased over time after testicular detorsion, reaching a maximum at 24 h.[16] We thus examined rats treated for 1 h ischemia with 720° TT followed by 24 h reperfusion.

The Johnsen score,[26] a histopathological grading system of spermatogenesis, has been widely used to evaluate histopathological testicular changes in animal models of TT.[4,5] In the present study, we also used the Johnsen score to evaluate spermatogenesis. However, the disruption in spermatogenesis after TT may emerge at later time points. Because the reperfusion time of our I/R rat model, 24 h, was relatively short, we also examined histopathological findings, which are not included in the Johnsen score, to evaluate acute testicular damage caused by I/R injury. Histopathological disorganization of seminiferous tubules has been reported in previous studies of testicular I/R injury.[4,5,36,37]

Table 2. Gene expression analysis.

		Bax	*Bcl2*	*Fas*	*Fasl*	*Casp3*	*Casp8*	*Casp9*	*Gpx1*	*Ho1*	*Sod1*	*Sod2*	*Prdx1*	*Prdx4*	*Hspa2*	*Shcbp1l*	*Tex14*	*Gapc*	*Ace*	*Bbs4*
RNA-seq[a]	Group 1	15.34 (1.94)	1.36 (0.10)	0.06 (0.06)	0.00 (0.00)	0.42 (0.14)	0.55 (0.44)	16.46 (0.98)	22.49 (2.37)	28.05 (1.00)	305.91 (1.53)	22.30 (0.75)	83.82 (8.61)	98.85 (3.35)	1718.60 (7.03)	257.07 (4.31)	40.96 (1.28)	13.84 (0.28)	448.71 (13.46)	16.71 (0.32)
	Group 3	23.25* (0.78)	0.94 (0.18)	0.74* (0.11)	0.14* (0.03)	1.63* (0.24)	0.37 (0.20)	11.02* (1.21)	43.05* (4.96)	45.87* (3.97)	374.77 (32.17)	40.55 (7.62)	133.80 (19.57)	123.93* (6.53)	1482.79* (37.28)	198.15* (5.27)	31.49* (1.44)	11.06* (0.64)	384.09* (16.34)	13.68* (0.93)
RT-PCR[b]	Group 1	1(0.06)	1 (0.01)	1 (0.08)	1 (0.08)	1 (0.04)	1 (0.04)	1 (0.02)	1 (0.04)	1 (0.06)	1 (0.07)	1 (0.03)	1 (0.03)	1 (0.02)	1 (0.06)	1 (0.19)	1 (0.03)	1 (0.04)	1 (0.15)	1 (0.13)
	Group 3	1.23* (0.05)	1.31* (0.07)	1.65 (0.38)	0.87 (0.07)	2.11* (0.23)	1.49* (0.02)	0.88 (0.07)	1.62* (0.14)	1.28* (0.03)	1.07 (0.00)	1.09 (0.08)	1.52 (0.31)	1.15 (0.06)	0.59* (0.11)	0.54 (0.06)	0.40* (0.09)	0.65* (0.09)	0.26* (0.08)	0.36* (0.07)

Group 1: sham operation, Group 3: 1 h testicular torsion then 24 h reperfusion.
Data are presented as mean (standard error of the mean).
Student's *t*-test was performed for the comparison between groups.
*$P < 0.05$ vs group 1.
[a]Gene expression levels determined by RNA-sequencing (RNA-seq) are presented as transcript per million.
[b]Gene expression levels in testis determined by real-time RT-PCR are presented as fold-change comparison. *Gapdh* was used as an internal control for RT-PCR analysis.

Histopathological examination showed that seminiferous tubule injury (germ cell sloughing, necrosis and vacuolation in seminiferous tubules) was significantly increased in the TT with reperfusion group compared with the TT without reperfusion and sham-operated groups. Germ cell sloughing was a histopathological finding observed at the early phase of autoimmune orchitis,[38] and it was reported to be caused by the disruption of the Sertoli cell cytoskeleton.[39] In the short-term TT model of the present study, germ cell sloughing was observed in more than 60% of the seminiferous tubules. Germ cell sloughing may reflect damage to Sertoli cells at the early phase of testicular I/R injury. Vacuolation in seminiferous tubules is an early indicator of Sertoli cell injury, similar to germ cell sloughing,[40] and it occurs after exposure to testicular toxicants.[39] The tubular vacuolation and necrosis observed in our rat model of TT with reperfusion was consistent with previous studies.[5,41] The extent of vacuolation and necrosis observed in the current TT with reperfusion model was classified as mild. Sloughing, vacuolation and necrosis in seminiferous tubules was also detected in tubules with mild injury. These findings of seminiferous tubule injury may be useful indicators for assessing testicular damage in future studies using TT animal models.

Testicular I/R injury leads to ROS production, resulting in germ cell apoptosis.[16,42,43] In our study, we found that 24 h of testicular reperfusion after TT increased germ cell apoptosis. Previous studies have shown that TT-induced germ cell apoptosis is induced by both intrinsic and extrinsic pathways.[16,44–46] In addition, eugenol and other antioxidant agents can prevent these apoptotic pathways.[5,37,47] Intrinsic stresses such as hypoxia and free radicals initiate the intrinsic pathway of apoptosis,[48] which results in the disrupted integrity of the mitochondrial membrane and induced release of cytochrome c from mitochondria.[44,49] Cytochrome c associates with APAF1 and activates caspase-9 (CASP9), an initiator caspase,[48] which activates caspase-3 (CASP3), an effector caspase that leads to apoptosis.[50] In the intrinsic pathway, the Bcl-2 family including BAX and BCL2 plays an important role in regulating mitochondrial membrane permeability.[48,49] BCL2 protects the integrity of the mitochondrial membrane, while BAX increases mitochondrial membrane permeability and promotes apoptosis.[50–52] The extrinsic pathway of apoptosis is initiated when FAS ligand (FASL) binds its receptor FAS, which then activates caspase-8 (CASP8), an initiator caspase.[16] Activated CASP8 activates CASP3, which induces apoptosis.[50] Our results showed that genes encoding BAX (*Bax*), BCL2 (*Bcl2*), CASP8 (*Casp8*) and CASP3 (*Casp3*) were significantly upregulated in the testis of the TT with reperfusion group compared with that of sham-operated group. Genes encoding proteins involved in apoptotic cascades have individual expression dynamics,[16,44] therefore, detailed time-course measurements may be needed to capture potential changes in the expression levels of other apoptosis-related genes that did not show significant differences in this study.

A large amount of ROS produced during I/R injury causes oxidative stress when its oxidative damage overwhelms the antioxidant system.[17,53] MDA, the end product of lipid peroxidation, is an indicator of oxidative stress.[42] Antioxidant enzymes such as SOD, glutathione peroxidase (GPX) and heme oxygenase 1 (HO-1) are part of the defense system against oxidative stress.[54–56] Previous studies reported that MDA levels were increased, and antioxidant enzyme levels or activities were altered in testicular tissues of testicular I/R models.[57] Similarly, some studies on ovarian torsion showed that I/R injury in ovary caused oxidative stress and apoptosis of ovarian tissue.[47,58,59] Various experimental studies have been conducted to validate the potential agents against oxidative stress. For example, vitamin C, vitamin E analog, and other antioxidant compounds ameliorated oxidative stress by preventing lipid peroxidation and increasing antioxidant enzymes in animal models of I/R injury.[47,60,61] In this study, mean testicular MDA levels were

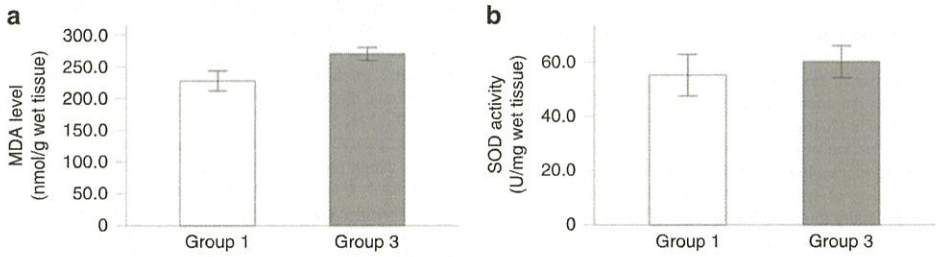

Fig. 4 Oxidative stress biomarkers in testis tissue. a Mean malondialdehyde (MDA) levels in testicular tissue (nmol/g wet tissue). **b** Mean superoxide dismutase (SOD) activities in testicular tissue (U/mg wet tissue). Group 1: sham operation, group 3: 1 h testicular torsion then 24 h reperfusion. Student's *t*-test was performed for the comparison between groups. Data are presented as mean and standard error of the mean.

slightly but not significantly increased in the TT with reperfusion group compared with the sham-operated group. We also found no significant difference in SOD activities. Pathway enrichment analysis of RNA-seq data showed the upregulation of a pathway associated with the oxidation-reduction process. We, therefore, examined the expression levels of genes encoding antioxidant enzymes by RT-PCR. There were no significant differences in the expression of genes encoding peroxiredoxin and SOD, but the expression levels of genes encoding glutathione peroxidase 1 (GPX1) and HO-1 were significantly increased in the TT with reperfusion group compared with the sham-operated group. GPX1 functions in the detoxification of hydrogen peroxide and lipid peroxides, and HO-1 regulates the production of ROS by degrading free heme.[54,55] Thus, these data demonstrate responses at the genetic level to oxidative damage in testes exposed to I/R injury, although no significant differences were observed in biological markers of oxidative stress. These results suggested that the oxidative damage occurred in our short-term TT model similar to previous studies of testicular or ovarian torsion.[17,58,59] The levels of biomarkers of oxidative stress change over time,[62] which may explain why we did not detect significant changes to MDA levels and SOD activities at the time point examined. In addition, testicular I/R injury in the current short-term TT model may not be severe enough to cause significant changes in MDA levels and SOD activities and may reflect the mild histopathological damage.

Consistent with previous studies,[4,5] the Johnsen score was significantly decreased in the TT with reperfusion group compared with the sham-operated group. However, the observed difference may have little clinical significance because the Johnsen score was greater than 8, indicating that spermatogenesis was maintained. The relatively high Johnsen score in this study may reflect the shorter duration of ischemia and reperfusion in the current I/R rat model. Notably, we detected the downregulation of pathways associated with spermatogenesis in the I/R model by RNA-seq and the pathway enrichment analysis. The process of spermatogenesis includes meiosis, acrosome biogenesis and flagellum formation.[22] The expression levels of genes associated with these stages of spermatogenesis, *Hspa2*, *Tex14*, *Gopc*, *Bbs4*, and *Ace*, were significantly downregulated in the current testicular I/R model. Heat shock protein family A member 2 (HSPA2) is a molecular chaperone that plays an important role in meiosis during spermatogenesis, and *Hspa2* knockout mice exhibited meiotic arrest at the G2/M phase and infertility.[63] The SHC binding and spindle associated 1 like (SHCBP1L) protein forms a complex with HSPA2 and maintains spindle stability during meiosis.[64] In the current study, the expression level of *Hspa2* was significantly downregulated, while *Shcbp1l* expression was unchanged. Testis expressed 14 (TEX14) is localized in the spermatogonial intercellular bridge and necessary for spermatogenesis; mice with disrupted *Tex14* exhibit germ cell arrest at the first meiotic division.[65] Golgi associated PDZ and coiled-coil motif containing (GOPC) protein is a Golgi-associated factor involved in vesicular transport from the Golgi apparatus in round spermatids. Mice with disrupted *Gopc* exhibit infertility and lack the sperm acrosome.[66] Bardet-Biedl syndrome 4 (BBS4) protein is essential for the formation of the sperm tail; *Bbs4* knockout mice were infertile due to the complete lack of flagella in mature spermatozoa.[67] The expression of *Ace*, encoding angiotensin-converting enzyme, was significantly downregulated in the TT with reperfusion group compared with the sham group. *Ace* encodes two isozymes, somatic and testis-specific angiotensin-converting enzyme, the latter being necessary for fertilization because of its function of sperm transport within the fallopian tube and sperm binding to the zona pellucida.[68] In the present study, the altered expression of transcripts related to spermatogenesis was observed 24 h after surgical detorsion of TT, although spermatogenesis was maintained at the histopathological level.

Both parametric and non-parametric statistical tests have been used for the analysis of RT-PCR data in previous literature. Azizollahi et al.[20] and Ramaswamy et al.[29] used the parametric test, while Hemmingsen et al.[69] and Lee et al.[37] used the non-parametric test. In this study, we used the parametric test for the analysis of RT-PCR data for the following reasons. First, the cycle threshold (Ct) calculated from the RT-PCR dataset is generally assumed to be normally distributed.[32] Second, non-parametric statistics are considered to be robust but inferior to parametric statistics in the power to identify differential gene expression.[33,70] Third, parametric statistics may be more appropriate than non-parametric statistics for small-scale and exploratory gene expression analysis.[34]

The limitations of our study are the relatively small sample size and the use of a model with a short reperfusion time. Further studies with a larger sample size and longer-reperfusion models are required. However, this is the first exploratory study to investigate the gene expression alterations associated with spermatogenesis after TT using RNA-seq. Our results demonstrated the downregulation of genes associated with various phases of spermatogenesis in the testis subjected to short-term TT followed by 24 h of reperfusion, although the severity of I/R injury was considered mild from histopathological findings. Furthermore, we identified the downregulation of *Hspa2*, *Tex14*, *Gopc*, *Bbs4*, and *Ace* in the testis subjected to TT. Further detailed studies are required to investigate the long-term effect of TT on the testicular gene expression profiles and spermatogenesis.

CONCLUSION

Our results suggest that TT and reperfusion injury caused testicular damage, impaired spermatogenesis, and altered the expression of transcripts related to spermatogenesis and sperm function, although the ischemic duration was short in the current model.

DATA AVAILABILITY

The RNA-seq dataset generated during and/or analyzed during the current study is available in the DDBJ Sequenced Read Archive (DRA) under the accession number DRA015199.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: H.K., R.S., S.S., M.U., T.M., T.F., A.T., and K.O. Drafting the article or revising it critically for important intellectual content: H.K., S.S., M.U., and R.S. Final approval of the version to be published: H.K., S.S., T.F., M.U., A.T., K.O., T.M., and R.S.

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COMPETING INTERESTS

The authors declare no competing interests.

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