〈Regular Article〉

Pathological analysis of spermatic dysfunction following testicular ischemia-reperfusion injury

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ABSTRACT **Introduction & Objectives**: Torsion, which may result in testicular ischemia, requires emergency surgery to restore testicular blood flow. However, the risk of spermatic dysfunction remains even if surgery is performed. The pathology of spermatic dysfunction in testicular ischemia-reperfusion injury (TIRI) remains unclear. A previous study showed the relevance of inflammation and oxidative stress in the other organs of ischemia-reperfusion injury. We hypothesized that inflammation and oxidative stress play key roles in causing spermatic dysfunction following TIRI. We investigated the pathophysiology of spermatic dysfunction in TIRI focusing on inflammatory changes using TIRI model mice.

Materials and Methods: The study used C57BL/6J male mice aged 10 to 15 weeks. To create TIRI model mice, the unilateral (left side) testicular vessels were clamped using Dieffenbach clamps (Bulldog clamps) for 1 hour and de-clamped. The bilateral testes were removed at 0 (ischemic state), 1, 3, and 5 weeks after creating the TIRI model mice. Spermatic changes following TIRI were investigated by analyzing the histology of the testes and semen and assessing levels of inflammation and oxidative stress. Semen was collected from the bilateral cauda epididymites and investigated using the sperm motility analysis system (SMAS).

Results: Histological analysis after hematoxylin-eosin staining showed tissue thickening in interstitial tissues at week 1 and 3 on the left (affected) testis, and week 1, 3 and 5 on the right (unaffected) testis. The infiltration of lymphocytes-predominant inflammatory cells were observed at week 1 and week 3 on the left (affected) testis. The destruction of ductal structures and giant cells were observed at weeks 3 and 5 on the left (affected) testis and week 5 on the right (unaffected) testis. SMAS showed significantly decreased spermatic concentration and motility in both testes of TIRI model mice compared with those of sham-operated mice at weeks 1, 3 and 5. Inflammation analysis using an inflammation-related proteome assay showed

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significantly increased levels of cytokines (IL-2, IL-3, IL-17A, and IL-23) and chemokines (CCL2, CCL5, CXCL1, and CX3CL1) at weeks 1, 3, and 5 in both testes of TIRI model mice. For the assessment of oxidative stress, enzyme-linked immuno-sorbent assay (ELISA) for 8-hydroxy-2'-deoxyguanosine (8-OHdG) was performed, which showed that levels of 8-OHdG were significantly increased in the left (affected) testis of TIRI model mice compared with that of sham-operated mice at all observation periods. Meanwhile, ELISA showed that levels of 8-OHdG in the right (unaffected) testis were significantly increased in TIRI model mice at weeks 3 and 5 compared with that of sham-operated mice.

Conclusions: Spermatic dysfunction following TIRI is induced by inflammation and oxidative stress. Inflammation and oxidative stress may be novel regulatory factors to prevent spermatic dysfunction following TIRI.

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Key words: Testicular ischemia-reperfusion injury, Spermatic dysfunction, Pathological analysis, Oxidative stress, Inflammation

INTRODUCTION & OBJECTIVES

Testicular torsion is an urgent urological condition that frequently occurs between the neonatal and pubertal periods[1]. Testicular torsion, which may cause testicular ischemia, requires emergency surgery within 6-12 hours of onset to restore testicular blood flow. More the risk of testicular dysfunction increases, the longer surgery is postponed[2-4]. However, even if testicular blood flow is restored following surgery, spermatic dysfunction may still occur. Reports showed that the unaffected testis may also develop testicular dysfunction even after the restoration of testicular blood flow[4-15]. Some studies also reported the impact of oxidative stress in the unaffected testis following restoration of testicular blood flow; however, the pathophysiology remains unclear[12-15]. In organs with a high risk of ischemia-reperfusion injury, such as the brain and heart, oxidative stress and inflammation play key roles in the pathophysiology of ischemia-reperfusion injury[16, 17]. Additionally, inflammation may also be associated with spermatic dysfunction following testicular ischemia-reperfusion injury (TIRI). Chakraborty *et al.*[7] and Dondero *et al.*[8] indicated that spermatic dysfunction in the unaffected testis may be caused by an immune reaction from the affected testis. Meanwhile, Merimsky *et al.*[9, 10] indicated the presence of an anti-sperm antibody. Although the identity of the anti-sperm antibody has not yet been clarified, above reports indicates that immune reaction, such as an inflammation, is induced in the affected side of testis, and subsequently immune reaction is influenced in the unaffected side of testis. From the above data, we speculated that pathophysiology of TIRI could be clarified by analyzing inflammatory changes as the main target, similar to the pathophysiology of ischemia-reperfusion injury in other organs, and hypothesized that inflammation in the affected testis causes oxidative stress that affects the unaffected testis. Thus, we investigated the pathophysiology of spermatic dysfunction following TIRI while focusing on inflammatory changes in the testes.

MATERIALS AND METHODS

Research approval and animals

The study was approved by the animal research committee of Kawasaki Medical School, Kurashiki, Japan (approval numbers 19-046 and 21-050) and was conducted according to the guidelines for the care and use of laboratory animals of Kawasaki Medical School. The study used C57BL/6J male mice (Jackson Laboratory, ME, USA) aged 10 to 15

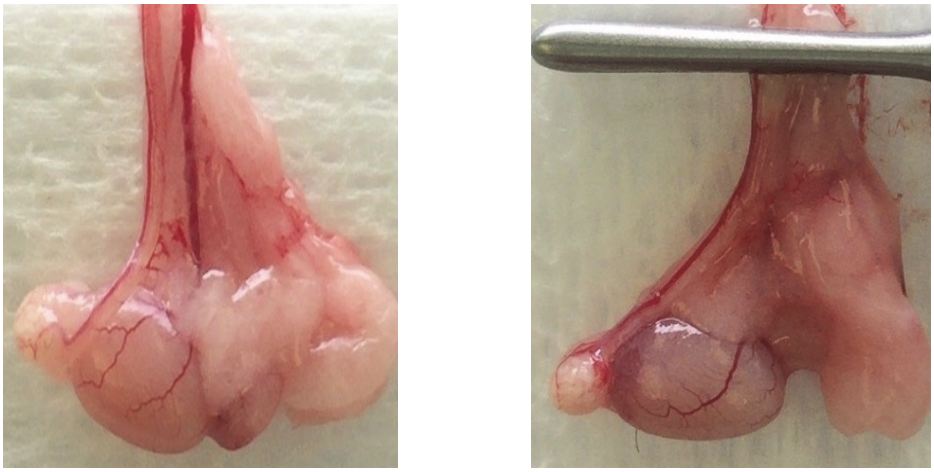

Fig. 1. Testicular ischemia model
Unilateral (left side) testicular vessels were clamped using Dieffenbach clamps (Bulldog clamps). Left: Normal testis. Right: Testicular ischemia after 1 hour. Animals: C57BL/6J male mice (10 to 15 weeks).

weeks. Mice were divided into a TIRI model group and a sham-operated group. Sedation was achieved with inhalation of sevoflurane (Pfizer Japan Inc., Tokyo, Japan). To create TIRI model mice, the unilateral (left side) testicular vessels were clamped using Dieffenbach clamps (Bulldog clamps, YDM, Tokyo, Japan) and de-clamped after 1 hour (Fig. 1).

Histological analysis

The bilateral testes were removed at 0 (ischemic state), 1, 3, and 5 weeks after creating TIRI model mice and were compared with those from sham-operated mice. The excised testes were fixed in Carnoy fixative fluid (Wako Pure Chemical, Osaka, Japan) for 3 hours. The fixed tissues were dehydrated in an ethanol series and finally in absolute ethanol. The dehydrated tissues were embedded in paraffin, cut into 5-μm sections, mounted on slides, and dried for 45 min at 45℃. Tissue slides were de-waxed and hydrated through graded ethanol solutions and water. Histological analysis following hematoxylin-eosin staining was performed using an Olympus BX-53 microscope (Olympus, Tokyo, Japan).

Semen analysis

Semen was collected from the bilateral cauda epididymites and diluted 200 times with TYH culture medium (LSI Medience, Tokyo, Japan). Semen analysis was performed using the sperm motility analysis system (SMAS) (DITECT, Tokyo, Japan).

Inflammatory analysis

Inflammatory changes in the testes were analyzed using a similar procedure to that described above. The testicular tissues from mice were homogenized with cell lysis buffer and PMSF, and tissue fragments were removed after centrifugation (15,000 × g, 5 min, 5℃).

A proteome assay using 111 types of inflammation-related proteomes (Proteome Profiler Mouse XL Cytokine Array, R&D Systems, MN, USA) was performed for the inflammatory analysis. The protein concentration of each sample was 300 μg/mL. The pixel density (PD) was calculated using an image analyzer (Image Quant LAS4000 mini, GE Healthcare Japan, Tokyo, Japan) and image analysis software (Image Quant TL, GE Healthcare Japan).

The calculated PD was corrected using endogenous control, while the negative and positive controls were set at PD 0 and 100, respectively. A significant difference ($p < 0.05$, Welch test) in the level of cytokines compared with sham-operated mice at all observation periods was set as the representative candidate for spermatic dysfunction in TIRI.

Oxidative stress analysis

Oxidative stress in the testes was analyzed using a similar procedure to that described above for testicular tissues. Enzyme-linked immuno-sorbent assay (ELISA) for 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Japan Institute for the Control of Aging, Shizuoka, Japan) was used to determine oxidative stress. The 8-OHdG assay was performed by setting the measurement wavelength to 540 nm and absorbance measurement with Varioskan Flash (Thermo Fisher Scientific, MA, USA).

Statistical analysis

Excel Statistics (Microsoft, WA, USA) was used for statistical analysis. Differences between the TIRI model and sham-operated mice were analyzed by the Welch test, with $p < 0.05$ considered as significant. The PD range was defined as 0 (negative control) to 100 (positive control) and expressed as the PD ratio, which was calculated as the TIRI model mice PD divided by the sham-operated mice PD.

RESULTS

TIRI model mice showed inflammation and destruction of ductal structures in both testes

Histological analysis showed tissue thickening in interstitial tissues at week 1 and 3 on the left (affected) testis, and week 1, 3 and 5 on the right (unaffected) testis (Fig. 2). The infiltration of inflammatory cells were observed at week 1 and week 3 on the left (affected) testis (Fig. 2). The infiltration of inflammatory cells were predominantly composed of lymphocytes (Fig. 3). The destruction of ductal structures and giant cells were observed at weeks 3 and 5 on the left (affected) testis and week 5 on the right (unaffected) testis (Fig. 2). The giant cells are multinucleate cells which indicated the germ cell degeneration such as some kinds of cell death (Fig. 3)[18].

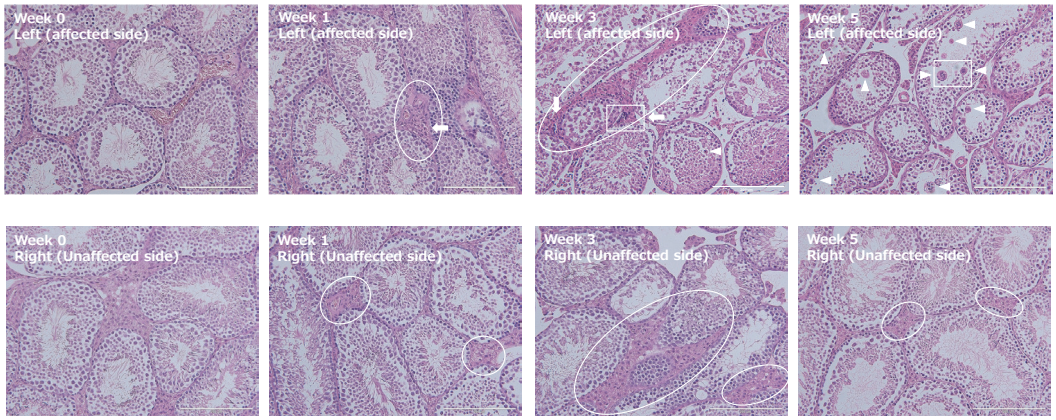

Fig. 2. Histological analysis using hematoxylin-eosin stain in testicular ischemia-reperfusion injury (TIRI) model mice
Tissue thickening in interstitial tissues (circle) are observed at week 1 and 3 on the left (affected) testis, and week 1, 3 and 5 on the right (unaffected) testis. Infiltration of inflammatory cells (arrow) are observed at week 1 and week 3 on the left (affected) testis. The destruction of ductal structures and giant cells (arrowhead) were observed at weeks 3 and 5 on the left (affected) testis and week 5 on the right (unaffected) testis.
Square: magnify parts to Fig. 3. Scale bar: 200 μm. Magnification: 200×. N = 5/week.

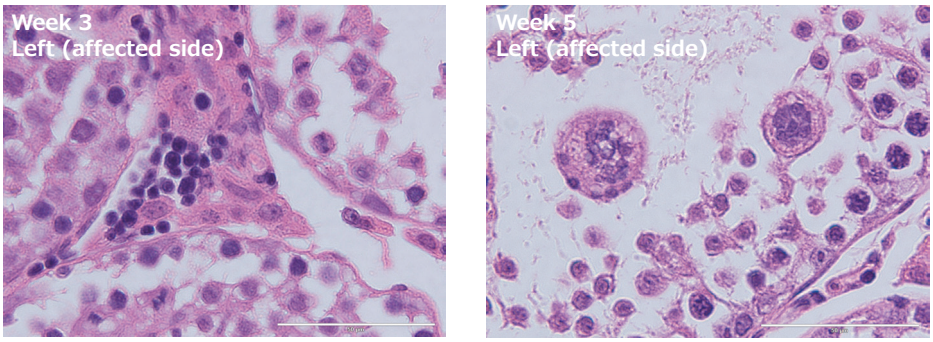

Fig. 3. Inflammatory cells and giant cells in testicular ischemia-reperfusion injury (TIRI) model mice
Infiltration of inflammatory cells (left figure) are predominantly composed of lymphocytes. The giant cells (right figure) are multinucleate cells which indicated the germ cell degeneration such as some kinds of cell death. Scale bar: 50 μm. Magnification: 1000×. N = 5/week.

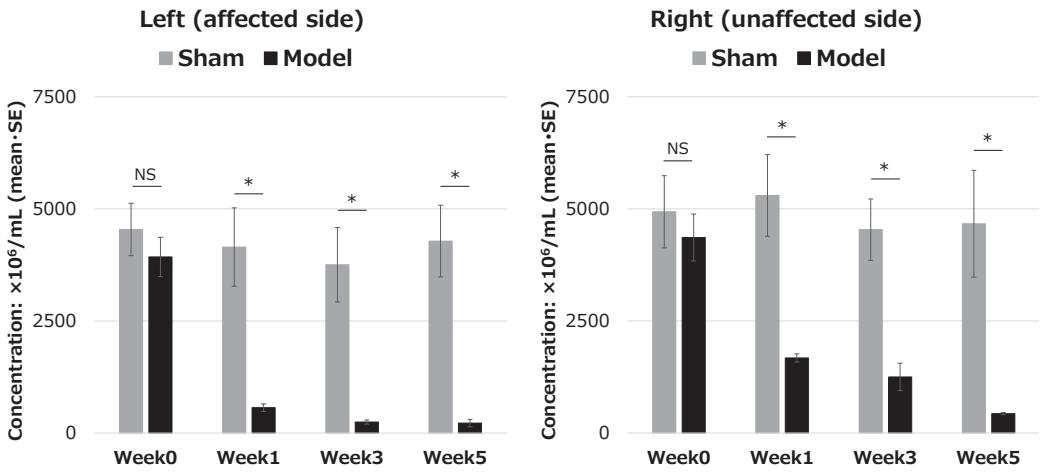

Fig. 4. Spermatic concentration analysis in testicular ischemia-reperfusion injury (TIRI) model mice
Semen analysis using the sperm motility analysis system showed significantly decreased spermatic concentration in both testes of TIRI model mice compared with those of sham-operated mice at weeks 1, 3, and 5. Indicated data show the mean and standard error (SE). NS (not significant) $p > 0.05$ and $^{*}p < 0.05$ for TIRI model mice vs sham operated mice (Welch test). N = 5/week.

TIRI model mice showed decreased spermatic concentration and motility in both testes

SMAS showed significantly decreased spermatic concentration (Fig. 4) and motility (Fig. 5) in both testes of TIRI model mice at weeks 1, 3, and 5 compared with those of sham-operated mice.

TIRI model mice showed increased levels of cytokines and chemokines in both testes

The proteome assay showed that levels of 8 types of cytokines and chemokines in the TIRI model mice were significantly elevated compared with those of sham-operated mice at all observation periods (except for week 0). Elevated levels of cytokines (IL-2, IL-3, IL-17A, and IL-23) and chemokines (CCL2, CCL5, CXCL1, and CX3CL1) were observed in TIRI model mice compared with sham-operated mice after model creation (Fig. 6). Levels of the cytokines and chemokines in the left (affected) testis increased at week 1, whereas

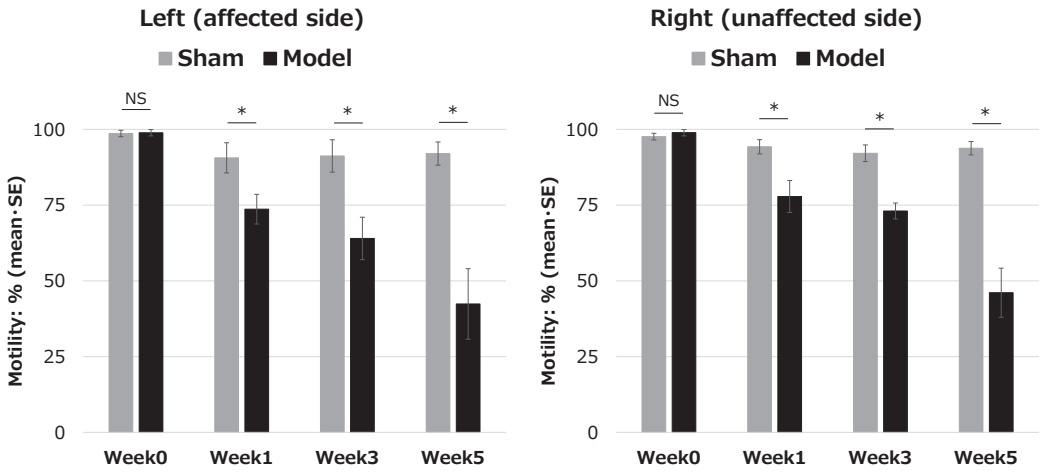

Fig. 5. Spermatic motility analysis in testicular ischemia-reperfusion injury (TIRI) model mice
Semen analysis using the sperm motility analysis system showed significantly decreased spermatic motility in both testes of TIRI model mice compared with those of sham-operated mice at weeks 1, 3, and 5. Indicated data was show the mean and standard error (SE). NS (not significant) $p > 0.05$ and $^{*}p < 0.05$ for TIRI model mice vs sham operated mice (Welch test). N = 5/week.

	IL-2		IL-3		IL-17A		IL-23		CCL2		CCL5		CXCL1		CX3CL1	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Week 0	3.8	3.1	4.0	3.3	5.5	1.3	1.2	1.2	5.2	2.5	4.6	3.5	5.0	1.7	4.0	1.1
Week 1	9.5	2.4	8.1	3.2	5.9	2.9	5.3	2.6	10.5	4.2	8.2	3.3	7.3	3.2	5.6	2.6
Week 3	5.6	3.6	8.0	4.2	3.9	2.4	3.5	1.9	7.8	4.4	7.2	4.4	6.0	3.2	4.0	3.6
Week 5	6.7	5.8	5.4	5.3	3.8	4.0	3.4	2.7	5.5	6.6	5.2	4.2	7.0	3.3	4.2	3.4

Fig. 6. Inflammatory analysis in testicular ischemia-reperfusion injury (TIRI) model mice
The inflammation-related proteome assay showed high levels of cytokines (IL-2, IL-3, IL-17A, and IL-23) and chemokines (CCL2, CCL5, CXCL1, and CX3CL1) in TIRI model mice compared with sham-operated mice at all observation periods (except for week 0). The PD ratio was calculated as the TIRI model mice PD divided by the sham-operated mice PD.

they gradually increased over time in the right (unaffected) testis.

TIRI model mice showed high oxidative stress in both testes

ELISA showed that the left testis of TIRI model mice had significantly increased levels of 8-OHdG compared with those of sham-operated mice at all observation periods (Fig. 7). On the other hand, the right testis of TIRI model mice had significantly increased levels of 8-OHdG at weeks 3 and 5 compared with those of sham-operated mice (Fig. 7).

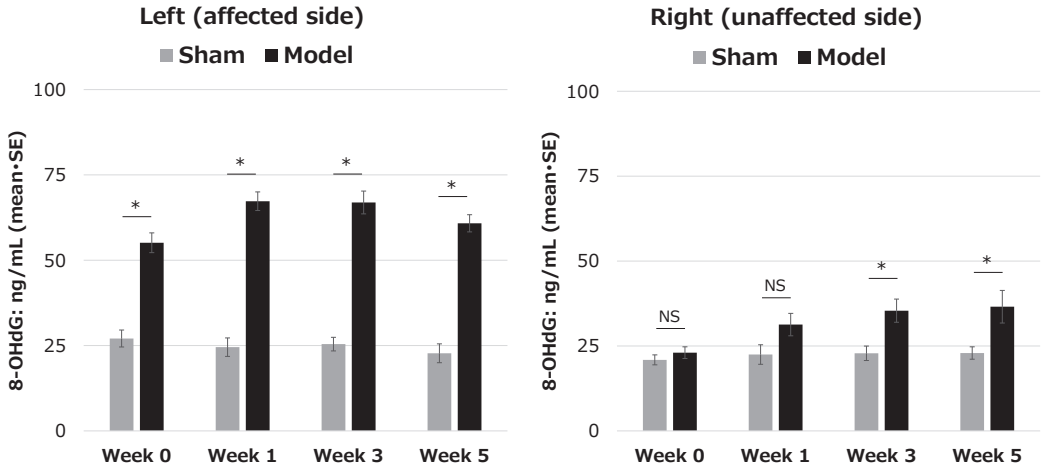

Fig. 7. Oxidative stress analysis in testicular ischemia-reperfusion injury (TIRI) model mice
ELISA showed that the left testis of TIRI model mice had significantly elevated levels of 8-OHdG compared with that of sham-operated mice at all observation periods. On the other hand, ELISA showed that the right testis of TIRI model mice had significantly elevated levels of 8-OHdG at weeks 3 and 5 compared with that of sham-operated mice. Indicated data show the mean and standard error (SE). NS (not significant) $p > 0.05$ and $^{*}p < 0.05$ for TIRI model mice vs sham operated mice (Welch test). N = 5/week.

DISCUSSION

Summary and novelty of the study

Although the presence of an anti-sperm antibody in TIRI was reported[9, 10], the pathophysiology of spermatic dysfunction following TIRI has not been sufficiently analyzed, because the therapeutic objective in clinical practice is simply to restore blood flow. Our research is a new study focusing on the spermatic dysfunction following TIRI and relevance between inflammatory changes and oxidative stress, which have been focused on the organs with a high risk of ischemia-reperfusion injury, and the pathology of spermatic dysfunction following TIRI which has not given much importance so far. Our study showed tissue thickening in interstitial tissues and lymphocytes-predominant inflammatory changes were observed at week 1 in the affected testis and gradually increased at over time in the bilateral testis. Oxidative stress was also immediately observed in the affected testis and gradually affected the unaffected testis starting on week 3. These changes can result in early to long-term testicular and spermatic dysfunction in the bilateral testes. Our results suggest that suppressing oxidative stress such as antioxidants and inflammatory changes such as IDO1 inhibition before reperfusion surgery may preserve the spermatic function in TIRI and long-term male fertility.

Creation method and validity of TIRI model mice

The recovery time from testicular ischemia to reperfusion and the degree of spermatic cord rotation are important factors that determine the degree of testicular dysfunction[4]. In human cases of spermatic cord rotation ranging from 180° to 360°, testicular atrophy does not occur until 12 hours after onset[19]. However, in cases wherein the spermatic cord rotates ≥ 360°, testicular atrophy may occur even if orchiopexy is performed within 4 hours after onset[19]. Furthermore, in cases wherein the spermatic cord rotates ≥ 360° and ≥ 24 hours has passed after onset, testicular atrophy will occur in all patients[19]. Animal studies of TIRI mainly used mice

and rats[20, 21]; in this study, we used C57BL/6J mice as a TIRI model animal. There are various methods to create an animal ischemic model, depending on the twisting method and duration of twisting[20, 21]. In our study, we used Dieffenbach clamps (Bulldog clamps) to induce ischemia and reduce variance from the degree of rotation among all mice, and the ischemia time was set for 1 hour. We determined that our TIRI model is valid as a previous study had a similar ischemia time and that our preliminary research[22] revealed that the testis will be completely necrotic following 2 hours or more of clamping.

Relationship between oxidative stress and inflammation in ischemic-reperfusion injury

Previous studies reported the relationship between oxidative stress and inflammation in the pathophysiology of ischemic-reperfusion injury[22–24]. Oxidative stress plays an important role in inducing necroptosis, which is programmed necrosis, in various conditions that cause ischemic-reperfusion injury[22–24]. Our preliminary examination[22] showed the relationship between pathophysiology of TIRI and various cell deaths which mainly composed of necroptosis. In addition, immunostaining (TUNEL staining) showed the concordance between cell death and giant cells[22]. Gliem *et al.*[24] reported that CCL2 is produced from ischemic brain cells in ischemic-reperfusion injury after cerebral infarction and activates macrophages after cerebral infarction. However, CCL2 receptor inhibition resulted in the suppression of macrophage infiltration[25]. Bonilla *et al.*[26] reported that IL-1β and IL-23 are produced from ischemic brain cells in ischemic-reperfusion injury after cerebral infarction. IL-1β is a precursor protein produced by activated macrophages, and IL-1β and IL-23 induce expression of IL-17, IL-21, and IL-22 by $\gamma\delta$T cells[27]. Nian *et al.*[28] reported that IL-1 and IL-6 were induced after myocardial infarction, causing ischemic-reperfusion injury. Our histological analysis showed the infiltration of lymphocytes-predominant inflammatory cells were observed in the left (affected) testis, but not observed in the right (unaffected) testis. However, tissue thickening in interstitial tissues and destruction of ductal structures were observed in bilateral testes. At the same periods, increase of T-cell related cytokines (IL-2, IL-3) and chemokines (CX3CL1), and macrophage-related cytokines (IL-17A, IL-23) and chemokines (CCL2, CCL5, CCL23) were observed in bilateral testes. Our results showed lymphocytes-predominant inflammatory changes were mainly related to the pathophysiology in spermatic dysfunction following TIRI. Though the histological presence of monocytes and macrophages as components of inflammatory cells was rarely observed, macrophage-related inflammatory changes may also be relevant to the pathophysiology in spermatic dysfunction following TIRI, and tissue thickening in interstitial tissues may play a role in some inflammatory changes.

Semen changes following TIRI

The spermatic dysfunction is caused by disorder of spermatogenesis, such as disorder of sperm production and maturation, and obstruction of seminal duct, when abnormalities occur in the spermatogenesis organs[29]. Our study suggests that the spermatic dysfunction is mainly caused by disorder of sperm production and maturation, and obstruction of seminal duct is negative because of the presence of semen in semen analysis. However, it is difficult to distinguish between probabilities sperm production or maturation. Aitken *et al.*[30] reported that oxidative stress in semen may adversely affect male fertility. Overproduction of reactive oxygen increases oxidative stress in semen and causes spermatic dysfunction due to DNA damage[30]; however, spermatic dysfunction can be prevented by suppressing oxidative stress[31–33]. Furthermore, Ho *et al.*[20] reported that spermatic

dysfunction can be prevented by suppressing inflammatory changes after TIRI. Our study revealed that oxidative stress and inflammatory changes are associated with spermatic dysfunction, which may be prevented by suppressing these changes.

CONCLUSIONS

Spermatic dysfunction in TIRI is induced by inflammation and oxidative stress. Inflammation and oxidative stress may be novel regulatory factors to prevent spermatic dysfunction in TIRI.

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CONFLICT OF INTEREST

None

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