Ischemic preconditioning and postconditioning are effective strategies to reduce testicular torsion-detorsion injury

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Key words: ischemic preconditioning, ischemic postconditioning, ischemia-reperfusion injury, testis, oxidative damage

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Abstract:

Purpose: The main pathophysiology of torsion-detorsion is associated with ischemia-reperfusion injury (I-R injury) in the testis caused by the twisted spermatic cord and its release, which is most likely mediated by oxygen free radicals. In this study, we investigated the effects of ischemic preconditioning (IPreC) and postconditioning (IPostC) on rat testicular I-R injury.

Materials and Methods: Eight-week-old male Sprague Dawley rats were divided randomly into four aged-matched groups: a sham-operated control~~ rats~~, 60 min ischemia/~~-~~120 min reperfusion (I-R)~~ rats~~, three cycles of 5 min ischemia/~~-~~5 min reperfusion and then 60 min ischemia/~~-~~120 min reperfusion (IPreC)~~ rats~~, and 60 min ischemia and then five cycles of 10 sec reperfusion/~~-~~10 sec ischemia and subsequently 120 min reperfusion (IPostC)~~ rats~~. After sacrifice, the levels of malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), myeloperoxidase (MPO), superoxide dismutase (SOD), catalase (CAT), HSP 70 protein and its mRNA, and DNA fragmentation were measured in the rat testis. The~~Histological analysis of~~ testicular tissue was also histologically analyzed~~performed~~.

Results: The levels of MDA, 8-OHdG, MPO, HSP 70 mRNA, SOD, CAT, DNA fragmentation, and apoptosis cells were significantly higher in the I-R group ~~significantly increased compared to~~than in the control group. IPreC reduces histological parameters including vacuolation and necrosis, and reduces MDA, 8-OHdG, MPO, HSP70 mRNA but not protein, SOD, CAT, DNA fragmentation and apoptosis compared to the I-R group, while IPostC ameliorates 8-OHdG, SOD, HSP70 mRNA, DNA fragmentation and apoptosis when compared to the I-R group.

Conclusions: Our data indicated that both IPreC and IPostC treatments ameliorated~~ed~~

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~~effect on~~ testicular damage induced by I-R injury.

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Testicular torsion is a common urologic emergency among infants and adolescents. Testicular damage after spermatic cord torsion is related to the period of ischemia and to the severity of the torsion.[1] The main pathophysiology of testicular torsion is ischemia-reperfusion injury (I-R injury) of the testis caused by the twisted spermatic cord and its release, which is most likely mediated by oxygen free radicals.[2] Mammalian testes are highly sensitive to oxidative free radical damage, and several antioxidant enzymes and antioxidant drugs have been reported to prevent testicular I-R injury.[3] Ischemic preconditioning (IPreC) is a~~the~~ phenomenon ~~that~~ whereby a prior ischemic stress renders the organ resistant to a subsequent ischemic insult.[4] It has been demonstrated that brief episodes of sublethal I-R and IPreC provide~~s~~ powerful tissue protection in different tissues such as heart, brain, skeletal muscle, lung, liver, intestine, kidney, retina, and endothelial cells.[5] However, to our knowledge, the protective effects of IPreC on testicular tissue have not been ~~not~~ investigated adequately.[6, 7]

A~~R~~ recent development in cardiac physiology has indicated that ischemic postconditioning (IPostC) is an interesting mechanism against reperfusion injury.[8] IPostC is defined as rapid intermittent interruptions of blood flow in the early phase of reperfusion; these interruptions~~and~~ mechanically alter~~s~~ the hydrodynamics of reperfusion.[9] It is a simple method ~~which~~ that provides a new tool to protect organs from I-R injury in the heart and brain.[10] However, it is unclear whether IPostC can protect the teste~~i~~s against I-R injury. The present study was thus planned to investigate whether or not IPreC and IPostC have a protective effect on testicular I-R injury.

MATERIALS AND METHODS

Animal model and experimental design

All animal experiments were performed in accordance with the guidelines set by the Tottori University Committee for Animal Experimentation. Eight-week-old male Sprague-Dawley rats weighing 260–300 g (SLC, Shizuoka, Japan) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). The rats were assigned to one of four groups based upon the intervention (n = 5–6 in each group). I-R group; 60 min ischemia/-120 min reperfusion ~~rats~~, IPreC group; three cycles of 5 min ischemia/-5 min reperfusion and then 60 min ischemia/-120 min reperfusion ~~rats~~, IPostC group; 60 min ischemia followed by~~and then~~ five cycles of 10 sec reperfusion/-10 sec ischemia and subsequently 120 min reperfusion, and Cont group; a sham-operated control ~~rats rats~~. T~~In order t~~o perform I-R in the testes, the right testicular artery was clamped with a small clip (Sugita standard aneurysm clip, holding force 145 g; Mizuho Ikakogyo, Tokyo) for 60 min, ~~and then removing the clip another~~after which the clip was removed for 120 min. T~~In order t~~o confirm these treatments in experimental testes, blood flow in the right testis was measured with a Laser Doppler Flow meter (BRL-100; Bioresearch Co., Nagoya, Japan) during the experimental period (Fig. 1), according to the method used in our previous report.[11] The rats were sacrificed with an overdose of pentobarbital (60 mg/kg, i.p.) at 120 min reperfusion. After the sacrifice, the testis was fixed in 10% phosphate-buffered formalin or immediately frozen~~,~~ and then stored at –80 °C until used.

Measurement of MDA concentration and~~,~~ 8-OHdG content in the testes

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T~~In order t~~o investigate oxidative damage in~~of~~ the testis during I-R, malondialdehyde (MDA) concentration, a marker of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG) content, a marker of oxidative DNA damage, ~~respectively~~ were each measured in the experimental rat testis using a commercially available kit. The MDA concentration in the testis was measured by colorimetric assay according to the manufacturer's instructions (BIOXYTECH MDA-586™ kits, OXIS International, Portland, OR). The 8-OHdG content in the extracted DNA solution was determined by the enzyme-linked immunosorbent assay (ELISA) method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Aging, Shizuoka, Japan).

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Measurement of MPO activity in the testes

Myeloperoxidase (MPO) activity in testicular tissue was detected using a spectrophotometric method (MPO ELISA kit, HyCult Biotechnology, Uden, the Netherlands), reflecting the number of polymorphonuclear neutrophils (PMN) in the tissue. This method uses 3, 3', 5, 5'-tetramethyl benzidine (TMB) as an oxidizable dye. T~~, and t~~he reaction was started by adding hydrogen peroxide (H_2O_2) to~~in~~ the medium.

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Measurements of SOD and CAT activities in the testes~~.~~

Superoxide dismutase (SOD) and catalase (CAT) are important members in the antioxidant enzymatic defense system, which converts the superoxide radical to H_2O_2. The procedures f~~t~~or quantifying SOD and CAT activity were carried out according to the descriptions provided with the~~of~~ Superoxide Dismutase Assay Kit and Catalase

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assay Kit (Cayman Chemical, Ann Arbor, MI~~, USA~~)~~-~~, respectively.

Measurements of HSP 70 protein and its mRNA level~~.~~

~~The expression of~~ HSP 70 expression was measured using a StressXpress HSP 70 enzyme-linked immunosorbent assay (ELISA) kit (Stressgen Biotechnologies, Victoria, BC, Canada) according to the manufacturer's instructions. HSP 70 mRNA in the experimental testis was measured by real-time polymerase chain reaction (PCR) methods. The RNA was purified by an RNeasy Mini Kit (Q~~u~~iagen, Valencia, CA) according to the manufacturer's instructions. A~~The~~ reverse~~-~~transcriptase (RT) mixture (20 μl) containing 2 μg of total RNA was made and incubated at 37°C for 60 min according to ~~by~~ a previously reported method.[12] Real-time PCR was carried out using a LightCycler thermal cycler system with a LightCycler SYBR Green I kit according to the manufacturer's instructions (Roche Diagnostics, Tokyo, Japan).[13] The following primers were used for HSP 70 (accession number NM_212504), forward, 5' ACA AGG GCG AGA ACC GGT C 3'; reverse, 5' TTC AGA CCC GCG ATC ACG 3'.

Protein assay

Protein was determined using a commercial kit (Protein Assay Rapid Kit~~wako~~, Wako Pure Chemical Industries, Osaka, Japan).

DNA fragmentation analysis

DNA fragmentation was assessed ~~with~~ using the Apoptotic DNA Ladder Extraction Kit (Biovision, Mountain View, CA, USA), and was analyzed by electrophoresis on a 1.2%

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agarose gel containing ethidium bromide in both gel and running buffer. Electrophoresis was run at 50 V for 60 min. DNA was visualized with UV light and photographed.

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Histological examination

After fixation, the tissues were embedded in paraffin. Five micron-thick tissue sections were cut from these paraffin blocks. The sections were deparaffinized and hydrated gradually, and then were examined by h~~H~~ematoxylin and e~~E~~osin (H&E) staining. Each section was viewed under a light microscope at a magnification of ×400. Histological examinations were performed under a light microscope by a pathologist blinded to the experiment. The testes were evaluated histologically with respect to ~~the following characteristics:~~ their vacuolation and~~,~~ necrosis characteristics. A five~~5~~-level original grading scale was used to quantify ~~for~~ each characteristic~~s~~. Histological grading was based on the following scale: 0, minimal or no evidence of injury; l, slight~~ly~~ injury; 2, mild injury; 3, moderate injury; 4, severe injury. Statistical evaluations also used ~~was made using~~ this scale.

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TUNEL assay

Testicular DNA fragmentation was evaluated with the TUNEL assay~~.~~ (Apop Ta~~q~~g Plus Peroxidase In Situ Apoptosis Detection Kit, Chemicon Laboratories, Temecula, CA, USA). Formalin-fixed, paraffin wax-embedded tissue sections (n=5-6 for each group) were deparaffinized, ~~and~~ stained by the TUNEL technique, and ~~was~~ used as a chromogen. TUNEL-positive cells displayed brown staining within the nucleus of

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apoptotic cells. TUNEL-positive cells were quantified under high-power magnification (x400) by an investigator who was blinded to the studies, and were expressed as numbers per seminiferous tubule. At least 100 seminiferous tubules on each slide were randomly examined to determine the number of TUNEL-positive cells.

Data analysis

A statistical comparison of differences between groups was performed with the use of analysis of variance and Fisher's multiple comparison tests. $P < 0.05$ was regarded as the level of significance.

Drugs and chemicals

All other chemicals were available commercially and reagent grade.

RESULTS

Measurements of oxidative damage and neutrophil infiltration in the testes

Table 1 shows l~~L~~evels of MDA concentration, 8-OHdG content, and MPO activity in the testes ~~respectively, are shown in Table 1~~. The MDA concentration, 8-OHdG content, and MPO activity ~~in the I-R group~~ were significantly higher in the I-R group than ~~those~~ in the sham-operated control group. The MDA concentration, 8-OHdG content, and MPO activity in the IPreC group were significantly decreased compared to those in the I-R group. The MDA concentration and MPO activity in the IPostC group were slightly, ~~but~~ not significantly, lower than~~decreased compared to~~ those in the I-R group. However, IPostC treatment significantly reduced the 8-OHdG content. Our data indicate that ~~treatment with~~ IPreC treatment ameliorated the increases in~~of~~ oxidative damage and neutrophil infiltration in the testis during I-R, and that IPostC treatment also ameliorated the increase in~~of~~ oxidative DNA damage.

Antioxidant enzyme activities

Table 2 shows the individual activities of SOD and CAT. ~~SOD and CAT~~These activities were significantly higher in the I-R group ~~higher~~ than ~~those~~ in the sham-operated control group. Furthermore, SOD and CAT activities were significantly lower~~decreased~~ in the IPreC group than in~~compared with~~ the I-R group. IPostC treatment also significantly reduced SOD activity compared with the I-R group. CAT activity was slightly, ~~but~~ not significantly, lower~~decreased~~ in the IPostC group than in ~~compared with~~ the I-R group.

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Expression levels of HSP 70 protein and its mRNA in the testes~~.~~

Figure 2 shows~~The~~ the data on the expression levels~~s~~ of HSP 70 protein and its mRNA in the testis~~ is shown in Fig 2~~. The expression of the HSP 70 protein was slightly but not significantly higher in the I-R group than in the control group. However, ~~the expression of the~~ HSP 70 mRNA expression was significantly higher in the I-R group than in the control group. IPreC and IPostC groups each had a slight~~ly~~ tendency~~d~~ to decrease the~~ expression of the~~ HSP 70 protein expression in the testis compared to that in the I-R group. The expression of the HSP 70 mRNA was significantly lower~~decreased~~ in the IPreC and IPostC groups than in~~compared with~~ the I-R group.

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Histological examination

The greatest significant histopathologic scores observed were in the I-R group. Extensive tubular vacuolation, necrosis, and loss of germ cell maturation~~ of germ cells~~ were observed in the I-R group. In contrast, IPreC treatment significantly reduced these I-R group changes~~ observed in the I-R group~~. Histopathologic scores ~~showed a~~ were dramatically decreased~~ score~~ in the IPreC group than in~~compared with~~ the I-R group. Histopathologic scores were slightly~~, but~~ not significantly, lower~~ decreased~~ in the IPostC group than in~~compared with~~ the I-R group (Fig. 3, Table 3).

DNA fragmentation and TUNEL assay

Apoptosis was evaluated by DNA fragmentation analysis and TUNEL assay (Fig. 4). A typical DNA laddering pattern was observed in the I-R group. I-R induced upregul~~r~~ation of DNA fragments was decreased by both the IPreC and IPostC

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treatments. In the TUNEL assay, a large number of TUNEL-positive germinal cells were observed in the seminiferous tubules of I-R injury testes, whereas TUNEL-positive cells were not detected in the seminiferous tubules of the sham-operated control group. However, it was difficult to distinguish if the TUNEL-positive cells were either Sertoli-cells or spermatocytes. Furthermore, we did not observed significant I-R-induced alterations neither in spermatids nor in spermatogoniums. The number of TUNEL-positive cells was significantly reduced in the seminiferous tubules by IPreC and IPostC treatments (Fig. 4). Our data indicate that IPreC and IPostC treatments had an anti-apoptotic effects on the testis during I-R.

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DISCUSSION

I-R injury in the testis is related to testicular tor~~i~~sion-detorsion, and~~ this I-R injury~~ is associated with overgeneration of reactive oxygen species (ROS).[14] I-R contributes to abnormal signal transduction or cellular dysfunction and initiates the cascade of apoptosis/necrosis, with subsequent inflammatory infiltration.[15] Reperfusion injury is an integrated response to the restoration of ~~the~~ blood flow after ischemia, and is initiated at the very early moments of reperfusion, lasting potentially for several days.[15]

Although some researchers have reported on~~that~~ IPreC's~~the~~ effect~~ of IPreC~~ on testicular I-R induced-damage, IPostC's~~ the~~ protective effects ~~of IPreC~~ on testicular tissue have not been investigated.[6, 7] Ceylan et al reported that there are no protective effects with IPreC in rat testis during 90 minutes of 720 degrees torsion, while Sahinkanat et al reported that IPreC provides tissue protection in testicular tissue.[6, 7] Although some reports indicate that IPreC and IPostC are effective especially in the reperfusion phase, these reports did not include~~lack of~~ observation in the reperfusion phase. Furthermore, IPreC is clinically feasible only when the occurrence of ischemia is predictable.[5] Compared to ischemia, ~~the onset of~~ reperfusion ha~~i~~s a more predictable onset. IPostC is a simple and harmless method that~~which~~ provides a new tool to protect organs from I-R injury.[10] The r~~R~~esults of~~from~~ these studies suggested that the early moments of reperfusion a~~we~~re important in the pathogenesis of postischemic injury, and that manipulation of this early reperfusion phase can reduce~~d~~ I-R injury. T~~he results of t~~he present study demonstrates that~~ the~~ IPreC and IPostC have protective effects against I-R-~~-~~induced biochemical and histological changes in~~ the~~ rat teste~~i~~s. To our knowledge, the present study provides the first evidence for the protective effect of IPostC against testicular I-R injury.

It is well known that the generation of excessive ROS during reperfusion plays a major role in I-R injury, and that increased production of ROS inflicts significant injury on ischemic tissue through oxidization of cell membrane lipids, protein, DNA leading to testicular dysfunction, and cell death.[3] Then, testicular torsion itself ~~causes a~~ significantly increases ~~in~~ neutrophil adhesion to the testicular venous endothelium.[16] It is reported that neutrophils recruited to the testis after torsion are potent generators of ROS.[16] In our study, testicular IPreC treatment significantly ameliorated the levels of MDA concentration, 8-OHdG contents, and MPO activity, suggesting ~~that there was~~ attenuated lipid peroxidation, DNA damage, and neutrophil infiltration, respectively. Testicular IPostC treatment ameliorated the level of 8-OHdG contents. It may explain that testicular 8-OHdG content is more sensitive marker of oxidative stress than the other markers used in this study. Our results showed that IPreC and IPostC inhibited oxidant generation and oxidant-mediated injury in testicular I-R injury. ~~Furthermore, our~~Our results also showed that the IPreC and IPostC significantly inhibited apoptosis caused by testicular I-R injury, ~~which was~~as proved by DNA fragmentation and TUNEL assay. The results of the present study demonstrate that ~~the~~ IPreC has preventive effects against I-R-induced biochemical and histological changes in the rat testis, and that ~~the~~ IPostC has protective effects against I-R-induced DNA damage. The protection achieved in the rat testis with ~~the~~ IPostC was not equivalent to the benefits gained by IPreC ~~in the rat testis~~. Therefore, strategically modifying early reperfusion events -to reduce reperfusion injury may not provide the same level of powerful testicular protection ~~by reducing reperfusion injury comparable to a~~provided by a pretreatment strategy such as IPreC. Because IPreC triggers protective pathways before ischemia while IPostC alters events after ischemia, the mechanisms and their timing ~~of those~~

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mechanisms are likely quite different between the two maneuvers. The involvement of "effectors" such as K_{ATP} channels and the mPTP in both IPreC- and IPostC would also suggest common pathways, but at which time (ischemia vs reperfusion) these pathways exert the organ-protection must differ. Although many of the pathways involved in IPostC have been identified in IPreC, some pathways, i.e., ERK1/2, may not be involved in IPreC. The timing of action of these pathways and other mediators of protection in IPostC differs from that of IPreC.[17, 18] In addition, it remains unclear whether deleterious mechanisms were attenuated or whether beneficial mechanisms were triggered by testicular IPreC and IPostC are still unclear.

Endogenous antioxidant systems (i.e., SOD, CAT) counteract the potential for injury to cellular structures by regulating the balance of individual ROS and their reactants.[19] Heat-shock protein (HSP) chaperones, which are also induced by stress, are essential cellular protective mechanismsachineries.[20] HSP 70 is an endogenous factor for protecting cell againstand tissue injury under various pathological conditions.[21] Data from this study demonstrated that I-R induced up-regulations of the expression ofs SOD, CAT, HSP 70, and its mRNA. Our study also revealed that IPreC and IPostC treatments decreased the I-R--induced upregulration of SOD, CAT, HSP 70 mRNA in the testis during I-R. Further studies will beare needed to clarify the mechanism responsible for these phenomenaons.

In our research, we only tested the IPostC using only five cycles of 10 sec of reperfusion followed by 10 sec ischemia. The interval (10 sec) referred towas chosen– previouslyliterature[22] and our limited preliminary experiments. Thus, the exact numbers of optimal intervals and cycles in testicular IPostC may need to be investigated in a further study.

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CONCLUSIONS

Our study provides~~ an~~ evidence ~~to demonstrate~~of the beneficial effects of testicular IPreC and IPostC in vivo. In particular, the intervention ~~of~~ by IPostC is very simple and may be applicable~~, which~~ for targeting~~s~~ the first few minutes of reperfusion. There is a possibility ~~to be~~of clinically ~~applicable in~~applying the treatment ~~o~~for testicular torsion in the near future.

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Figure legends

Fig. 1. Hemodynamic data during experimental period in ~~the~~ rat testes.

Fig. 2. Expression levels of HSP 70 protein and its mRNA in ~~testis of~~ rat~~s~~ testes. Data are shown as mean ± SEM of five ~~to~~ or six separate determinations in each group. ~~Expression of~~ HSP 70 protein expression was normalized with protein content. ~~Expression of~~ HSP 70 mRNA expression was normalized with that of beta-actin mRNA.

* significantly different from control~~.~~ (p<0.05).

† significantly different from I-R (ischemia-reperfusion)~~.~~ (p<0.05).

Fig. 3. Histological changes evaluated by hematoxylin-eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining.

TUNEL-positive germinal cells in the seminiferous tubules (black arrows).

Original magnification: ×400.

Fig. 4. A: DNA fragmentation analysis. B: T~~t~~he apoptosis index was calculated as the number of apoptotic cells per 100 seminiferous tubules.

* significantly different from control~~.~~ (p<0.05).

† significantly different from I-R (ischemia-reperfusion). (p<0.05)

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Table 1. Measurements of MDA concentration, 8-OHdG content and MPO activity in rat testes.

	MDA (nmol/mg protein)	**8-OHdG (ng/mg DNA)**	**MPO (ng/mg protein)**
Cont	1.02 ± 0.09	0.50 ± 0.03	0.07 ± 0.01
I-R	3.69 ± 0.17*	1.66 ± 0.20*	0.91 ± 0.15*
IPreC	2.91 ± 0.15*†	0.85 ± 0.15*†	0.38 ± 0.10†
IPostC	3.30 ± 0.39*	1.10 ± 0.10*†	0.77 ± 0.26

Data are shown as mean ± SEM of five to six separate determinations in each group. MDA concentration and MPO activity were normalized with protein content. 8-OHdG content was normalized with DNA content.

* significantly different from cont (control). ($p<0.05$)

† significantly different from I-R (ischemia-reperfusion). ($p<0.05$)

Table 2. Antioxidant enzymatic activities of SOD and CAT in rat testes

	SOD (U/mg protein)	CAT (U/mg protein)
Cont	0.85 ± 0.03	2.16 ± 0.19
I-R	1.22 ± 0.03*	3.11 ± 0.22*
IPreC	0.87 ± 0.11†	2.31 ± 0.13†
IPostC	0.94 ± 0.11†	2.82 ± 0.16‡

Data are shown as mean ± SEM of five to six separate determinations in each group. SOD (superoxide dismutase) and CAT (catalase) activities were normalized with protein content.

* significantly different from cont (control). ($p<0.05$)

† significantly different from I-R (ischemia-reperfusion). ($p<0.05$)

‡ significantly different from IPreC (ischemic preconditioning). ($p<0.05$)

Table 3. Overall testicular injury scores.

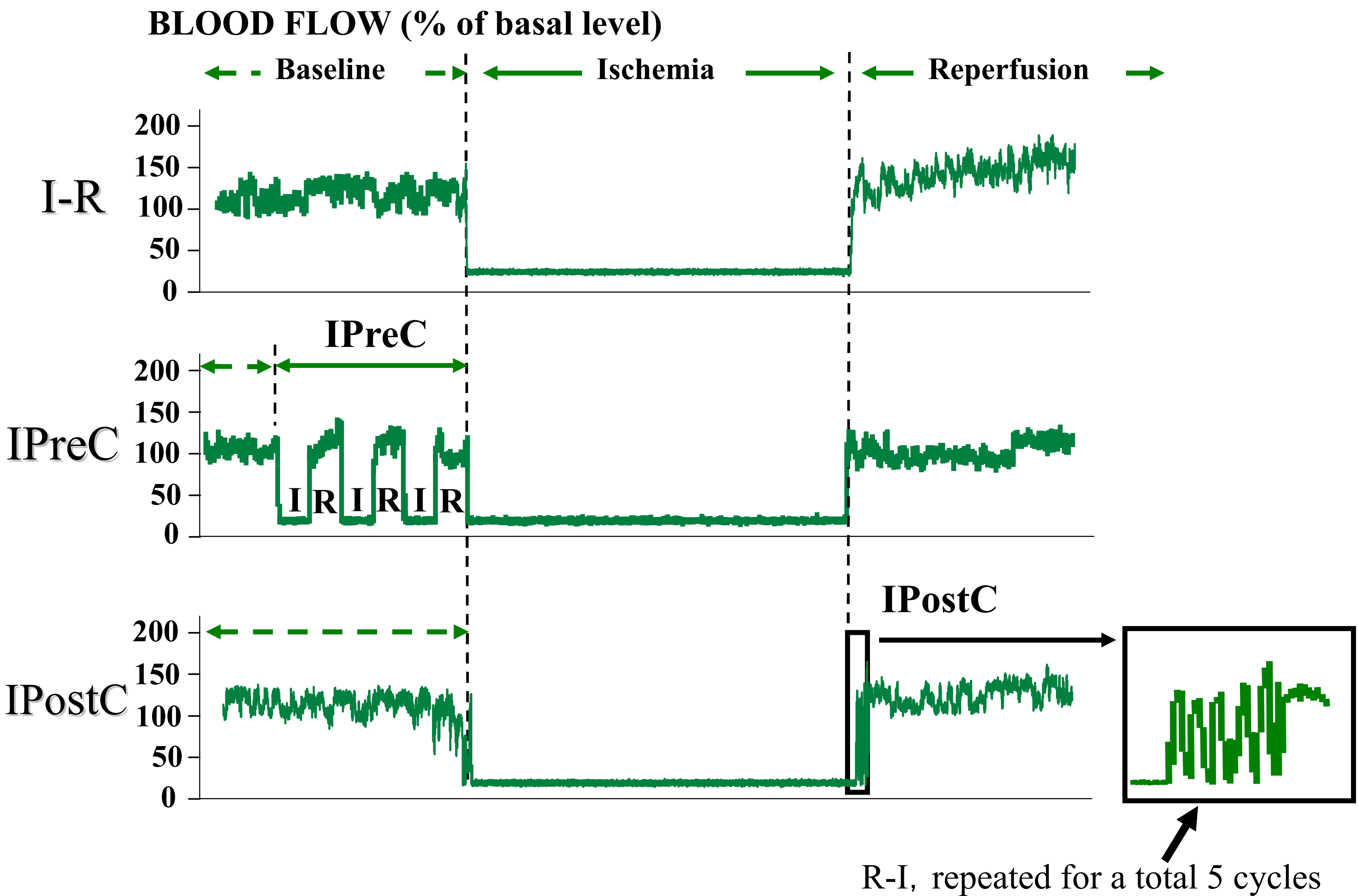
	Vacuolation	Necrosis	Total score
Cont	0.17 ± 0.17	1.00 ± 0	1.17 ± 0.17
I-R	3.17 ± 0.31*	2.67 ± 0.21*	5.83 ± 0.31*
IPreC	1.20 ± 0.25*†	1.20 ± 0.20†	2.40 ± 0.40*†
IPostC	3.40 ± 0.24*‡	1.80 ± 0.20*†	5.20 ± 0.20*‡

Data are shown as mean ± SEM of five to six separate determinations in each group.

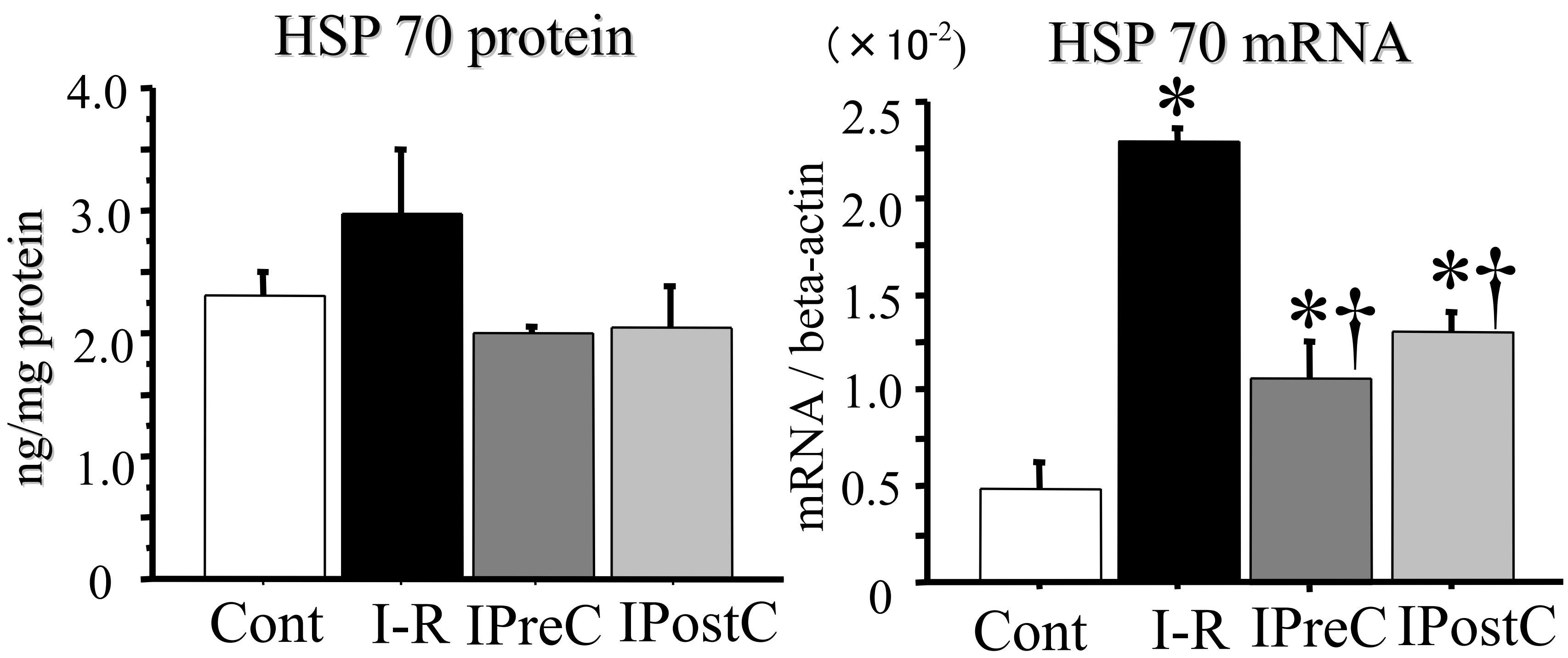
A 5-level original grading scale was used to quantify for each characteristics. Histological grading was based on the following scale: 0, minimal or no evidence of injury; l, slightly injury; 2, mild injury; 3, moderate injury; 4, severe injury.

* significantly different from cont (control). ($p<0.05$) † significantly different from I-R (ischemia-reperfusion). ($p<0.05$)

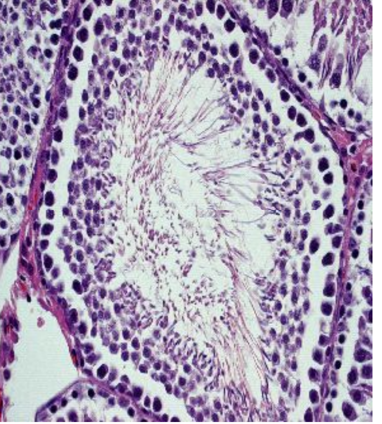
‡ significantly different from IPreC (ischemic preconditioning). ($p<0.05$)

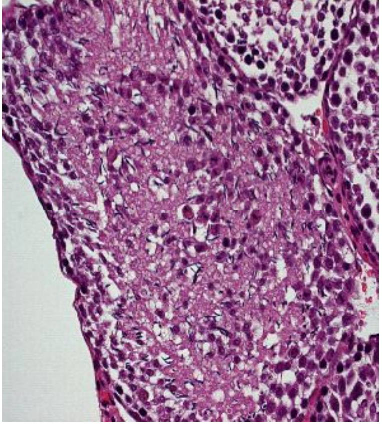


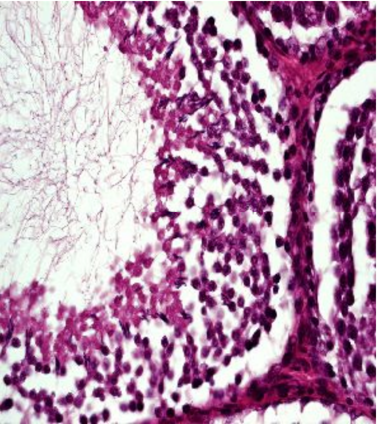
BLOOD FLOW (% of basal level)
Baseline
Ischemia
Reperfusion
I-R
200
150
100
50
0
IPreC
I
R
I
R
I
R
IPostC
R-I, repeated for a total 5 cycles

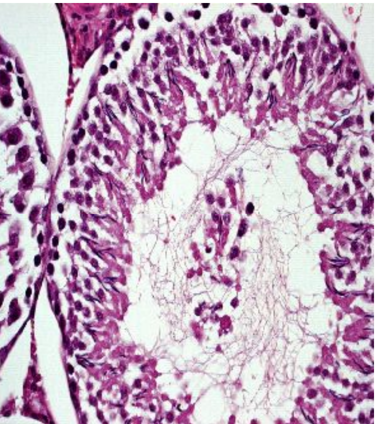

HSP 70 protein
ng/mg protein
4.0
3.0
2.0
1.0
0
Cont
I-R
IPreC
IPostC
HSP 70 mRNA
(×10-2)
mRNA / beta-actin
2.5
2.0
1.5
1.0
0.5
0
*
*†
*†
Cont
I-R
IPreC
IPostC

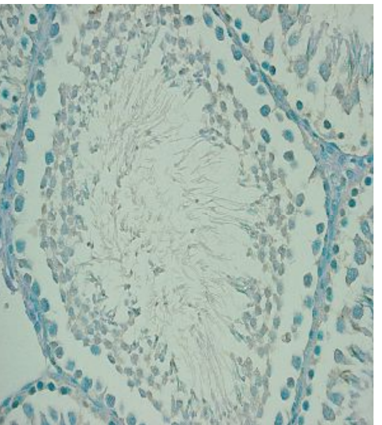
Cont
I-R
IPreC
IPostC
HE
TUNEL

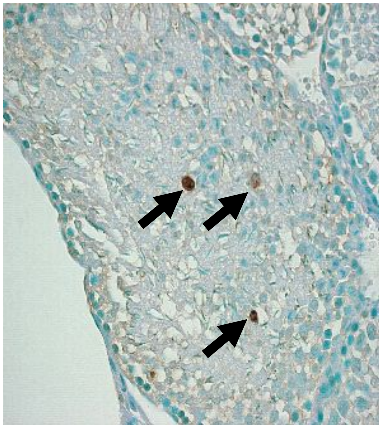


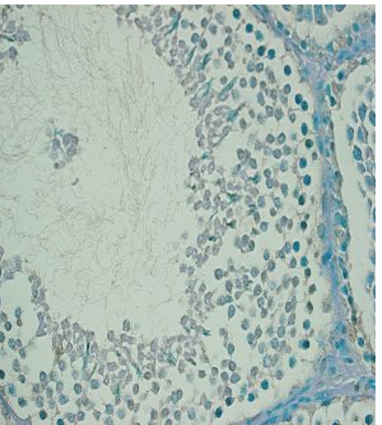


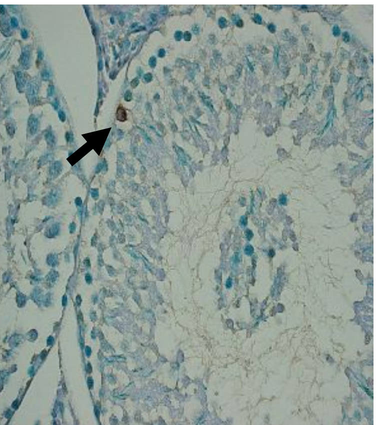












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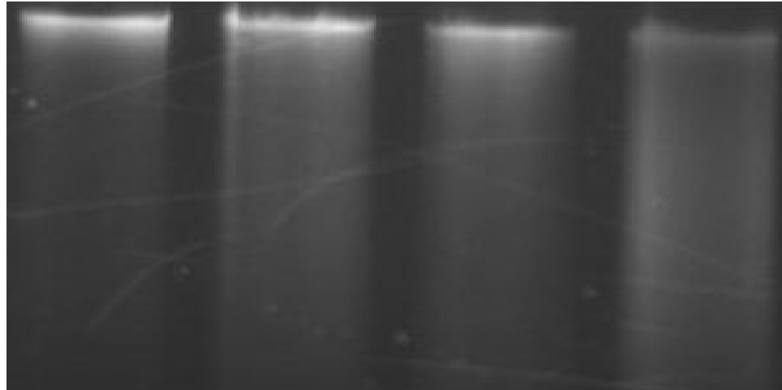

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