Molecular and Cellular Biochemistry **286:** 139–145, 2006.
DOI: 10.1007/s11010-005-9105-3

Real-time monitoring of nitric oxide and blood flow during ischemia-reperfusion in the rat testis

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Received 10 Octorber 2005; accepted 5 December 2005

Abstract

In the present study, we attempted to clarify the role of nitric oxide (NO) and its release during the ischemia-reperfusion rat testis. Eight-week-old male Sprague-Dawley rats were divided into seven groups: age-matched control rats, ischemia (30 minutes)-reperfusion (30 minutes) rats without N^G-nitro-L-arginine methyl ester (L-NAME) and L-arginine (L-Arg) treatment, ischemia (30 minutes)-reperfusion (30 minutes) rats treated with L-NAME (10, 30, and 100 mg/kg), ischemia-reperfusion rats treated with L-Arg (10 and 30 mg/kg). Sixty minutes prior to induction of ischemia, L-NAME or L-Arg was administrated intraperitoneally. Real-time monitoring of blood flow and NO release were measured simultaneously with a laser Doppler flowmeter and an NO-selective electrode, respectively. NO_2-NO_3 and malonaldehyde (MDA) concentrations were measured in the experimental testes. Furthermore, we investigated possible morphological changes in the testis. Clamping of the testicular artery decreased blood flow to 5–20% of the basal level measured before clamping. Immediately following clipping of the artery, NO release rapidly increased. After removing the clip, NO release gradually returned to the basal level. This phenomenon was enhanced by treatment with L-Arg and inhibited by treatment with L-NAME. NO_2-NO_3 concentrations were increased by treatment with L-Arg and decreased by treatment with L-NAME, while MDA concentrations were increased by treatment with L-NAME and were decreased by treatment with L-Arg. In histological studies, the ischemia-reperfusion caused infiltration of leukocytes and a rupture of microvessels in the testis. Our data suggest that NO has cytoprotective effects on ischemia-reperfusion injury in the rat testis. (Mol Cell Biochem **286:** 139–145, 2006)

Key words: nitric oxide, ischemia, reperfusion, testis, NO monitor, laser Doppler flowmeter

Introduction

The presentation of a child or adolescent with acute scrotal pain, tenderness, or swelling should be looked upon as an emergency situation requiring prompt evaluation, differential diagnosis, and potentially immediately surgical exploration. One of the main causes of acute scrotum in child and adolescent is testicular torsion. The occurrence of testicular torsion has been estimated to be as high as 1 in 4,000 males by the age of 25, and has been implicated in testicular injury and infertility [1]. The main etiology of torsion-detorsion is associated with ischemia-reperfusion in the testis.

Nitric oxide (NO) has been shown to be an important paracrine messenger and neurotransmitter that helps the body maintain vascular tone and thus blood pressure, and that promotes homeostasis and various functions in many tissues [2, 3]. NO and NO synthases (NOS) are widely localized in the testis. NO has been implicated as a crucial regulator

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in inflammation, and NOS is a regulator of the endocrine system in the testis. NO and NOS are thought to regulate an array of functions, including sperm motility, maturation and germ cell apoptosis, Sertoli cell tight junction dynamics, and Leydig cell steroidogenesis. High concentrations of NO mediate DNA deamination, oxidation, or nitration via the interaction of NO with either oxygen or surperoxide radicals, and low concentrations of NO directly interact with soluble guanylate cyclase to induce synthesis of cGMP and to induce MAP kinase signaling pathways in the testis [4].

Reperfusion of ischemic tissue leads to a sequence of events that, paradoxically, injure tissues. Superoxide and hydroxyl radicals are considered to be major reactive oxygen species contributing to ischemia-reperfusion injury in many tissues. These radicals attack and damage many biological molecules, thereby increasing lipid peroxides in membranes. Peroxynitrite, reacting with NO and superoxide radicals, has also been reported to be a highly reactive compound that can have harmful effects on various cells and tissues [5, 6]. On the other hand, NO has been reported to be cytoprotective in many tissues in ischemia-reperfusion. Ischemia rapidly decreases the extracellular pH, and the decrease in extracellular pH is maintained during the ischemic condition [7]. The reduction of NO_2 to $NO^{\cdot}$ is promoted by ischemia-induced acidosis as well as enzymatic catalysis. Primarily, $NO^{\cdot}$ is considered to be protective in many tissues, including heart and kidney [8, 9]. $NO^{\cdot}$ donors protect against ischemia-reperfusion damage in in vitro models of these tissues. However, Ozokutzan and associates have reported that NO plays a harmful role in ischemia-reperfusion in the testis [10]. In contrast to their report, some reports indicate that NO plays a cytoprotective role in ischemia-reperfusion in the testis [11–13]. Thus, the role of NO in ischemia-reperfusion testis is complicated and confusing. In the present study, we attempted to clarify the relations of NO release and blood flow during ischemia-reperfusion and the role of NO in ischemia-reperfusion in the rat testis.

Materials and Methods

Preliminary studies

In order to examine the effects of N^G-nitro-L-arginine methylester (L-NAME) and L-arginine (L-Arg) in the testis, L-NAME or L-Arg was intraperitoneally administrated 10, 30, and 100 mg/kg or 10 and 30 mg/kg, respectively, and real-time blood flow and NO release in the rat testis were simultaneously monitored (n = 5). In each rat, L-NAME or L-Arg was administrated and cumulative dose-response curves were constructed in a stepwise manner after the NO release response to the previous dose had reached a plateau. The precise methodology was detailed in the production of the animal model, measurement of blood flow in the testis, and in vivo real-time monitoring of NO release in the testis sections.

Ischemia-reperfusion model

All animal experiments were performed in accordance with the guidelines set by the Tottori University Committee for Animal Experimentation. Male Sprague-Dawley rats (8 weeks old, weighting 240–280 g, SLC, Shizuoka, Japan) were divided randomly into seven groups: age-matched control rats (A group), ischemia-reperfusion rats without either L-NAME or L-Arg treatments (B group), ischemia-reperfusion rats treated with L-NAME (10, 30, and 100 mg/kg, i.p.; C, D, E groups, respectively), and ischemia-reperfusion rats treated with L-Arg (10 and 30 mg/kg, i.p.; F and G groups, respectively)(in each group, n = 6–8). Sixty minutes prior to induction of ischemia, L-NAME or L-Arg was administrated intraperitoneally. Under pentobarbital anesthesia (30 mg/kg, i.p.), lower abdominal midline incision was performed and bilateral testes were exposed outside of the body. In order to perform ischemia and reperfusion in the testis, bilateral testicular arteries were clamped with small clip (Sugita standard aneurysm clip, holding force 145 g; Mizuho Ikakogyo, Tokyo) for 30 min in the B, C, D, E, F, and G groups. Subsequently, these groups were exposed to 30 minutes of reperfusion in the bilateral testes. The protocol of this experiment was shown in Fig. 1. After ischemia-reperfusion, the left testis was immediately isolated, frozen in liquid nitrogen, and stored at −80°C until use for biochemical studies, and the right testis was immediately fixed with 10% formalin for histological studies.

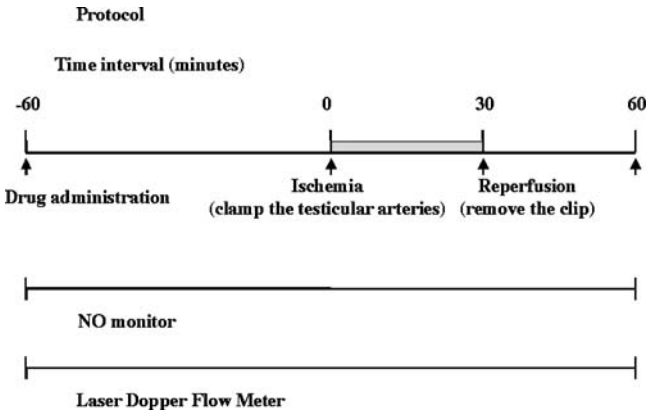

Fig. 1. Protocol of the experiments.

Measurement of blood flow in the testis

Blood flow in the rat testis was measured with a laser Doppler flowmeter (BRL-100, Bioresearch Co., Nagoya, Japan) as previously reported, with a minor modification [14–16]. Briefly, under pentobarbital anesthesia, bilateral testes were accessed via central incision of the lower abdomen and exposed outside of the body. A needle probe (tip diameter of 1 mm) was put into the rat left testis (approximately 5 mm) and the probe was fixed with glue. Subsequently, blood flow in the testis was measured before, during, and after ischemia in the B, C, D, E, F, and G groups. Blood flow is expressed as a percentage of the basal level.

In vivo real-time monitoring of NO release in rat testis

Real-time monitoring of NO release in the rat testis was conducted in the B, C, D, E, F, and G groups as reported previously, with minor modifications [14–16]. In brief, an NO-selective electrode (NOE-47, tip diameter of 0.2 mm; Inter Medical Co., Nagoya) was inserted into the left testis, and the tip of the electrode was fixed in the testis. A reference electrode was placed in the subcutaneous tissue. In addition, real-time monitoring of blood flow and NO release in the rat testis were measured simultaneously with a laser Doppler flowmeter and an NO-selective electrode, respectively. Production of NO was measured with an NO-monitor (Model NO-501; Inter Medical Co., Nagoya) and expressed in terms of current in pico amperes (pA). NO production during ischemia-reperfusion is expressed as a percentage of the basal level.

Measurement of NO_2-NO_3 concentrations in the testis

The final products of NO in vivo are NO_2-NO_3. The relative proportion of NO_2-NO_3 is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both nitrite and nitrate. NO_2-NO_3 concentrations in the testis were measured by means of the Griess method. In short, the tissues were homogenized in PBS (pH 7.4) with the Multi-beads Shocker® (YASUIKIKI, Osaka, Japan) and centrifuged at 10,000 × g for 20 minutes. The supernatant solutions were recentrifuged at 100,000 × g for 30 minutes. Then the supernatant solutions were used to perform the NO_2-NO_3 assay. NO_2-NO_3 concentrations were measured by colorimetric assay according to the manufacturer's instructions (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical Co., Ann Arbor, MI). The absorbance was measured at 540 nm. The values were estimated per amount of protein in the tissue. Protein was determined using a commercial kit (Protein Assay Rapid Kit $_{wako}$, Wako Pure Chemical, Osaka, Japan).

Measurement of malonaldehyde (MDA) concentrations in the testis

In order to investigate lipid peroxidation of the testis during ischemia-reperfusion, MDA, a marker of lipid peroxidation, concentrations were measured in the experimental rat testis. The tissues were chopped into small pieces and the pieces were then homogenized in 4 volumes of PBS buffer in 5mM BHT with two 15-second bursts and 30-second intervals for cooling using a POLYTRON® (KINEMATICA AG, Switzerland) with the speed set at 7 (70% of maximum power). Then the MDA concentrations in the testis were measured by colorimetric assay according to the manufacturer's instructions (BIOXYTECH MDA-586™ kits, OXIS International, Portland, OR). The absorbance was measured at 586 nm. The values were estimated based on the amount of protein in the tissue. Protein was determined using a commercial kit (Protein Assay Rapid Kit$_{wako}$, Wako Pure Chemical, Osaka, Japan).

Histological examination of the rat testis

After fixation, the tissues were embedded in paraffin. Five micron-thick tissue sections were cut from these paraffin blocks. All of the testis specimens were stained using Hematoxylin and Eosin (H & E) staining in accordance with previous reports [15]. Each section was viewed under a light microscope at a magnification of ×40–400.

Data analysis

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

Drugs and chemicals

N^G-nitro-L-arginine methylester (L-NAME) was purchased from Sigma (St. Louis, MO). L-arginine (L-Arg) was purchased from Wako Pure Chemical, (Osaka, Japan). All other chemicals were of reagent grade.

Result

Effects of L-NAME and L-Arg on NO release and blood flow in the normal rat testis

Table 1 shows the effects of L-NAME on NO release and blood flow in the rat testis. Treatment with L-NAME significantly decreased the NO release and blood flow in the testis. In contrast to the treatment with L-NAME, treatment

Table 1. Effect of L-NAME on testicular Blood flow and NO release

	Blood Flow (% of basal level)	NO release (% of basal level)
0 mg/kg	100 ± 0	100 ± 0
10 mg/kg	67.0 ± 11.0*,**	75.5 ± 5.1*
30 mg/kg	50.9 ± 9.2*	69.6 ± 4.8*
100 mg/kg	27.5 ± 5.6*	62.6 ± 4.3*

0, 10, 30 and 100 mg/kg mean 8-week-old SD rats treated with 0, 10, 30 and 100 mg/kg of L-NAME , respectively. Data are shown as mean± SEM of six to eight separeted determinations in each group. *Significantly different from 0 mg/kg group. **Significantly different from 100 mg/kg group.

with L-Arg did not change the NO release and blood flow in the rat testis. Our data indicate that NO regulates circulation of the testis under normal conditions.

Measurement of blood flow during ischemia-reperfusion in the testis

Figure 2 demonstrates the blood flow during ischemia-reperfusion in the rat testis. Clamping of the rat left testis artery decreased blood flow to 5–20% of the basal level measured before clamping. After removal of the clip, the blood flow recovered to 50–100% of the basal level within 1 minute in all groups. Our data indicated that there was no tendency of blood flow levels during reperfusion between treatments with L-NAME and L-Arg.

In vivo NO release during ischemia-reperfusion in the testis

Released NO levels in the rat testis before, during, and after the induction of ischemia are shown in Fig. 2. Immediately following clipping of the testicular artery, NO release rapidly increased, and reaching a plateau within approximately 30 minutes. When a NO electrode was inserted into rat testis treated with 10, 30 and 100 mg/kg of L-NANE, NO release was decreased in a dose-dependent manner (groups C, D and E, respectively). In contrast to treatment with L-NAME, L-Arg treatment (10 and 30 mg/kg) significantly increased NO release in a dose-dependent manner (groups F and G). After removing the clip, although NO release treated with L-NAME returned to almost basal levels, NO release treated with L-Arg (10 and 30 mg/kg) remained significantly higher than basal levels during these 30 minutes. These data suggest that treatment with L-NAME (10, 30, and 100 mg/kg) significantly decreased NO release during ischemia-reperfusion, and that treatment with L-Arg (10 and 30 mg/kg) significantly increased NO release during ischemia-reperfusion.

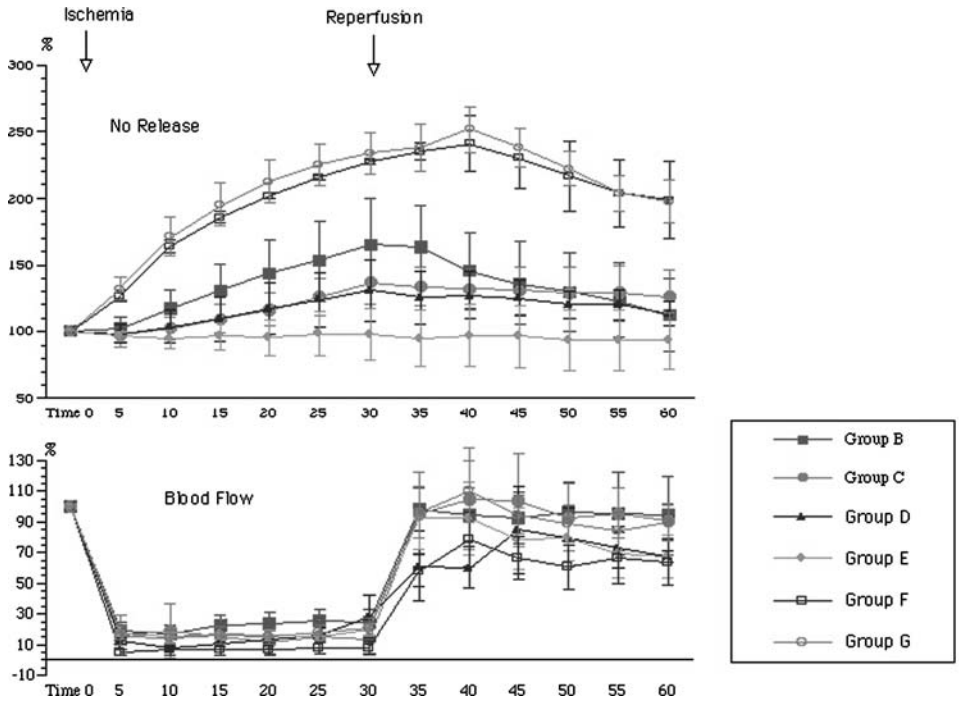

Fig. 2. Blood flow and NO release during ischemia-reperfusion in the testis. B: 30 minutes ischemia-30 minutes (I-R) reperfusion rats C: I-R rats treated with L-NAME (10 mg/kg) D: I-R rats treated with L-NAME (30 mg/kg) E: I-R rats treated with L-NAME (100 mg/kg) F: I-R rats treated with L-Arg (10 mg/kg) G: I-R rats treated with L-Arg (30 mg/kg). Blood flow and NO release were expressed as % of the basal level. Data are shown as mean ± SEM of six to eight separated determinations in each group.

Table 2. Data of NO_2-NO_3 and MDA concentrations in the testis

	NO_2-NO_3 concentrations (nmol/mg protein)	MDA concentrations (nmol/mg protein)
A	1.08 ± 0.08	0.13 ± 0.02
B	$2.01 \pm 0.38^{*}$	$0.21 \pm 0.03^{*}$
C	$1.82 \pm 0.19^{*,**}$	$0.21 \pm 0.02^{*,**}$
D	$1.54 \pm 0.16^{*,**}$	$0.23 \pm 0.03^{*,**,***}$
E	$1.51 \pm 0.13^{*,**}$	$0.25 \pm 0.02^{*,**,***}$
F	$2.13 \pm 0.14^{*}$	$0.18 \pm 0.02^{*}$
G	$2.61 \pm 0.28^{*}$	0.16 ± 0.01

MDA: malonaldehyde. A: control rats, B: 30 minutes ischemia-30 minutes (I-R) reperfusion rats C: I-R rats treated with L-NAME (10 mg/kg) D: I-R rats treated with L-NAME (30 mg/kg) E: I-R rats treated with L-NAME (100 mg/kg) F: I-R rats treated with L-Arg (10 mg/kg) G: I-R rats treated with L-Arg (30 mg/kg). Data are shown as mean± SEM of six to eight separated determinations in each group. *Significantly different from A group. **Significantly different from G group. ***Significantly different from F group.

NO_2-NO_3 concentrations in the testis

NO_2-NO_3 concentrations in the testis are shown in the Table 2. The NO_2-NO_3 concentration in the testis of group B was significantly higher than that of group A. Treatment with L-NAME (groups C, D, and E) significantly decreased in NO_2-NO_3 concentrations in the testis compared to that of group B. In contrast to the treatment with L-NAME, L-Arg treatment (30 mg/kg) tended to increase in NO_2-NO_3 concentrations in the testis compared to that of the group B. However, NO_2-NO_3 concentrations in the testis of group G were significantly higher than those in groups C, D, and E. These data support the data of real-time monitoring of NO release in the testis.

MDA concentrations in the testis

The tissue concentrations of MDA are also shown in Table 2. The MDA concentrations in the testis of group B were significantly higher than those of group A. Treatment with L-NAME (groups C, D, and E) tended to increase the MDA concentrations in the testis compared with group B. In contrast to the treatment with L-NAME, L-Arg treatment (groups F and G) tended to decrease the MDA concentrations in the testis compared with group B. The MDA concentrations in the testis of group G were significantly smaller than those in groups C, D, and E. Our data indicate that treatment with L-NAME increased MDA concentrations in the testis, while treatment with L-Arg decreased these MDA concentrations during ischemia-reperfusion.

Histological examination of the rat testis

Figure 3 shows H&E staining of the rat testis. In control rats (group A), slow levels of spermatogenesis and immature sperms were observed. In the ischemia-reperfusion testis (group B), histological damage was observed, with many red blood cells being observed due to vessel extravasations, ruptures of microvessels, and leukocyte infiltrations in the testis compared to the control. This damage was primarily observed under tunica albugenia. In groups C, D, E, F, and G, vessel extravasations, ruptures of microcirculation, and leukocyte infiltration in the testis were also observed. Significant protective effects of NO during ischemia-reperfusion were not observed in histological examinations of groups C, D, E, F, and G.

Discussion

Ischemia-reperfusion injury in the testis has been associated with testicular torsion, often required emergent surgery. Following testicular torsion, atrophy of the testis and male infertility are often observed [1]. To avoid these complications, it is important to investigate effective drug therapies against ischemia-reperfusion injury in the testis. Recent studies demonstrated that NO was an important role on ischemia-reperfusion injury in many tissue. In the present study, we demonstrated the role of NO in ischemia-reperfusion injury in the testis, simultaneously measuring both blood flow and NO release. Under normal conditions, NO was found to regulate blood flow in the testis. Clamping of the testicular artery induced a rapid increase in NO release, and removing the clip induced a gradual decrease in NO release in the testis. Treatment with L-NAME increased the MDA concentrations in the testis and treatment with L-Arg decreased MDA concentrations in the testis during ischemia-reperfusion. These data clearly demonstrate that NO has cytoprotective role in the testis during ischemia-reperfusion.

The role of NO during ischemia-reperfusion is complicated and sometimes has been controversial. Ozokutan and associates have reported that NO plays an important role in damaging the testis in ischemia-reperfusion [10]. In contrast, some reports have indicated that increased in NO levels in the testis significantly prevent ischemia-reperfusion or torsion-detorsion injury [11–13].

Possible mechanisms of testicular dysfunction in ischemia may include the impairment of oxygen and nutrient supplies and the removal of waste products through the circulatory system. Decreases in the intracellular content of ATP and glycogen and an increase in intracellular Ca^{2+} may play important roles in testicular injury. Generation of these reactive oxygen species is attributed to ischemia-reperfusion injury. Peroxynitrite has also been reported to be a powerful oxidative product with deleterious effects on various cells and tissues [5, 6]. In contrast, ischemia rapidly decreases the extracellular pH during the ischemic condition. The reduction of NO_2 to $NO^{\cdot}$ is promoted by ischemia-induced acidosis as well as

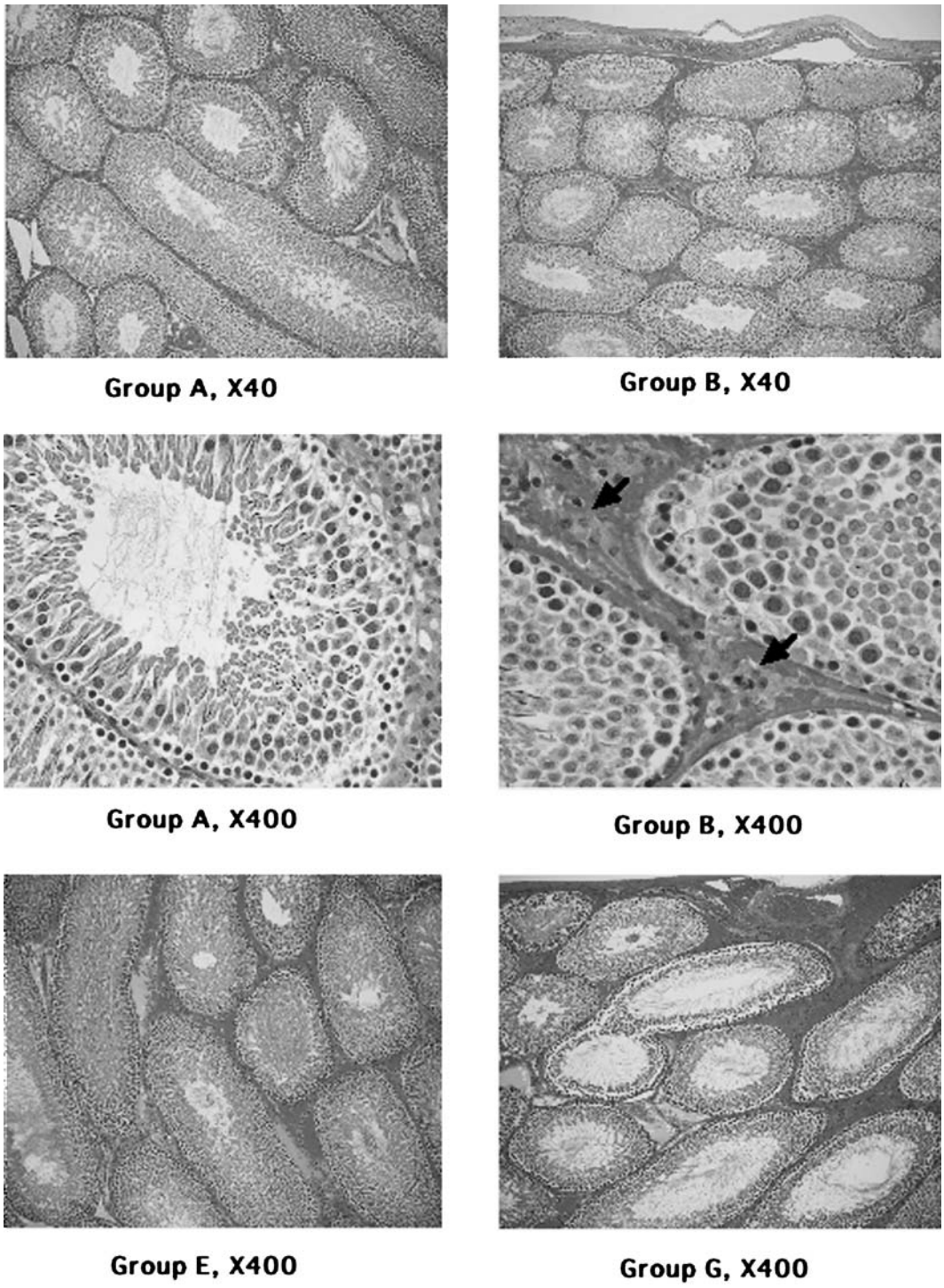

Fig. 3. Histological examinations in the testis. A: control rats, B: 30 minutes ischemia-30 minutes (I-R) reperfusion rats E: I-R rats treated with L-NAME (100 mg/kg) G: I-R rats treated with L-Arg (30 mg/kg). Spermatogenesis was observed slightly and immature sperms were observed (Group A). In ischemia-reperfusion testis, histological damages were observed, where in a lot of red blood cells were observed due to vessel extravasations, ruptures of microcirculation, and leukocyte infiltrations (See arrows) in the testis compared to the control. These damages were mainly observed under perrididymis (Group B, E and G).

enzymatic catalysis. $NO^{\cdot}$ donors protect against ischemia-reperfusion damage in in vitro models of these tissues [7–9]. Moreover, reactive oxygen species inactivate the relaxing NO and promote a reduction in blood flow. Attenuation of NO-mediated vasodilation has been reported to be dependent on the inactivation of NO by O_2^- anions generated by endothelial XO [17]. Because NO interacts with free oxygen radicals, the decreased NO release during reperfusion may be the result of oxidant species that react with NO, possibly causing a decrease in NO concentrations in the reperfused testis [11]. Interestingly, in the real-time monitoring of blood flow, we demonstrated that the blood flow levels of reperfusion were less than 100% of basal levels regardless of treatment with L-NAME or L-Arg. Generally, the levels of reperfused blood flow are higher than basal levels in many systems. As the testis is covered by tunica albugenia, it may be difficult to dilate the microvessels in the testis during reperfusion. It is also possible that mechanically reduced blood flow during reperfusion causes a decrease in NO concentration in the testis.

Although we suspect that NO plays an important role in ischemia-reperfusion in the testis, the movement of NO levels in the testis has not been examined. In the present study, we attempted to directly and simultaneously monitor NO release and blood flow in the rat testis during ischemia-reperfusion. The ischemia caused an increase in NO release in the testis, and reperfusion immediately returned NO release to basal levels. The pattern of NO release in the testis was similar to that of the stomach, kidney, and urinary bladder [14–16]. We also confirmed that the NO release during ischemia-reperfusion was blocked by L-NAME and enhanced by L-Arg. The changes in NO release during ischemia-reperfusion suggest that the testis may maintain the necessary blood supply via the circulatory system by dilating arteries with released NO during ischemia, and that the reperfusion may utilize the accumulated NO as a source of $NO^{\cdot}$ formation in the testis. The formed $NO^{\cdot}$ might protect the testis from additional damage.

Janetschek et al have reported that torsion-detorsion induces hemorrhagic infarction in the testis [18]. Since torsion-detorsion injury is mainly induced by ischemia-reperfusion, our data support this report. In histological examinations, we observed leukocyte infiltrations in the testis. In the histological study, however, cytoprotective effects of NO in ischemia-reperfusin injury were not observed.

In conclusion, immediately following clipping of the artery, NO release rapidly increased. After removing the clip, NO release gradually returned to the basal level. This phenomenon was enhanced by treatment with L-Arg and inhibited by treatment with L-NAME. In the present study, we demonstrated that NO has cytoprotective effects during ischemia-reperfusion in the testis.

Acknowledgements

This study was supported by a grant from the Ministry of Education, Science, and Culture of Japan (#14704041).

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