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Role of Nitric Oxide in Ischemia-Reperfusion Injury and Acute Rejection in Rat Intestinal Transplantation

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ABSTRACT

Objective. This study was designed to evaluate the role of nitric oxide (NO) in ischemia-reperfusion injury (IRI) and acute rejection (AR) in rat intestinal transplantation, using administration of the NO inhibitor N^G-nitro-L-arginine methyl ester (L-NAME).

Materials and Methods. Rats that underwent orthotopic allogeneic intestinal transplantation were assigned to 2 groups. In the normal allograft group (Wistar to Sprague-Dawley rats), L-NAME 0 mg/kg/d (group 1-1), 4 mg/kg/d (group 1-2), 8 mg/kg/d (group 1-3), or 12 mg/kg/d (group 1-4) was injected intraperitoneally. In the high responder allograft group (Dark Agouti to Lewis rats), L-NAME 0 mg/kg/d (group 2-1) or 8 mg/kg/d (group 2-2) was injected intraperitoneally. Survival times were observed and maltose absorption tests performed as well as light microscopic examination of the grafts.

Results. The mean survival time of group 1-3 was significantly prolonged compared with group 1-1 ($P < .01$). In group 2, the survival time of group 2-2 was significantly prolonged compared with group 2-1 ($P < .01$). Histological changes showed IRI was attenuated in group 1–2 compared with group 1-1, whereas it was aggravated in groups 1-3 and 1-4. Treatment with L-NAME (8 mg/kg/d) attenuated the graft damage of AR in groups 1 and 2. Maltose absorption tests showed that inhibition of NO impaired maltose absorption.

Conclusion. This study suggested that NO plays a dual role as both a cytotoxic and a cytoprotective factor in IRI, and may serve as a kind of cytotoxic medium in AR in rat intestinal allotransplantation.

THE CLINICAL APPLICATION of intestinal transplantation has been less successful than that of other organs. The sequential events of severe ischemia-reperfusion injury (IRI) and intense acute rejection (AR) are the main obstacles in intestinal allotransplantation.

Substantial studies suggest that the physiological and pathological functions of nitric oxide (NO) in the intestine closely relate to IRI and AR. Under physiological conditions, NO has many beneficial effects in the intestine, such as scavenging oxygen free radicals, maintaining normal vascular permeability, and inhibiting platelet aggregation.[1] However, NO overproduction may aggravate tissue damage under many pathological conditions. The role of NO in IRI remains a matter of controversy and the function of NO in AR is not known in intestinal transplantation.

Regulation of NO may be a promising strategy against IRI and AR in intestinal allotransplantation. In some animal transplantation models, inhibition of NO by the NO synthase (NOS) blocker N^G-nitro-L-arginine methyl ester (L-NAME) causes tissue dysfunction,[2–4] whereas it provides benefits in other models.[5,6] In light of these conflicting studies, we speculated that the effects of inhibiting NO varied with the doses of L-NAME and the animal model. We evaluated the effects of NO in intestinal IRI and AR using various doses of L-NAME and different rat combinations.

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MATERIALS AND METHODS

Animals and Experimental Groups

Male Lewis rats (250–300 g) were purchased from Vitalriver Laboratory Animal Technology Company (Beijing, China). Female Dark Agouti (DA) rats (190–210 g), female Wistar rats (200–250 g), and male Sprague-Dawley (SD) rats (250–300 g) were purchased from The Supplying Base of Medical Laboratory Animals of Heilongjiang Province (Harbin, China).

Animals were assigned to 2 groups. Group 1 was an allogeneic transplant group of Wistar rat donors and SD recipients. Group 2 was a high responder allogeneic transplant group with DA donors and Lewis recipients. From 30 minutes before transplantation to the completion of the experiment, L-NAME (Beyotime Institute of Biotechnology, China) was dissolved in 0.5 mL 0.9% saline given as a single daily intraperitoneal (IP) injection in the following doses: group 1—0 mg/kg/d (group 1-1; n = 20), 4 mg/kg/d (group 1-2; n = 20), 8 mg/kg/d (group 1-3; n = 26), or 12 mg/kg/d (group 1-4; n = 16); and group 2—0 mg/kg/d (group 2-1; n = 18) or 8 mg/kg/d (group 2-2; n = 20).

Operative Procedures

Orthotopic intestinal transplantation was performed as previously described.[7] Both donors and recipients were fasted for 8 to 10 hours allowed free access to water before the operation. They were anesthetized by IP injection of sodium pentobarbital (30–40 mg/kg) and ether inhalation. The proximal intestine (50%) of the donor was removed on a vascular pedicle consisting of the superior mesenteric artery with an aortic cuff and portal vein. The vascular bed and intestinal lumen were flushed with 4°C saline. The cold storage time was 30 minutes. The donor portal vein and aortic cuff were anastomosed to the recipient infrarenal IVC and aorta, respectively. After resection of 50% of the recipient's intestine, enteric continuity was restored by proximal and distal end-to-end anastomoses. Cefazolin sodium (100 mg/kg/d) was given intramuscularly for 5 days postoperatively. The survival time of each subgroup was observed in 6 transplants.

Maltose Absorption Test and Sampling

At 30 minutes after reperfusion and on postoperative days (POD) 1, 3, 5, 7, 9, 11, 14, and 17 a biopsy (or enthareria) for sampling was performed in 5 to 6 rats and a maltose absorption test was performed in 5 to 6 rats in each subgroup, as previously described.[7] The rats were fasted for 8 to 10 hours before the biopsy. A laparotomy was performed under ether inhalation anesthesia. Basal blood glucose level was measured. Maltose (10 mg) dissolved in 1 mL normal saline (1%), was infused into the proximal 10 cm of intestine with both ends clamped. At 30 minutes after the maltose infusion, a second blood glucose level was measured to analyze the blood glucose absorptive level. The segmental graft was severed and an intestinal anastomosis was performed; the interval between the 2 samplings was at least 3 days. Cefazolin sodium (100 mg/kg/d) was given intramuscularly for 3 days postoperatively.

When diffuse adhesions in the abdomen were hard to mobilize, or the rat was dying, it was sacrificed for sampling. If the death was due to blood vessel or intestinal anastomotic complications, the rat was discarded from the study.

Histological Assessment of Tissue Damage

The specimen was fixed in 4% paraformaldehyde. After being embedded in paraffin, the sample was stained with hematoxylin-eosin (H&E) for evaluation by light microscopy. Histological examination was performed in a blinded manner by a single pathologist. The grade of IRI was evaluated using a scale from 0 to 8 as described by Park et al.[8] The degree of AR was diagnosed using a scale from 0 to 3 as described by Schmid et al[9] and Rosemurgy and Schraut.[10]

Data Analysis and Statistics

All data are expressed as mean values ± SD. Statistical analyses were performed using the Kruskal-Wallis test and Mann-Whitney U test with $P < .05$ considered significant.

RESULTS

Graft Survival

The L-NAME 8 mg/kg/d group showed significantly prolonged survival but with a higher mortality rate due to complications which mainly occurred within POD 3 (Table 1). In group 1, the mean survival time of group 1-1 was 11.67 ± 1.21 days and that of group 1-3 was prolonged to 17.33 ± 1.86 days ($P < .01$ vs group 1-1). The survival time of group 1-4 was 3.33 ± 0.82 days ($P < .01$ vs group 1-1). Among group 2, the mean survival time of group 2-1 was 6.83 ± 0.75 days and that of group 2-2 was prolonged to 10.17 ± 0.98 days ($P < .01$ vs group 2-1).

Histological Findings

The degree of IRI was attenuated using L-NAME at 4 mg/kg/d, whereas it was aggravated by L-NAME at 8 and 12 mg/kg/d (Table 2). At 30 minutes after reperfusion, the mean grade of IRI in group 1-1 was 2.67 ± 0.52; the grade of IRI in group 1-2 decreased to 1.33 ± 0.82 ($P < .05$ vs group 1-1). The grades of IRI among groups 1-3 and 1-4 were significantly increased to 4.17 ± 0.26 ($P < .01$ vs group 1-1) and 4.83 ± 0.98 ($P < .01$ vs group 1-1), respectively. Thereafter, the grades of IRI were reduced gradually in all groups. On PODs 1 and 3, compared with group 1-1, the grade of IRI in group 1-2 was decreased ($P > .05$); the grades of IRI in groups 1-3 and 1-4 were significantly increased ($P < .01$). Similar to group 1, from 30 minutes to POD 1, the grades of IRI in group 2-2 were significantly greater than those in group 2-1 ($P < .05$).

Table 1. Graft Survival and Mortality Due to Complications

Group	Survival (d)	N0	N1	N2	N3	N	Mortality (%)
1							
1-1	11.67 ± 1.21	6	14	2	4	26	23.1 (6/26)
1-2	12.33 ± 1.51	6	14	1	3	24	16.7 (4/24)
1-3	17.33 ± 1.86*	6	20	5	6	37	29.7 (11/37)
1-4	3.33 ± 0.82†	6	10	4	3	23	30.4 (7/23)
2							
2-1	6.83 ± 0.75	6	12	2	3	23	21.7 (5/23)
2-2	10.17 ± 0.98‡	6	14	4	4	28	28.6 (8/28)

N, total number; N0, to observe survival time; N1, survived the biopsy of design; N2, lost due to complications of transplantation; N3, lost due to biopsy.
*$P < .01$ vs group 1-1.
†$P < .01$ vs group 1-1.
‡$P < .01$ vs group 2-1.

Table 2. Histological Results of IRI (Grade, Mean ± SD)

Group	30 Minutes (n = 6)	POD 1 (n = 6)	POD 3 (n = 6)
1			
1-1	2.67 ± 0.52	1.83 ± 0.41	0.42 ± 0.49
1-2	1.33 ± 0.82*	1.17 ± 0.75	0.17 ± 0.41
1-3	4.17 ± 0.26†	3.33 ± 0.61†	1.58 ± 0.38†
1-4	4.83 ± 0.98‡	4.75 ± 0.27‡	2.92 ± 0.20‡
2			
2-1	2.50 ± 0.55	1.67 ± 0.26	0.83 ± 0.52
2-2	3.83 ± 0.41§	3.00 ± 0.63§	1.25 ± 0.61

The grade of IRI was evaluated using a scale from 0 to 8 based on Park's grading system.[8]
*$P < .05$ vs group 1-1.
†$P < .01$ vs group 1-1.
‡$P < .01$ vs group 1-1.
§$P < .05$ vs group 2-1.

The damage caused by AR was attenuated by the use of L-NAME 8 mg/kg/d either in group 1 or in group 2 (Table 3). In group 1-1, AR of phases 1 and 2 occurred on POD 5 and POD 7, respectively; AR of phase 2–3 occurred on POD 9. From POD 3 to POD 7, the histological changes in group 1-2 were similar to those of group 1-1. From POD 9 to POD 11, the tissue damage in group 1-2 was slightly attenuated compared with group 1-1. In group 1-3, the progress of AR was delayed and tissue damage attenuated compared with group 1-1. In group 2-1, AR of phases 1, 2, and 3 occurred on POD 3, POD 5, and POD 7, respectively. Tissue damage in group 2-2 was attenuated compared with group 2-1.

Maltose Absorption Tests

Maltose absorption was decreased by the use of L-NAME in group 1 (Fig 1A). Compared with pretransplantation, from 30 minutes after reperfusion to POD 1, the blood glucose absorption curves decreased in a dose-dependent manner. After POD 1, the absorption curve in group 1-1 was obviously increased, reaching a peak on POD 5 (58.7 ± 9.10 g/L) which did not recover to the pretransplantation level. The absorption curve in group 1-2 increased slowly, showing a peak on POD 5 (32.93 ± 7.72 g/L). The absorption curve in group 1-3 was similar to that in group 1-2, but showed a peak on POD 9 (38.3 ± 7.69 g/L). The absorption peaks in groups 1-2 and 1-3 were lower than those in group 1-1. The absorption curve in group 1-4 remained at an extremely low level. The absorption curves increased even though the graft showed phase 1 AR by microscopy. When phase 2 AR occurred, the absorption curves were obviously decreased.

Table 3. Histological Results of Acute Rejection Damage (Phase)

Group	POD 3	POD 5	POD 7	POD 9	POD 11	POD 14	POD 17
1							
1-1	0	1	2	2–3	3	—	—
1-2	0	1	2	2	2–3	—	—
1-3	0	0–1	1	1	1–2	2	3
1-4	0	—	—	—	—	—	—
2							
2-1	1	2	3	—	—	—	—
2-2	0–1	1	2	2–3	—	—	—

The phase of AR was diagnosed using a scale from 0 to 3 as described by Schmid et al[9] and Rosemurgy and Schraut.[10]

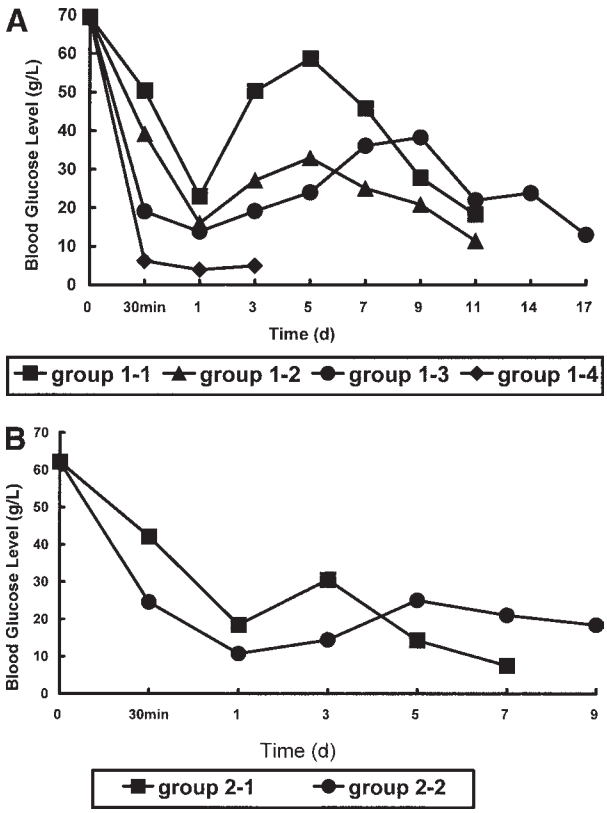

Fig 1. Maltose absorption curves in group 1 **(A)** and group 2 **(B)**.

The absorption curves in group 2 were lower than those in group 1 (Fig 1B). From 30 minutes after reperfusion to POD 1, the absorption curves in groups 2-1 and 2-2 were obviously decreased. After POD 1, the absorption curve in group 2-1 showed a low absorption peak on POD 3 (30.52 ± 5.79 g/L). The absorption curve in group 2-2 gradually increased, reaching a peak on POD 5 (25.02 ± 6.48 g/L).

DISCUSSION

IRI remains one of the major obstacles in intestinal transplantation. The role of NO in IRI is still a matter of considerable controversy. Takada et al[5] showed that inhibition of NO by L-NAME reduced lactate dehydrogenase (LDH) leakage induced by ischemia-reperfusion and decreased IRI. In contrast, Kubes[2] demonstrated that L-NAME greatly exacerbated intestinal IRI, increasing mucosal barrier dysfunction associated with ischemia-reperfusion.

In group 1, the administration of L-NAME 4 mg/kg/d attenuated IRI. Increasing the dose of L-NAME accentuated IRI, increasing the early postoperative complications. The mucosa of grafts in hosts treated with L-NAME 12 mg/kg/d showed massive loss of villi, hemorrhage, and

ulceration by light microscopy at 30 minutes after reperfusion. The rats displayed severe diarrhea postoperatively with a survival time of merely 3.33 days. Autopsy and microscopy demonstrated the cause of death to be due to severe IRI. The study confirmed that NO plays a dual role as both a cytotoxic and a cytoprotective factor in intestinal IRI. Moderate inhibition of NO provides a protective effect, whereas excessive inhibition of NO aggravates tissue damage. The harmful effects of high L-NAME dosages are mainly due to abolishing the physiologically protective functions of NO. In addition, it probably is related to up-regulated expression of the inflammatory cytokine CINC.[4]

The AR in intestinal transplantation is more difficult to control effectively than that in other organs. In a rat kidney allotransplantation model, the administration of aminoguanidine (AG), an inhibitor of inducible NOS (iNOS) may improve kidney function.[11] In our preliminary study, the use of AG did not obviously affect AR in rat intestinal transplantation. L-NAME, the more potent NOS inhibitor, was used for the above reason.

Our results showed that tissue damage in AR was slightly attenuated with L-NAME 4 mg/kg/d. The 8 mg/kg/d dose of L-NAME delayed the development of AR; tissue damage was attenuated and graft survival significantly prolonged in both the normal and the high responder allograft groups, whereas the administration of L-NAME neither completely controlled nor reversed AR responses. The results suggested that NO may be involved in the function of immune regulation and may be a kind of cytotoxic medium in intestinal AR. The pathways and mechanisms by which inhibition of NO on various levels affords protective effects require further study.

Commonly, maltose absorption tests reflect the degree of mucosal damage by decreasing maltose absorption levels.[7] In our study, the unparallel phenomenon was shown between tissue damage and decreased maltose adsorption levels. Treatment with L-NAME 4 mg/kg/d or 8 mg/kg/d attenuated the tissue damage caused by IRI and AR, respectively, whereas the absorption curves in both groups remained at low levels with obviously decreased peak values. There is evidence that intestinal perfusion is dependent on NO production; inhibition of NO by L-NAME significantly decreased mucosal blood flow.[12] We speculated that the impaired maltose absorption by L-NAME is attributed to decreased mucosal blood flow.

In conclusion, our results suggested that NO plays a dual role as both a cytotoxic and a cytoprotective factor in IRI in rat allogeneic intestinal transplantation. NO inhibition of various levels may be a strategy to contravene IRI and AR in intestinal transplantation.

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