

# Protective effects of L-arginine on reperfusion injury after pancreaticoduodenal transplantation in rats

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**BACKGROUND:** Post-transplantation pancreatitis and graft thrombosis are two major complications of pancreas transplantation that contribute to morbidity, mortality, and graft loss. Nitric oxide (NO) is a potent vasodilator agent formed when L-arginine (L-Arg) is converted to L-citrulline by the action of NO synthase (NOS), and plays a major role in microcirculatory changes. We therefore investigated the effect of L-Arg on reperfusion injury following pancreaticoduodenal transplantation in rats.

**METHODS:** The homologous male Wistar rat model of heterotopic total pancreaticoduodenal transplantation was used. The L-Arg-treated rats received the intravenous injection of L-Arg 5 minutes before and after reperfusion at a dose of 200 mg/kg while the N-Nitro-L-arginine methyl ester (L-NAME)-treated rats at a dose of 10 mg/kg. The amount of NO in the pancreas graft was measured. Serum concentration of cytokine-induced neutrophil chemoattractant (CINC) was determined by enzyme-linked immunosorbent assay, the expression of CINC mRNA was detected by Northern blot assay in the pancreas graft, and the activity of myeloperoxidase (MPO) was measured. Histological examination was performed.

**RESULTS:** The amount of NO was higher in the L-Arg group than in the control group, while it was lower in the L-NAME group than in the control group ( $P < 0.05$ ). The peak of serum CINC concentration occurred 3 hours after reperfusion with the difference among the groups being significant. The expression peak of CINC mRNA in the pancreas graft occurred 3 hours after reperfusion. The expression level in the L-Arg group ( $7.66 \pm 1.53 \mu\text{g/L}$ ) was lower than in the control group ( $26.31 \pm 2.01 \mu\text{g/L}$ ), while in the

L-NAME group ( $34.18 \pm 3.12 \mu\text{g/L}$ ) it was higher than that in the control group ( $P < 0.05$ ). The activity of MPO in the L-Arg group was obviously decreased as compared with in the other groups. The pancreas inflammation was ameliorated when L-Arg was administered, whereas the pancreas damage was aggravated when L-NAME was administered.

**CONCLUSIONS:** L-Arg can increase the amount of NO and inhibit the elevation of CINC, the CINC mRNA expression and early neutrophil accumulation in the pancreas. NO has protective effects on ischemia/reperfusion injury in pancreaticoduodenal transplantation.

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**KEY WORDS:** pancreas transplantation; reperfusion; L-arginine

## Introduction

It is well known that ischemia/reperfusion associated with organ transplantation leads to tissue injury.<sup>[1,2]</sup> Ischemia/reperfusion generates mediators including oxygen-free radicals (OFR) and causes release of inflammatory factors.<sup>[3,4]</sup> Nitric oxide (NO) is a diffusible intercellular messenger molecule that does not require special membrane carriers,<sup>[5]</sup> and a highly reactive substance with a very short half-life due to its chemical instability as a radical species.<sup>[6,7]</sup> In this sense, NO reacts with the superoxide anion-generating peroxynitrite that is then transformed into nitrate releasing hydroxyl radical.<sup>[8,9]</sup> We therefore studied the effect of NO donor (L-Arg) on reperfusion injury after pancreaticoduodenal transplantation in rats.

## Methods

### Animals

Male Wistar rats were obtained from the Experimental Animal Center, the China Medical University, Shenyang, China. The animals were kept in standard conditions and fed with water and rodent chow ad libitum. Their

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weight ranged from 250 to 300 g.

### Techniques of pancreaticoduodenal transplantation

Anesthesia for operation and subsequent postoperative care were consistent with the National Institute of Health guidelines for the care and use of animals. The animals were fasted for the 24 hours before surgery, but allowed free access to water. The rats were anesthetized with ether. Heterotopic pancreaticoduodenal transplantation was performed according to a modification of a previously described technique.<sup>[10]</sup> After shaving and disinfecting the abdomen with 75% alcohol, a midline incision was made. The donor pancreas was isolated on an aortic segment branching off the celiac axis and the superior mesenteric artery. The venous outflow was provided by the portal vein. The pancreas grafts were flushed with and stored in cold (4 °C) heparinized lactated Ringer's solution. Heterotopic intra-abdominal transplantation was performed by end-to-side anastomoses between the aortic segment of the graft and the recipient infrarenal aorta. The graft portal vein was anastomosed using the same technique to the recipient vena cava. Enteric diversion of exocrine graft secretion was accomplished by end-to-side duodenojejunosomy. The abdomen was closed in two layers with 2-0 silk suture. A single intramuscular injection of 5 mg of cefamandole was given postoperatively and the rats were kept under warming lamps until they became active.

### Sham-operated control

Sham-operated control animals underwent identical preparations except that the infrarenal aorta and vena cava clips were not applied. The abdominal wall was then closed with 2-0 silk suture.

### Pancreaticoduodenal transplantation in diabetic recipients

Syngeneic pancreaticoduodenal transplantation was performed in diabetic recipients to assess whether islet cell function is preserved after surgery. Diabetes was induced by intravenous administration of streptozotocin (Sigma) at a single dose of 55 mg/kg body weight. Only those rats were used with stable blood sugar levels above 200 mg% on two successive determinations within 2 weeks after streptozotocin injection. Once the rats were established as diabetics, they were subjected to pancreaticoduodenal transplantation. Diabetes mellitus was confirmed by repetitive nonfasting blood glucose measurements above 200 mg/100 ml. Endocrine graft function was studied by daily determination of blood glucose levels after transplantation. Serum concentration of glucose was measured by an Exac Tech blood glucose meter in samples collected from the cut tip of the tail.

### Pancreaticoduodenal transplantation in nondiabetic

### recipients

Further experiments were carried out using syngeneic pancreaticoduodenal transplantation in nondiabetic recipients to investigate the effect of L-Arg on ischemia/reperfusion injury.

### Experimental protocol

Recipient animals were divided into three groups. Animals of L-Arg group ( $n=6$ ) received grafts stored for 12 hours. Five minutes before reperfusion and 5 minutes after reperfusion, L-Arg was applied as a bolus injection (200 mg/kg i. v. Sigma) twice, animals of L-NAME group ( $n=6$ ) received grafts stored for 12 hours. Instead of L-Arg, L-NAME was applied as a bolus injection (5 mg/kg i. v. Sigma) 5 minutes before reperfusion and 5 minutes after reperfusion. In animals of saline group ( $n=6$ ), instead of L-Arg, comparable amount of saline was used as a bolus injection twice at the same time.

### Blood chemistry

Serum concentrations of amylase and lipase were measured with a multianalyzer (Clinilizer, CL-7150, Nippon Denshi, Tokyo, Japan).

### Measurement of CINC activity

A sensitive ELISA for rat CINC using biotin-conjugated anti-CINC rabbit IgG was established. The biotin-streptavidin sandwich ELISA detected CINC at concentrations from 3 pg/ml to 30 ng/ml.

### RNA isolation and Northern blot analysis

Pancreas specimens were obtained from animals killed at various times after reperfusion, quick-frozen in liquid nitrogen, and stored at -70 °C prior to RNA extraction. Total RNA was prepared according to the method described by Chromczynski et al.<sup>[11]</sup> Twenty mg of RNA was subjected to electrophoresis on a 1% agarose-formaldehyde denaturing gel with 1×MOPS buffer containing 200 mmol of morpholinopropanesulfonic acid, 50 mmol of sodium acetate, and 10 mmol of EDTA (pH 7.0). After being transferred to nylon filters in 20×SSC by Northern blotting, <sup>32</sup>P-labeled DNA probes were hybridized at 42 °C in 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.05 mol of Tris-HCl, 1.0 mol of NaCl, 0.1% sodium pyrophosphate, 1.0% sodium dodecyl sulfate, 10% dextran sulfate, and 100 µg/ml of salmon sperm DNA. After hybridization, filters were washed as above using deionized, distilled, and diethyl pyrocarbonate-treated water. The hybridization signal was assessed by radioisotope counting and autoradiography.

### MPO activity

Pancreas MPO was measured as described previous-

ly. In brief, the pancreas graft was homogenized with an apolytron homogenizer using 6 ml of homogenization buffer (50 mmol, pH 6.0) containing 0.5% hexadecyl-trimethyl ammonium bromide (Sigma) and 5mmol of EDTA. The homogenized samples were then sonicated and centrifuged ( $3000 \times g$ , 30 minutes) at 4 °C. The activity of MPO in the supernatant was assayed by measuring the change of A460 caused by the decomposition of  $H_2O_2$  in the presence of o-dianisidine.

### Histology

Pancreas specimens were harvested 24 hours after transplantation. Pancreatic tissue was fixed in 4% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin/eosin or chloroacetate esterase for light microscopic analysis. Since the cytochemical method for chloroacetate esterase has been reported to be useful for the identification of neutrophils. The number of neutrophils was measured and reported as per square millimeter.

### Preparation of peritoneal macrophages

The peritoneal cavities of naive control rats were washed with 80–100 ml of phosphate-buffered saline (PBS). The cells were centrifuged ( $300 \times g$ , 20 minutes) through a discontinuous Percoll gradient (density: 1.077, Sigma) to separate the macrophages from other cells. The macrophages were collected from the interface layer and were washed in RPMI 1640 medium (Sigma). Cell viability was determined by trypan blue exclusion. The cells were then resuspended in RPMI 1640 medium with 10% fetal calf serum (Sigma) at a density of  $1 \times 10^5$  cell/well, and incubated in a humidified atmosphere of 5%  $CO_2$  and 95% air at 37 °C for 2 hours to allow attachment. Nonadherent cells then were removed by two rinses with warm PBS. An addition aliquot of adherent cells was used after 2 hours of attachment to assess their identity by Wright-Giemsa staining and nonspecific esterase staining. By this method, at least 90% of adherent cells appeared to be macrophages.

### CINC production by peritoneal macrophages in response to L-Arg

Peritoneal macrophages were incubated with L-Arg, L-NAME or saline. Cell-free supernatants were collected after a 6-hour incubation, centrifuged and assayed at least three times using macrophages from six rats.

### Statistical analysis

The data are presented as the mean  $\pm$  standard deviation. Statistical significance was determined using Student's *t* test. A *P* value less than 0.05 was considered significant.

## Results

Graft ischemic time was found  $39.1 \pm 4.4$  minutes (mean  $\pm$  standard deviation). The cross-clamp time of the infrarenal aorta and vena cava was  $38.6 \pm 3.7$  minutes. No postoperative bleeding was encountered from the vascular anastomoses. The serum glucose concentration returned to normal in the diabetic recipients within 24 hours after syngeneic pancreaticoduodenal transplantation. Thus, islet cell function was well preserved by this surgical technique.

Syngeneic pancreaticoduodenal transplantation was performed in nondiabetic rats to investigate the effects of L-Arg on ischemia/reperfusion injury. No significant differences were noted in the serum amylase concentrations among the three experimental groups. On the other hand, L-Arg significantly decreased the peak serum lipase concentration after reperfusion, whereas L-NAME increased it (Table 1). Sham-operated control animals showed no significant changes in the serum concentration of amylase or lipase.

The serum CINC concentration increased rapidly in the L-NAME group and saline group, peaked at 3 hours after reperfusion, and decreased gradually thereafter. And the concentration in the L-NAME group was higher than in the saline group. Pretreatment with L-Arg inhibited the elevation in serum CINC concentration after reperfusion. Sham-operated control animals showed no significant changes in serum CINC concentrations (Table 1).

Northern blot analysis of CINC mRNA revealed abundant transcripts in the pancreas specimens obtained 3 hours after reperfusion in the L-NAME group and saline group animals. In contrast, the animals treated with L-Arg demonstrated lower levels of CINC mRNA transcripts 3 hours after reperfusion than did the other two group animals (Fig.).

Serum CINC concentration peaked at 3 hours after reperfusion with a significant difference among the groups. Expression peak of CINC mRNA in the pancreas graft occurred 3 hours after reperfusion. The expression level in the L-Arg group was lower than in the saline group, whereas it was higher in the L-NAME group than in the saline group ( $P < 0.05$ ).

**Table 1.** The serum concentration of amylase and lipase and CINC 3 hours after transplantation (mean  $\pm$  SD)

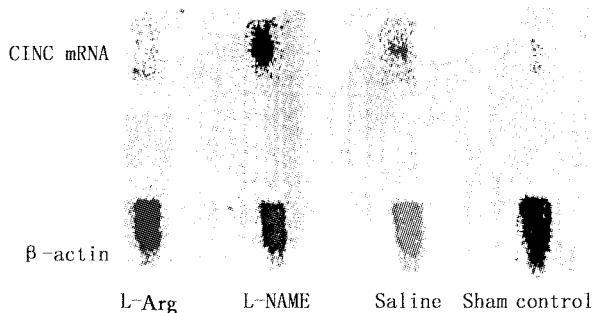
Group	n	Amylase (IU/L)	Lipase (IU/L)	CINC ( $\mu$ g/L)
Sham control	6	297 $\pm$ 23	107 $\pm$ 23	3.22 $\pm$ 0.84
Saline	6	13220 $\pm$ 370	268 $\pm$ 19	26.31 $\pm$ 2.01
L-Arg	6	12380 $\pm$ 930	147 $\pm$ 11 *	7.66 $\pm$ 1.53 *
L-NAME	6	13680 $\pm$ 1120	292 $\pm$ 26 *	34.18 $\pm$ 3.12 *

L-Arg: L-arginine; L-NAME: N-Nitro-L-Arginine methyl ester; CINC: cytokine-induced neutrophil chemoattractant. \*:  $P < 0.05$  vs saline-treated group.

**Table 2.** The NO concentration 1 hour after transplantation and the MPO activity and the number of neutrophils infiltrating 24 hours after transplantation in pancreas grafts (mean  $\pm$  SD)

Group	n	NO ( $\mu$ mol/L)	MPO (U/g)	Neutrophils (cells/mm <sup>2</sup> )
Sham control	6	0.11 $\pm$ 0.05	0.84 $\pm$ 0.07	2.01 $\pm$ 0.18
Saline	6	0.28 $\pm$ 0.07	1.66 $\pm$ 0.16	25.36 $\pm$ 4.12
L-Arg	6	0.37 $\pm$ 0.09 *	1.17 $\pm$ 0.09 *	9.36 $\pm$ 2.25 #
L-NAME	6	0.19 $\pm$ 0.10 *	2.21 $\pm$ 0.23 *	29.33 $\pm$ 4.14 *

MPO: myeloperoxidase; NO: nitric oxide; L-Arg: L-arginine; L-NAME: N-Nitro-L-arginine methyl ester; CINC: cytokine-induced neutrophil chemoattractant. \*:  $P < 0.05$  vs saline-treated group. #:  $P < 0.01$  vs saline-treated group.



**Fig.** Northern blot analysis. Expression of CINC mRNA transcripts in pancreas specimens from L-Arg, L-NAME, Saline groups and sham control group 3 hours after reperfusion. L-Arg: L-arginine; L-NAME: N-Nitro-L-arginine methyl ester; CINC: cytokine-induced neutrophil chemoattractant.

The amount of NO was higher in the L-Arg group than in the saline group, but it was lower in the L-NAME group than in the saline group ( $P < 0.05$ ). Pretreatment with L-Arg significantly decreased the MPO activity in the pancreas graft 24 hours after reperfusion compared to the other two groups. In addition, the number of neutrophils accumulated in pancreas grafts 24 hours after transplantation in the animals treated with L-Arg was lower than in the other two groups (Table 2). Pancreas inflammation was ameliorated when L-Arg was administered, while the pancreas damage was aggravated when L-NAME was given.

## Discussion

In the present study, the application of L-Arg during early reperfusion improved pancreatic nutritive perfusion and attenuated inflammatory tissue damage of pancreatic grafts after 12 hours of cold storage.<sup>[12]</sup> These results strongly suggest that the administration of L-Arg during reperfusion may be a useful therapeutic maneuver in the clinical setting of pancreas transplantation.<sup>[13,14]</sup>

Importantly we observed that NO donor L-Arg effectively attenuated leukocyte emigration into the pan-

creatic tissue elicited by cold ischemia and reperfusion.<sup>[15,16]</sup> This observation is consistent with the fact that NO was shown to be an antiadhesion molecule both in vitro and in vivo.<sup>[17]</sup> Mechanism such as the prevention of neutrophil activation/adherence and the modulation of expression of adhesion molecules,<sup>[18,19]</sup> such as CD11/CD18 on neutrophils and intercellular adhesion molecule-1 on endothelial cells, may account for the decrease of leukocytic tissue infiltration by L-Arg.<sup>[20]</sup> Inactivation of endothelial cell-derived and leukocyte-derived superoxide anions, which are known to promote leukocyte adhesion, by NO can also be proposed as an operative mechanism for the observed attenuation of pancreatic tissue infiltrates.<sup>[21,22]</sup> Moreover, in line with the supposed effects of NO, pancreatic tissue edema formation, serving as an early indicator of graft injury, was found to be significantly reduced upon L-Arg treatment, similarly as described for the effect of NO donors in attenuation of the increased mucosal permeability of the feline small intestine after 6 hours of hypothermic ischemia and 2 hours of normothermic reperfusion.<sup>[23,24]</sup>

In contrast to the results of this study, it should be noted that the augmentation of NO by L-Arg was also reported to increase posts ischemic injury, in a cardiac model of normothermic hypoxia followed by reoxygenation, which clearly differs from hypothermic ischemia and reperfusion.<sup>[25-27]</sup> Despite the dual nature of nitric oxide and its cytotoxic effects, the beneficial effects of nitric oxide that have been outlined above seem to play much more important roles in the vivo environment after hypothermic ischemia and reperfusion, which was present in our model.<sup>[28]</sup> Moreover, the cytotoxic effect of NO determining pancreatic tissue damage was shown to be relevant only after a short 30-minute, but not after a 12-hour, period of cold ischemia of the pancreas.<sup>[29,30]</sup>

In rats, CINC has been found to be a potent neutrophil chemoattractant and to belong to the IL-8 superfamily. Serum concentration of CINC significantly increased after reperfusion in pancreaticoduodenal transplantation.<sup>[31-33]</sup> In addition, a large number of neutrophils infiltrated into the pancreas grafts in the untreated animals.<sup>[34,35]</sup> Nevertheless, pretreatment with L-arginine significantly decreased the serum concentrations of CINC and the neutrophil accumulation in pancreas grafts.<sup>[36,37]</sup>

In conclusion, our data suggest that the supplement of NO with NO donors during reperfusion of pancreatic isografts seems to be a new strategy for the prevention of organ injury because NO donors improve posts ischemic reperfusion and markedly attenuate leukocyte-dependent tissue injury. L-Arg can increase the amount of NO and inhibit the elevation of CINC, CINC mRNA expression and early neutrophil accumulation in the pancreas. L-Arg has protective effects on the ischemia/reperfusion injury in pancreaticoduodenal transplantation.

## Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Of all my verse, like not a line; But like my title, for it is not mine.  
That title more and better than I stole; Ah, how much better, had I  
stol' n the whole!

— Robert Louis Stevenson