Glycine intravenous donor preconditioning is superior to glycine supplementation to low-potassium dextran flush preservation and improves graft function in a large animal lung transplantation model after 24 hours of cold ischemia

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Objectives: The potential role of glycine in combination with standard lung preservation with low-potassium dextran solution in lung ischemia-reperfusion injury has not been investigated in a preclinical porcine transplant model.

Methods: In a control group (n = 6), donor lungs were flushed with 1 liter of low-potassium dextran solution. In a second group (LPD-glyc, n = 6), low-potassium dextran solution was supplemented with 3.75 g of glycine. In a third group (IV-glyc, n = 6), donor preconditioning was performed by intravenous administration of 3.75 g glycine 1 hour before low-potassium dextran preservation. Grafts were stored in low-potassium dextran at 4°C for 24 hours. Posttransplant graft function was assessed throughout a 7-hour observation period.

Results: In the control group, 2 recipients died of right-sided heart failure caused by severe ischemia-reperfusion injury. All animals of the glycine groups survived the entire observation period. Pulmonary vascular resistance remained significantly ($P < .01$) lower in both glycine groups when compared with controls. At the end of the observation period pulmonary vascular resistance in the control group was higher ($P < .01$) compared with the glycine groups (1310 ± 319 dyn × sec × cm^{-5} vs 879 ± 127 dyn × sec × cm^{-5} [LPD-glyc] vs 663 ± 191 dyn × sec × cm^{-5} [IV-glyc]). Changes of lung tissue water content were lower in the IV-glyc group compared with the LPD-control ($P < .01$) and LPD-glyc lungs ($P < .05$). Oxygenation (Po_2/Fio_2) was higher in the IV-glyc group compared with the LPD-glyc and control lungs (445 ± 110 mm Hg vs 388 ± 124 mm Hg [$P < .01$] vs 341 ± 224 mm Hg [$P < .001$], respectively).

Discussion: Modification of low-potassium dextran solution with glycine or donor preconditioning ameliorates ischemia-reperfusion injury in lung transplantation. This intriguing approach merits further evaluation with respect to the mechanisms involved and may improve results in clinical lung preservation.

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Ischemia-reperfusion (I/R) injury is a major contributor to early graft failure after lung transplantation.[1] The clinical consequences of I/R injury include prolonged ventilatory requirements, requirement for high Fio_2, and pulmonary edema.[2] An understanding of the pathophysiology of I/R injury is evolving, and the following mechanisms play a role: cytokine production, neutrophil activation, free radical generation, lipid peroxidation, and decreased number of endogenous free radical scavengers.[3,4] The therapeutic or preventive approach to I/R injury has been designed to directly counter these pathologic processes (eg, antitumor necrosis factor-α antibodies)[5] or to augment defense systems with the addition of novel agents (eg, calcium channel blockers)[6]or endogenously produced substances (eg,

Abbreviations and Acronyms
I/R = ischemia-reperfusion
LPD = low-potassium dextran
PVR = pulmonary vascular resistance

superoxide dismutase).[7] Many of these treatments have resulted in prolonged lung preservation with good posttransplantation graft function in experimental models, but the elimination of I/R injury in clinical lung transplantation still remains a challenge, especially with regard to prolonged ischemic times or the consideration of marginal donor lungs for transplantation.

Our approach to ameliorating I/R injury uses glycine, a nonessential amino acid. Glycine is nontoxic and has been shown to protect proximal tubules and hepatocytes from hypoxia[8] and surgical manipulation after donor intravenous (IV) pretreatment.[9] Glycine also prevents nephrotoxicity caused by cyclosporine A.[10] Furthermore, glycine added to a graft rinse solution reduced reperfusion injury and improved initial graft function and survival after liver transplantation.[11] Moreover, glycine improved the hepatic microcirculation and reduced liver injury in a low-flow, reflow perfusion model.[12]

Glycine has not been investigated in lung transplantation. The mechanism, however, by which glycine exerts its protective effect in the liver is controversial and likely complex. Many have held glycine, which is an amino acid component of glutathione, as an example of a free radical scavenger.[13] Zhang and colleagues[14] showed inhibition of Kupffer cells in the liver graft by glycine.[14] The underlying mechanisms here are provided by Wheeler and associates,[10] who reported that glycine can activate the glycine-dependent chloride channel, which causes chloride inflow. This itself leads to a superpolarization of the cell membrane and consequently inhibits the openness of potential dependent calcium channels, thus inhibiting the activation of Kupffer cells and, ultimately, the release of proinflammatory cytokines.[10] In a study on hepatocyte serum-free cryopreservation, Muller and colleagues[15] impressively demonstrated that glycine improved the viability of cryopreserved porcine hepatocytes by preventing damage of the genomic DNA leading to improved recovery of living cells to approximately 55% of the initial cell number after thawing. In that study, glycine was added to both the thawing and the freezing solution. In another study, Schemmer and colleagues[16] applied glycine to human liver preservation and showed reduced I/R injury after transplantation.

In this study, we hypothesized that (1) the addition of glycine to the lung flush preservation solution and (2) IV donor pretreatment with glycine would both improve lung graft function after 24 hours of cold ischemic preservation and transplantation in a preclinical large animal model.

Materials and Methods

The purpose of this study was to evaluate initial lung function after 24 hours of cold ischemic preservation with low-potassium dextran (LPD) or glycine-enriched LPD solution (LPD-glyc) followed by a 7-hour observational reperfusion period. In addition, we tested the effect of IV donor pretreatment with glycine (IV-glyc) 1 hour before lung retrieval on posttransplant graft function after 24 hours of cold ischemic preservation. The results in these 2 study groups were compared with a control group in which lungs were flushed with and stored for 24 hours in LPD and then transplanted.

Animal Care

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" and the "Guide for the Care of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. The study was reviewed and all experiments were approved by the local ethics committee of the Hannover Medical School, Hannover, Germany.

Donor Procedure

In all 18 donor animals (female pigs, German Landrace, 25-35 kg), anesthesia was induced by the application of azaperone (5 mg/kg), atropine (5 mg total dose, intramuscular), and pentobarbital (1 mg/kg, IV). Animals were intubated and ventilated in a pressure-controlled mode with a peak inspiratory pressure of 30 cm H_2O, a positive end-expiratory pressure of 5 cm H_2O, and an F_{IO_2} of 0.5. Anesthesia was maintained by continuous infusion of pentobarbital (5 mg · kg · h) and fentanyl (1 mg · kg · h). After median sternotomy, the inferior and superior venae cavae were encircled with ties and the pulmonary artery was dissected from the ascending aorta. After systemic application of heparin (300 IU/kg), a 5-mm cannula was inserted into the pulmonary artery. Right-sided heart inflow occlusion was performed, and the left atrial appendage was excised. The pulmonary artery was clamped, and either 1 liter of 4°C cold LPD solution (Perfadex, Vitrolife, Gothenburg, Sweden) supplemented with 0.3 mL Tris-buffer (control group and IV-glyc group) or 1 liter of LPD solution supplemented with 3.75 g of glycine (LPD-glyc) plus 0.7 to 1.1 mL Tris-buffer was infused. The pH of the preservation solution was adjusted using Tris-buffer to a target level of 7.5. The intrapulmonary artery pressure was recorded throughout the entire flushing period. A mean perfusion pressure of 16 mm Hg was maintained. The harvested lungs were stored in a semi-inflated state in LPD at 4°C for 24 hours.

Porcine Single Lung Transplantation

Six transplant procedures were performed in each of the 3 study groups. In all 18 recipient animals (female pigs, German Landrace, 25-36 kg) anesthesia was induced and maintained as described above. Recipient arterial pressure was monitored by a carotid artery catheter, and pulmonary artery hemodynamics were monitored by a Swan-Ganz catheter (7.5 F, Baxter Healthcare, Irvine, Calif). Cardiac output and extravascular lung water were recorded using a femoral artery thermodilution catheter connected to the cardiac output recording device (PiCCO Systems, Pulsion Medical Systems AG, Munich, Germany). The chest was entered through a left lateral thoracotomy in the fourth intercostal space. Left pulmonary artery, lung veins, and bronchus were dissected. After

systemic administration of heparin (300 IU/kg), a left-sided pneumonectomy was performed. The graft was transplanted using running polypropylene sutures for all 3 anastomoses. After 10 minutes of reperfusion the right pulmonary artery and right main bronchus were crossclamped through the left-sided thoracotomy.

Assessment of Hemodynamics and Lung Function

In all experiments, atrial, systemic arterial, and pulmonary arterial pressures were recorded online. Dynamic lung compliance was monitored continuously with a modified ventilator (Dräger, Lübeck, Germany). Arterial blood gas analysis was performed after placement of catheters and every 30 minutes during the reperfusion period. At these intervals, pulmonary vascular resistance (PVR) was calculated after measurement of cardiac output with the transfemoral thermodilution catheter, which was connected to the cardiac index computer (PiCCO Systems). After calibration by injection of cold 0.9% saline solution into the jugular vein, the system calculated the cardiac index online by pressure curve analysis. By means of the same thermal bolus injection that was distributed into intravascular blood volume and extravascular lung water, the extravascular lung water index (arithmetic value divided by body weight in kilograms) was computed.

Wet-to-Dry Lung Weight Ratios

After flush perfusion, immediately before cold ischemic storage, a 4 × 4-cm biopsy was taken from the lower segments of the upper lobe of the right donor lung to avoid damage to the graft before storage and transplantation. The wet-to-dry ratio of that biopsy was considered as the baseline measure of the prestorage lung water content. At the end of the 7-hour reperfusion period the lingula of the transplanted lung was resected and used for the wet-to-dry ratio calculation. All specimens were taken at the same site of the right native donor lung or the pulmonary grafts to achieve the most comparable conditions. All biopsies were weighed and then placed in an oven at 100°C for 72 hours. After this drying procedure, the portion was reweighed and the ratio of the weight before and after drying was calculated. This measure reflects lung water or pulmonary edema.

Statistical Analysis

All data are expressed as mean ± standard deviation. Continuous data were analyzed by repeated-measures analysis of variance. For data without repeated measurement, analysis of variance was applied. Data obtained from the 2 animals that died during the observation period were included in statistical analyses up to the point at which death occurred. All data were analyzed with SPSS software (SPSS for MS Windows, version 11.5; SPSS Inc, Chicago, Ill).

Results

Donor and recipient pigs were matched for size in each study group. The mean baseline Pao_2 levels in arterial blood in donor animals before retrieval in each group were not different. Two of the 6 recipient animals in the control group died during the reperfusion period from severe right-sided heart failure at 2 and 4.5 hours after initiation of reperfusion. All 12 animals in the 2 glycine groups survived for the entire observation period.

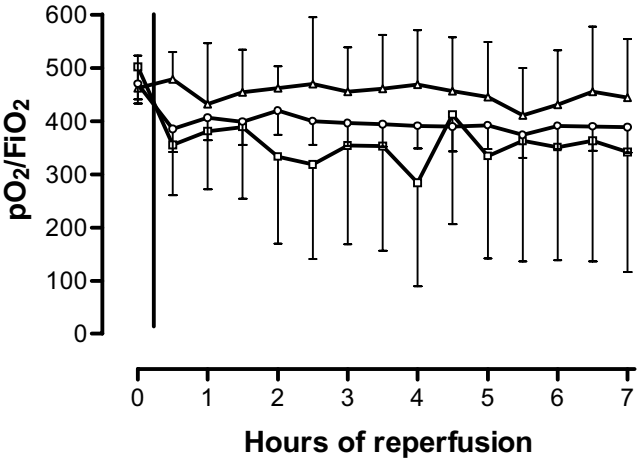

Figure 1. Pao_2/Fio_2 ratios in group comparison. *Open triangles,* Intravenous glycin; *open circles,* glycin; *open circles,* control.

Assessment of Posttransplantation Lung Function and Ischemia-Reperfusion Injury

Glycine as an additive to low-potassium dextran preservation solution versus low-potassium dextran. After completion of the 7-hour reperfusion period, a favorable trend toward higher Fio_2/Pao_2 ratios was seen in the LPD-glyc group (388 ± 124 mm Hg) compared with the control group (341 ± 224 mm Hg, $P = .05$) (Figure 1). PVR was significantly lower in the glycine group throughout the observational period than in the control cohort ($P < .01$). Figure 2 illustrates the changes in PVR with time in the LPD-glyc and control groups. Before termination of the experiment, PVR was significantly higher in the IV-glyc control (control) group compared with the LPD-glyc group with 1310 ± 319 dyn × sec × cm^{-5} vs 879 ± 127 dyn × sec × cm^{-5} ($P < .01$). The mean pulmonary arterial pressure was also significantly lower in the LPD-glyc group compared with control lungs (Figure 3) ($P < .01$). As mentioned above, to evaluate the content of lung water at the end of the reperfusion period reflecting postreperfusion pulmonary edema, wet-to-dry weight ratios were calculated. After 7 hours of reperfusion, wet-to-dry ratios were significantly lower in LPD-glyc lungs (7.0 ± 0.5) compared with control lungs (10.1 ± 0.8), representing less fluid accumulation over the posttransplant reperfusion period ($P < .05$). The cardiac output was also significantly higher in the LPD-glyc group over time compared with control animals ($P < .01$). Systemic vascular resistance, systemic arterial blood pressure, and extravasal lung water content were not statistically different between groups; however, a trend toward a lower extravasal lung water content ($P = .06$) was observed in the LPD-glyc group.

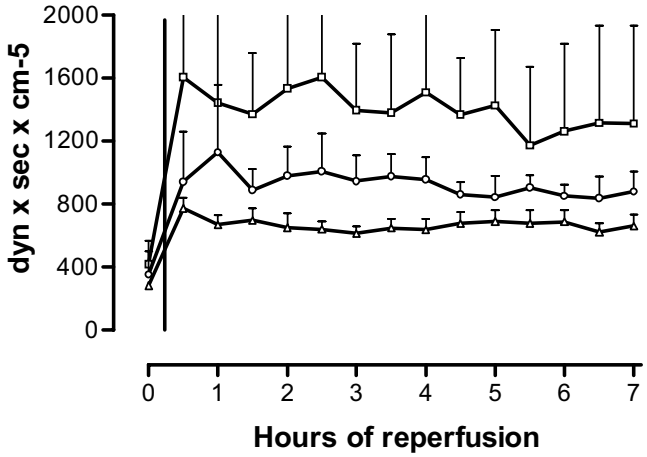

Figure 2. Changes in PVR in study groups throughout graft reperfusion ($P < .05$: LPD-glyc and IV-glyc vs LPD). *Open triangles,* Intravenous glycin; *open circles,* glycin; *open circles,* control.

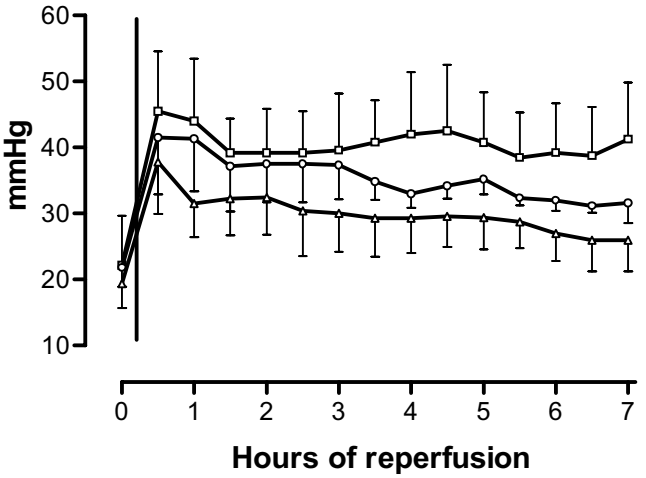

Figure 3. Changes in mean pulmonary artery pressure in study groups during reperfusion ($P < .05$, LPD-glyc vs all other groups). *Open triangles,* Intravenous glycin; *open circles,* glycin; *open circles,* control.

Glycine for intravenous donor pretreatment versus low-potassium dextran. Compared with control lungs, the IV-glyc group showed significantly higher F_{IO_2}/Pa_{O_2} ratios (341 ± 224 mm Hg vs 445 ± 110 mm Hg, respectively; $P < .001$) (Figure 1). As in the LPD-glyc group, the PVR was significantly lower in the IV-glyc group throughout the observational period compared with the control cohort ($P < .01$) (Figure 2). Also, before the end of the reperfusion period, the PVR was significantly higher in the IV-glyc (control) group (1310 ± 319 dyn × sec × cm^{-5} vs 663 ± 191 dyn × sec × cm^{-5}) ($P < .01$). In addition, wet-to-dry lung weight ratios were significantly lower at the end of the reperfusion period in the IV-glyc group than in the control group ($P < .01$). The mean pulmonary artery pressure was also significantly lower in the IV-glyc group compared with control lungs (Figure 3) ($P < .01$). The cardiac output was also significantly higher in the IV-glyc group over time compared with control animals ($P < .01$). Again, systemic vascular resistance, systemic arterial blood pressure, and extravasal lung water content were not statistically different between groups; however, a lower extravasal lung water content ($P = .06$) was observed in the IV-glyc group.

Intravenous glycine donor pretreatment is superior to glycine supplementation of the flush preservation solution. When the 2 glycine study groups were compared statistically, significantly higher F_{IO_2}/Pa_{O_2} ratios were seen in the IV-glyc group (445 ± 110 mm Hg vs 388 ± 124 mm Hg, respectively; $P < .001$) (Figure 1). PVR was significantly lower in the IV-glyc group throughout the observational period than in the LPD-glyc cohort ($P < .01$) (Figure 2). Wet-to-dry lung weight ratios were not statistically different between the 2 groups (LPD-glyc 7.0 ± 0.5 vs IV-glyc 6.3 ± 0.3). The mean pulmonary artery pressure, however, was significantly lower in the IV-glyc group compared with LPD-glyc lungs (Figure 3) ($P < .05$). The cardiac output was also significantly higher in the IV-glyc group over time compared with LPD-glyc animals ($P < .01$). Systemic vascular resistance, systemic arterial blood pressure, and extravasal lung water content were not statistically different between the 2 study groups.

Discussion

We used our well-established and previously described preclinical porcine single lung transplantation model of I/R injury[17] to evaluate the protective effects of glycine as an additive to the preservation solution or as donor IV pretreatment. We found that the addition of glycine to the pulmonary flush solution during cold ischemia resulted in improved oxygenation, reduced PVR, and reduced pulmonary artery pressures and wet-to-dry weight ratios measured after 24 hours of cold storage and 7 hours of reperfusion when compared with LPD preserved lungs. IV donor pretreatment with glycine, however, was superior over the supplementation of glycine to the flush solution.

The mechanism by which glycine exerted these protective physiologic effects remains elusive and will be further investigated in ongoing studies in our laboratories including surfactant function analysis.

This model has proven useful to study interventions to protect against I/R injury after lung transplantation.[17] We preserved the lungs for an extended period of 24 hours to ensure that we had sufficiently severely injured lungs. We chose to examine the severity of the I/R injury 7 hours after

the start of reperfusion because this corresponds to a time in the clinical setting when we frequently see evidence of this injurious process.

Glycine was selected because it has been shown in other models of I/R injury, mainly after liver transplantation, to improve functional and biochemical measures when added to the flush solution.[14,16] Barros-Schelotto and colleagues[18] have proven glycine to reduce post-liver transplant I/R injury in porcine grafts retrieved from non–heart-beating donors.[18] Moreover, Schemmer and associates[19] reported that IV glycine administration improves survival in rat liver transplantation. We chose LPD as our preservation solution because it has become the current clinical and experimental standard of lung preservation for transplantation worldwide.[20,21]

Glycine has been shown to have a wide range of activities, and it is likely that its ability to improve graft function after I/R injury is the result of one of these other mechanisms. Bogdanova and coworkers[13] were able to show the ability of glycine to scavenge free oxygen radicals in hepatocytes. In their study, treatment of hepatocytes with mercaptopropionyl glycine, a scavenger of OH*−, abolished hypoxia-induced inhibition of the Na+/K+ adenosine triphosphatase. Also, some centers, including our own program, now use glutathione-based preservation solutions. Glutathione is a potential oxygen free radical scavenger. We previously showed that Celsior, a low-potassium glutathione-based preservation solution, led to comparable outcome in a porcine single lung transplant model of I/R injury when compared with LPD.[22] However, PVR and neutrophil sequestration were both significantly reduced in the Celsior group in this previous study. Glycine is an amino acid component of glutathione and therefore exerts its action as an oxygen free radical scavenger.

Others have shown that glycine is protective in I/R injury models of liver transplantation because of its ability to protect the graft from brain death-related injury when the donor was pretreated with glycine.[14] The underlying mechanism was the inhibition of Kupffer cells by glycine. In that study they also demonstrated with electron microscopy that the inhibition of glycine by strychnine led to severe injury of the graft. Wheeler and colleagues[10] reported that on a subcellular level glycine activates glycine-dependent chloride channels, which causes chloride inflow into the cell leading to superpolarization of the cellular membrane. As a consequence, the openness of potential dependent calcium channels is inhibited and proinflammatory cytokine release is reduced. In a study on cryopreserved hepatocytes, Muller and collaborators[15] determined that glycine improved the viability of cryopreserved porcine hepatocytes by preventing damage of the genomic DNA. Thereby, glycine improved the recovery of living hepatocytes to approximately 55% of the initial cell number after thawing. Southard and colleagues demonstrated improved viability of human hepatocytes when glycine was added to both the freezing and the thawing solution in another cryopreservational study, as reported by Muller and colleagues.[15] Especially in liver transplantation, it is well known that surgical organ manipulation during harvest significantly harms the organ. Once again, Schemmer and colleagues[23] were able to show that donor pretreatment with glycine totally prevented the effects of organ manipulation on survival and enzyme release. In another study by Schemmer and colleagues,[9] using the rat liver transplantation model, IV donor pretreatment improved survival after transplantation.

In this study, we noted no systemic hemodynamic differences (diastolic and systolic blood pressures) between the groups. This suggests that glycine added to the preservation solution, as well as IV donor pretreatment, did not affect systemic arterial hemodynamics. However, a significant reduction in pulmonary artery pressure and PVR, and increases in cardiac output were seen, which may support a local regulatory effect of glycine in the pulmonary vasculature. The answer to why IV pretreatment with glycine is superior to supplementation of the flush solution remains elusive. However, with regard to the potential action of glycine in organ preservation and thereby in consideration of the evidence in the literature, it can be speculated that the donor IV pretreatment with glycine inhibits injurious mechanisms of brain death to the lung, which would not be possible if the drug is administered only at the time of flushing. This is supported by Zhang and colleagues,[14] who reported less graft injury after transplantation of livers from brain-dead donor animals. However, this is certainly not the mechanism of action in this study, because only lungs from healthy, non–brain-dead donor animals were transplanted.

Conclusion

We showed that glycine either in the flush preservation solution or as IV donor pretreatment in a porcine single lung transplant model decreased the severity of I/R injury after 24 hours of cold ischemic preservation and 7 hours of reperfusion. This is primarily represented by improved oxygenation and decreased pulmonary artery pressure, PVR, and posttransplant pulmonary edema. We were unable to ascertain which mechanism was responsible for the beneficial effects seen. Future studies at our program will include the effect of glycine on the preservation of surfactant function and the role of glycine on injurious mechanisms of brain death to the transplanted lung. Our ultimate goal is to be able to apply this simple modification of lung preservation solutions or the IV pre-retrieval donor treatment with glycine to the clinical setting to improve lung function after transplantation.

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