

Sildenafil extends survival and graft function in a large animal lung transplantation model[☆]

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Abstract

Objective: Restoring intracellular cGMP and inducing NO-synthesis attenuates ischemia-associated early pulmonary allograft dysfunction. Phosphodiesterase-5 (PDE), predominantly expressed in lung tissue, plays a pivotal role in modulating the cGMP/NO-synthase pathway in endothelial and epithelial cells. In this study, we evaluate the effect of employing sildenafil (Viagra[®]), a specific inhibitor of PDE-5, to counteract ischemia/reperfusion (I/R) injury in a single lung transplantation model of extended ischemia. **Methods:** Donor animals (weight matched outbred pigs, 28–35 kg) in the treatment group (I) ($n = 5$) were injected with 0.7 mg sildenafil/kg into the pulmonary artery (PA) prior to inflow occlusion. For perfusion, Perfadex[®], containing 0.7 mg sildenafil/l was used, and the graft stored at 1 °C in the perfusion solution. After 24 h ischemia, unilateral left lung transplantation was performed. Starting at reperfusion, group I received continuous sildenafil (0.7 mg sildenafil/kg), over 6 h. Except for the sildenafil application, the control group (II) ($n = 4$) was treated identically (PGE1 was injected into the PA). One hour after reperfusion, the right main bronchus (MB) and right PA were occluded. Over the next 5 h, cardiopulmonary parameters (systemic arterial, PA, central venous, left atrial pressure, $p\text{CO}_2$, $p\text{O}_2$) were measured, including extravascular lung water (EVLW). Thiobarbituric acid-reactive substance assay (TBARS) and myeloperoxidase (MPO) analysis from lung tissue were run. **Results:** All recipients of group I survived the 6-h reperfusion period; in contrast, all control animals died within 1–2 h after occlusion of the right side. In comparison to a marked rise in pulmonary vascular resistance (PVR) in group II (>1000 dyne s cm^{-5}), PVR in group I remained stable, moderately elevated from baseline (baseline: 150–180 dyne s cm^{-5} vs endpoint: 1000 dyne s cm^{-5}). EVLW in group I did not increase during reperfusion (baseline: 6.75 ± 1.4 mg/kg vs endpoint: 6.7 ± 1.0 mg/kg), in contrast to group II, where pulmonary edema at 2-h reperfusion preceded terminal graft failure (group I: 9.7 ± 0.1 mg/kg vs group II: 6.48 ± 1.8 mg/kg). Tissue reactive free radicals at endpoint measurement in group I did not differ significantly from native tissue. Yet, when compared to specimen taken from group II at time of terminal graft failure, a significant increase in free radicals was noted (group I: 13.8 ± 1.6 pmol/g vs group II: 18.5 ± 3.0 pmol/g, $p < 0.05$). **Conclusion:** Sildenafil treatment prevents terminal early graft failure, allowing lung transplantation after 24-h ischemia time. Reperfusion edema was strikingly diminished, preserving pulmonary structural and functional integrity while prolonging graft ischemia time. Employing the established PDE-5 inhibitor sildenafil during lung perfusion, storage, and implantation, ischemic tolerance may be extended and early graft function improved.

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1. Introduction

Clinical lung transplantation is limited by the scarcity of suitable grafts and low organ-specific ischemic tolerance. Extensive ischemia/reperfusion (I/R) injury may lead to early graft dysfunction following pulmonary transplantation in ca. 15% of recipients. The hallmarks of I/R injury in the lung are an increase of vascular permeability, permeating pulmonary

edema, endothelial desquamation, and neutrophil sequestration. The course of I/R injury can be attenuated by induction of the NO- and prostaglandin-synthesis pathways, where cAMP and cGMP function as second messengers.

Through hydrolysis of these cyclic nucleotides, phosphodiesterases (PDE) play a pivotal role in cGMP- and cAMP-mediated signal transduction. Varying in terms of tissue and substrate specificity, 11 families of phosphodiesterases exist [1], with high concentrations of PDE-4 and -5 in pulmonary parenchyma [2,3]. By metabolizing cAMP, PDE-4 modulates prostaglandin synthesis. Recently, we have preserved lung allograft function in a model of prolonged ischemia by employing a novel PDE-4 inhibitor (PDI747) [4].

Phosphodiesterase-5 has been identified as the primary cGMP-hydrolyzing enzyme in human corpus cavernosal and

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pulmonary tissue [5]. High intracellular concentrations of cGMP, which are converted from GTP by NO-activated guanyl cyclases, initiate protein phosphorylation, with ensuing bronchial and vascular dilation [5]. The importance of the cGMP/NO-synthesis-pathways in targeting I/R injury has been demonstrated by our group: simultaneous administration of 8-Br-cGMP (second messenger of NO) and tetrahydrobiopterin (coenzyme of NO-synthase) preserved pulmonary graft function in the pig after 30-h ischemia [6].

Given the predominant distribution of PDE-5 in the lung, and on the basis of the crucial role of cGMP in maintaining cellular functionality during reperfusion after extended ischemia, we investigated the influence of selective PDE-5 inhibition with sildenafil (Viagra®) on graft survival after extended ischemia.

2. Materials and methods

2.1. Animals and procedures

Nine weight-matched pairs of outbred pigs, randomized into control and study group, served as donors and recipients. Harvest and left lung transplantations were performed as previously reported [4,6]. Lungs were flushed with 1.0 l of LPD solution (Perfadex, Vitrolife AB, Kungsbacka, Sweden) and stored at a temperature of 1 °C for 24 h. One hour after reperfusion, the right contralateral lung was excluded from perfusion and ventilation. All animals received humane care in compliance with the European Convention on Animal Care. The protocol was approved by the local animals study committee.

2.2. Study groups

Two groups were studied. In group I ($n = 5$), sildenafil (0.7 mg/kg, Pfizer, Zurich, Switzerland) was injected into the pulmonary artery (PA) before inflow occlusion. In addition, LPD containing 0.7 mg sildenafil/l was used as flush solution. During backtable preparation, the isolated left lung was perfused retrograde with 500 ml LPD containing 0.7 mg/l sildenafil. Furthermore, recipients of these grafts received continuous i.v. infusion with 0.7 mg/kg sildenafil over 5 h, starting at reperfusion. In group II ($n = 4$), 8 µg/kg prostaglandin E1 (PGE1) (Prostin VR Pediatric; The Upjohn Company, Kalamazoo, MI, USA) was injected into the PA before flushing, as in clinical use. For in vivo and backtable perfusion, LPD was employed. Preservation time of groups I and II donor lungs was 24 h.

2.3. Assessment

One hour after reperfusion of the transplanted lung, the right PA (including the branch to the upper lobe) and the right main bronchus were ligated to assess isolated allograft function for the following 5 h after reperfusion. During the assessment period, anaesthesia was maintained with Isoflurane 1.5%. FiO₂ was 1.0 tidal volume 5.5 l at a respiratory rate of 20 min⁻¹ and a PEEP of 5 mm H₂O. Systemic arterial, PA, central venous, and left atrial pressure were recorded continuously. Arterial and mixed venous blood was collected

for gas analysis every 60 min. Upper lobe allograft samples were collected and snap frozen at -80 °C in liquid nitrogen for tissue MPO and TBARS assay. Time of tissue harvest was at time of spontaneous death (2–3 h after reperfusion for the control animals) and at 5 h after reperfusion for the treated recipients (endpoint). All values are given as mean ± standard deviation of the mean (SD).

2.4. Extravascular lung water

Extravascular lung water as direct assessment of reperfusion edema was measured as previously described [6]. A fiberoptic catheter (System Cold Z-021, Pulsion, Munich, Germany) was advanced via the external carotid artery into the descending aorta. The indicator bolus consisted of two components: indocyanine green served as an intravascular marker and ice cold 5% glucose as a thermal intra- and extravascular indicator. The bolus was injected via the external jugular vein with a temperature-controlled injector. The dilution curves for dye and temperature were recorded simultaneously in the descending aorta with the thermistor-tipped fiberoptic catheter. Thoracic intravascular and extravascular fluid volumes were determined based on the measurement of the mean transit times for thermal and dye indicators and of the decay time volumes calculated from the indicator dilution curves. The lung water computer (System Cold Z-021, Pulsion) determined the mean transit time for the thermal indicator and for the dye indicator and calculated total thermal volume (ITTV), intrathoracic blood volume (ITBV), and extravascular thermal volume (ETV). The extravascular thermal volume (ETV) was calculated as follows: ETV = ITTV – ITBV. All measurements were made in triplicate. The mean value was used for analysis.

2.5. Myeloperoxidase assay

Donor and recipient lung samples were frozen immediately and stored at -80 °C until assessment. MPO levels were measured using a myeloperoxidase assay kit (Cytostore, Calgary, Canada). Enzyme activity was expressed as change in optical density units per milligram of tissue protein per minute ($\Delta OD / (mg \text{ min})$).

2.6. TBARS assay

Thiobarbituric acid-reactive substance (TBARS) levels in lung tissue were measured with the NWLSS kit from Northwest (Northwest Life Sciences Specialities, Vancouver, Canada) following the company protocol. Briefly a 10% (w/v) was prepared and TBARS levels were determined by reference to a standard curve of 1,1,3,3-tetramethoxypropane and the results were expressed as nanomoles of malondialdehyde (MDA) per gram of wet lung.

2.7. Hemodynamic parameters and gas exchange

Systemic arterial, PA, central venous, and left atrial pressure were recorded continuously with a hemodynamic monitor system (Hellige, Freiburg, Germany). Measurement of cardiac output was necessary for the lung water

assessment (System Cold Z-021, Pulsion). Pulmonary vascular resistance was calculated in CGS units according Poiseuille's law. Arterial and mixed venous blood was collected for gas analysis every 60 min.

3. Results

No differences between groups were noted in donor weight (group I: 32.4 ± 3.1 kg vs group II: 30.8 ± 2.2 kg), recipient weight (group I: 34.2 ± 3.9 kg vs group II: 34.5 ± 2.8) and total preservation time (group I: 1557 ± 35 min vs group II: 1526 ± 23 min).

All five recipients from group I survived until the end of the experiment (1 h of bilateral perfusion, followed by 5 h of isolated graft ventilation/perfusion). In contrast, all four recipients of group II died within 1–2 h after occlusion of the right side, with signs of severe pulmonary edema and acute right heart failure.

Extravascular lung water in group II was markedly increased by 1 h following occlusion of the right side (9.7 ± 0.1 mg/kg), in comparison to the grafts in group I (6.48 ± 1.8 mg/kg). Since all recipients in group II died between 2 and 3 h after reperfusion (1–2 h after occlusion of the right side), continuous EVLW measurements over 360 min could only be obtained for group I. During the entire observation period, EVLW remained stable in group I without a significant increase during 5 h of isolated allograft perfusion/ventilation (6.75 ± 1.4 mg/kg vs 6.7 ± 1.0 mg/kg) (Fig. 1).

Neutrophil migration, based on assessing myeloperoxidase activity in the tissue, did not differ significantly between the two groups (group I at endpoint: 3.6 ± 0.7 U/ μ g vs group II at time of terminal graft failure: 3.7 ± 1.4 U/ μ g; native tissue: 0.6 ± 1.5 U/ μ g). However, free radical tissue damage expressed as MDA concentration per gram wet lung was significantly lower in group I compared to group II (group I at endpoint: 13.8 ± 1.6 μ M vs group II at time of terminal graft failure: 18.5 ± 3.0 μ M, $p < 0.05$; native tissue: 12.4 ± 0.9 μ M) (Fig. 2).

In the treated recipients, arterial oxygenation deteriorated moderately following occlusion of the right side, but remained stable throughout the observation period (baseline: 41.6 ± 20.1 kPa vs endpoint: 16.8 ± 2.1 kPa). Pulmonary vascular resistance (PVR) in group II increased markedly within 1 h after exclusion of the right side, and all animals died within the following 1–2 h with values over 1000 dyne s cm^{-5} . In contrast, all recipients from group I survived a 6-h reperfusion period, displaying a moderately increased PVR (400 – 500 dyne s cm^{-5}) at endpoint measurement (Fig. 3).

4. Discussion

Sildenafil application during pig lung allograft procurement and reperfusion attenuated I/R injury and abolished fatal early graft dysfunction after 24-h preservation time. Employing the novel and specific PDE-5 inhibitor sildenafil prevented reperfusion-associated pulmonary edema, stabilized recipient pulmonary vascular resistance, and significantly reduced free radical tissue damage. This is the first study to report on the successful pharmacological preconditioning (PC) with sildenafil of grafts undergoing extended ischemia in a large animal lung transplantation model.

Extending the clinical potential of sildenafil, recent studies investigate its therapeutic significance in treating pulmonary arterial hypertension (PAH) and in attenuating I/R injury in models of tissue preservation [5]. Accumulating evidence from various mechanistic studies indicate a multifactorial mode of action, where sildenafil (i) induces iNOS expression [7], (ii) activates protein kinase C [8], (iii) inhibits the catalytic hydrolysis of cGMP [9], and (iv) opens mitochondrial K_{ATP} channels [10].

Brief episodes of ischemia [11], or administration of certain pharmacological agents, i.e., adenosine, bradykinin, opioids, and free radicals [12], have been shown to reduce tissue necrosis and preserve myocardial contractile function [5]. The vasodilation induced by sildenafil can mediate this effect of ischemic preconditioning: release of adenosine/

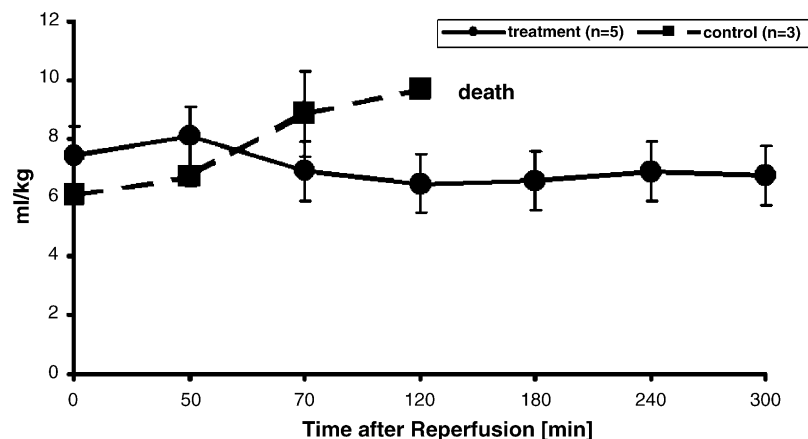


Fig. 1. Extravascular lung water (EVLW) measurements for both groups during observation time. Timepoints: 0 min (at reperfusion), 60 min (after occlusion of the right side), and 70–360 min (measurements every 60 min). Group I (treated) remained stable without significant increase during the whole observation period (baseline: 6.75 ± 1.4 mg/kg vs endpoint: 6.7 ± 1.0 mg/kg). In group II, grafts were markedly increased by 1 h following occlusion of the right side (9.7 ± 0.1 mg/kg), in comparison to the grafts in group I (6.48 ± 1.8 mg/kg). Since all recipients in group II died between 2 and 3 h after reperfusion (1–2 h after occlusion of the right side), continuous EVLW measurements over 360 min could only be obtained for group I.

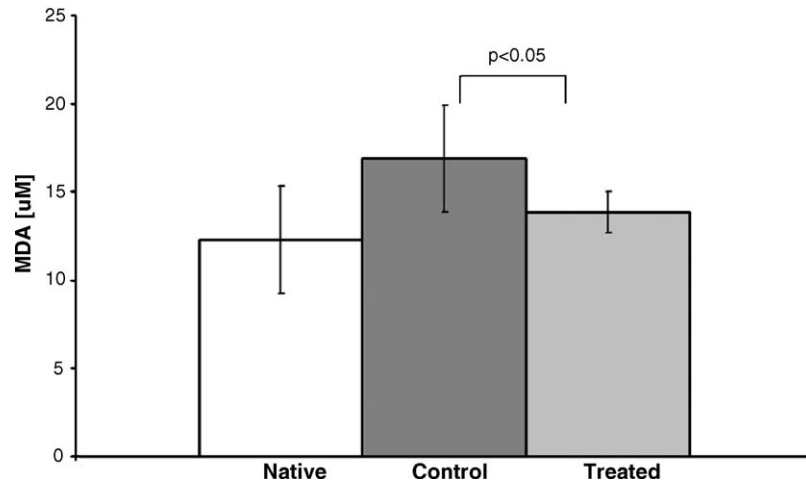


Fig. 2. Lipid peroxidation. Thiobarbituric acid reactive substance (TBARS) levels in the graft as measurement for lipid peroxidation 6 h after reperfusion (group I) and 2–3 h after reperfusion at terminal graft failure (group II). Specimen from treated recipients (group I) showed significantly lower free radical damage than untreated animals (group I: $13.8 \pm 1.6 \mu\text{M}$ vs group II: $18.5 \pm 3.0 \mu\text{M}$, $p < 0.05$; native tissue: $12.4 \pm 0.9 \mu\text{M}$).

bradykinin triggers a signaling cascade, leading to the phosphorylation of nitric oxide synthases (eNOS/iNOS), with subsequent release of NO. Nitric-oxide-induced guanylcyclase-activation eventually raises cGMP levels, in turn activating protein kinase G (PKG), which can open $\text{mitoK}_{\text{ATP}}$ channels. The opening of $\text{mitoK}_{\text{ATP}}$ channels will in part recompensate the electrochemical gradient for ATP synthesis and Ca^{2+} transport, thus maintaining intracellular energy status [5].

In our study, sildenafil has been used for direct organ-specific preconditioning and, during reperfusion, systemically. To appropriately evaluate the influence of this novel PDE-5-inhibitor on early graft function, both the local and the systemic effect have to be taken into consideration. Given the pivotal role of phosphodiesterases in modifying cellular cGMP concentration, the predominance of PDE-5 in pulmonary parenchyma [3] supports the rationale of targeting this enzyme with a specific inhibitor. When sildenafil is injected into the pulmonary circulation, it affects both endothelial and epithelial cells. First, sildenafil has a central role as a

competitive substrate analogon of cGMP for the catalytic epitope of PDE-5. Inhibiting enzymatic hydrolysis of cGMP preserves high intracellular levels of this cyclic nucleotide, which are pivotal in mediating further tissue protection. As a direct hemodynamic consequence, an increasing cGMP pool triggers protein kinase G-induced protein phosphorylation, lowering intracellular Ca^{2+} concentrations, leading to vascular dilation. Second, in a mouse heart I/R model, sildenafil administration induced a transient increase in intra-organ eNOS and iNOS mRNA expression, resulting in a significant rise of cardiac NO-synthase protein levels after 24 h [7]. Third, systemically, sildenafil lowers pulmonary artery systolic pressure and significantly increases cardiac output, which is clinically being exploited in treating pulmonary arterial hypertension [13].

We have observed a significant attenuation of free radical tissue damage in the sildenafil-treated group. Interestingly, this effect has been noted in another in vivo study, where doxorubicin-associated cardiotoxicity in mice, mediated through the generation of reactive oxygen species, could

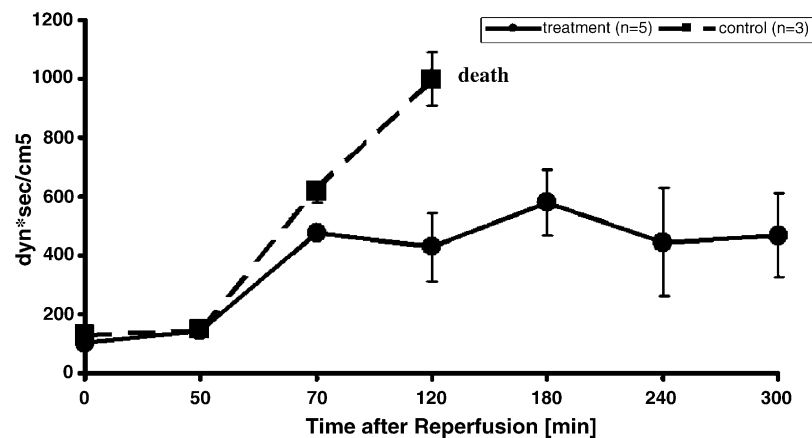


Fig. 3. Pulmonary vascular resistance (PVR) measurements for both groups during observation time. Timepoints: 0 min (at reperfusion), 60 min (after occlusion of the right side), and 70–360 min (measurements every 60 min). PVR in group II increased markedly within 1 h after exclusion of the right side, and all animals died within the following 1–2 h with values over $1000 \text{ dyne s cm}^{-5}$. All recipients from group I survived the 6-h reperfusion period, displaying a moderately increased PVR ($400\text{--}500 \text{ dyne s cm}^{-5}$) at endpoint measurement.

be prevented by sildenafil application. In this model, employing the same dose of 0.7 mg/kg sildenafil three times over the course of eight weeks, cardiomyocyte apoptosis was inhibited, preserving myocardial histomorphology and left ventricular function. It has been shown that this effect depends on NO-synthase and the opening of mitoK_{ATP} channels [14]. These results may indicate a broader therapeutical horizon for employing sildenafil in clinical lung transplantation. In addition to its efficacy in preventing early graft dysfunction, an intermediate, antiapoptotic effect may be postulated. So far, this has only been observed in cardiomyocytes [14,15], so further studies are needed to assess this effect on long-term graft endothelial and epithelial cell integrity.

Monitoring of arterial oxygenation in treated recipients in this model has been shown in the past to reflect the course of graft performance not adequately [6]. A large intraindividual variability and the sensitivity of the porcine cardiopulmonary circulation to surgical manipulation may in part obscure the clinical picture. In spite of the fact that pO₂ is often used as a critical parameter to assess lung function in other studies, the lung water computer employed in this model allows us a precise and extensive assessment of the dynamic changes of pulmonary edema following implantation. The pronounced effect on reducing lung water has been recently described for another specific PDE-5 inhibitor (E4201), in near term fetal lambs. Direct infusion of E4201 showed a NOS-independent decrease of net liquid production [16].

Taken together, employing the novel-specific PDE-5 inhibitor sildenafil for graft perfusion, storage and during the early reperfusion period preserves pulmonary functional and structural integrity, and enables lung allograft transplantation after an extended ischemic period of 24 h. This pharmacologically safe and well-established PDE-5 inhibitor may find a place for attenuating early graft dysfunction in clinical lung transplantation in the future.

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Appendix A. Conference discussion

Dr R. Schmid (Berne, Switzerland): I know the model very well. First of all, why did you put the prostaglandin in the flush solution in the control group and not in the treatment group?

Dr Korom: Well, we did not put it in the flush solution. We injected it into the pulmonary artery.

Dr Schmid: It was not clear, because you wrote Perfadex plus prostaglandin.

Dr Korom: I apologize if this was not made clear.

Dr Schmid: So you compared prostaglandin injection versus ...

Dr Korom: Sildenafil injection.

Dr Schmid: Did you do that because the animals in the control group did not survive?

Dr Korom: No. We performed the regular explantation, and before inflow occlusion, prostaglandin was injected into the PA.

Dr Schmid: But you didn't do that in the other group.

Dr Korom: Not the PGE. We used sildenafil instead.

Dr Schmid: And the second thing is the endpoint assessment. When the animals die after 3 h and you assess neutrophil migration in one group, with the MPO assay, in one group you measure it 3 h after perfusion, and in the other group at sacrifice. You cannot compare the data at all because the reperfusion is 4 h longer.

Dr Korom: It's hard to compare. However, we had these dying animals and we were trying to get some data in that situation. So it is a problem, yes.

Dr Schmid: But this is not valuable data. The second thing is, did you measure extracorporeal lung water? This can be done repetitively, and when the animal is dying, you have some data you can compare at a specific time point.

Dr Korom: I did show it. It was the second slide, but we can go over it later on.

Dr N. Pizanis (Essen, Germany): What was the direct problem? Why did all of your control animals die within 3 h? Was it reperfusion edema, or what was it?

Dr Korom: Graft failure due to pulmonary edema and finally cardiac arrest. We have had control animals for 30 h of ischemia surviving in our laboratory basically with the same settings, with the same surgeons. So this is just an inter-animal difference we were observing. But it was striking that from the same batch of animals, all the sildenafil-treated showed no problems.

Dr Pizanis: Did the problem start after clamping of the right pulmonary artery? We have this model, too, and I know that this is a tricky moment.

Dr Korom: Right, this is a tricky moment. But usually in the treated group, they all leveled out, but that's when conditions with the control animals started to deteriorate.

Dr Pizanis: Did you have hemodynamic data from a Swan-Ganz catheter?

Dr Korom: Yes.

Dr Pizanis: And did you react with catecholamines or anything in order to counteract ...

Dr Korom: We tried to save them, but it was no use.

Dr D. Van Raemdonck (Leuven, Belgium): We have also used this model and we know that it's not an easy model, because after clamping of the right pulmonary artery, you force the whole cardiac output through the transplanted postischemic lung. We have found that by modifying the arterial anastomosis from an end-to-end anastomosis to a more side-to-side anastomosis with a

patch technique this was much better tolerated by the animals. So my question to you is, what type of arterial anastomosis did you use, and have you used the same technique in both groups?

Dr Korom: Yes. It's end-to-end and the same in both groups for the artery.

Dr S. Aharinejad (Vienna, Austria): We were students of your group when we came to Zurich ...

Dr Korom: Well, you were guests.

Dr Aharinejad: We were guests, and we thank you all for teaching it. It's interesting looking at this laboratory. We went back to Vienna. We transplanted the rat model, Fisher-Lewis, and we did 40 transplantations. All the rats died. We had several problems. We managed to overcome the problems, technical problems, which were our fault, and all the animals had edema, and according to the protocol utilized in your laboratory, in your institution, the animals were not supposed to receive PGE, and then we started prostaglandin and the animals are doing perfect.

Dr Korom: This is a very interesting observation in another species. We have never seen these problems with the rats. I'm not in Zurich anymore, but maybe my co-workers should come to Vienna and have a look.

Dr M. Kamler (Essen, Germany): I just want to come back to the technique of the anastomosis. Maybe you should try to do pressure gradients before and after the anastomosis, because we found the same problem when we did the pilot experiments.