Pre-arrest Administration of the Cell-permeable Free Radical Scavenger Tempol Reduces Warm Ischemic Damage of Lung Function in Non–Heart-beating Donors

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Background: Lungs retrieved from non–heart-beating donors (NHBDs) may alleviate the shortage of suitable organs for transplantation. The critical point is the preservation of lungs during warm ischemia, when severe damage is caused by free radicals. We investigated the effect of ventilation, pre-arrest administration of heparin, and the cell-permeable free radical scavenger, tempol, on the function of NHBD grafts.

Methods: Six experimental and two control groups ($n = 6$ per group) were established. All experimental groups underwent a protocol of NHBD lung harvesting, which included 1 hour of warm ischemia after pentobarbital euthanasia followed by 90 minutes of cold ischemia. The groups were constructed as follows: Group An—non-ventilated during warm ischemia, no heparin; Group Av—room-air ventilated during warm ischemia, no heparin; Group Hn—non-ventilated, heparin added pre-arrest; Group Hv—ventilated, heparin; Group Tn—non-ventilated, heparin and tempol added pre-arrest; Group Tv—ventilated, tempol, heparin; Group Ac—control group, no warm and cold ischemia, lungs harvested immediately after euthanasia; and Group Tc—controls with tempol added pre-arrest. The lungs were then perfused ex vivo and the perfusion pressure, lung weight and arteriovenous difference in oxygen partial pressure were measured.

Results: We found that room-air ventilation during warm ischemia caused severe pulmonary edema during reperfusion. Heparinization prevented an increase in perfusion pressure and ameliorated the oxygen transport ability. Pre-arrest administration of tempol prevented edema formation after ventilation during warm ischemia and had a positive effect on the oxygen transport ability of the lungs.

Conclusions: The free radical scavenger tempol, which has a very good ability to permeate biologic membranes, contributes to better preservation of lungs retrieved from NHBDs. J Heart Lung Transplant 2008;27:890–7. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

Utilization of lungs retrieved from non–heart-beating donors (NHBDs) appears to be a a promising way to increase the pool of suitable lung donors. The crucial difference between the routinely used transplantation technique, when the lungs are harvested from brain-dead, heart-beating donors (HBDs), and the NHBD transplantation process takes place during the warm ischemia period. Warm ischemia occurs after circulatory arrest when the organ, left at room temperature, is not perfused. Whereas for HBDs organ perfusion is maintained until the harvesting itself, NHBD lungs are retrieved after cessation of circulation, and with a significant period of warm ischemia.

An major advantage when utilizing lung tissue is the possibility of maintaining oxygen extraction from the alveoli, even after cessation of circulation, which allows ongoing aerobic metabolism.[1] It has been experimentally proven that lungs retrieved from donors after cardiac arrest or after myocardial infarction can survive for at least 1 hour.[2–4] Also, it is possible that there is a protective effect of ventilation during warm ischemia. However, artificial ventilation of non-perfused lungs creates a potential risk of tissue damage by reactive oxygen species (ROS).

The aim of this study was to assess the effect of ventilation during warm ischemia and, considering the controversy regarding the need for heparin administration,[5,6] to verify the putative benefit of pre-arrest hep-

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Table 1. Experimental Groups

	Group	Warm ischemia	Heparin	Ventilation	Tempol	No. of rats
Experiment A: Effect of ventilation	Ac, controls	−	+	−	−	6
	An	+	−	−	−	6
	Av	+	−	Room air	−	6
Experiment B: Effect of heparin	Hn	+	+	−	−	6
	Hv	+	+	Room air	−	6
Experiment C: Effect of tempol	Tc, controls	−	+	−	+	6
	Tn	+	+	−	+	6
	Tv	+	+	Room air	+	6

arinization. Because we had found that pressure-controlled ventilation deteriorates pulmonary function, we tested the protective effect of pre-arrest administration of tempol, an intracellular ROS scavenger.

METHODS

Study Design

The study was performed using adult male rats (AnLab, Czech Republic) in three separate experiments (Table 1). In Experiment A we investigated the effect of room-air ventilation during warm ischemia; in Experiment B the effect of pre-arrest heparinization on lung function was assessed; and in Experiment C we studied the possible protective effect of pre-arrest administration of tempol.

All experiments were performed in accordance with the European Community and NIH guidelines for use of experimental animals and approved by the animal studies committee at our institution. All drugs and chemicals were from Sigma (Prague, Czech Republic) unless stated otherwise.

Experiment A: Effect of Ventilation During Warm Ischemia

Two experimental groups (Groups An and Av; Table 1) underwent the protocol of lung harvesting from NHBDs. Rats were anesthetized with by intraperitoneal administration of sodium thiopental (50 mg/kg), and a tracheal cannula was introduced into the trachea. All animals were then euthanized by overdose with sodium thiopental (250 mg/kg).[7,8] The animals were kept at room temperature for another 60 minutes (warm ischemia). During warm ischemia, animals in Group Av were ventilated (Model 683 Harvard Rodent Ventilator, Harvard Apparatus, Inc., Holliston, MA) with room air at 50 breaths/min, with peak inspiratory pressure 10 cm H_2O, whereas rats from the other experimental group (Group An) were left untouched. Ventilation (in the ventilated groups) was then stopped and two cannulas were introduced into the thoracic cavity. The animals were placed into a cold thermostatic chamber and in situ topical cooling of the lungs was started with cold saline solution (12°C) that contained 4% Ficoll.

After 90 minutes, the lungs were isolated and perfused as described previously.[9,10] The tracheal cannula was reconnected to the ventilator and the lungs were ventilated with a gas mixture containing 21% O_2 + 5% CO_2 + 74% N_2. In the opened chest, the main pulmonary artery and the left atrium were cannulated. Using a peristaltic pump, the lungs were perfused in a constant flow mode (4 ml per 100 g body weight per minute) with a saline solution containing 4% Ficoll and meclofenamate (17×10^{-6} mol/liter). The lungs were suspended by the trachea in a heated (38°C) humidified chamber and allowed to stabilize for 15 minutes before measurement of lung function (see later). The outflow from the left atrial cannula was recirculated into the perfusate reservoir, and the outflow pressure was set to −2 mm Hg.

Control group. Control animals (Group Ac) were anesthetized and isolation of the heart–lung block was performed in the same manner as described previously. Perfusion of the lungs started immediately after isolation, and therefore the lungs did not undergo warm and cold ischemia.

Experiment B: Effect of Pre-arrest Heparinization

Under thiopental anesthesia, the rats in Groups Hv and Hn (Table 1) were given 600 IU of heparin intrahepatically.[7] A tracheal cannula was introduced and, 30 minutes after administration of heparin, the animals were euthanized by overdose of sodium thiopental (250 mg/kg). The lungs then underwent warm and cold lung ischemia in the same way as in Experiment A. During warm ischemia, the animals of Group Hn were left untouched, whereas Group Hv animals were ventilated with room air. The control group for Experiment B was identical to the control group for Experiment A (Group Ac).

Experiment C: Effect of Pre-arrest Amination of Tempol

Tempol (4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl) freely permeates biologic membranes and, as an intracellular scavenger of superoxide anions and hydroxyl radicals, it has a protective effect with regard to ischemia–reperfusion injury, inflammation and multiple-organ failure.[11–14]

Tempol (50 mg/kg) was administered intraperitoneally immediately after the start of thiopental anesthesia. The rats in Groups Tv and Tn (Table 1) in Experiment C were given heparin as in Experiment B. A tracheal cannula was introduced and, after thiopental euthanasia, the lungs underwent a further 60 minutes of warm ischemia as in both previous experiments. Considering the results of Experiment B (see later), and because there was no assumption of either potentiation or inhibition of the effect of the combination of heparin plus tempol, we used heparin pre-arrest in all the experimental groups. To elucidate the effect of ROS production during artificial ventilation, Group Tv rats were ventilated as in Experiment A, whereas Group Tn animals were left untouched during this period. After 90 minutes of cold ischemia the heart–lung block was prepared as described earlier and lung function measurement was performed.

The controls were identical to the control group in Experiment A. To investigate the effect of tempol on pulmonary function we established Group Tc, a tempol-treated control group. The rats in this group were anesthetized and treated with tempol and heparin in the same way as in the experimental groups. Preparation of the heart–lung block started immediately after thiopental euthanasia, and therefore, similar to the control group, warm or cold ischemia was not performed.

Measurement of Lung Function

We assessed pulmonary function for 180 minutes from the beginning of perfusion of the isolated lungs. The pulmonary perfusion pressure was monitored via a transducer connected to the inflow cannula (PowerLab, ADI Instruments) and the lung weight gain was measured continually via a force transducer. After a 20-minute equilibration period, the first measurement of oxygen transport ability was performed. We took two samples of perfusion solution: an arterial sample from the inflow and a venous sample from the outflow cannula. Analysis of oxygen partial pressure was performed immediately after sampling (ABL 5, Radiometer Medical A/S, Copenhagen, Denmark). Because we used isolated lungs, it was necessary to desaturate the perfusate in the inflow artificially by bubbling the perfusate in the reservoir with 5% CO_2 + 95% N_2 for 5 minutes. Thus, the increase in oxygen partial pressure between the inflow and outflow cannula (ΔPo_2) was a measure of the ability of the lung to transport oxygen from the alveoli into the perfusate.

After a 10-minute recovery period, the reactivity to angiotensin II (0.4 μg) and to acute hypoxia (0% O_2 + 5% CO_2 + 74% N_2) was tested in two cycles. At 30, 90, 120 and 180 minutes after start of perfusion, the lung transport ability for oxygen was measured in the same way as described earlier and, during these time intervals, the values of perfusion pressure and lung weight gain were taken.

Statistical Analysis

For statistical evaluation we used repeated-measures analysis of variance (ANOVA), Fisher's PLSD test and the Games/Howell post hoc test, as appropriate. Values are presented as mean ± SEM in the figures.

RESULTS

Analysis of Lung Survival

All lungs that were not ventilated during warm ischemia (Groups An and Hn) and all control lungs (Group Ac) survived until the end of the functional assessment. This means that all measured pulmonary functions were not altered and pulmonary edema was not present. The lungs also displayed significant vasoconstrictor responses to angiotensin II and acute ventilatory hypoxia. However, almost all (i.e., 5 of 6) lungs that were ventilated during warm ischemia, both with (Group Hv) and without (Group Av) pre-arrest heparin addition, did not survive for more than 30 minutes due to massive edema formation that started immediately after the beginning of reperfusion (Figure 1).

In contrast to room-air–ventilated groups in Experiments A and B, in Experiment C all lungs from both the experimental (Groups Tv and Tn) and the control group (Group Tc) survived until the end of the functional assessment after pre-arrest tempol administration, regardless of ventilation.

Functional Assessment

Effect of ventilation and heparinization (Experiments A and B). There was no difference in lung weight gain among both the non-ventilated (Groups An and Hn) and control (Group Ac) groups.

Because of massive pulmonary edema in both groups that were ventilated with room air during warm ischemia, the perfusion pressure and the oxygen transport ability was assessed only in the non-ventilated groups (Groups An and Hn) and in the control group (Group Ac). The perfusion pressure of the non-ventilated group without heparin (Group An) was significantly higher compared with both the non-ventilated heparinized group (Group Hn) and controls (Group Ac) (Figure 2).

The best oxygen transport ability was observed in the controls (Group Ac). ΔPo_2 was significantly higher in the control group (Group Ac) compared with the

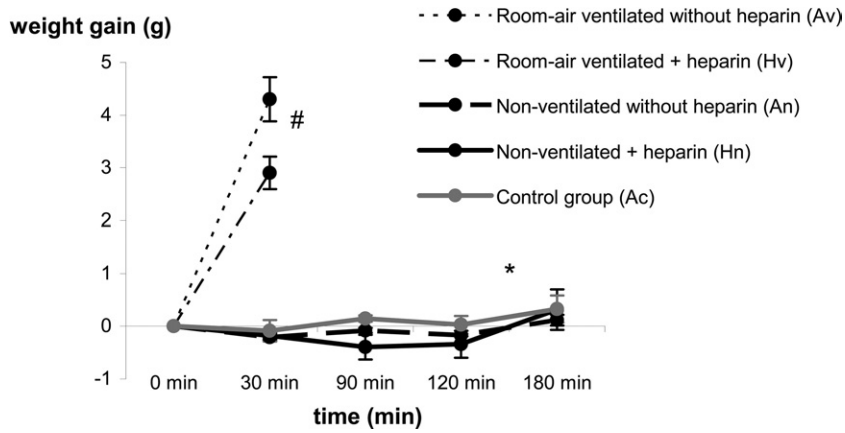

Figure 1. Ventilation with room air caused pulmonary edema. #A significant increase in lung weight gain indicating pulmonary edema in groups ventilated during warm ischemia with room-air. *In the last 60 minutes there was a significant increase in lung weight gain in the non-ventilated and control groups ($p < 0.001$).

non-ventilated groups (Groups An and Hn). The difference in oxygen transport ability was much more significant in the non-ventilated group without heparin (Group An) compared with controls (Group Ac) ($p < 0.005$) than the difference between controls and the heparin-treated group (Group Hn) ($p < 0,05$). When comparing non-ventilated groups, we observed significantly higher values of ΔPo_2, indicating better oxygen transport ability, in the lungs obtained from heparinized animals (Group Hn) (Figure 3).

Effect of pre-arrest administration of tempol (Experiment C). We did not observe any significant differences between the control group (Group Ac) and the tempol-treated control group (Group Tc).

Tempol prevented edema formation after room-air ventilation during warm ischemia. With regard to lung weight gain, tempol-treated non-ventilated (Group Tn) or room-air–ventilated (Group Tv) groups did not differ compared with either controls (Group Ac) or tempol-treated controls (Group Tc) (Figure 4).

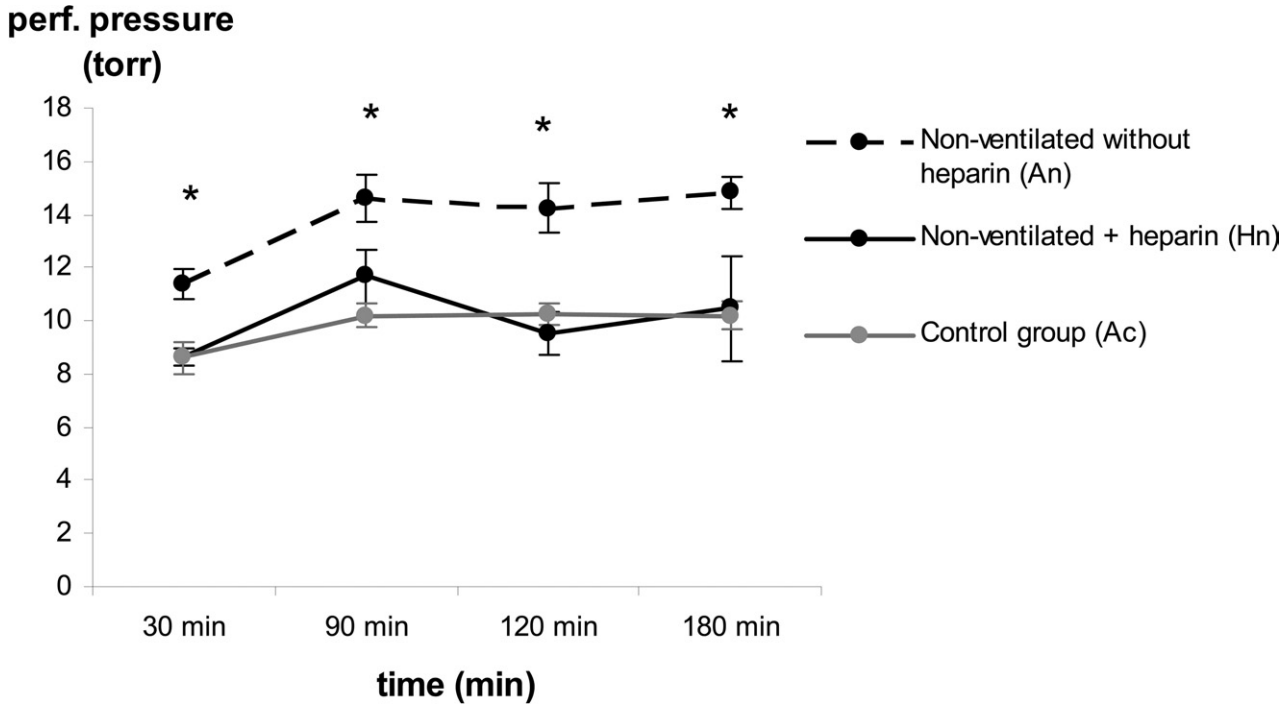

Figure 2. Heparinization prevented the increase of perfusion pressure. We observed a significant increase in perfusion pressure in the non-ventilated group without heparin (Group An) compared with both the heparinized (Group Hn) and control (Group Ac) groups (*$p < 0.005$).

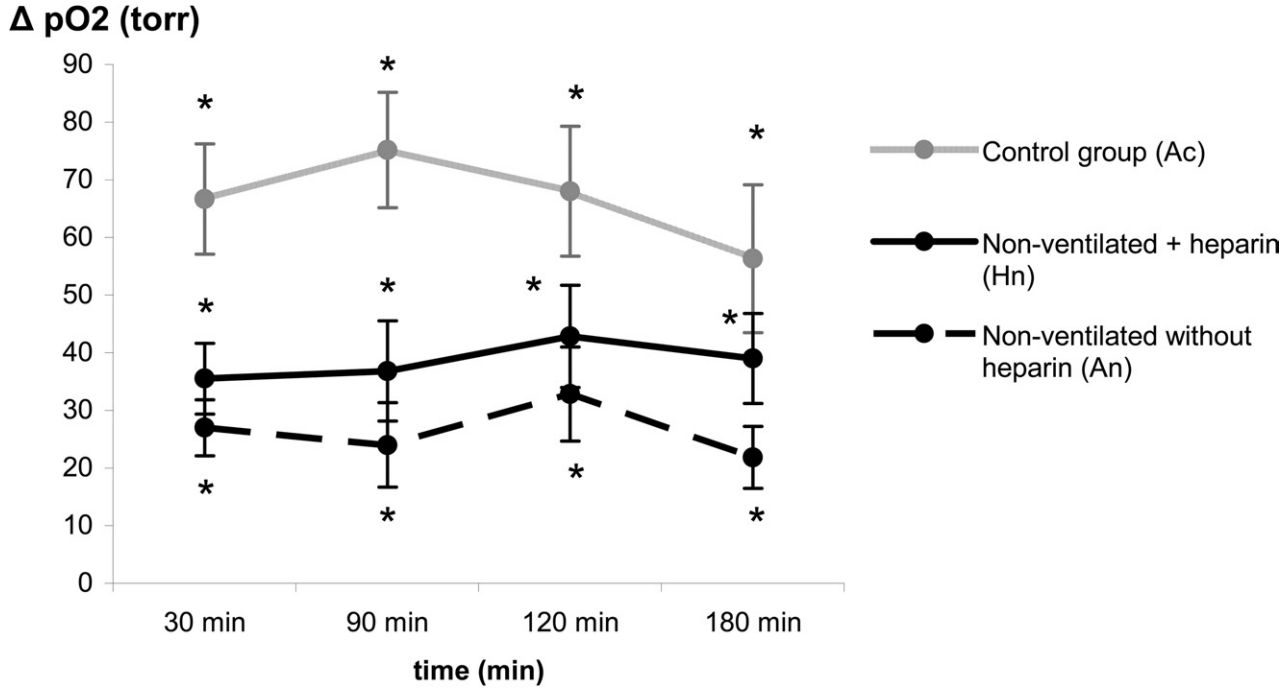

Figure 3. Pre-arrest administration of heparin partially prevented the decrease of oxygen transport ability in the lungs harvested from non-ventilated rats.

We found only a slightly higher perfusion pressure in the room-air–ventilated group (Group Tv) compared with controls (Group Ac) ($p < 0.05$); no differences were observed among other groups (Figure 5).

We also did not find any differences in ΔPo_2 among tempol-treated groups (Groups Tc, Tv and Tn) (Figure 6). This was in contrast to the significantly worse oxygen transport ability of the non-ventilated groups without tempol (Groups An and Hn) compared with controls (Group Ac), as described earlier.

DISCUSSION

We showed that heparinization before 1 hour of warm ischemia prevents an increase in perfusion pressure and improves oxygen transport ability after lung reperfusion. Our findings are in agreement with Boglione et al[6] and others,[7,15] who described the protective effect of pre-arrest heparinization on lung function in NHBDs. In the first successful clinical transplantation from an NHBD, by Steen and co-workers, heparin was administered via a central venous catheter and chest compressions were used for its distribution.[16] Wittwer et al[17] observed a continuous elimination of blood clots via the pulmonary artery in lungs from NHBDs treated using retrograde preservation with Perfadex solution, suggesting that coagulation during warm ischemia is a very active process. Some investigators, however, still question the need for heparinization.[4,5]

Another frequently discussed point, covered in our study, is the effect of ventilation during warm ischemia. Van Raemdonck et al described good graft function in deflated lungs after 1 hour of warm ischemia.[18] Further, Steen et al used no ventilation during warm ischemia in their study using a pig model[19] nor in their clinical case study of a lung transplant from an NHBD.[16] Rega et al[5] showed that animals ventilated and administered 3 hours ($Fio_2 = 0.5$) of warm ischemia had a significantly higher pulmonary vascular resistance and wet to dry ratio compared with HBDs (where pulmonary function was measured immediately after death of animals). In contrast, there were no significant differences observed between HBD controls and NHBDs topically cooled for 3 hours. All these findings correlate well with our observation that pressure-controlled room-air ventilation of lungs during 1 hour of warm ischemia had a negative effect on pulmonary function and led to pulmonary edema.

Other studies have indicated a protective effect of short-term ventilation. Boglione et al concluded that ventilation with room air during warm ischemia works as a protective mechanism.[6] Loehe et al[20,21] described good graft function during 5 hours of in situ reperfusion when lungs were inflated with oxygen in a single action before warm ischemia. We assume that interpretation of their results may be based on the protective effect of the initial distension of alveoli rather than on the benefit of oxygen supply. Albert et al showed that, at higher vascular pressure, lung inflation reduces filtration and edema formation.[22] This opinion was also supported by Ulicny et al,[23] who proposed that mechanical ventila-

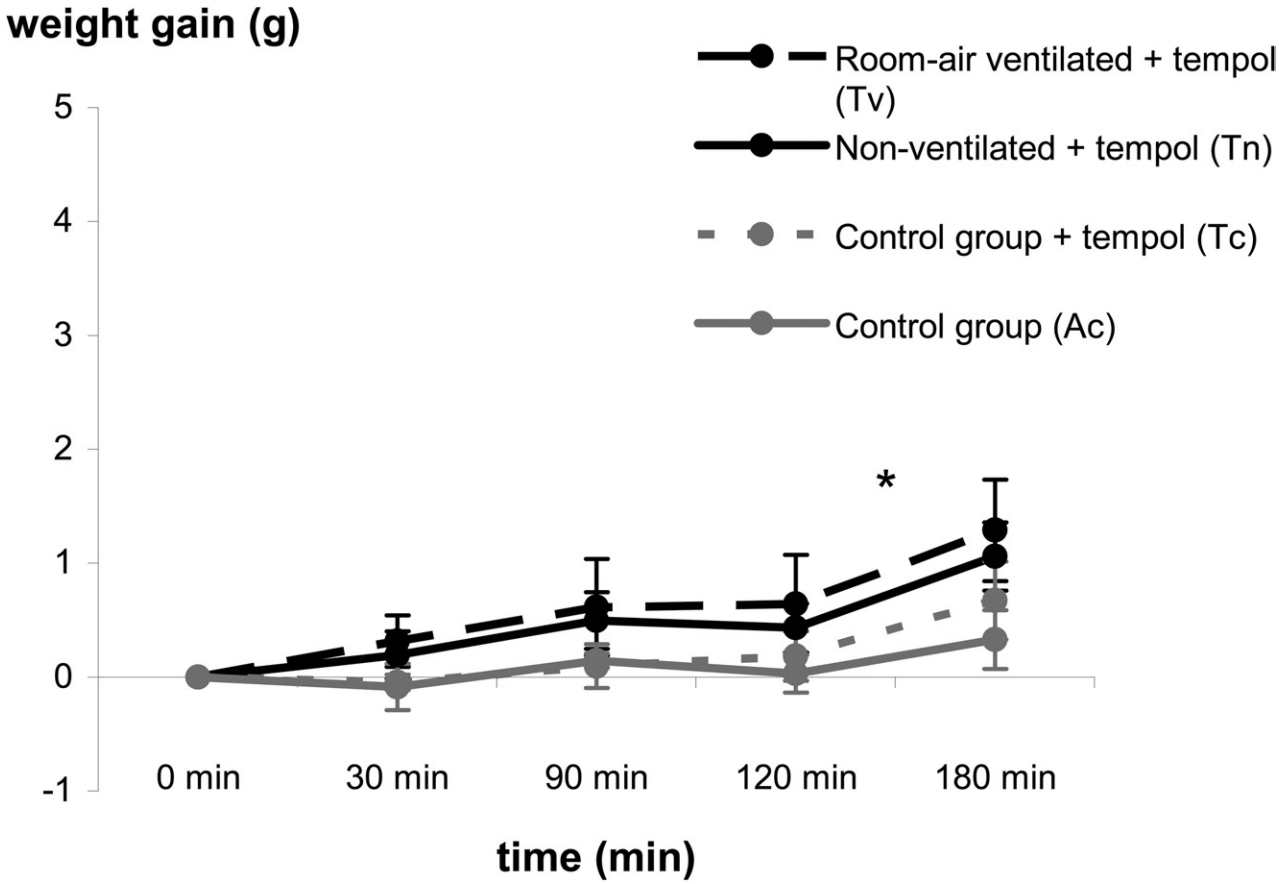

Figure 4. Tempol prevented edema formation. After pre-arrest administration of tempol we did not observe any differences between the experimental (warm ischemia) and control (no warm ischemia) groups. The pulmonary edema presented in room-air–ventilated rats without tempol (Experiments A and B; see Figure 1) was not observed here. After 120 minutes of reperfusion, lung weight increased in all groups (*$p < 0.001$).

tion itself reduces edema formation by maintaining alveoli distended, and this mechanism is independent of a continual supply of oxygen. Van Raemdonck also suggested that the critical factor in protecting lungs from warm ischemia damage might be the prevention of alveolar collapse rather than the actual oxygen delivery caused by ventilation.[8]

In contrast to our study and those of others,[4,18] Wittwer et al[17] found that ventilation in a pressure-controlled mode with $F_{IO_2} = 0.5$ for up to 300 minutes during warm ischemia had no harmful effect on pulmonary function. We have no explanation for this discrepancy.

Based on our results, we expected the pressure-controlled ventilation with room air to play a key role in ROS-mediated damage of lung function. In the absence of circulation, the lung tissue is exposed to a higher partial pressure of oxygen. Therefore, large amounts of ROS are produced, even under ischemic conditions during ventilation with an oxygen-containing gas mixture. When blood flow is re-established, a burst of free radicals occurs, leading to the parenchymal damage. This was described by Koyoma et al,[24] who also reported a beneficial effect of superoxide dismutase on protection against pulmonary edema formation. Also, administration of the scavenger and inhibitor of hydroxyl radicals, MCI-186, had a significant protective effect on pulmonary ischemia–reperfusion injury by inhibiting lipid peroxidation.[25]

We observed a clear and significant positive effect of tempol administration. When added before cardiac arrest, tempol showed a protective effect against pulmonary edema development in lungs ventilated with room air during warm ischemia. Furthermore, tempol prevented the impairment of oxygen transport ability that was observed in the first two experimental phases. It is important to point out that we did not observe any effect of tempol on the control groups. Tempol was administered before cardiac arrest, which allowed its distribution into the pulmonary vascular bed and a good effect during warm ischemia. In our recent study[26] we did not find any effect of tempol given at the beginning of reperfusion in an attempt to prevent ischemia–reperfusion injury. Therefore, the beneficial effect of tempol is restricted to pre-arrest administration only. In view of this, tempol reduced the harmful effect of warm ischemia,

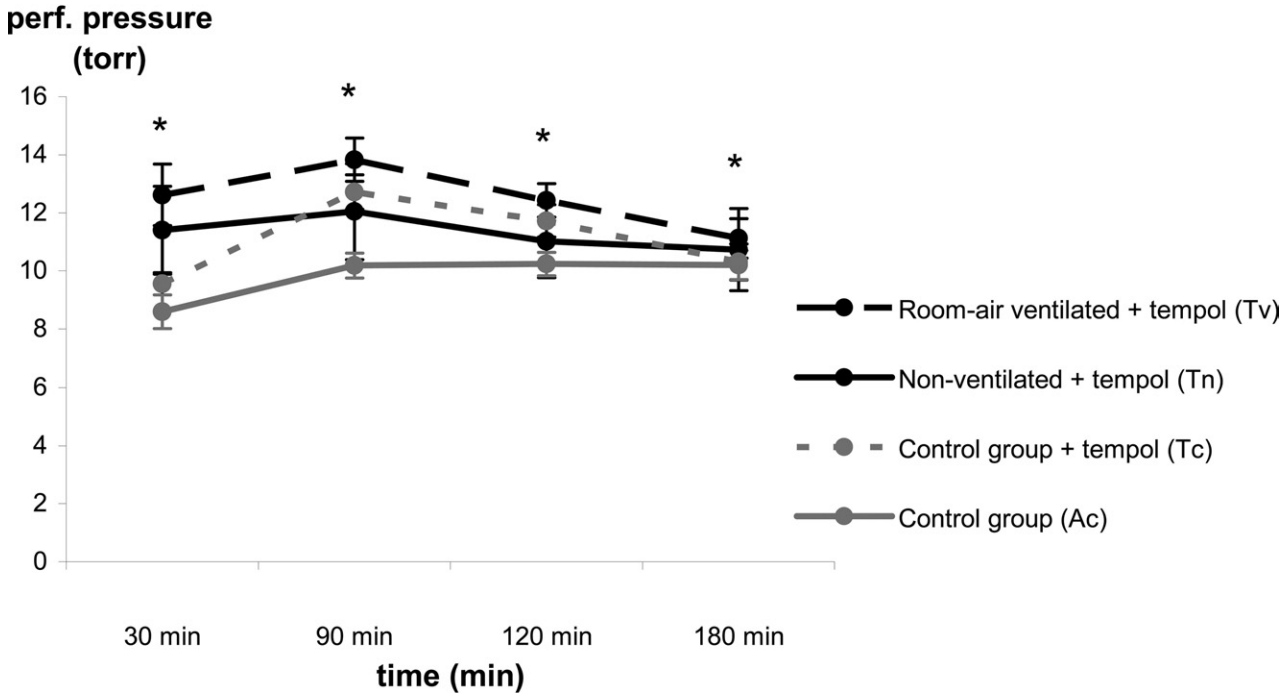

Figure 5. The effect of pre-arrest administration of tempol on perfusion pressure. Perfusion pressure in the group ventilated during warm ischemia with room air was slightly elevated (*$p < 0.05$, by repeated-measures ANOVA). No differences were observed among the other groups.

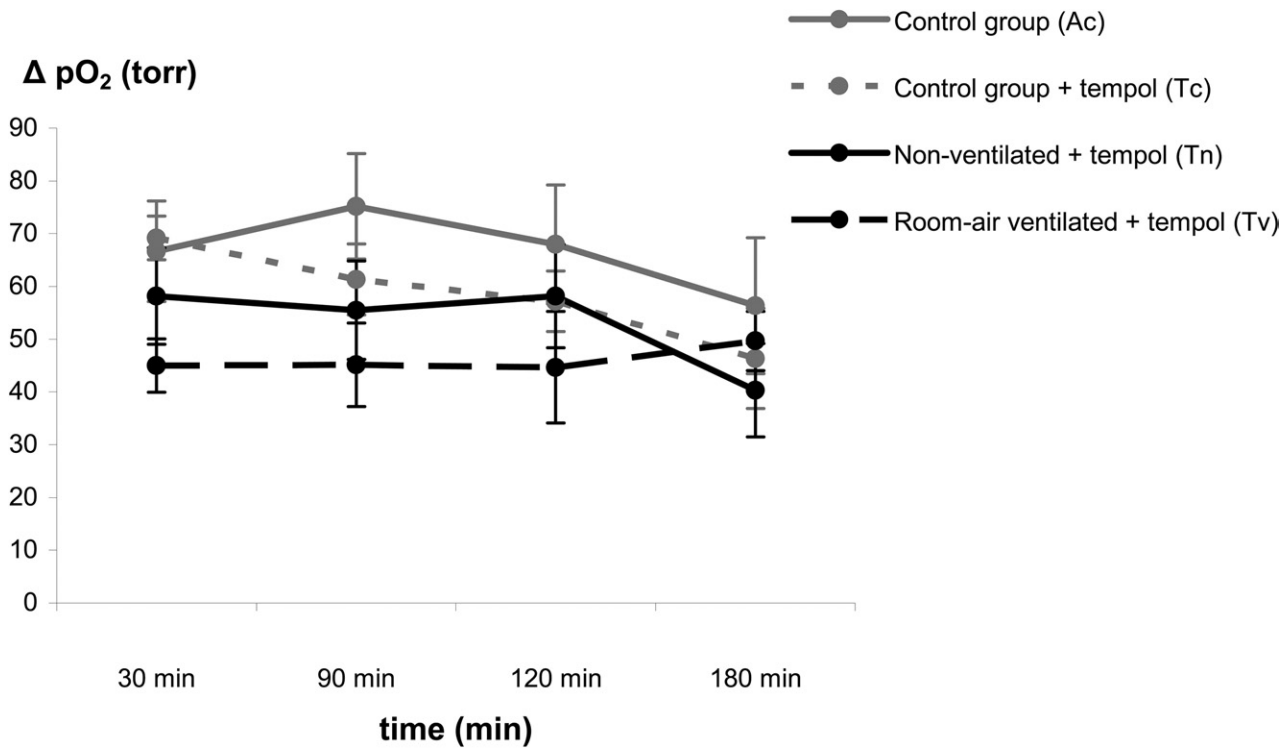

Figure 6. Tempol prevented the decrease of oxygen partial pressure in lungs harvested after warm ischemia. No differences were found in oxygen transport ability among tempol-treated groups.

which was manifested even more in the presence of ventilation with an oxygen-containing gas mixture.

There are several complementary effects of tempol-mediated reduction of ROS damage. Tempol attenuates the harmful effect of superoxide anions in vitro[27,28] and also reduces the formation of hydroxyl radicals generated in the Fenton reaction by reducing the intracellular levels of ferrous iron.[29] Although tempol probably does not scavenge H_2O_2, it is likely that it diminishes H_2O_2 injury by preventing the effects of hydroxyl radicals.[14]

The principal findings of this study are as follows: (1) heparin caused lower perfusion pressure and better oxygen transport ability in lungs retrieved from non–heart-beating rats; (2) room-air ventilation during warm ischemia resulted into massive pulmonary edema after reperfusion; and (3) pre-arrest administration of tempol protected the lungs from the damage caused by room-air ventilation. Tempol also prevented the deterioriation of ΔPo_2 in lungs harvested after warm ischemia.

The results of this study support the crucial importance of ROS in warm ischemia damage. We propose that the radical scavenger tempol, which has an excellent ability to permeate biologic membranes, can contribute to better preservation of lungs retrieved from NHBDs. Our findings further indicate that early administration of heparin and tempol, as well as avoidance of ventilation during warm ischemia, may be recommended for NHBD lung transplantation.

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