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# Effects of lung preservation solutions on PMN activation *in vitro*

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**Abstract** Polymorphonuclear leukocyte (PMN) activation and PMN-endothelial cell interactions may cause graft failure due to ischemia-reperfusion injury after lung transplantation. We investigated the effects of Euro-Collins solution (EC), low-potassium dextran solution (LPD), and EC plus pentoxifylline (EC-PTXF) on adhesion molecule (CD11b/CD18 and L-selectin) expression, chemotaxis, and oxidative burst of PMN. PMN from healthy human volunteers were incubated with EC, LPD, and EC-PTXF, and, in controls, without preservation solution. LPD exerted a suppressive effect on PMN chemotaxis as compared to EC ($P < 0.05$), but had no attenuating effect on the increase of CD11b/CD18, the shedding of L-selectin, and intracellular oxidant generation. EC-PTXF attenuated the expression of CD11b/CD18 and the oxidative burst as compared to EC alone ($P < 0.05$). These effects of LPD and PTXF on PMN function may contribute to successful organ preservation in transplantation.

**Key words** Lung preservation solution, neutrophil, chemotaxis, adhesion molecule

## Introduction

Lung transplantation has been developed as a viable therapeutic option for selected patients with terminal lung disease [5]. Although early results have improved, graft dysfunction due to ischemia-reperfusion injury remains a significant and unpredictable problem associated with lung transplantation in the early postoperative phase [6, 25]. At present, flush-perfusion with modified Euro-Collins solution (EC) and hypothermic storage continue to be used as the standard preservation method for donor lungs [21]. With the currently applied method, the safe period for reliable preservation is limited to 6 h [21]. To prolong the storage period and to prevent ischemia-reperfusion injury, the development of optimal lung preservation methods is necessary.

Although the pathogenesis of ischemia-reperfusion injury after lung transplantation remains unclear, activation of polymorphonuclear leukocytes (PMN) and interaction between PMN and pulmonary vessels have been implicated in the development of ischemia-reperfusion injury [36]. The concept of lung injury after ischemia-reperfusion is that PMN first roll on and adhere to activated vascular endothelial cells, and then migrate to alveo-

lar air spaces through the interstitium, releasing various mediators including reactive oxygen species as well as proteinases. PMN-induced damage of pulmonary endothelium and interstitium may finally lead to the development of edema [9].

Recently, some investigators reported that lung preservation with low-potassium dextran solution (LPD) provided better function of lung grafts in the early phase after transplantation than EC, however, the mechanisms responsible for the improved storage with LPD [21, 33, 35] are unclear.

Meanwhile, several reports have demonstrated that both oxygen radical scavengers and inhibitors of PMN activation can effectively prevent ischemia-reperfusion injury [20, 21]. Pentoxifylline (PTXF) is a methylxanthine derivative which can inhibit PMN activation and PMN-endothelial cell interactions [16]. Indeed, PTXF has ameliorated ischemia-reperfusion injury after lung transplantation in several animal models [4, 23]. These results suggest that the use of PTXF during donor lung flush and preservation would have favorable effects on pulmonary vessels and PMN after reperfusion, however, the *in vitro* effect of PTXF added to preservation solutions has not yet been clarified.

Several *in vitro* studies of the effects of cold preservation solutions have been performed using fibroblasts [30], cultured pulmonary endothelial cells [7, 8], and isolated type II pneumocytes [15]. However, little investigation has been performed to elucidate the direct effects of lung preservation solutions on PMN activation *in vitro*. Investigations clarifying the effect of lung preservation solutions on PMN activation would provide additional information for the selection of lung preservation solutions.

To understand the role of lung preservation solutions in PMN activation, we first investigated the effect of EC and LPD on PMN adhesion molecule expression, chemotaxis, and oxidative burst *in vitro;* EC being an intracellular crystalloid solution, and LPD a solution with extracellular ion composition. Then, in order to evaluate the effect of PTX added to the flush perfusion solution, we compared the effect of PTXF added to EC with that of EC alone.

## Materials and methods

### Materials

Pentoxifylline (PTXF), RPMI-1640 medium (RPMI), human serum albumin, fMLP, phorbol myristate acetate (PMA), recombinant human tumor necrosis factor-alpha (TNF), ferricytochrome-*c*, and superoxide dismutase (SOD) were purchased from Sigma Chemie (Deisenhofen, Germany). Mouse $IgG_{2a}$ monoclonal antibody, anti-leu-15, a mAb directed against the alpha-chain (CD11b) of the CD11b/CD18 adhesion receptor complex, and a lysing medium for flow cytometry (FACS lysing solution) were obtained from Becton Dickinson (Heidelberg, Germany). Anti-TQ-1, an antibody directed against CD62L (L-selectin), was from Coulter Electronics (Krefeld, Germany). Mouse $IgG_{2a}$ and anti-leu-15 antibodies had been conjugated with phycoerythrin, and anti-TQ-1 with Red Dye. 2,7-Dichlorofluorescein diacetate (DCFH-DA) was from Serva (Heidelberg, Germany). Polymorphoprep™, a ready-made solution containing 13.8% sodium metrizoate and 8.0% dextran 500 for the isolation of PMN, was from Nycomed Pharma AS (Oslo, Norway). The tetrazolium salt for the colorimetric assay (Cell Proliferation Reagent WST-1) in the chemotaxis experiments was from Boehringer Mannheim (Mannheim, Germany). Modified Euro-Collins solution (EC) was prepared by the Department of Pharmacy at our institution (Apotheke, Klinikum Innenstadt, Universität München). Perfadex from Kabi Pharmacia AB was used as low-potassium dextran solution (LPD). All preservation solutions were stored at 4 °C. Pentoxifylline was added to modified Euro-Collins solution at a concentration of 300 μg/ml (EC-PTXF). The composition of EC, LPD and EC-PTXF are shown in Table 1. The experiments were performed at a steady temperature, 20 °C, in clean, ventilated cages.

**Table 1** Compositions of used lung preservation solutions

| | | EC | EC-PTXF | LPD |
|---|---|---|---|---|
| $Na^+$ | (mmol/L) | 10 | 10 | 138 |
| $K^+$ | (mmol/L) | 115 | 115 | 6 |
| $CA^{2+}$ | (mmol/L) | (–) | (–) | (–) |
| $Mg^{2+}$ | (mmol/L) | (–) | (–) | 0.8 |
| $Cl^-$ | (mmol/L) | 15 | 15 | 142 |
| $SO_4^{2-}$ | (mmol/L) | (–) | (–) | 0.8 |
| $HCO_3^-$ | (mmol/L) | 10 | 10 | (–) |
| Phosphate | (mmol/L) | 57.5 | 57.5 | 0.8 |
| Dextran-40 | (g/L) | (–) | (–) | 50 |
| Glucose | (g/L) | 35.7 | 35.7 | 0.91 |
| Osmolarity | (mOsm/L) | 375 | 375 | 292 |
| PTXF | (μg/ml) | (–) | 600 | (–) |

*EC* Euro-Collins' solution,
*PTXF* pentoxifylline,
*LPD* low-potassium dextran solution

### Experimental design

*Experiment 1 Comparison of LPD with EC*

All experiments were performed using whole blood or isolated cells from at least six donors. All volunteers gave their informed consent prior to sampling. Each measurement was performed in triplicate. Experimental groups consisted of EC-, LPD-, and control groups, according to the lung-preservation solution used: EC, LPD and phosphate-buffered saline (PBS), respectively, mixed with blood or isolated neutrophils. PMN adhesion molecule expression, chemotaxis, and oxidative burst were studied in these three groups.

*Experiment 2 Evaluation of effect of PTXF added to EC*

In order to evaluate the effect of PTXF added to flush perfusion solution, the second experimental groups consisted of EC alone, EC-PTXF, and control groups, according to the lung-preservation solution used: EC, EC-PTXF and PBS, respectively, mixed with blood or isolated neutrophils. Sampling of whole blood, isolation

of neutrophils, and triplicate measurements were performed similarly to experimental design 1. Assays of adhesion molecules, chemotaxis and oxidative burst was performed similarly to design 1.

## Methods of evaluating PMN activation

### *Expression of adhesion molecules*

*Whole blood incubation.* From healthy human subjects ($n = 9$) 10 ml venous blood was drawn with a syringe containing 50 U/ml heparin as an anticoagulant. The blood samples were diluted with PBS to a concentration of $2 \times 10^6$ leukocytes/ml. Lung preservation solution (EC, LPD or EC-PTXF) or PBS (Control) was added to the diluted blood, which was then incubated at 4 °C for 2 h in a sterile 5-ml screw-capped tube. The tube was gently agitated every 15 min to avoid sedimentation of cells. After preincubation, blood samples were subsequently incubated at 37 °C for 30 min with fMLP ($10^{-7}$ mol/L) or PMA ($10^{-7}$ mol/L) as agents to stimulate neutrophils.

*Flow cytometry.* To 200 µl pretreated whole blood 5 µl anti-leu-15 or anti-TQ-1 antibodies were added. After incubation for 30 min at 4 °C in the dark, 2 ml lysing medium (FACS lysing solution) was added for 10 min to fix leukocytes and to lyse erythrocytes. The samples were centrifuged, washed, resuspended in PBS, and stored at 4 °C in the dark until flow cytometric analysis. Class-matched irrelevant phycoerythrin-conjugated monoclonal antibodies (mouse $IgG_{2a}$) were added to determine non-specific antibody binding. Single red color immunofluorescence analysis was performed with a FACSort flow cytometer (Becton Dickinson) with an excitation wavelength of 488 nm and collection of fluorescence signals at a wavelength of 595 ± 30 nm (red fluorescence). Antibody binding was determined as mean channel fluorescence intensity after gating neutrophils by their characteristic volume and side scatter properties [14, 27].

### *Chemotaxis*

*Preparation of neutrophil suspension.* From healthy human subjects ($n = 17$) 20 ml venous blood was drawn, with addition of 50 U heparin/ml blood. Over 5 ml Polymorphoprep™ (density = 1.113) 5 ml blood was layered. After centrifugation at 500 g for 35 min, two leukocyte bands were visible. The lower band, which consisted of PMN, was harvested and washed twice with PBS. Contaminating erythrocytes were lysed hypotonically by adding 7 ml deionized water for 30 s. Isotonicity of the cell suspension was restored with 7 ml 1.8% NaCl. PMN were resuspended in RPMI to a cell count of $2 \times 10^6$/ml, which contained 2% human serum albumin. Lung preservation solution (EC, LPD, or EC + PTXF) or PBS (control) was added to the cell suspension to a cell count of $1 \times 10^6$/ml, and the suspension was incubated at 4 °C for 2 h. After preparation, the purity of the PMN suspension was > 90%, and cell viability was > 99% as assessed by trypan blue exclusion.

*Chemotaxis assay.* Chemotaxis of neutrophils was assessed by the method of Shi and coworkers [29] with minor modifications. For the colorimetric assay, we used tetrazolium salt (Cell proliferation reagent WST-1), which is cleaved to formazan dye (dark red) by the succinate-tetrazolium reductase system in the mitochondria of viable cells [19]. A 96-well chemotaxis chamber (Neuro Probe Inc., USA) was used. Each well of a microtiter plate was alternately filled with $10^{-9}$ mol/L fMLP plus $10^{-2}$ ng/ml TNF as a chemoattractant or PBS alone as the control without stimulus. The microtiter plate was placed in the recess of the lower compartment of the chemotaxis chamber. A framed polycarbonate filter (3 µm pore size, Neuro Probe Inc., USA) was then placed on the top of the filled microtiter plate with the adhesive side down. The upper compartment of the chamber with 96 holes was then closed on the filter and latched to the lower compartment.

The experiment was divided into two parts, which consisted of a study for comparison among the control, EC- and LPD groups ($n = 9$), and a study among the control-, the EC-, and the EC-PTXF groups ($n = 8$). First, 150 µl PMN suspension ($1 \times 10^6$/ml) from each group was added to the wells of the upper compartment alternately in triplicate, both with and without chemoattractant. The chamber was then incubated at 37 °C in 5% $CO_2$/95% air for 1 h. After incubation, the cells that had migrated into the filter and into the lower plate were collected by centrifugation at 500 g for 10 min for WST-1 assay. Then, 10 µl cell proliferation reagent WST-1 was added to each microtiter plate well containing cells collected in 100 µl RPMI-1640 medium. The microtiter plate was then incubated at 37 °C for 1 h and read immediately after incubation with a microtiter plate ELISA reader (Titertek Multiskan, Germany) using a filter for a wavelength of 450 nm.

### *Oxidative burst*

*Flow cytometry: intracellular oxidative burst.* PMN were isolated from six healthy volunteers and incubated with or without lung preservation solution by the same methods as described in experiment 2 (Chemotaxis). The intracellular production of reactive oxygen species (ROS) was measured by flow cytometry in the before mentioned manner [1] with minor modifications. First, 50 µM DCFH-DA was added to 250 µl PMN suspension ($1 \times 10^6$ cells/ml) incubated with preservation solution. The suspension was then incubated at 37 °C with gentle horizontal agitation for 15 min. After incubation, 50 µl PMA adjusted to a concentration of 1 mM was added to the neutrophil suspension, and then the suspension was incubated at 37 °C for 15 min. Single green color fluorescence of 2,7-dichlorofluorescin (DCF) produced by the intracellular oxidation of DCFH was analyzed by a FACSort flow cytometer at a wave length of 530 ± 15 nm. Oxidative burst activity was determined as mean channel fluorescence intensity after gating neutrophils by their characteristic volume and side scatter properties.

*Cytochrome-c Reduction: Extracellular Release of Superoxide.* PMN were isolated from seven healthy volunteers and incubated with or without lung preservation solution by the same methods as described in Experiment-2 (Chemotaxis). Then, 50 µM ferricytochrome-c was added to PMN suspensions, and then the tubes were preincubated for 5 min at 37 °C. The cells were stimulated to produce superoxide by the addition of 100 nM PMA. SOD (90 units/$10^6$ cells) was added to similarly stimulated cells to confirm that ferricytochrome-*c* reduction was due to superoxide production. After incubation at 37 °C for 10 min, the reaction was stopped by reducing the temperature to 4 °C. Absorbance at a wavelength of 550 nm was spectrophotometrically measured using cell suspensions containing SOD as blanks. The total production of $O_2^-$ (nmol/$10^6$ cells/10 min) after stimulation was calculated using a coefficient of 21.1 [17].

### *Statistical analysis*

All data are presented as mean ± standard error of the mean (SEM). In the two chemotaxis experiments, results were analyzed by the nonparametric Friedman test among cells from the same donors, and the Wilcoxon signed rank test was used for comparison of

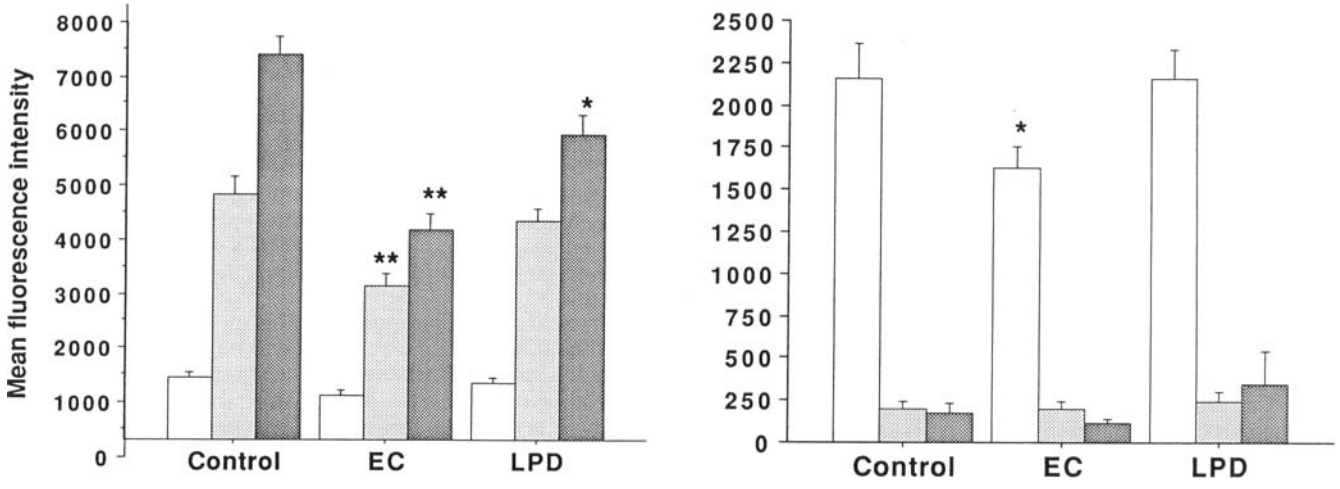


**Fig. 1** CD11b/CD18 *(left side)* and CD62L (L-selectin) adhesion receptor expression on PMN without stimulation, and with stimulation by fMLP and by PMA in the Control-, EC-, and LPD groups. Data are shown as mean ± standard error (SEM). There was a significant difference in CD11b/CD18 expression upon stimulation by PMA between the control- and LPD groups (* $P < 0.05$, versus Control group). However, levels of CD11b/CD18 expression after stimulation by both fMLP and PMA in the EC group were significantly lower than in the Control and LPD groups (** $P < 0.01$, versus Control- and LPD group). There were significant differences in L-selectin presentation levels without stimulation between the EC group and the Control- and LPD group (* $P < 0.05$). □ No stimulus, ▨ fMLP, ■ PMA

data. As regards the expression of adhesion molecules and oxidative burst assays, one-way analysis of variance (ANOVA) and Fisher's least-significant difference test were used to analyze differences among groups. Differences of $P < 0.05$ were considered statistically significant. Exact $P$-values are given as occasion demands. Analyses were performed on a personal computer using a statistical software program (Stat View 4.02; Abacus Concepts, Inc).

## Results

Experiment 1 Comparison of LPD with EC

*Expression of adhesion molecules as presented in Fig. 1*

Stimulation with fMLP and PMA resulted in a significant increase in the binding of anti-leu-15 on the surface of neutrophils, suggesting upregulation of CD11b/CD18. After stimulation with fMLP, the level of CD11b/CD18 increase was significantly lower in the EC-group as compared to that of the control- and the LPD groups ($P < 0.01$). Level of CD11b/CD18 increase induced by PMA stimulation was significantly lower in the EC group than in the control- and the LPD groups.

Upon stimulation of PMN with fMLP and PMA, a significant decrease of anti-TQ-1 binding was observed in all groups, suggesting a shedding of L-selectin from the cell surface. After cell incubation without stimuli, the binding of anti-TQ-1 to PMN in the EC group was significantly lower than that in the control- and the LPD groups, suggesting reduced levels of presentation of L-selectin on the cell surface. Furthermore, the decrease of L-selectin in the EC group was smaller than in the LPD group ($P < 0.05$). After stimulation with fMLP or PMA, there was no significant difference among any of the groups.

*Chemotaxis as presented in Fig. 2*

Stimulation with fMLP plus TNF caused a significant increase in PMN chemotaxis in all groups ($P < 0.01$). The levels of chemotaxis in the EC- and the LPD groups were reduced as compared to the control group ($P < 0.05$). Chemotaxis after stimulation in the LPD group was lower than in the control- and EC groups ($P < 0.05$).

*Oxidative burst*

*Intracellular generation of oxidants:* There was no significant difference in the spontaneous intracellular generation of oxidants among all groups. After stimulation, PMN oxidative burst activity was significantly increased in all groups ($P < 0.01$). Levels of oxidative burst activity after stimulation among the control, the EC- and the LPD groups revealed no significant differences.

*Cytochrome-c reduction: extracellular release of superoxide:* Superoxide ($C_2^-$) production upon stimulation with PMA was lower in the LPD group (2.7 ± 0.6 nmol/$10^6$ cells/10 min) than that in the control- (4.9 ± 1.3) and EC- (3.4 ± 0.9) groups (Fig. 3). The difference among groups failed to reach statistical significance by ANOVA ($P = 0.1025$).

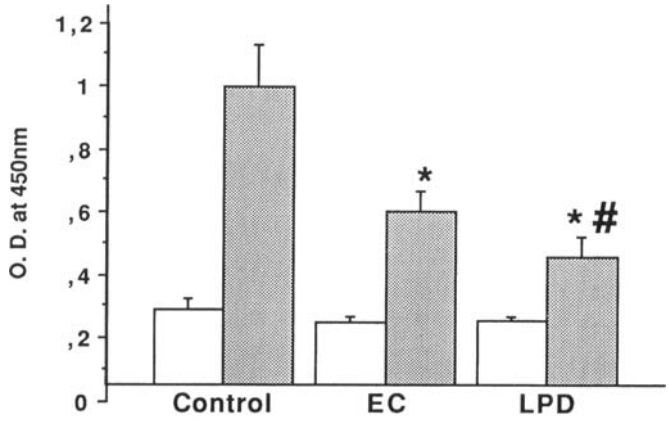


**Fig. 2** Results of PMN chemotaxis experiments comparing Control-, EC-, and LPD groups ($n = 9$). Data are shown as mean ± standard error (SEM). In the EC- and LPD groups, levels of chemotaxis after stimulation were lower than those in the Controls (* $P < 0.05$ versus Control). Chemotaxis after stimulation in the LPD group was significantly lower than in EC group (# $P < 0.05$ versus EC). □ Before, ▨ after stimulus

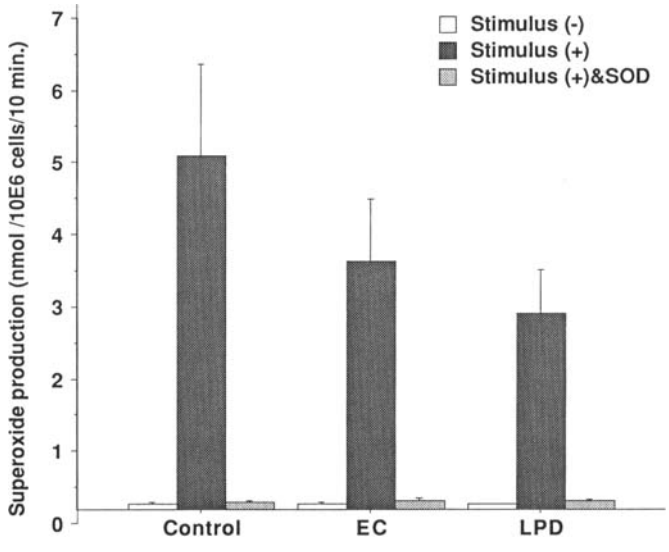


**Fig. 3** Extracellular release of superoxide, which was measured as cytochrome-c reduction of PMN after stimulation by PMA, in Control-, EC-, and LPD groups. Data are shown as mean ± standard error (SEM). Mean value of superoxide production in LPD group was lower than that in Control and EC groups

Experiment 2 effect of PTXF added to EC

*Expression of adhesion molecules as presented in Fig. 4*

After stimulation with fMLP, levels of CD11b/CD18 increase were significantly lower in the EC- and EC-PTXF groups compared to those of the control group ($P < 0.01$). Furthermore, CD11b/CD18 increase after fMLP stimulation in the EC-PTXF group was lower than that in the EC group ($P < 0.01$). However, there was no significant difference between the EC- and EC-PTXF groups after PMA stimulation.

In all groups we observed a shedding of L-selectin following stimulation of PMN with fMLP and PMA, represented by a significant decrease of anti-TQ-1 binding. After cell incubation without stimuli, the binding of anti-TQ-1 to PMN in the EC- and in the EC-PTXF groups was significantly lower than in the control group, suggesting reduced levels of presentation of L-selectin on the cell surface. After stimulation with fMLP or PMA, there was no significant difference among any of the groups.

*Chemotaxis*

The levels of chemotaxis in the EC- and EC-PTXF groups were reduced as compared to the control group ($P < 0.05$). However, there was no significant difference between the EC- and the EC-PTXF groups.

*Oxidative burst*

*Intracellular generation of oxidants:* In the EC-PTXF group, oxidative burst activity after stimulation was significantly lower than in the control group ($P < 0.05$), whereas the oxidative burst was not attenuated in the EC group (Fig. 5). The mean value of oxidative burst levels upon stimulation in the EC-PTXF group ($95.5 \pm 12.5$) was lower than that in the EC group ($137.4 \pm 11.8$) ($P < 0.05$).

*Cytochrome-c reduction: extracellular release of superoxide:* Superoxide ($O_2^-$) production upon stimulation with PMA in the EC-PTXF group ($1.8 \pm 0.4$ nmol/$10^6$ cells/10 min) was lower than that in the control ($4.9 \pm 1.3$) and EC ($3.4 \pm 0.9$) groups (Fig. 6). The difference between the control- and the EC-PTXF groups was statistically significant ($P < 0.05$).

## Discussion

Experiment 1 comparison of LPD with EC

It has been demonstrated that EC can cause potassium-induced pulmonary vasoconstriction [4, 12, 24] and that it has less protective effect on cultured pulmonary endothelial cells during extended preservation [7]. LPD exerted a suppressive effect on PMN chemotaxis after stimulation, compared to EC alone, which suggests that LPD may prevent PMN migration (Fig. 2). The mechanisms for the suppressive effect of LPD are unknown [24],

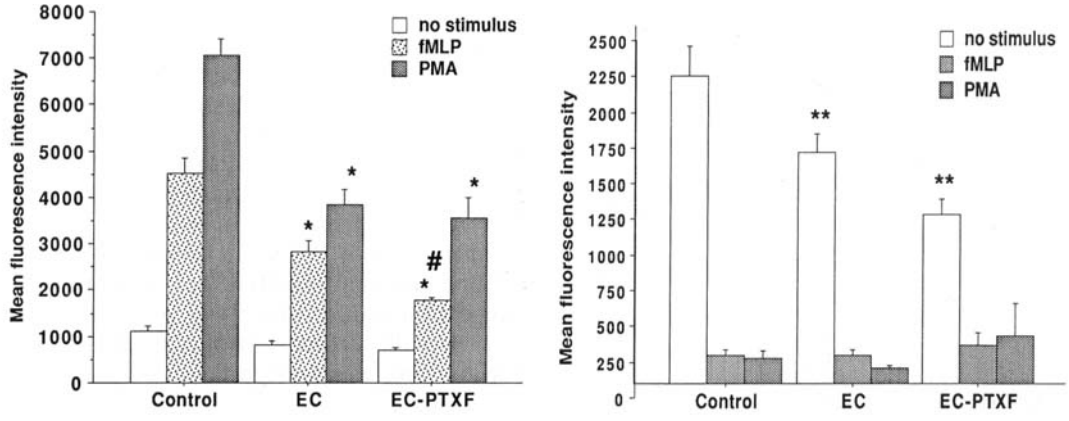


**Fig. 4** CD11b/CD18 (left side) and CD62L (L-selectin) adhesion receptor expression on PMN without stimulation, and with stimulation by fMLP and by PMA in the Control-, EC-, and EC-PTXF groups. Data are shown as mean ± standard error (SEM). Levels of CD11b/CD18 expression after stimulation by both fMLP and PMA in the EC- and EC-PTXF groups were significantly lower than in the Controls (* $P < 0.01$ versus Control). Furthermore, there was a significant difference between the EC and EC-PTXF groups after stimulation by fMLP (# $P < 0.01$). There were significant differences in L-selectin presentation levels without stimulation between the Control group and the EC- or the EC-PTXF group (** $P < 0.05$)

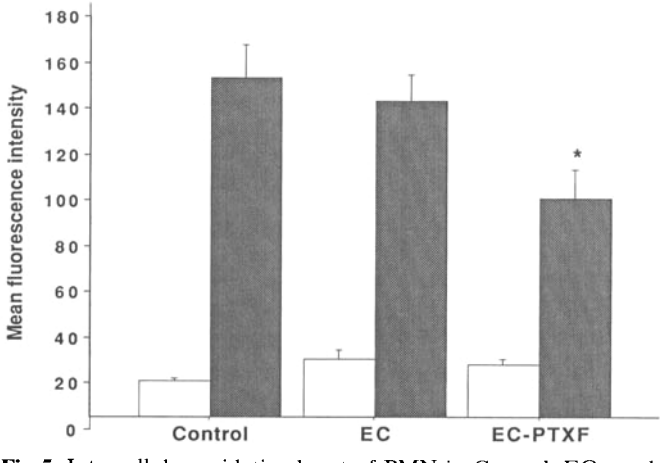


**Fig. 5** Intracellular oxidative burst of PMN in Control, EC-, and EC-PTXF groups. Data are shown as mean ± standard error (SEM). In the EC-PTXF group, level of oxidative burst after stimulation was lower than that in Control- and EC groups (* $P < 0.05$ versus Control- or EC-group). □ Before stimulus, ▨ after stimulus

but there are a number of possible explanations. It is conceivable that the fluid composition of electrolytes, osmotic or oncotic pressure, which may stabilize cell membranes or cytoplasm, and dextran, may all contribute to these results [11, 35].

The intracellular generation of oxidants was not attenuated in the LPD group as compared to the control- or EC group, although superoxide production upon stimulation with PMA was lower in the LPD group than in the control- and the EC groups (Fig. 3). It has been suggested that dextran has a scavenging effect on toxic oxygen metabolites [3]. Keshavjee et al. [11], however, reported that dextran 40 had no superoxide radical-scavenging effect. These authors also stated that protective or scavenging effects of dextran related to the generation of other toxic oxygen metabolites cannot be ruled out. Recently, we reported that lung preservation with LPD reduced lung lipid peroxidation during 18 h of ischemia, as well as ischemia plus reperfusion, in a pig model [28]. Further investigations are needed to elucidate whether LPD has any protective effects on the production of reactive oxygen species.

Some studies indicate that LPD provides better immediate function of the preserved lung than EC solution [21, 28, 33, 35]. The advantage of LPD has been explained partially by its low concentration of potassium, preventing pulmonary vasoconstriction, which leads to an increase in pulmonary vascular resistance, and, in consequence, impaired distribution of the perfusion solution [13]. Furthermore, since dextran may act as an oncotic agent, it may reduce pulmonary edema and improve lung function after reperfusion [35]. Previous reports suggested that dextran attenuates microvascular ischemia-reperfusion injury through reduction of post-ischemic leukocyte-endothelium interaction, which includes sticking and rolling of leukocytes [22, 31]. In our study, LPD did not ameliorate the expression of adhesion molecules (Fig. 1) or the oxidative burst. These results are nevertheless consistent with previous reports, as the osmolarity of LPD is lower than that of EC, and the lower osmolarity may accelerate the expression of adhesion molecules and cell swelling [22]. Extracellular type solutions including LPD provided better cell preservation of human lung fibroblasts [30] and rat isolated alveolar type II cells [16] *in vitro*. However, the mechanisms responsible for improved storage with LPD are not

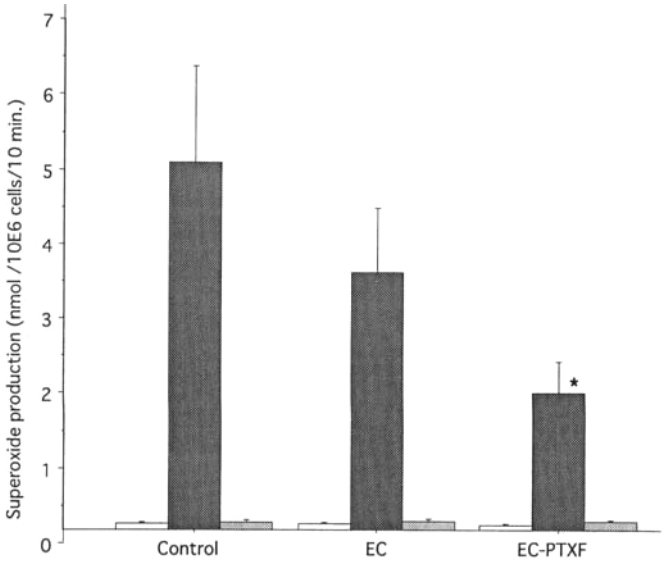


**Fig. 6** Extracellular release of superoxide, which was measured as cytochrome-c reduction of PMN after stimulation by PMA, in Control-, EC-, and EC-PTXF groups. Data are shown as mean ± standard error (SEM). In EC-PTXF group, level of superoxide production after stimulation was lower than that in the Control groups (* $P < 0.05$ versus Control). ☐ Stimulus (–), ▩ stimulus (+), ▩ stimulus (+) & SOD

known, and the benefits of the use of extracellular fluid-type solutions, such as LPD, remain controversial [21]. The results of our chemotaxis experiments additionally support the use of LPD for lung preservation to prevent PMN-mediated lung injury.

## Experiment 2 evaluation of effect of PTXF added to EC

PTXF added to EC reduced the presentation of CD11b on PMN (Fig. 4). PTXF has inhibitory effects on PMN *in vitro* when they are preexposed to inflammatory cytokines such as IL-1 and TNF [16, 32]. Several studies report that PTXF reduces the adhesion of PMN to cultured umbilical vein endothelial cells and attenuates the expression of molecules on PMN, including CD11b/CD18 and L-selectin [2, 14, 32]. PTXF can increase intracellular cyclic AMP, decrease intracellular free ionized calcium, and inhibit the signal chain between the receptor and the functional activities of PMN [32]. Our results indicate that PTXF inhibits the upregulation of CD11b/CD18 induced by stimulation with fMLP, but not with PMA, as compared to EC alone. These discrepancies can be explained by different activation sites on PMN [18].

PTXF had no attenuating or deteriorating effect on PMN chemotaxis as compared to EC alone. PTXF has been reported to increase directed migration of PMN when added to PMN preincubated with inflammatory cytokines [32]. In the present study, such an effect of PTXF may have been masked by the mixing with EC, because EC alone also showed some attenuating effect on chemotaxis as compared to the controls.

PTXF inhibits cytokine-induced superoxide production by PMN by decreasing the level of free intracellular calcium and increasing cAMP level [32]. Our results indicate that the intracellular oxidative burst upon stimulation with PMA can be attenuated by PTXF added to EC, but not by EC alone (Fig. 5). This suggests that PTXF may suppress the intracellular production of reactive oxygen species in other ways than by receptor and post-receptor signal transduction. The effects of PTXF *in vitro* shown in this study are similar to those that have been previously described. Previous reports, however, do not examine the effect of PTXF added to organ preservation solution. Our study demonstrates that PTXF added to EC, intracellular electrolyte and crystalloid solution, effectively further suppresses the PMN inflammatory response.

Several studies about PTXF using animal models have demonstrated that PTXF can reduce acute lung injury or ischemia-reperfusion injury [4, 10, 23, 26]. Yamashita et al. reported that PTXF ameliorated lung allograft reperfusion injury when it was administered in the lung-flush solution, but not when it was systemically administered to recipients before and during reperfusion [34]. The present data are consistent with these reports, and support the use of PTXF added to flush-perfusion solutions for donor-lung preservation. At present, however, PTXF has failed to gain clinical acceptance as an additive to preservation solutions. These conflicting results may be due to differences of species, storage duration, and the dose of PTXF used. Further study is needed to clarify the clinical usefulness of PTXF as an additive in preservation solutions for cold-ischemic lung storage and ischemia-reperfusion injury, as well as to elucidate the effect of PTXF added to organ preservation solutions *in vitro*.

## Limitations of this study and conclusions

There are several limitations of the present *in vitro* study of PMN and preservation solutions as regards relating the results to actual lung preservation and reperfusion injury. First, the composition of the preservation solution should be changed by various degrees dependent on the hours of the different diffusion processes, the various kinds of cell debris, and the release of cellular molecules. Thus, after implantation of the lung and reperfusion of the graft, recipient PMN would not contact the original preservation solution, but a solution of a changed composition.

The effect of temperature on preservation fluids and PMN interaction is also a crucial problem. Donor lungs

and preservation solutions should be cooled during preservation and storage, and the temperature of the preservation solution should be returned to body temperature during reperfusion. Therefore, PMN were mixed with preservation solutions at 4 °C and incubated with stimulating agents, fMLP, TNF and PMA, at 37 °C in this study. The effects of these preservation solutions on PMN should be further examined at various temperatures and after various storage periods.

Whether the interaction between recipient PMN and preservation solution is significant for the mechanism of ischemia-reperfusion injury in lung transplantation should be elucidated further. The pulmonary vessels and interstitium of donors are submerged in preservation solution during pulmonary flush perfusion and ischemic storage. After reimplantation and during reperfusion of donor lungs, circulating PMN may come into direct contact with pulmonary vessels and the preservation solution. However, the volume of preservation solution within the lung at the time of reperfusion is relatively low, particularly if controlled pressure reperfusion is utilized.

On the other hand, large numbers of PMN may accumulate in the lungs at times of stress such as during donor-lung excision. Although the majority of PMN sequestered in the lung can be flushed out with a flush perfusion solution, a small number of residual PMN may be activated during preservation and reperfusion. In these residual donor neutrophils, various inflammatory processes, including adhesion to the endothelium, chemotaxis into the interstitium, and superoxide release cause ischemia-reperfusion injury. The present work may present data on the interaction between preservation fluids and donor PMN, and may provide additional information for the selection of lung preservation solutions to prevent ischemia-reperfusion injury.

In conclusion, we demonstrated first that LPD has a suppressive effect on PMN chemotaxis as compared to EC, but no attenuating effect on the expression of adhesion molecules and the intracellular oxidative burst of PMN. Second, PTXF added to EC ameliorates CD11b/CD18 expression on PMN as compared to EC alone, and reduces the intracellular oxidative burst. The detrimental process of ischemia-reperfusion injury may not be totally prevented solely by inhibition of PMN activation. However, the present study *supports the view that* LPD or addition of PTXF to preservation solutions may be effective in attenuating ischemia-reperfusion injury due to PMN activation.

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