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Better lung protection following death due to rapid exsanguination in rats

Sıçanlarda hızlı eksanguinasyon ile ölüm sonrasında akciğerin daha iyi korunması

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Background: This study aims to investigate the effects of death due to rapid exsanguination on the viability of lung tissue.

Methods: Fourty-six Sprague-Dawley male rats with a weight range of 310-370 g were included in the study. Rats were divided into six groups: *(i)* ischemic alone (I group; n=8); *(ii)* passive exsanguination group of whose major abdominal veins were cut following death (PE group; n=8) *(iii)* group of whose major abdominal veins were cut and sacrified with rapid exsanguination (RE group; n=8); *(iv)* lung perfusion group with saline (SP group; n=8); *(v)* lung perfusion group with Perfadex (PP group; n=8) and *(vi)* control group (C group; n=6). Rats in all experiement groups except rapid exsanguination ones and all in the control group were euthanized with intrahepatic pentobarbital. Lungs were removed following euthanasia in the controls. In all study groups, lungs were ventilated in the cadavers at room temperature for 120 minutes and kept in warm ischemia.

Results: Myeloperoxidase (MPO) activity, luminol chemiluminescence (CL) values and non-viable cell rate were higher in the ischemia group. The PE group had increased MPO activity, lucigenin CL values and non-viable cell rate, whereas the RE group had reduced MPO activity and luminol CL values, compared to ischemia group. MPO activity, lucigenin CL levels and non-viable cell rate were lower in the RE group, compared to PE. The PP had lower MPO activity and luminol CL values, compared to SP or ischemia group, whereas non-viable cell rate increased.

Conclusion: Death following rapid exsanguination results in better preservation of lung viability and minimal oxidative injury. This may be explained by rapid loss of platelets and inflammatory cells in the tissue and shift of extravascular fluid to intravascular compartment.

Key words: Cadaveric lung; donor lung; ischemic injury; lung transplantation; organ preservation.

Amaç: Bu çalışmada hızlı eksanguinasyon ile ölümün akciğer dokusu canlılığı üzerindeki etkisi araştırıldı.

Çalışma planı: Çalışmaya ağırlıkları 310-370 g arasında değişen 46 adet Sprague-Dawley cinsi erkek sıçan alındı. Sıçanlar altı gruba ayrıldı; *(i)* sadece iskemide tutulan grup (I grubu; n=8), *(ii)* ölüm sonrası majör abdominal damarların kesildiği pasif eksanguinasyon grubu (PE grubu; n=8), *(iii)* sıçanların majör abdominal damarlar kesilerek hızlı eksanguinasyonla sakrifiye edildiği grup (RE grubu; n=8), *(iv)* akciğerlerin serum fizyolojikle perfüze edildiği grup (SP grubu; n=8), *(v)* akciğerlerin Perfadex ile perfüze edildiği grup (PP grubu; n=8) ve *(vi)* kontrol grubu (C grubu; n=6). Hızlı eksanguinasyon grubu dışındaki deney gruplarında ve kontrol grubundaki tüm sıçanlara intrahepatik pentobarbital ile ötenazi uygulandı. Kontrol grubunda ötenazi sonrası akciğerler çıkartıldı. Tüm çalışma gruplarında akciğerler ölü sıçanın vücudunda 120 dakika süreyle oda ısısında ventile edilerek sıcak iskemide tutuldu.

Bulgular: Miyeloperoksidaz (MPO) aktivitesi, lüminol kemilüminesans (CL) değerleri ve ölü hücre oranları iskemi grubunda yüksek bulundu. İskemi grubuyla karşılaştırıldığında PE grubunda MPO aktivitesi, lüsigenin CL değerleri ve ölü hücre oranları yüksek iken, RE grubunda MPO aktivitesi ve lüminol CL değerleri düşük bulundu. PE grubu ile karşılaştırıldığında da RE grubunda MPO aktivitesi, lüsigenin CL değerleri ve ölü hücre oranları düşük bulundu. SP ve iskemi gruplarıyla karşılaştırıldığında, PP grubunda MPO aktivitesi ve lüminol CL değerleri düşük, ölü hücre oranları ise yüksek bulundu.

Sonuç: Hızlı eksanguinasyon ile ölümde akciğer canlılığı daha iyi korunur ve oksidatif hasar minimaldir. Bu durum trombositlerin ve inflamatuvar hücrelerin hızla dokudan uzaklaşması ve ekstravasküler sıvının intravasküler kompartmana kayması ile açıklanabilir.

Anahtar sözcükler: Kadavra akciğeri; donör akciğeri; iskemik hasar; akciğer transplantasyonu; organ korunması.

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Lung transplantation has become the standard treatment of choice for patients with end-stage pulmonary diseases. The most limiting factor for this treatment continues to be the shortage of suitable donor organs.[1,2] The use of lungs from non-heart-beating donors (NHBDs) has been advocated to cope with donor shortage.[3] Lung parenchymal cells do not solely rely on perfusion for aerobic cellular metabolism as oxygen is present in the airways and alveoli. Therefore, unlike other organs, the lung may be considered a suitable organ for retrieval after circulatory arrest.[4-7] However, there is always a delay between circulatory arrest and the initiation of cold in situ flush, and as the warm ischemic interval prolongs, the lung injury becomes more prominent, which decreases the chance of a successful transplantation.[7,8] Techniques like topical cooling with cold crystalloid solution flush through the pleural cavity via chest tubes and deliverance of high-flow endobronchial cooled humidified air have been shown to be effective in preserving the lung parenchyma during the warm ischemia period.[8,9]

Most of the studies on NHBDs are focused on ischemia-reperfusion injury, primarily regarding events occurring after reperfusion: however, several inflammatory and cellular changes occur during warm ischemia, depending on the length of the ischemic period.[7]

Inflammatory cells contribute to reactive oxygen species (ROS) formation in the lung tissue which cause oxidative stress and injury.[10] Reactive oxygen species are mainly produced by phagocytes as well as polymorphonuclear, alveolar, bronchial, and endothelial cells. Activated phagocytes synthesize and release both superoxide ($O_2^{\cdot-}$) and hypochlorous acid (HOCl). The myeloperoxidase (MPO) enzyme oxidizes Cl^- found within the cytoplasm or in the extracellular fluid, into HOCl. A reaction between the HOCl and $O_2^{\cdot-}$ further increases hydroxyl radical ($^{\cdot}OH$) concentration thus causing tissue damage.[11]

Chemiluminescence (CL) is an accepted and accurate method of estimating ROS generation.[12,13] This technique, which measures light production as a byproduct of oxidative metabolism, has been utilized to demonstrate the involvement of ROS in various conditions. Luminol and lucigenin are widely used CL probes with different selectivity. Luminol quantifies a group of reactive species, including hydrogen peroxide (H_2O_2), $^{\cdot}OH$, and hypochlorite (OCl^-), whereas lucigenin is mostly selective for superoxide measurements.[14]

As most of the inflammatory agents causing ischemic injury are components of blood, we hypothesized in this study that washing out the blood from the lungs with various techniques may reduce oxidative injury during the warm ischemic period. We investigated the role of exsanguination on the viability of lung tissue with biochemical and histochemical analysis.

MATERIALS AND METHODS

Fourty-six male Sprague-Dawley rats weighing between 310 and 370 g were included in the study. They were housed in a temperature-controlled room (22±2 °C) with standardized light/dark (12/12 h) cycles, and the relative humidity (65-70%) was kept constant. The rats were fed with standard rat pellets and tap water ad libitum. The experiments were approved by the Marmara University Animal Care and Use Committee.

Experimental protocol

The rats were divided into six groups, one being the control group and five being experimental groups consisting of six to eight rats each.

The animals were anesthetized by intraperitoneal administration of 35 mg/kg pentobarbital sodium (IE Ulagay, İstanbul, Turkey). Briefly, a small laparotomy incision was performed, and 600 U heparin (Mustafa Nevzat, İstanbul, Turkey) was injected intrahepatically. An incision was made to expose the trachea, and the animal was intubated with a tracheotomy. Other than the rapid exsanguination (RE) group, the rats in the other experimental groups were euthanized with an intrahepatic pentobarbital sodium (120 mg/kg) injection. On the other hand, the rats in RE group (n=8) were sacrified by rapid exsanguination via division of the abdominal aorta and inferior vena cava in the abdomen. Cardiac arrest was documented by observation of absence of cardiac motion transmitted through the diaphragm and by palpation in all experimental groups. At this point, the heart-lung blocks were harvested via median sternotomy in the control (C) group (n=6). In the study groups, immediately after death, the lungs were ventilated through a cannula (8F) by a rodent ventilator (Harvard rodent ventilator Model 683; Harvard Apparatus Co. Millis, MA) at a tidal volume of 3 cc at a rate of 60 breaths/min and a positive end-expiratory pressure of 2 cm H_2O for 120 minutes at room temperature. The heart-lung blocks were left in situ in an effort to simulate the cadaveric donor as closely as possible.

In the ischemia (I) group (n=8), the rats were ventilated for 120 minutes. In the passive exsanguination (PE) group (n=8), division of the inferior vena cava and abdominal aorta was performed for passive exsanguination at the beginning of the ventilation period. A median sternotomy was performed on the rats in the saline perfusion (SP) group (n=8) and the

Perfadex perfusion (PP) group (n=8). The venae cavae were ligated, the abdominal aorta was divided, and a 20-gauge intravenous catheter was inserted into the main pulmonary artery. At room temperature, a physiological saline (0.9% NaCl) or Perfadex (with 1 mEq/l and 60mg $CaCl_2$ added; Vitrolife Sweden, Kungsbacka, Sweden) solution was infused through this catheter from a reservoir positioned 30 cm above the heart until a clear perfusate was obtained from the divided aorta. The sternotomy and laparotomy were then stapled, and ventilation was continued for 120 minutes in both groups.[8,15]

After this warm ischemia period, the heart-lung blocks were harvested in all study groups via median sternotomy. The following steps were common in all six groups: the right hilar structures were clamped, the main pulmonary artery was catheterized as described above, and a left atriotomy was performed. Trypan blue dye dissolved in Krebs-Henseleit bicarbonate buffer (150 ml 500 mM) was infused, followed by 150 ml of fixative (2% gluteraldehyde plus 2% paraformaldehyde in 0.1 mol/L Sorenson's buffer), both from the reservoir positioned 30 cm above the heart. During this infusion, mechanical ventilation was established briefly in all the study groups with the same parameters previously used to permit perfusion of the left lung. The mid-portion of the left lung was then excised and stored at 4 °C in the same fixative used in the routine histological preparation.[4]

The right lung was resected and stored at -80 °C for subsequent measurement of MPO activity. The ROS formation in the tissue samples was monitored using a CL technique with luminol and lucigenin probes.

Measurement of myeloperoxidase (MPO) activity

Myeloperoxidase is a heme protein stored within the azurophilic granules of leukocytes and found within circulating neutrophils, monocytes, and some tissue macrophages.[16,17] Tissue MPO activity is frequently utilized to estimate tissue polymorphonuclear leukocyte (PMN) accumulation in inflammed tissues, which has been shown to correlate significantly with the number of PMNs determined histochemically.[18] Tissue-associated MPO activity was determined in 0.2 to 0.5 g samples that were homogenized in 10 volumes of ice-cold potassium phosphate buffer [phosphate buffer saline (PBS), 20 mM dipotassium hydrogen phosphate (K_2HPO_4) pH 7.4]. The homogenate was centrifuged at 12.000 rpm for 10 min at 4 °C, and the supernatant was discarded. The pellet was then re-homogenized with an equivalent volume of K_2HPO_4 containing 0.5% (w/v) hexadecyltrimethylammonium bromide (HETAB). Myeloperoxidase activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianizidine.2HCl. One unit enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0 unit/min at 460 nm and 37 °C and is expressed in units per gram of tissue.[19]

Chemiluminescence (CL) assay

To assess the contribution of ROS in pulmonary inflammation, luminol and lucigenin CLs were measured as indicators of radical formation. Luminescence of the tissue samples was recorded at room temperature using a Mini Lumat Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing a PBS–HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (0.5 mol/l PBS containing 20 mmol/l HEPES, pH 7.2). The ROS were quantitated after the addition of the enhancers, lucigenin or luminol, for a final concentration of 0.2 mmol/l. Counts were obtained at one-minute intervals, and the results are given as the area under the curve for a counting period of five minutes. Counts were corrected for wet tissue weights and expressed as relative light units (rlu) per milligram of tissue.

Histological evaluation of cell viability

For light microscopic analysis, samples from the lung were dehydrated in an alcohol series, cleared in toluene, and embedded in paraffin. Tissue sections (5 mm) were stained with eosin only. Hematoxylin was avoided because its blue color would have interfered with the interpretation of the trypan blue dye.

Each glass slide was delineated into four quadrants and then viewed with a light microscope (Olympus BX 51, Tokyo, Japan) at x 400 magnification. The histological counting of the non-viable cells was performed by an experienced histologist who was unaware of either the control or experimental groups. In each section, five different areas were selected randomly, and 50 parenchymal cell nuclei were identified and counted in each of the four quadrants. The color of each nucleus was recorded. Cell nuclei were identified as either pink (viable) or blue (non-viable), with the latter color representing the trypan blue which stains the nuclei of non-viable cells. An eyepiece graticule (0.0785 mm^2) was used to avoid overlapping of counting areas. The number of blue nuclei in each quadrant was added together, and a percentage was derived. The percentage of non-viable cells in each rat lung specimen was used to calculate the mean percentage of non-viability in each group.[4]

Statistical analysis

All data was expressed as mean ± standard error of the mean (SEM). Groups of data were compared with

an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Results were considered significant when p was less than 0.05. Calculations were made using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Tissue MPO activity

Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration to the inflamed tissue, was significantly higher in the lung tissues of the I group (23.2±3.1 U/g) compared with the C group (12.4±1.5 U/g; p<0.05), (Figure 1). Myeloperoxidase activity in the PE (24.6±3.1 U/g) and SP (33.5±6.9 U/g) groups was not different than in the I group. On the other hand, in the RE (12.9±1.7 U/g; p<0.05) and PP (13.9±1.0 U/g; p<0.05) groups, lung MPO activity was significantly depressed to a level that was not different from that of the C group. Also, deaths in the RE group from the abdominal aorta and inferior vena cava significantly decreased the lung MPO activity when compared with the PE group which had the same type of deaths (p<0.05). Likewise, MPO activity in the PP group was significantly lower than in the SP group (p<0.05).

Chemiluminescence levels in the lung tissue

The lung luminol CL level showed a marked increase in the I group (11.4±0.7 rlu/mg protein; p<0.001) when compared with the C group (6.3±0.6 rlu/mg protein). Both the RE and PP groups abolished the elevation in lung luminol CL (8.5±0.8 rlu/mg protein; p<0.05 and 7.3±0.7 rlu/mg protein; p<0.01, respectively). On the other hand, neither the PE group (9.1±068 rlu/mg protein) nor the SP group (9.4±1.0 rlu/mg protein) changed the luminol CL levels (Figure 2).

The lucigenin CL level in the I group (7.9±0.4 rlu/mg tissue) was not different than the level in group C (6.8±1.2 rlu/mg tissue) while the level in the PE group (10.6±0.7 rlu/mg tissue) increased significantly when compared with the I group (p<0.05). In the RE group, the lucigenin CL level (6.5±0.3 rlu/mg tissue) was significantly lower than in the PE group (p<0.001) (Figure 3). On the other hand, the lucigenin CL levels in the perfusion groups were not significantly altered (saline, 9.7±0.6 rlu/mg tissue and Perfadex, 7.6±0.8 rlu/mg tissue), and the CL levels were not different from those of the C group either.

Histological analysis of cell viability

The two-hour warm ischemia period resulted in an extensive number of non-viable cells (10.7±1.8%) compared with the C group (0.5±0.2%; p<0.05), (Figure 4). Non-viable cells were found to be extremely high in the PE group (40.2±4.7%) when compared with the I group (p<0.001). Similarly, there were high numbers of non-viable cells recorded in the perfusion

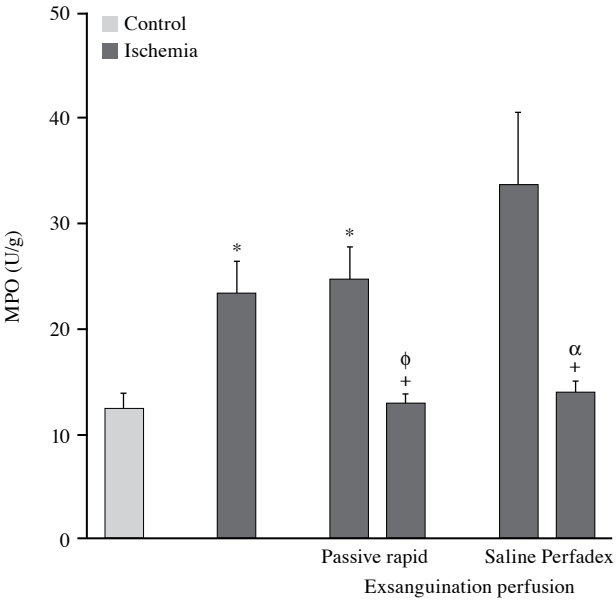

Figure 1. Tissue associated myeloperoxidase (MPO) activity of the control, ischemia alone and exsanguination and perfusion groups with ischemia. * p<0.05 compared to the control group; + p<0.05 compared to the I group; ϕ p<0.05 compared to the PE group; α p<0.05 compared to the SP group.

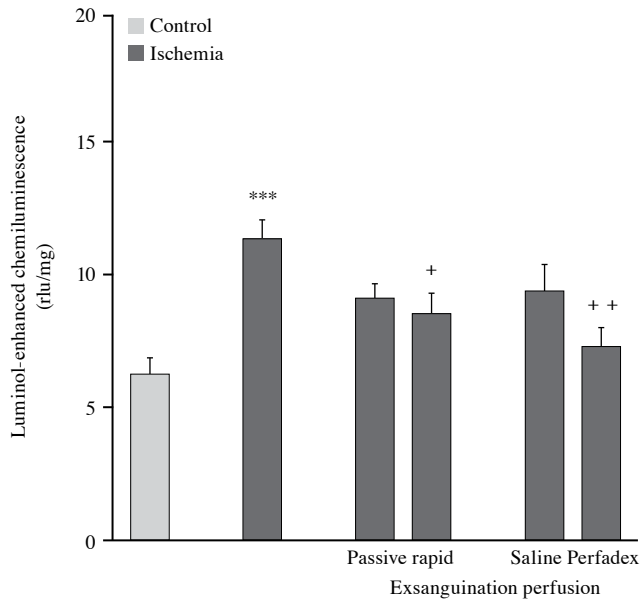

Figure 2. Luminol chemiluminescence (CL) levels of the control, ischemia alone and exsanguination and perfusion groups with ischemia. rlu: Relative light units. *** p<0.001 compared to the C group; + p<0.05 and ++ p<0.01 compared to the I group.

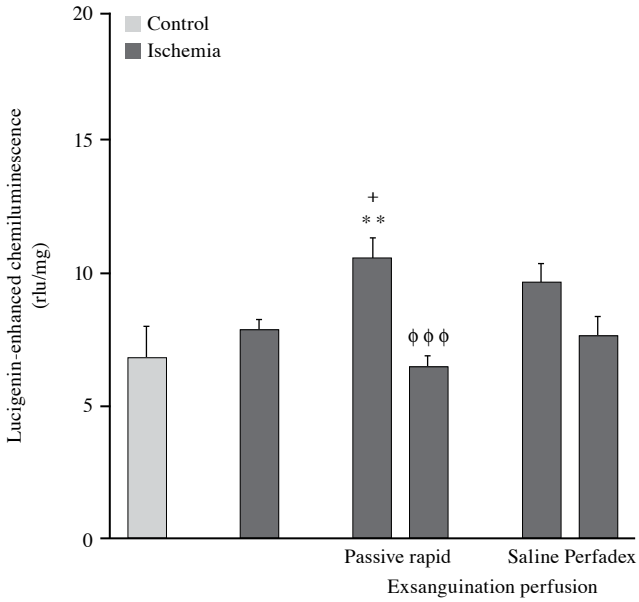

Figure 3. Lucigenin chemiluminescence (CL) levels of the control, ischemia alone and exsanguination and perfusion groups with ischemia. rlu: Relative light units; ** p<0.01 compared to the C group; + p<0.05 compared to the I group; ϕϕϕ p<0.001 compared to the PE group.

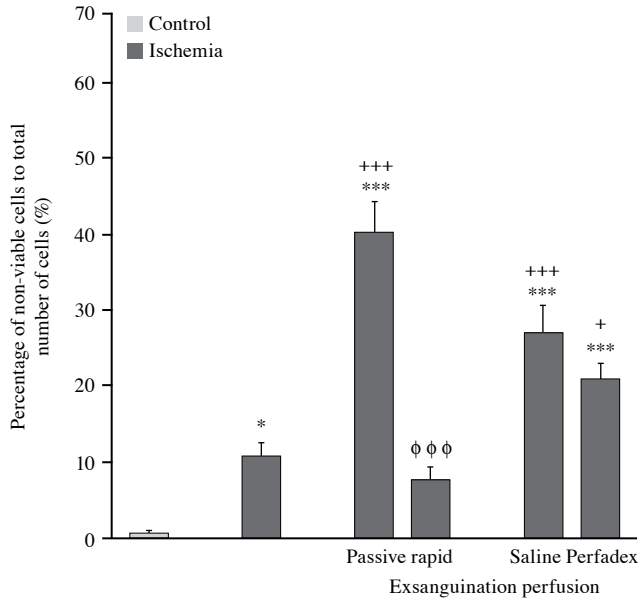

Figure 4. Number of non-viable cells in the control, ischemia alone and exsanguination and perfusion groups with ischemia. * p<0.05 and *** p<0.001 compared to the control group; +p<0.05 and +++ p<0.001 compared to the I group; ϕϕϕ p<0.001 compared to the PE group.

groups (saline, 27.0±3.6%; p<0.001) and (Perfadex, 20.7±2.2%; p<0.05). Fewer non-viable cells were found in the RE group (7.5±1.9%) when compared with the PE group (p<0.001), but the number of non-viable cells was not different from that of the C or I groups, and the perfusion groups did not differ from each other.

Microscopic examination of the C group revealed viable cells with pale pink nuclei in the lung parenchyma (Figure 5a). In the ischemia group, a slightly increased number of non-viable cells with blue nuclei was observed (Figure 5b). In the PE group, microscopic examination revealed a severely increased number of non-viable cells (Figure 5c). On the other hand, non-viable cells were mildly increased in the RE group, but this increase did not reach statistical significance (Figure 5d). Both the SP (Figure 5e) and PP (Figure 5f) groups showed a moderate increase in the number of non-viable cells in the lung parenchyma.

DISCUSSION

Animal studies have shown reasonable cadaveric graft function up to two hours after sudden death by drug administration or myocardial fibrillation.[7,8,20] However, to our knowledge, the role of death with rapid exsanguination without a hypotensive period on cadaveric lung viability has not been previously studied.

Almost all of the studies on ischemia-reperfusion injury in NHBDs are focused on the changes occurring in the reperfusion phase while the inflammatory and cellular changes during the warm ischemic period remain poorly understood.[21-24] Geudens et al.[7] showed that the cellular changes already occur during warm ischemia, and these were correlated with the length of the ischemic period in the mouse model. In this study, it was demonstrated that the number of macrophages and lymphocytes in bronchoalveolar lavage increases with longer warm ischemic intervals, and a significant rise occurs between 60 and 90 minutes of ischemia. In our study, we preferred a period of 120 minutes of warm ischemia for a demonstrative inflammatory reaction to produce ROS and investigate the effects on lung viability by measuring the MPO activity as well as the luminol- and lucigenin-enhanced CL on the lung tissue.

Reactive oxygen species are important contributors to tissue destruction. Although it is difficult to quantitate ROS because of their reactive nature and short lives, the CL method used in the present study is a simple and reproducible technique. The two CL probes, luminol and lucigenin, differ in selectivity. Lucigenin is particularly sensitive to superoxide radicals whereas luminol detects H_2O_2, OH^-, the hypochlorite ion OCl^-, peroxynitrite, and lipid peroxyl radicals.[14] The luminol-enhanced CL data of the current study demonstrates that a period of 120 minutes of warm ischemia involves toxic oxygen metabolites. Rapid exsanguination and Perfadex

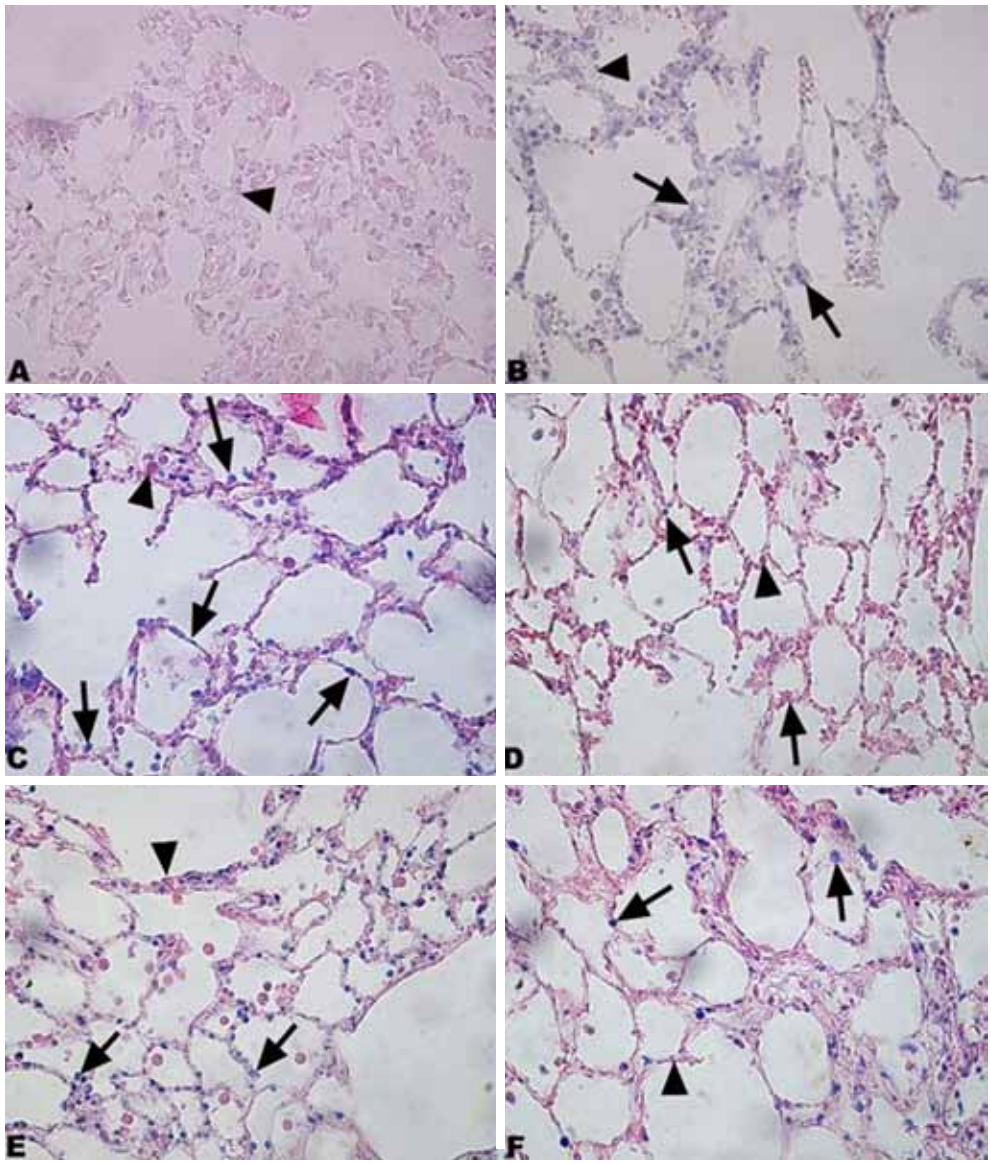

Figure 5. Micrographs illustrating the viable cells (▶) with pale pink nuclei and non-viable cells (➡) with blue nuclei of the lung parenchyma in control group **(a)**, I group **(b)**, PE group **(c)**, RE group **(d)**, SP group **(e)** and PP group **(f)** Stainings and original magnifications: Trypan blue and eosin staining, x 400.

perfusion act as antioxidant methods to reduce the generation of ROS.

It is well documented that one of the sources of ROS in several lung injuries is the activated neutrophils.[25] The tissue associated MPO, which is known as the index of neutrophil infiltration, plays a fundamental role in oxidant production by neutrophils.[26] At the site of the ischemic injury, MPO content is a marker of the magnitude of neutrophil infiltration and activation and serves as a quantitative index of injury severity. In our observation, elevated MPO levels in the lung tissue indicate that neutrophil accumulation contributes to the ischemia-induced oxidative injury, and rapid exsanguination and Perfadex perfusion appear to have preventive effects through the inhibition of neutrophil infiltration. These findings are in parallel with the luminol-enhanced CL data, which defines the role of neutrophils in the release of ROS.

In accordance with the CL and MPO results, the percentage of non-viable cells in the RE group also decreased, although it did not reach statistical significance. The highest number of non-viable cells was in the PE group, which may be a sign of cell death due to the oxidative damage caused by the inflammatory

cells and ROS in the remaining blood in the lung parenchyma. Tremblay et al.[27] showed in an isolated rat lung reperfusion model that a period of hypotension and hemorrhagic shock caused by exsanguination before death severely impairs cadaveric lung viability through activation and pulmonary sequestration of neutrophils along with the release of inflammatory mediators. However, our findings showed that rapid exsanguination may provide a total washout of the blood cells; leukocytes which cause inflammation, thrombocytes which bring about clot formation, and erythrocytes with strong oxidative agents, such as hemoglobin, from the lungs. Thus, there is less inflammatory reaction and ROS formation in situations where passive exsanguination after death is not sufficent enough to clear the inflammatory cells of the lungs. This results in a higher degree of ischemic injury.

In conclusion, this study examined the effects of rapid exsanguination on lung viability following warm ischemia. We showed that death following rapid exsanguination results in better preservation of lung viability and minimal oxidative injury. This may be due to the loss of inflammatory cells and platelets and the shift of extravascular fluid to the intravascular compartment. Further studies on this issue are needed for developing strategies to condition the NHBD lungs against ischemia-reperfusion injury. In clinical practice, these studies may lead to the conclusion that an NHBD lung harvested from a donor who "bled to death" rapidly can have a better outcome following transplantation.

Declaration of conflicting interests

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