An experimental rat model of ex vivo lung perfusion for the assessment of lungs regarding histopathological findings and apoptosis: low-potassium dextran vs. histidine-tryptophan-ketoglutarate*

Modelo experimental de perfusão pulmonar ex vivo em ratos: avaliação histopatológica e de apoptose celular em pulmões preservados com solução de baixo potássio dextrana vs. solução histidina-triptofano-cetoglutarato

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Abstract

Objective: To compare histopathological findings and the degree of apoptosis among rat lungs preserved with low-potassium dextran (LPD) solution, histidine-tryptophan-ketoglutarate (HTK) solution, or normal saline (NS) at two ischemia periods (6 h and 12 h) using an experimental rat model of ex vivo lung perfusion. **Methods:** Sixty Wistar rats were anesthetized, randomized, and submitted to antegrade perfusion via pulmonary artery with one of the preservation solutions. Following en bloc extraction, the heart-lung blocks were preserved for 6 h or 12 h at 4°C and then reperfused with homologous blood for 60 min in an ex vivo lung perfusion system. At the end of the reperfusion, fragments of the middle lobe were extracted and processed for histopathological examination. The parameters evaluated were congestion, alveolar edema, alveolar hemorrhage, inflammatory infiltrate, and interstitial infiltrate. The degree of apoptosis was assessed using the TdT-mediated dUTP nick end labeling method. **Results:** The histopathological examination showed that all of the lungs preserved with NS presented alveolar edema after 12 h of ischemia. There were no statistically significant differences among the groups in terms of the degree of apoptosis. **Conclusions:** In this study, the histopathological and apoptosis findings were similar with the use of either LPD or HTK solutions, whereas the occurrence of edema was significantly more common with the use of NS.
Keywords: Organ preservation; Organ preservation solutions; Lung transplantation; Reperfusion injury; Apoptosis.

Resumo

Objetivo: Comparar os achados histopatológicos e de apoptose em pulmões de ratos preservados em soluções *low-potassium dextran* (LPD, baixo potássio dextrana), *histidine-tryptophan-ketoglutarate* (HTK, histidina-triptofano-cetoglutarato) ou salina normal (SN) em 6 h e 12 h de isquemia pela utilização de um modelo experimental de perfusão pulmonar ex vivo. **Métodos:** Sessenta ratos Wistar foram anestesiados, randomizados e submetidos à perfusão anterógrada pela artéria pulmonar com uma das soluções preservadoras. Após a extração, os blocos cardiopulmonares foram preservados por 6 ou 12 h a 4°C, sendo então reperfundidos com sangue homólogo em um sistema de perfusão ex vivo durante 60 min. Ao final da reperfusão, fragmentos do lobo médio foram extraídos e processados para histopatologia, sendo avaliados os seguintes parâmetros: congestão, edema alveolar, hemorragia alveolar, hemorragia, infiltrado inflamatório e infiltrado intersticial. O grau de apoptose foi avaliado pelo método *TdT-mediated dUTP nick end labeling*. **Resultados:** A histopatologia demonstrou que todos os pulmões preservados com SN apresentaram edema alveolar após 12 h de isquemia. Não houve diferenças em relação ao grau de apoptose nos grupos estudados. **Conclusões:** No presente estudo, os achados histopatológicos e de apoptose foram semelhantes com o uso das soluções LPD e HTK, enquanto a presença de edema foi significativamente maior com o uso de SN.

Descritores: Preservação de órgãos; Soluções para preservação de órgãos; Transplante de pulmão; Traumatismo por reperfusão; Apoptose.

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Introduction

Lung transplantation is the only definitive treatment modality for selected patients with end-stage lung disease.

Over two decades, there has been a significant increase in the number of transplant centers, as well as in the number of transplant recipients or patients on waiting lists,[1] the mean annual number of lung transplants performed being higher than 1,500.[2] However, pulmonary transplant-related mortality remains significant. Graft dysfunction is considered the most common cause of early mortality, and its etiopathogenesis lies in ischemia-reperfusion injury.[3,4]

Donor lung preservation plays a key role in early graft function after transplantation.[5] The presence and severity of ischemia-reperfusion injury are influenced by factors pertaining to the donor and to the preservation techniques.[3] Ischemia-reperfusion injury is associated with cell death in various organ systems, and there is a correlation between the degree of apoptosis and ischemia-reperfusion injury.[6] During apoptosis, a well-regulated, energy-dependent cascade of events activates specific endonucleases, such as caspases, which are cysteine proteases specifically involved in the initiation and execution phases of apoptosis.

Under the clinical lung transplant program in our hospital, low-potassium dextran (LPD) solution is used for lung preservation. However, the cost of the solution and the logistical difficulties in obtaining it led us to seek alternatives that are logistically more efficient and provide similar clinical benefits. Histidine-tryptophan-ketoglutarate (HTK) solution is commonly used in heart transplantation at our institution. Therefore, the exclusive use of HTK solution under both transplant programs would be desirable in order to facilitate and reduce the cost-benefit ratio of preserving both organs.

To date, there have been no experimental rat models of ex vivo lung perfusion with the use of HTK as the preservation solution, although there have been studies demonstrating the advantages of HTK use for heart, liver, kidney, and pancreas preservation.[7-9]

Ex vivo lung perfusion models for the evaluation of new strategies for organ preservation have been shown to be effective and reliable in lung preservation studies.[10] We have recently demonstrated that the use of either HTK or LPD solutions at two ischemia periods (6 and 12 h) produced no significant differences among rat lungs in terms of physiological parameters, except for weight gain in the lungs undergoing 12 h of ischemia.[11]

The objective of the present study was to compare histopathological findings and the degree of apoptosis among rat lungs preserved with HTK solution, LPD solution, or normal saline (NS, 0.9% saline solution), at two different periods of hypothermic ischemia using an experimental rat model of ex vivo lung perfusion.

Methods

In the present study, 60 male Wistar (weight, 250-300 g) were used. The animals were randomized into groups (10 rats/group) according to the different periods of ischemia and the perfusion solution used: LPD-6 group (LPD, 6 h of ischemia); LPD-12 group (LPD, 12 h of ischemia); HTK-6 group (HTK, 6 h of ischemia); HTK-12 group (HTK, 12 h of ischemia); NS-6 group (NS, 6 h of ischemia); and NS-12 group (NS, 12 h of ischemia). The composition of the preservation solutions are described in Table 1. The study was approved by the local animal research ethics committee. After having been anesthetized (pentobarbital, 50 mg/kg i.p.), the animals were tracheostomized and connected to a ventilator (IL2 - Isolated Perfused Rat or Guinea Pig Lung System; Harvard Apparatus, Holliston, MA, USA) set to a tidal volume of 10 mL/kg, a RR of 70 breaths/min, and a positive end-expiratory pressure (PEEP) of 3 cmH_2O, on room air. The animals were anticoagulated (heparin, 1,500 IU, administered to the inferior vena cava). Further details on extraction and perfusion have been described elsewhere.[10,12]

According to the randomization, perfusion was achieved with 20 mL of LPD solution (Perfadex®; Vitrolife, Kungsbacka, Sweden), HTK solution (Custodiol®; Franz Kohler Chemie GMBH, Bensheim, Germany), or NS, at 4°C, administered under a constant pressure of 20 cmH_2O by raising the flask. At the end of the perfusion, the heart-lung block was extracted and preserved for 6 h or 12 h, according to the predetermined randomization, being prepared and connected to the ex vivo lung perfusion system (IL2 - Isolated Perfused Rat or Guinea Pig Lung System; Harvard Apparatus, Holliston, MA, USA; Hugo Sachs Elektronik, Hugstetten, Germany).

Table 1 - Composition of the preservation solutions used in the study.

HTK[a]		LPD[b]	
Composition	Concentration	Composition	Concentration
Sodium chloride	15 mmol/L	Sodium chloride	138 mmol/L
Potassium chloride	9 mmol/L	Potassium chloride	6 mmol/L
Potassium hydrogen 2-ketoglutarate	1 mmol/L	Magnesium	0.8 mmol/L
Magnesium chloride hexahydrate	4 mmol/L	Phosphate	0.8 mmol/L
Histidine.HCl.H_2O	18 mmol/L	Chloride	142 mmol/L
Histidine	180 mmol/L	Glucose	5 mmol/L
Tryptophan	2 mmol/L	Dextran 40	50 g/L
Mannitol	30 mmol/L	-	-
Calcium chloride	0.015 mmol/L	-	-
pH	7.40-7.45 (4°C)	pH (buffer solution)	7.4 (4°C)
Osmolality	310 mOsmol/L	Osmolality	295 mOsmol/L

HTK: histidine-tryptophan-ketoglutarate; and LPD: low-potassium dextran. [a]Custodiol®; Franz Kohler Chemie GMBH, Bensheim, Germany. [b]Perfadex®; Vitrolife, Kungsbacka, Sweden.

The block was reperfused with homologous blood obtained from 3 similarly anesthetized animals, and the volume was increased by adding enough NS to achieve a hematocrit of 15-20%. Reperfusion was performed via cannulation of the pulmonary artery/left atrium and tracheal ventilation for 60 min. The circuit was filled with blood, which was heated and recirculated through the system for 10 min with a gas mixture (90% nitrogen and 10% carbon dioxide at a flow of 200 mL/min) administered through a membrane oxygenator (D150 MediSulfone® Hemofilter; MEDICA s.r.l. Medolla, Italy). The heart-lung block was perfused (5-7 mL/min) and ventilated (tidal volume = 10 mL/kg, RR = 70 breath/min, and PEEP = 3 cmH_2O). After 5-10 min, we started data collection, which was performed every 10 min for 60 min. We collected data on hemodynamics and respiratory mechanics, as well as blood gas analysis (ABL 800; Radiometer, Copenhagen, Denmark) results. At the end of the reperfusion, fragments of the middle lobe were extracted, fixed in 4% formaldehyde buffered solution, and embedded in paraffin blocks. Subsequently, 5-µm sections were cut and stained with H&E. We conducted a qualitative and semi-quantitative analysis by assessing the following: congestion; alveolar edema; alveolar hemorrhage; interstitial hemorrhage; inflammatory infiltrate; and interstitial infiltrate. For the semi-quantitative assessment of changes such as alveolar hemorrhage, interstitial hemorrhage, interstitial inflammatory infiltrate and congestion, we considered the histological compartmentalization, i.e., alveolar spaces, (axial, septal, and peripheral) interstitium, capillary bed, and extent of changes. Each change (event) was separately semi-quantified and received a score ranging from zero to three, as follows: no event (score 0); event occurring in less than 33% of the affected compartments (score 1); event occurring in 33-66% of the affected compartments (score 2); and event occurring in more than 66% of the affected compartments (score 3).[13] Histological examination was performed by a single observer. The parameter "edema" was evaluated in two different compartments, namely the interstitial compartment and the bronchoalveolar compartment. Alveolar edema was scored with the semi-quantitative scoring system described above.

Apoptosis was assessed using the TdT-mediated dUTP nick end labeling (TUNEL) method. The fragments of the middle lobe were processed in accordance with the instructions provided in the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) and the methods described by Gavrieli et al.[14] and Rösl.[15] After deparaffinization in xylene, slides were pretreated with proteinase K (20 mg/mL) for 30 min in a humidified chamber at room temperature, washed in PBS, and treated with a solution of 3% oxygen peroxide in methanol for 30 min at room temperature. Subsequently, the fragments were washed in PBS twice, and, after the area around the sample was dried, 50 µL of the TUNEL reaction mixture—5 µL of the enzyme solution (violet tube) and 45 µL of the labeling solution (blue tube)—were added to each lung

fragment, followed by incubation under Parafilm for 60 min in a humidified chamber at 37°C. The assay is employed to label DNA strand breaks (with TdT) in each fragment. The assay catalyzes the polymerization of labeled nucleotides at the 3'-OH ends of DNA independently (TUNEL reaction) per sample, therefore allowing the detection of DNA strand breaks in the early stages of lung cell apoptosis. After the incubation period (TUNEL reaction), the slides were washed in PBS three times (2 min each). The area around the fragment was dried, and the slides were mounted with glycerin solution and PBS (1:1), were coverslipped and protected from light, and were immediately analyzed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany), through which labeled apoptotic cells were quantified in five random fields (magnification, ×40). The readings were performed by two investigators, who were blinded to the TUNEL reaction quantification. Microscopic field selection was random, the microscopic fields being always in the alveolar region. Five microscopic fields were selected, as it is done when reading H&E-stained slides. The identified cells were specific, and counts included the total number of cells in the selected region.

In the statistical analysis, we used mixed ANOVA and the Bonferroni test for parametric variables. For non-parametric quantitative data, we used the Kruskal-Wallis test and the Mann-Whitney test. For qualitative data, we used the chi-square test and Fisher's exact test. The level of significance was set at 5% ($p < 0.05$). The statistical analyses were performed with the Statistical Package for the Social Sciences, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Histopathological examination revealed no significant differences among the LPD-6, HTK-6, and NS-6 groups or among the LPD-12, HTK-12, and NS-12 groups, as well as revealing no significant ischemia-related differences between the groups (i.e., LPD-6 vs. LPD-12, HTK-6 vs. HTK-12, and NS-6 vs. NS-12), in terms of the following parameters: pulmonary congestion; alveolar hemorrhage; interstitial hemorrhage; and interstitial inflammatory infiltrate (Table 2). In all groups, the occurrence of alveolar edema was significantly more common after 12 h of ischemia than after 6 h of ischemia (Figure 1). Analysis of the frequency distribution of edema among the groups revealed that the proportion of edema was highest in the NS-12 group, and that the NS-12 group was the only group in which all of the lungs showed alveolar edema at the end of the reperfusion; in addition, the difference among the LPD-12, HTK-12, and NS-12 groups was significant ($p = 0.007$). The same did not occur in the comparison among the LPD-6, HTK-6, and NS-6 groups ($p = 0.08$).

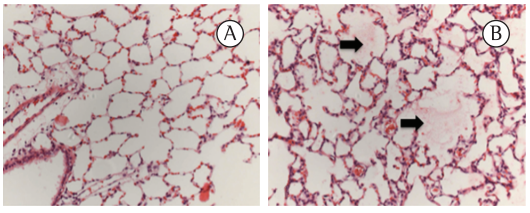

Figure 1 - Histological sections of rat lung tissue after 6 h (in A) and 12 h (in B) of ischemia in the normal saline group. The arrows show the presence of alveolar edema in B (H&E; magnification, ×100).

Table 2 - Comparison of histopathological findings among the groups studied.

Parameter	Values of p in the comparison among the groups[a]				
	LPD-6 vs. HTK-6 vs. NS-6	LPD-12 vs. HTK-12 vs. NS-12	LPD-6 vs. LPD-12	HTK-6 vs. HTK-12	NS-6 vs. NS-12
Congestion	0.797	0.135	0.370	1.000	0.370
Alveolar hemorrhage	0.077	0.701	0.087	1.000	1.000
Interstitial hemorrhage	0.103	0.943	0.087	1.000	0.303
Inflammatory infiltrate	0.278	0.441	0.350	1.000	0.582
Interstitial infiltrate	0.067	0.315	0.303	0.350	1.000

LPD: low-potassium dextran; HTK: histidine-tryptophan-ketoglutarate; and NS: normal saline. [a]Groups: LPD-6: LPD solution and 6 h of ischemia; LPD-12: LPD solution and 12 h of ischemia; HTK-6: HTK solution and 6 h of ischemia; HTK-12: HTK solution and 12 h of ischemia; NS-6 = NS solution and 6 h of ischemia; and NS-12: NS solution and 12 h of ischemia.

Nevertheless, in the HTK-6 group, the frequency of edema was 30%, whereas, in the LPD-6 group, it was 55.6%; however, the difference was not significant (p = 0.08; Figure 2).

There was no significant difference between the LPD-6 and LPD-12 groups in terms of the number of apoptotic cells (p = 0.319). The same occurred in the comparisons between the HTK-6 and HTK-12 groups (p = 0.258) and between the NS-6 and NS-12 groups (p = 0.226). In addition, no significant differences were found among the LPD-6, HTK-6, and NS-6 groups (p = 0.580) or among the LPD-12, HTK-12, and NS-12 groups regarding the number of apoptotic cells (p = 0.591; Figure 3).

Discussion

The present study demonstrated that the histopathological characteristics were similar between the lungs perfused with either LPD or HTK preservation solutions. These findings are in accordance with the physiological data from the same study, which were published previously. The relative oxygenation capacity of the lungs undergoing 12 h of ischemia was found to be reduced, as were the respiratory mechanics parameters. The physiological data were recently published and demonstrated that the lungs preserved with either LPD or HTK had a similar functional performance.[11]

Ischemia-reperfusion injury is a factor limiting donor organ viability, as well as being implicated as a contributing factor in the development of bronchiolitis obliterans.[16] The combination of hypothermia with preservation solutions remains the most widely used preservation method.

The LPD solution was developed nearly two decades ago,[17,18] and its experimental results have been shown to be superior to those of most solutions.[19] The development of preservation solutions for exclusive use in lung preservation has reduced the incidence of acute graft failure from 30% to less than 15%.[3] Despite the evidence, controversy remains regarding the benefits of the LPD solution in terms of late graft performance and one-year mortality after transplantation.[20] More recently, the introduction of ex vivo lung reconditioning[21] and donation after cardiocirculatory death[4] have reignited the controversy and underscored the need to re-evaluate the preservation solutions that are currently used.

The HTK solution is used as a cardioplegic solution, as well as being used for heart preservation for transplantation. Since its development, several changes have been made to its formulation, making it more effective during longer periods of ischemia.[22] However, the use of HTK solution for lung preservation had not been tested in an ex vivo model. If the solution proved effective for this purpose, it would be possible to use a single solution for cardiopulmonary extraction.

In the present study, we used an ex vivo lung perfusion model to evaluate HTK, comparing it with an extracellular solution commonly used in lung transplantation (i.e., LPD). We used an ex vivo rat lung preparation in which the lungs were perfused with homologous venous blood obtained by exsanguination of animals of the same species.[22,23] The ex vivo perfusion system is simple and reproducible, which is why it is widely used in studies of lung preservation for

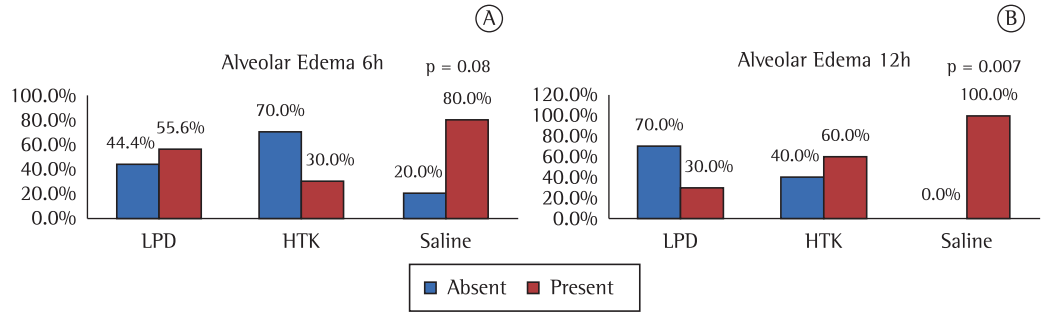

Figure 2 - Frequency of alveolar edema in lungs preserved with low-potassium dextran (LPD) solution, histidine-tryptophan-ketoglutarate (HTK) solution, or normal saline at two ischemia periods: 6 h (in A) and 12 h (in B). Note that the occurrence of alveolar edema was significantly more common in the groups undergoing 12 h of ischemia, the incidence being highest in the normal saline group.

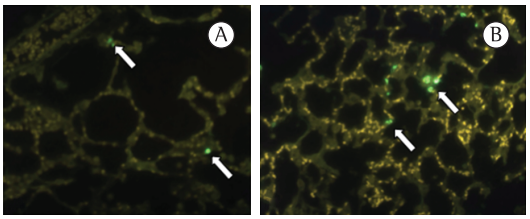

Figure 3 - Photomicrographs of rat lung tissue labeled by the TdT-mediated dUTP nick end labeling (TUNEL) method. In A, lung tissue undergoing 6 h of hypothermic ischemia with the use of low-potassium dextran solution and containing few apoptotic cells (arrows). In B, lung tissue undergoing 12 h of hypothermic ischemia with the use of saline and containing an increased number of apoptotic cells (TUNEL; magnification, ×40).

transplantation. We used homologous blood obtained by exsanguination of 2 or 3 rats per heart-lung block and diluted in NS. Although hemodilution affects gas exchange, we obtained reliable and consistent blood gas analysis results.

Hemodilution and its effects on reperfusion injury are well known. Puskas et al.[24] employed a similar ex vivo perfusion model, in which hemodilution was performed with a crystalloid solution, having found a reduction in ischemia-reperfusion injury following ischemia. In the present model, it is possible that hemodilution was greater in the lungs in the NS-6 and NS-12 groups, given that the vascular bed of those lungs had been filled with NS before reperfusion. Therefore, in our laboratory, NS was recently replaced with Krebs solution, given that the solution was found to be more effective in producing hemodilution, especially in experiments with prolonged periods of ischemia.

In the present study, even the lungs undergoing 6 or 12 h of ischemia provided consistent data for analysis; other authors have limited the periods of ischemia to 2-4 h.[25] Although NS is not employed in clinical or experimental lung preservation, our experimental design included an NS group. The choice to include an NS group was based on the capacity of NS to cause lung edema on reperfusion, a factor that allowed NS to be used as a parameter of edema in the comparison with the other solutions. Using the same model, we previously demonstrated that lungs preserved with NS for 12 h showed consistently worse performance than did those preserved with the other solutions for the same period of ischemia.[11] The histopathological findings of the present study confirmed this impression, given that all of the lungs in the NS group were edematous.

In our previous study, we found increased lung compliance in the lungs undergoing 6 h of ischemia and increased lung resistance in those undergoing 12 h of ischemia. We also found that there was no difference in relative oxygenation capacity between the lungs in the LPD-6 and LPD-12 groups or among the 12-h ischemia groups.[11]

We focused the present study on histopathological examination, with the conviction that our findings of morphological changes and apoptosis would support our previous physiological findings. We chose to include morphological parameters whose changes are relevant and related to vascular endothelial injury and to changes in the lung parenchyma, all of which are representative of postischemic reperfusion injury. We found no significant differences among the groups in terms of congestion, alveolar hemorrhage, interstitial hemorrhage, inflammatory infiltrate, or interstitial infiltrate. However, we found an increase in alveolar edema in the NS-12 group. This confirms the assumption that the use of NS solution in lung preservation causes severe edema on reperfusion, especially in prolonged periods of ischemia.

Cell death in ischemia-reperfusion-induced injury is caused by necrosis and apoptosis (programmed cell death). Apoptosis can be activated during the initial phases of reperfusion after lung ischemia.[18] Apoptosis differs from necrosis not only from a morphological standpoint but also with regard to mediators and biological mechanisms of injury. During apoptosis, there is a regulated, energy-dependent cascade of events, which activate specific endonucleases, such as caspases, which are involved in the initiation and execution phases of apoptosis.[26,27]

The duration of ischemia directly influences the degree of apoptosis in the tissues. The study of the biological pathways of apoptosis in various tissues suggests that the cascade of events begins during the period of ischemia. However, signs of apoptosis appear only during reperfusion, whereas cell necrosis occurs during ischemia.[26]

There is nevertheless unequivocal evidence that ischemia alone is not sufficient to trigger apoptosis. This has been demonstrated in cardiomyocytes, in which ischemia without reperfusion led only to cell death by necrosis. Burns et al. used the

TUNEL method to study apoptosis before and after reperfusion in kidney transplantation and found that the degree of apoptosis was lesser before reperfusion, suggesting that apoptosis occurs predominantly after reperfusion.[28] The induction of lung ischemia by pulmonary artery ligation in pigs resulted in chronic lung ischemia and overexpression of pro-apoptotic factors. The addition of reperfusion after chronic ischemia triggers massive apoptosis, leading to endothelial injury.[29] Various studies have demonstrated that the inhibition of apoptosis by caspase inhibitors results in reduced lymphocyte infiltration and cell death, which leads to improved lung function.[6,30]

In the present study, the assessment of apoptosis by the TUNEL method revealed no significant differences in the number of apoptotic cells between the 6-h period of ischemia and the 12-h period of ischemia in the LPD, HTK, or NS groups after 60 min of lung reperfusion. This finding can be interpreted in two different ways: first, the lack of differences might be due to severe ischemic injury, which was similar among the groups; second, the LPD and HTK solutions might be similar in terms of the quality of preservation (cell integrity). Therefore, the degrees of apoptosis associated with the two solutions might be similar. The lack of differences between the NS group and the other groups (LPD and HTK) in terms of the degree of apoptosis might be more closely related to ischemia itself, regardless of the protective effects of the preservation solutions. Nevertheless, judging by the degree of edema in the NS group, we can infer that the quality of preservation was inferior, as we had anticipated.

In conclusion, the rat lungs preserved with either LPD or HTK showed comparable histopathological and apoptosis findings after 60 min of reperfusion, whereas those preserved with NS showed a greater degree of edema on reperfusion. The findings of the present study support our previously reported physiological findings in the same model and underscore the need for further studies involving models of lung transplantation to determine whether the functional performance of lungs preserved with HTK is similar to that of those preserved with LPD.

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