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Effects of ischaemia and preservation on the ultrastructure of the bronchiolar epithelium

A quantitative electron microscopic study of human and canine lungs

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Abstract In ten cases of clinical human single-lung transplantation, the nontransplanted Euro-Collins-preserved contralateral lungs were examined using electron microscopy to determine the effects of ischaemia on the bronchiolar epithelium. Existing structural damage at the time of transplantation was characterized using this approach, and nine nonpreserved canine single lungs were also investigated to identify the impact of ischaemia. The study revealed a significant correlation between the duration of ischaemia and the mitochondrial surface-to-volume ratio, which can serve as a morphometric criterion for mitochondrial damage, in canine lungs. However, this correlation was not found in the human donor lungs. Further examination of human donor lungs showed slight to moderate damage to the endoplasmic reticulum and nuclear chromatin. In addition, various degrees of damage to mitochondrial structure, ranging from inconspicuous to severe, were found. The mitochondrial surface-to-volume ratio can be considered to be a suitable criterion for the quantification of ischaemic damage of the bronchiolar epithelium under experimental conditions. Ultrastructural analysis of human donor lungs revealed intact bronchiolar epithelial cell structures at the time of transplantation, reflecting adequate organ preservation with Euro-Collins solution.

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Introduction

In the last decade, lung transplantation has become an effective therapeutic option for patients with otherwise terminal end-stage lung disease [10, 16]. The most significant long-term complication is the development of an obliterative bronchiolitis [11, 12, 15, 41], the aetiology of which is uncertain [3]. The preponderance of evidence, however, favours the hypothesis that obliterative bronchiolitis is the result of immunological damage, that is to say, a form of chronic rejection [35]. To reduce the risk of chronic rejection, extended ischaemic tolerance would be helpful to allow more carefully matched donor choice [38]. Today's clinical preservation procedure allows for ischaemic periods of up to 6–8 h [2, 13].

In general, ischaemic stress results in a variable degree of tissue injury, which may be reversible or irreversible. To characterize cell damage, such ultrastructural features as margination of nuclear chromatin, dilatation of endoplasmic reticulum and swelling of mitochondria can be used [9, 30, 37]. Morphometric studies of myocardial tissue showed an increase in mitochondrial volume as a function of ischaemic time [37]. A suitable parameter for evaluation of mitochondrial swelling in cardiac myocytes is the surface-to-volume ratio (S_V ratio) of mitochondria [31].

The aim of this study was to investigate the effects of ischaemia on the bronchiolar epithelium. Human donor lungs that could not be matched for transplantation to another recipient were studied by scanning and transmission electron microscopy. Since the lungs were obtained during clinical single-lung transplantations, we were able to look for correlations between structural data collected from the nontransplanted donor lung and postoperative clinical data characterizing the transplanted twin lung. Additionally, canine lungs from an experimental study

on the effect of the duration of ischaemia on pulmonary ultrastructure [5, 25] were examined by transmission electron microscopy.

The respiratory bronchioles of human lungs are lined by a surface epithelium consisting of ciliated and nonciliated cells. Nonciliated cells are frequently termed Clara cells [23, 26]. Rogers et al. [28] differentiated between two populations of nonciliated cells, both containing secretory granules, which, however, differed in size and number of the granula. Therefore, in this study the more general term nonciliated cell is used, in preference to Clara cell. Besides semiquantitative investigations of cell structures, for example, nuclear chromatin, mitochondria and endoplasmic reticulum, the S_V ratio was determined as a quantitative indicator of mitochondrial swelling. By means of these criteria the existing damage to the bronchiolar epithelium of the donor lung at the time of transplantation, caused by ischaemia, was characterized.

Materials and methods

In ten cases of single-lung transplantation, the nontransplanted contralateral donor lungs were studied by means of scanning electron microscopy (SEM), transmission electron microscopy (TEM) and morphometry. Only donor organs that could not be matched by The Eurotransplant Foundation Centre for transplantation purpose to another recipient were used in this study. The donor data are shown in Table 1.

As described by Wahlers et al. [39], a pulmonary artery flush with modified Euro-Collins solution was used for organ preservation. While one single lung was transplanted, the contralateral donor lung, which could not be allocated to another recipient, was fixed as soon as it became available (93±95 min after transplantation) by instillation via the bronchial system at a pressure of 25 cmH_2O with a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.35, buffer osmolality 300 mosmol/kg) as described earlier by Fehrenbach et al. [7]. The ischaemia lasted for 121–515 min.

All tissue samples were collected according to a standardized systematic random sampling procedure [4]. Tissue samples for TEM (size: 1×1×1 mm^3) were processed according to Fehrenbach et al. [6] using an automated tissue processor (Histomat, Bio-med, Theres, Germany). Briefly, after osmication and en bloc staining with aqueous uranyl acetate (4%), overnight, samples were dehydrated in a graded ethanol series, transferred via propylene oxide to Araldite and finally embedded in Araldite. Tissue samples for SEM (size: 5×5×2 mm^3) were processed according to a modified osmium tetroxide-thiocarbo-hydrazide method [21], followed by dehydration in a graded ethanol series. After critical-point drying the specimens were fixed with a conductive adhesive on aluminium stubs and, avoiding metal sputtering, directly examined at 15 kV with a Zeiss DSM 960. For TEM analysis, semithin sections were made from 10–15 randomly chosen tissue blocks of each lung. The sections were stained with methylene blue and azur II and examined by light microscopy. For qualitative and quantitative investigations, ultrathin sections of the tissue blocks that showed parts of the most distal bronchioles, defined by direct continuation of the airway into one or more alveolar ducts, were employed. Ultrathin sections were stained with uranyl acetate and lead citrate. Each ultrathin section was examined according to the systematic quadrate subsampling procedure [40] with a Zeiss EM

Table 1 Clinical data of human donors

Case No.	Donor data				
	Age (years)	Sex	Time of intubation (h)	pO2: FiO2, preoperatively	Time until fixation (min)
91/01	24	Male	70	327	209
91/02	31	Female	28	654	245
92/01	41	Male	24	270	306
92/04	17	Male	57	368	333
92/05	52	Male	24	3316	458
92/06	51	Female	48	437	121
93/01	37	Male	96	467	515
94/01	56	Female	33	497	284
95/01	41	Male	48	433	384
95/02	32	Male	–[b]	–[b]	–[b]
94/03 [a]	15[a]	Male	48[a]	–[b]	7458[a]
Mean±SD	38.1±12.4		47.6± 24.1	418.8± 116.2	317.2± 122.6

[a] Not included in the statistics
[b] Data not available

Table 2 Grading for the degree of damage to ultrastructural features in human contralateral donor lungs

Score of ultrastructural damage	Ultrastructural feature		
	Endoplasmic reticulum	Nucleus	Mitochondria
1=Inconspicuous	Inconspicuous	Inconspicuous	Inconspicuous
2=Slight alteration	Slight dilatation	Slight clumping and margination of chromatin	Clearing of matrix and separation of cristae (increase of the intracristal space)
3=Moderate alteration	Moderate dilatation and slight vesiculation	Moderate clumping and margination of chromatin with central clarification	Fragmentation of cristae
4=Severe damage	Severe dilatation and vesiculation	severe margination and clumping of chromatin	Cristolysis

10A at a primary magnification of ×12,500. The test fields which hit bronchiolar epithelium were evaluated.

As a measure for mitochondrial swelling the surface to volume ratio of mitochondria (S_V $ratio_{Mi}$), was determined morphometrically [31]. A 72-point lattice system with test lines was superimposed on a TV-monitor for on-line recordings. The S_V $ratio_{Mi}$ was determined according to the formula:

$$S_V\ ratio_{Mi} = 2I/(P_{Mi} \times Z/3)\ [31]$$

where I is the total number of intersections of the test lines with the circumferences of the mitochondria, P_{Mi} the total number of test points overlying the mitochondria and Z is the true length of the test lines. In each cell hit by a test field, structural damage was graded in four categories using the following parameters: (1) margination of nuclear chromatin, (2) dilatation of endoplasmic reticulum and (3) swelling of mitochondria (Table 2). In addition, damage to ciliated and nonciliated cells was distinguished.

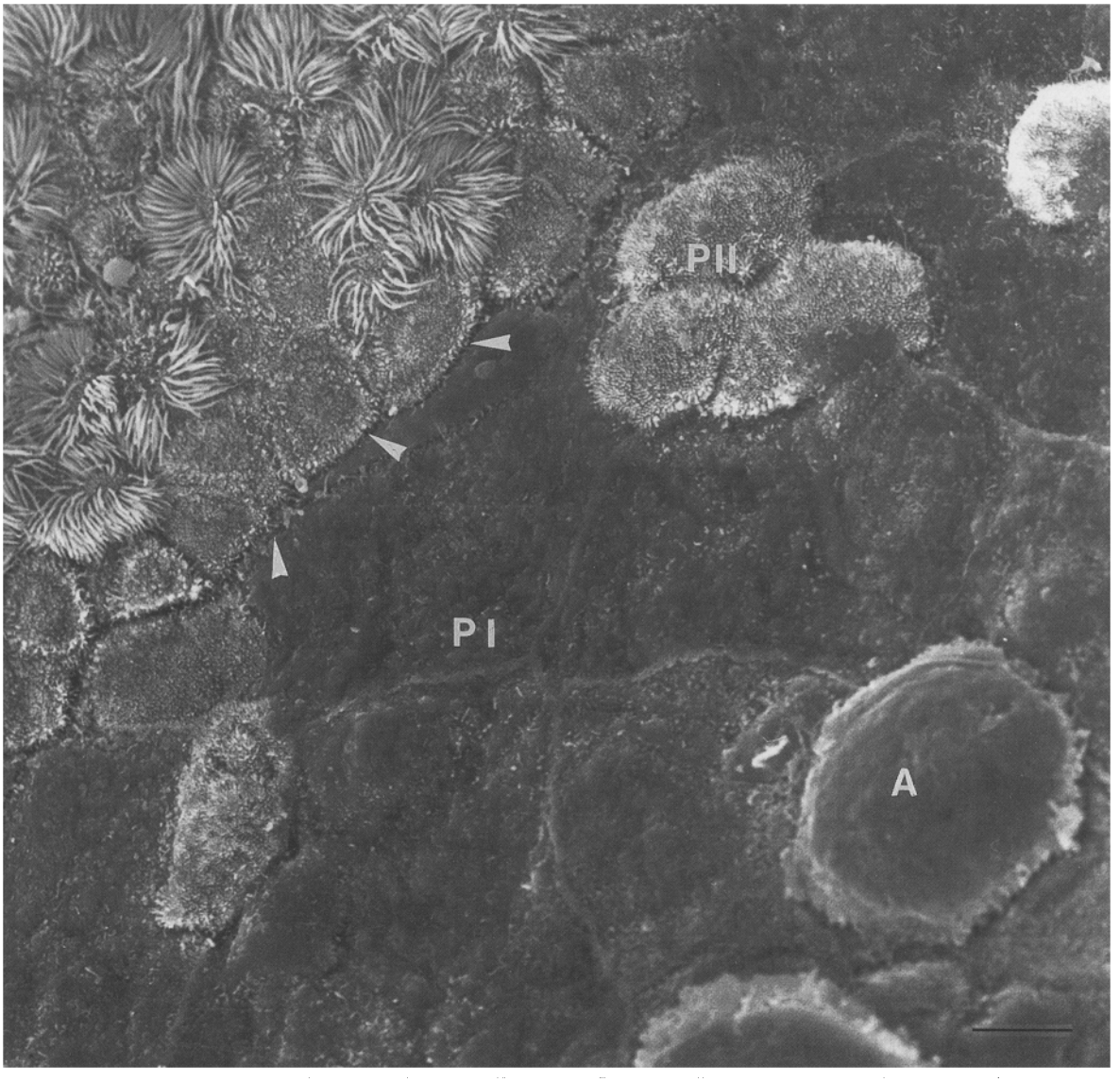
S_V ratio and structural damage to each lung were correlated with the donor age, intubation time of the donor and preoperatively measured oxygenation rate, and additionally with the postoperative intubation time of the recipient and oxygenation rates measured 6 h, 12 h and 24 h after surgery.

In the experimental study, the lungs of nine mongrel dogs weighing 20–30 kg were used. Following a previous experiment [20], the lungs were excised 5–10 min after cessation of the heart action. In group A (n=3) the lungs were fixed immediately with the fixative mentioned above by bronchial instillation, in group B (n=3) after having remained in Tutofusin (Pfrimmer, Erlangen, Germany) for 4 h and in group C (n=3) after 12 h of storage in Tutofusin at a temperature of approximately 4°C. Tissue samples for TEM investigations were collected in the same way as described for human donor lungs. After osmication and en bloc staining with aqueous uranyl acetate (1.5%) overnight, tissue samples were dehydrated in a graded acetone series and then embedded in Araldite [25].

Evaluation of the S_V ratio of the bronchiolar epithelium of canine lungs was performed as described for human donor lungs.

All results are given as mean values±SD unless indicated otherwise. A one-way analysis of variance was performed, and in the case of significant differences pairwise multiple comparisons were made using the Student-Newman-Keuls method. Correlations between variables were analysed by the Spearman rank order correlation (Sigma Stat, Jandel Scientific, Erkrath, Germany). P-values less than 0.05 were considered to be significant.

Fig. 1 SEM of human bronchiolar epithelium (case 92/01). A clear border between the bronchiolar and the alveolar epithelium is visible (*arrowheads*). The bronchiolar epithelium of this region consists of scattered ciliated cells between nonciliated cells (*PI* type I pneumocytes, *PII* type II pneumocytes, *A* alveolar macrophage). *Scale bar* 1 µm



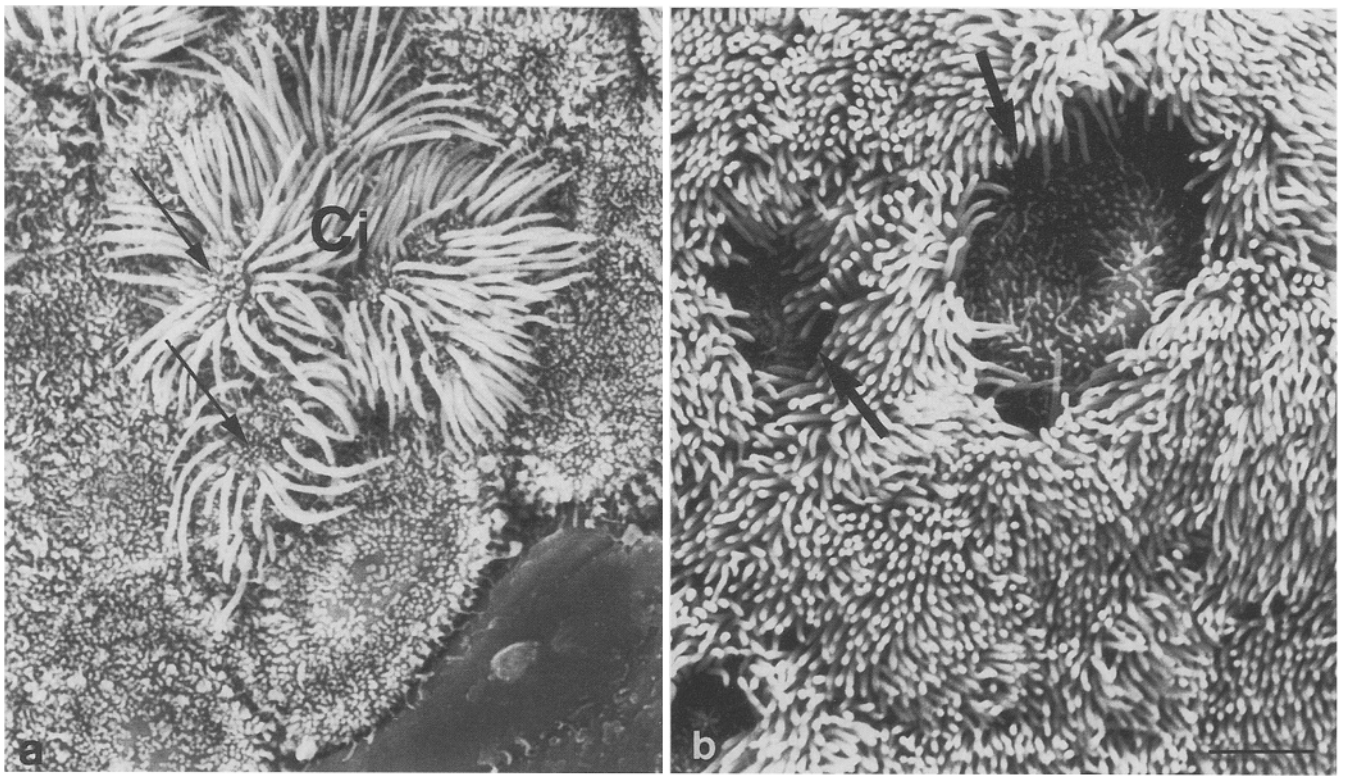
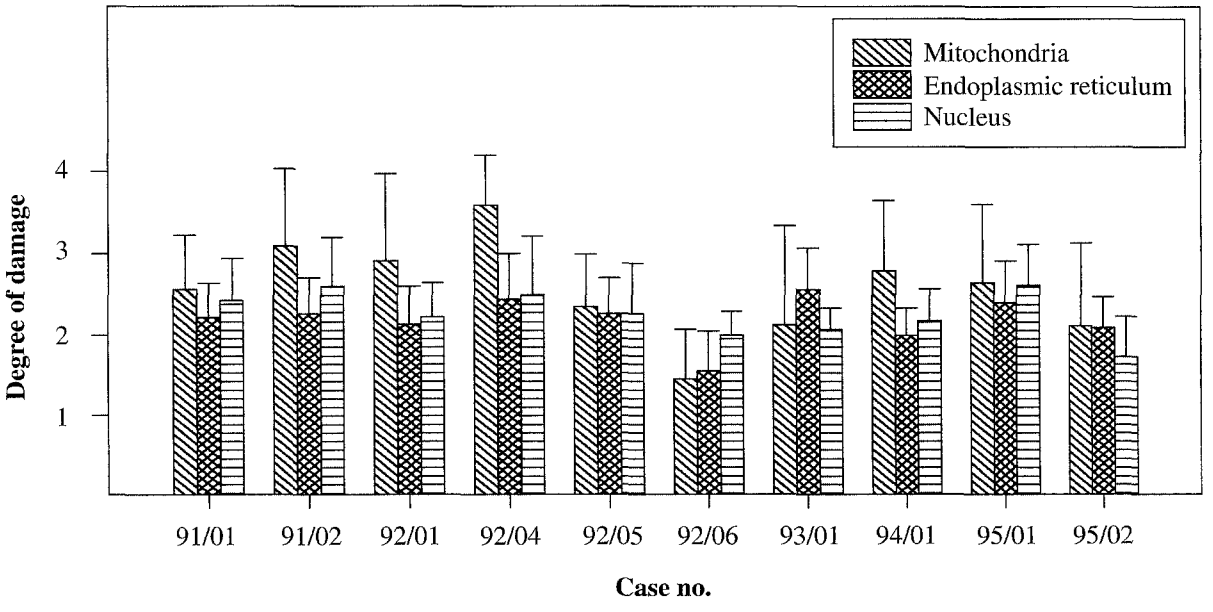


Fig. 2 **a** Higher magnification of Fig. 1. The surface of the ciliated cells shows cilia (*Ci*) and microvilli (*arrows*). **b** More proximal region of bronchiolar epithelium. Few nonciliated cells (*arrows*), covered by microvilli, can be seen. The surface of ciliated cells is covered with so many cilia that microvilli can not be distinguished (case 95/02). *Scale bar* 1 μm

Results

At SEM examination most of the human donor lungs showed no residual mucus on the bronchiolar epithelium. This was due to instillation fixation, and differentiation between ciliated and nonciliated cells was therefore possible. The shapes of the nonciliated cells from the individual lungs varied from round to flat. A clear border was visible between bronchiolar and alveolar epithelium (Fig. 1). In the bronchioli the number of the ciliated cells and also the number of cilia per cell decreased from proximal to distal (Fig. 2), so that in the region of transi-

Fig. 3 Degree of damage to ultrastructural features in the individual human contralateral donor lungs. TEM data (mean±SD) using the score described in Table 2



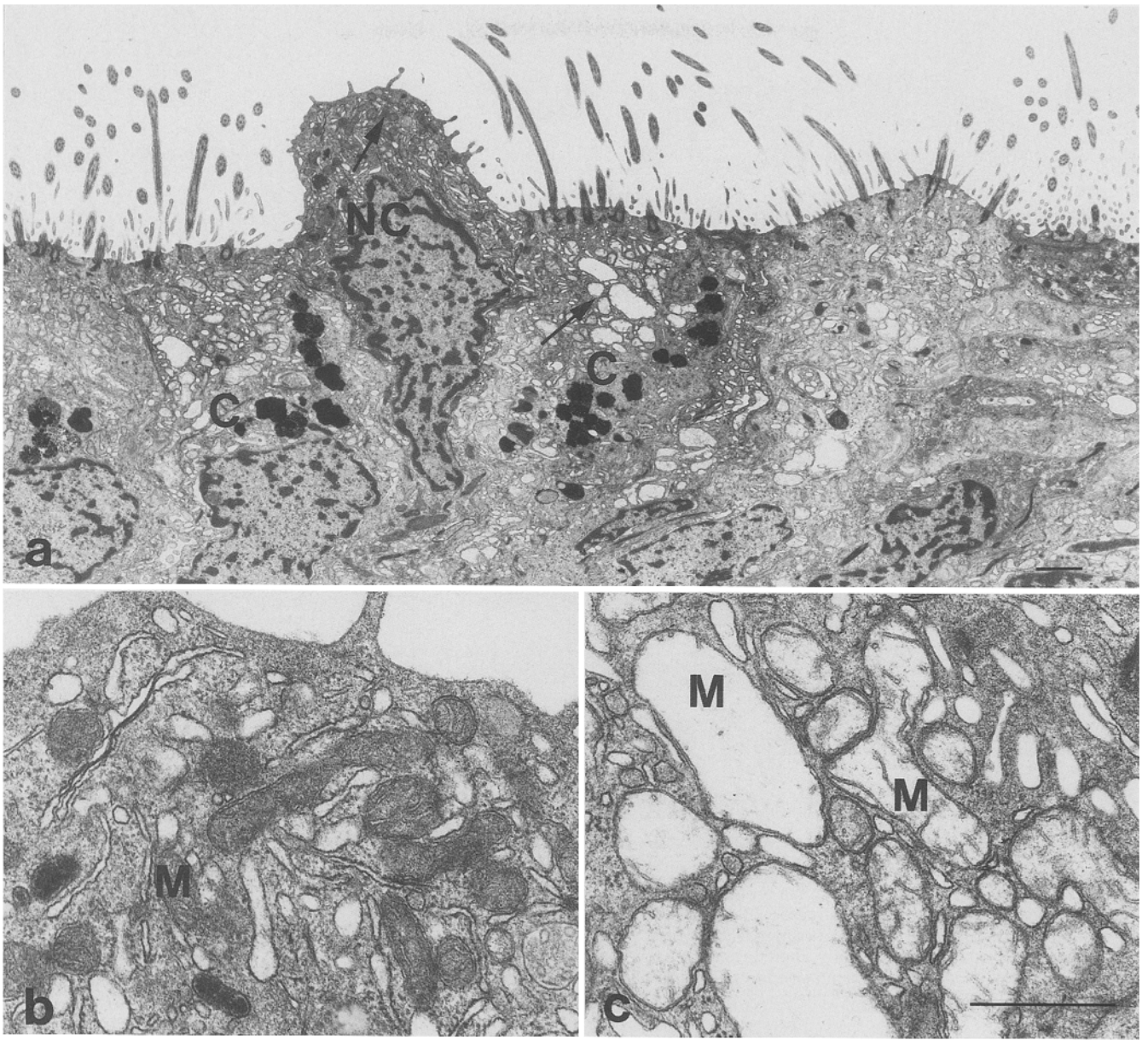


Fig. 4a–c TEM of human bronchiolar epithelium (case 93/01). **a** Ciliated (*C*) and nonciliated (*NC*) cells of proximal bronchiolar epithelium after an ischaemic period of 515 min. The mitochondria (*arrows*) of the nonciliated cell show hardly any alterations. The mitochondria of the adjacent ciliated cells are moderately swollen and show severe damage with fragmentation of cristae and cristolysis. *Scale bar* 1 µm. **b** Higher magnification of the nonciliated cell with inconspicuous mitochondria (*M*). **c** Higher magnification of the ciliated cell. Mitochondria (*M*) with fragmentation of cristae and cristolysis. *Scale bar* 5 µm

tion between alveolar and bronchiolar epithelium (distal regions) ciliated cells were scattered and showed only isolated cilia between a large number of microvilli, on their surface. In contrast, the proximal regions of the bronchioli showed a large number of ciliated cells with few nonciliated cells. Here each ciliated cell was covered by many cilia, so that microvilli could only be seen on the surface of the sparse, scattered nonciliated cells. In none of the lungs could obvious alterations of cilia or microvilli be observed.

On TEM examination, the alterations in the cell structures showed high inter- and intraindividual variations (Fig. 3). In some individuals, the degree of damage in the different test fields ranged between 1 and 4, while others had more homogeneous damage ranging from 2 to 3 in degree. On average, the human donor lungs showed slight (n=9) to moderate (n=1) dilatation of the endoplasmic reticulum and slight (n=8) to moderate (n=2) margination of the nuclear chromatin. With respect to the structure of mitochondria, a wide variation in the degree of damage was revealed (inconspicuous, n=1; slight alteration, n=3; moderate alteration, n=5; severe damage, n=1). Ultrastructurally, the damage ranged from clearing of the matrix structure, enlargement of the intracristal space and fragmentation of cristae up to cristolysis (Fig. 4). Although severe stages

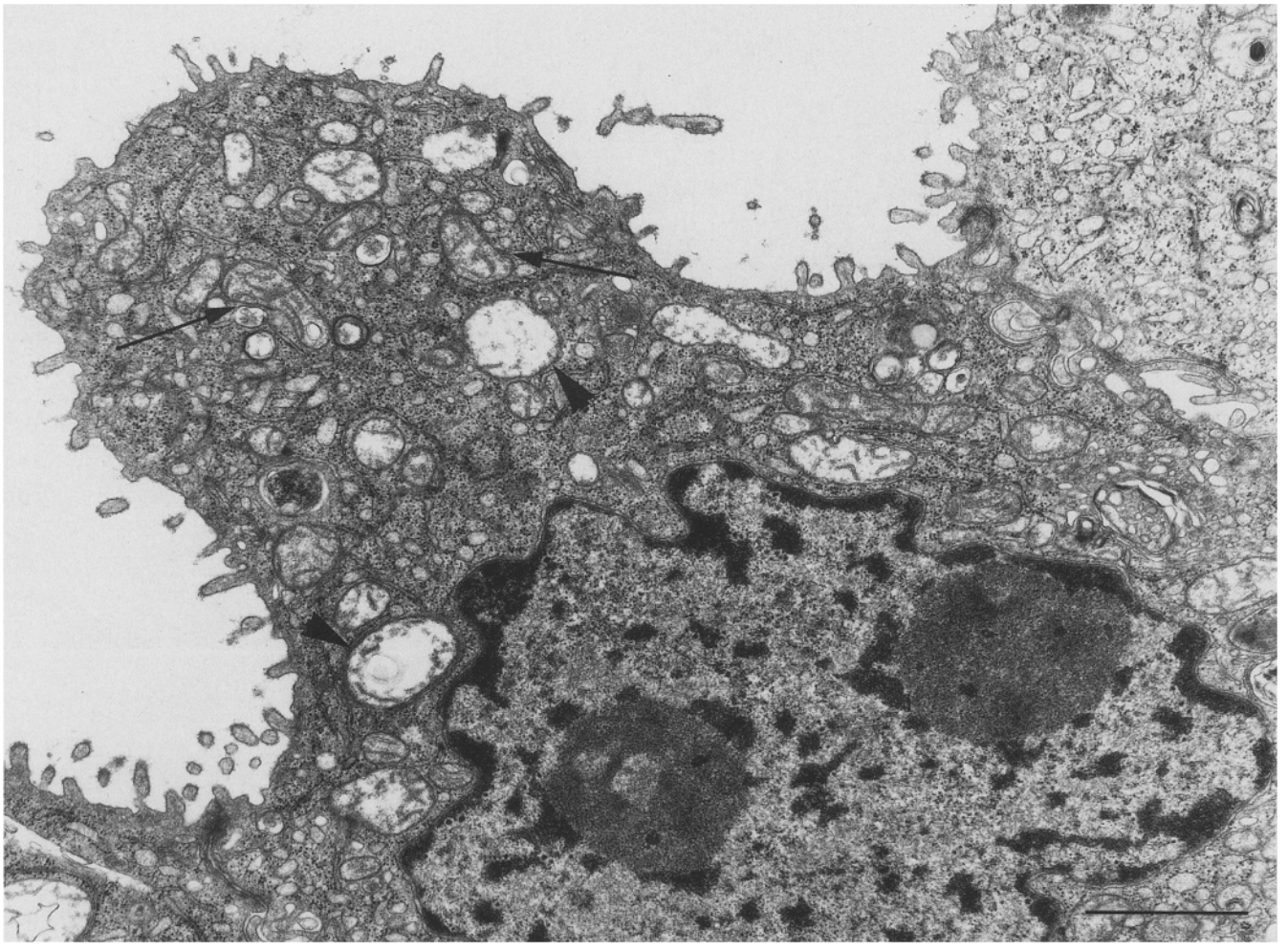


Fig. 5 TEM of a nonciliated cell in human bronchiolar epithelium after an ischaemic period of 333 min (case 92/04). The cell shows mitochondria with different degrees of damage. Some of the mitochondria are inconspicuous or slightly altered (*arrows*), and some are moderately swollen with fragmentation of cristae and cristolysis (*arrowheads*). *Scale bar* 1 μm

of cristolysis were observed frequently, only a small number of mitochondria showed disruption of the outer membrane. An extremely rare phenomenon was the occurrence of intramitochondrial woolly densities, also called amorphous material deposits or dense material deposits [9]. The variation in the ultrastructural damage did not only exist in different regions, but also within single small areas of the individual lungs. In some parts, different degrees of damage to mitochondria could be seen in the same cell or in the same test field (Fig. 5). There was no correlation between the features "mitochondrial structure" or "margination and clumping of nuclear chromatin" and the duration of ischaemia. Only the degree of damage to the endoplasmic reticulum correlated with the increase in duration of ischaemia (P=0.0121). On average, ciliated and nonciliated cells did not show any significant differences in their ultrastructural damage.

Correlation of the features "mitochondrial structure", "dilatation of endoplasmic reticulum" and "margination of nuclear chromatin" was positive for the degrees of damage to mitochondrial structure and nuclear chromatin (P=0.0186).

No statistically significant correlations were found on consideration of clinical donor variables, including donor age, intubation time and the preoperatively measured oxygenation rate, and of recipient data, including postoperative intubation time of the recipient and oxygenation rates measured at 6 h, 12 h and 24 h after surgery.

In the experimental study, nine lungs of mongrel dogs, divided into three groups with different periods of global ischaemia (0 h, 4 h, 12 h), were investigated. Morphometric evaluation of the mitochondrial S_V ratio in these three groups revealed a significant correlation between the duration of ischaemia and the mitochondrial S_V ratio of the bronchiolar epithial cells (P=0.0007). An increase in global ischaemia periods resulted in decrease in the mitochondrial S_V ratio (Fig. 6).

Values of the S_V ratio measured in the human donor lungs reached similiar values to those revealed in the experimental lungs of dogs (Fig. 7). However, with respect to the duration of ischaemia, in most cases the S_V ratios were lower in human donor lungs than in the canine

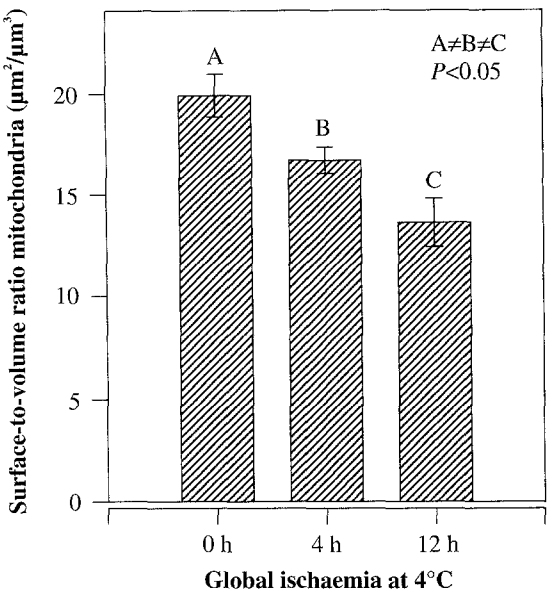


Fig. 6 Relationship between the duration of global ischaemia and the surface-to-volume ratio of mitochondria (mean±SD, n=3) in the animal model; *group A* immediate fixation (0 h), *group B* fixation after 4 h global ischaemia, *group C* fixation after 12 h global ischaemia (group B and C kept in Tutofusin at 4°C)

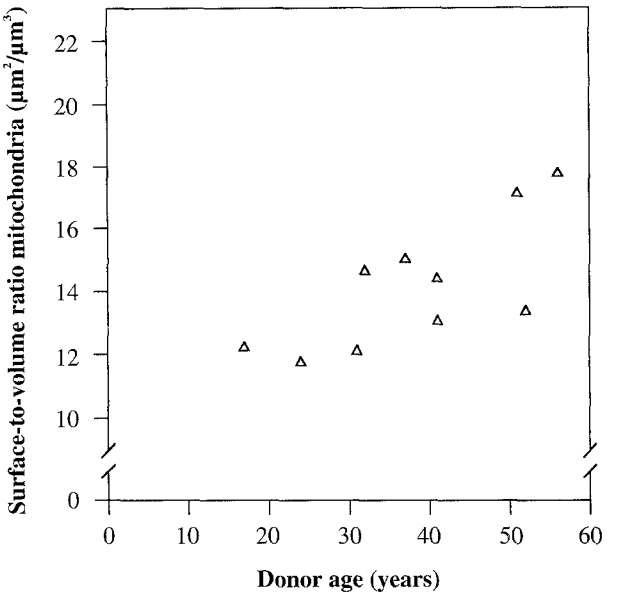


Fig. 8 Relationship between the surface-to-volume ratio (mean per individual lung) of mitochondria and donor age. An increase in donor age correlates with an increase in the mitochondrial surface-to-volume ratio

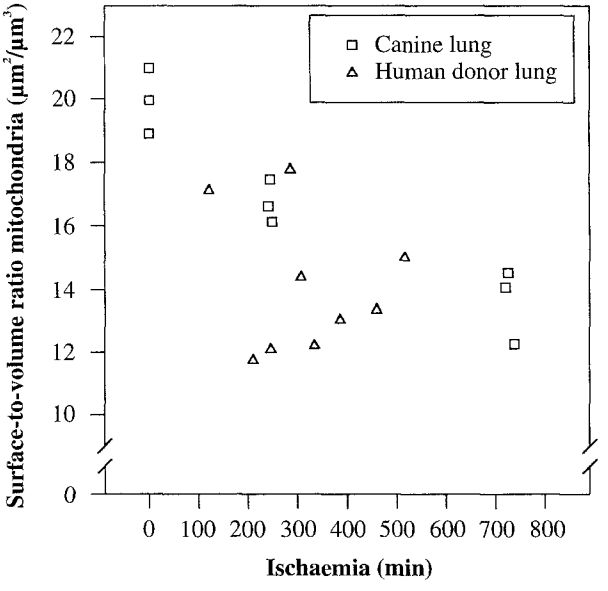


Fig. 7 Surface-to-volume ratio (mean per individual lung) of mitochondria after different periods of ischaemia. Combined graph of data evaluated under experimental (canine lung) and clinical (human donor lung) conditions. In human donor lungs there is no correlation between the surface-to-volume ratio and duration of ischaemia, but for similar periods of ischaemia the surface-to-volume ratio values are mostly lower than in the canine lungs

lungs. In the human donor lungs, no correlation between the mean S_V ratio and the duration of cold ischaemia was observed. However, a human donor lung additionally investigated with an extremely long period of cold ischaemia (7500 min) showed the lowest value of the S_V ratio (10.87 $\mu m^2/\mu m^3$).

For the human donor lungs, analysis of correlations of the S_V ratio and the different parameters of ultrastructural damage showed one relationship with statistical significance. A decrease in S_V ratio correlated with an increase in the degree of margination of nuclear chromatin (P=0.00523). In addition, a positive correlation between the S_V ratio and donor age was evident (P=0.0186) (Fig. 8).

Discussion

In 75% of single-lung, 80% of double-lung and 88% of heart-lung transplantations performed at Hannover Medical School between 1987 and 1992, the patients were able to return to work or school within approximately 1 year after the operation [15]. However, complications, including steroid-induced osteoporosis and especially the development of obliterative bronchiolitis, are very serious problems in the long-term course after primary successful lung or heart-lung transplantation [15], as has often been reported [11, 41]. Several factors, such as denervation, loss of bronchial artery supply, inadequate preservation during organ harvesting, immunological damage and repeated infections, could have resulted in alteration of the structure and function of the bronchial epithelium [27]. As an important source of early morbidity and mortality, ischaemic bronchial complications have been discussed [29]. However, as demonstrated in investigations of bronchoscopic brushings of transplant recipients, proximal and distal to the anastomosis, no differences in the epithelial structure and function were found between single-lung and heart-lung recipients in whom the bronchial artery supply was preserved [27].

There are some study reports describing the cell structures of human lung parenchyma [8] and some on the upper airway epithelium and mucociliary function after transplantation [14, 22, 27, 32]. However, to date no data on the preservation of the distal bronchiolar epithelial cell structure at the time of transplantation have been presented. Surface structure and cell types of the bronchiolar epithelium of the ten human donor lungs examined corresponded to the descriptions of human bronchiolar epithelium given in other studies [26, 28, 34, 36]. Cell injury caused by ischaemia has been described in detail for various tissues, including myocardium [30, 37] and pancreas [17]. Mitochondria are rapidly affected by ischaemia [30]. Schmiedl et al. [31] pointed out a significant correlation between the surface-to-volume ratio and the duration of ischaemia in heart muscle cells, and showed that the S_V ratio can be used as a suitable parameter for evaluating mitochondrial swelling in myocardial tissue. Since a significant correlation was also demonstrated in the experimental study, the S_V ratio may also be used as a parameter for mitochondrial swelling in bronchiolar epithelium. Comparison of canine and human lungs with similar ischaemic times revealed lower S_V ratio in human donor lungs. Although it must be taken into account that the experimental canine lungs and human donor lungs had been preserved differently, this still indicates a higher degree of mitochondrial swelling. In considering the variability in human donor age, a positive correlation between higher donor ages and higher S_V ratio was found, indicating that ageing might influence mitochondrial sensitivity. In alveolar type II cells of monkey lungs, lower numbers of mitochondria per cell and lower volume densities of mitochondria were described in older than in younger monkeys [33]. For this reason, we conclude that under complex clinical conditions ischaemia is only one of several factors, including patient history, pretreatment, time from brain death to harvesting and temperature during ischaemia, that may influence the S_V ratio.

Generally, the S_V ratio of mitochondria in cells of the bronchiolar epithelium determined in this study reached higher values than have been reported for myocardial tissue. Loss of matrix structure and fragmentation of cristae occurred at an S_V ratio of about 5.8, cristolysis at 5.5–5.6 and amorphous matrix densities at an S_V ratio of less than 5.5 $\mu m^2/\mu m^3$ in mitochondria of heart muscle [31]. Such a correlation was not seen in the mitochondria of the human donor lungs. In each donor lung, there was wide variation in the degree of damage to the mitochondria, so that even in individual cells or in individual test fields the degree of damage to the individual mitochondria was different (Fig. 5). Therefore, no relationship could be found between the two variables of structural damage and S_V ratio of the mitochondria when the mean values of each donor lung were compared. However, despite the variation in the structural preservation of mitochondria, the degree of damage to most lungs could be characterized as slight to moderate, and only in case 92/04 were the mitochondria severely damaged, showing a high rate of cristolysis. Although Trump et al. [37] described interruption of the outer mitochondrial membrane in moderate stages of reversible cell injury in myocardial tissue, such an alteration was rarely observed in the human donor lungs.Woolly densities, as described in irreversibly injured ischaemic myocardial tissue, were extremely rare [1, 18, 19] even in swollen mitochondria in reversible phases of cell injury [37]. Such woolly densities were not observed in the human donor lung with an extremely long period of ischaemia. Ghadially [9] reported that in promptly collected and fixed human biopsy material (neoplastic or nonneoplastic), woolly densities were not seen except in odd degenerated or necrotic cells, but if there had been a prolonged delay between collection and fixation intramitochondrial woolly densities were frequently encountered. Cooling down to a temperature of 4°C also retarded the number of intramitochondrial densities [24]. Other features, such as nuclear chromatin and endoplasmic reticulum showed a slight degree of damage with only little inter- and intraindividual variation. This is in agreement with investigations on other organs, in which margination and clumping of nuclear chromatin and dilatation and vesiculation of endoplasmic reticulum were described in the reversible phase of cell injury [9, 37]. Human donor pancreas showed well-preserved cell structures after up to 24 h of cold storage, with variable ischaemic tolerance of the different cell types [17]. Thus, all in all the distal bronchiolar epithelium of all human donor lungs, even those with longer periods of ischaemia revealed acceptable ultrastructural preservation. As the transplanted single lungs and the contralateral donor lungs investigated were subjected to the same conditions, one may assume that at the time of transplantation no irreversible damage of the bronchiolar epithelium was present. This agrees with the study of the lung parenchyma of six human donor lungs investigated by Fehrenbach et al. [8], in which an overall good to excellent preservation of lung parenchyma in all six lungs was found. Three of these lungs were also included in this study. In cases 92/01 and 92/06, Fehrenbach et al. [8] observed swollen vesicles and cisternae of the endoplasmic reticulum in type II pneumocytes. In the bronchiolar epithelium of these two lungs no peculiarities in the structure of the endoplasmic reticulum were observed, in comparison with the bronchiolar epithelium of the other donor lungs. Considering the clinical findings, no correlations with the degree of damage to the ultrastructural features existed, and in a retrospective analysis no significant effects of clinical donor data, including donor age, time from brain death to harvesting and ischaemic time, on postoperative lung function were revealed [39].

In conclusion, the surface-to-volume ratio of mitochondria is a suitable parameter for estimating ischaemic damage to the bronchiolar epithelial cells under defined experimental conditions, as established in canine lungs. In human donor lungs preserved under clinical conditions, in most cases lower S_V ratio values than those established under experimental conditions were

found. However, there was no significant correlation between the duration of cold ischaemia and the S_V ratio. This finding may be the result of the more complex conditions in clinical lung transplantations. In general, ultrastructural analyses of the bronchiolar epithelium of ten human contralateral donor lungs, preserved under clinical conditions of single-lung transplantations with periods of cold ischaemia up to 515 min, showed good preservation of the bronchiolar epithelial cell structures. Pulmonary ultrastructure in lungs treated in the same way as the transplantated donor lungs showed no significant alterations, which might favour late complications.

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