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The Journal of
Heart and Lung
Transplantation

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ORIGINAL CLINICAL SCIENCE

Prophylaxis with nebulized liposomal amphotericin B for *Aspergillus* infection in lung transplant patients does not cause changes in the lipid content of pulmonary surfactant

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KEY WORDS:
inhaled liposomal amphotericin;
Aspergillus;
lung transplantation;
pulmonary surfactant;
antifungal prophylaxis;
fungal infection;
Aspergillosis

BACKGROUND: Prophylaxis with inhaled liposomal amphotericin B has proven to be safe and effective for preventing infection due to *Aspergillus* spp in lung transplant recipients. However, the liposome contains a large quantity of phospholipids, and inhalation of these substances could potentially change the composition of pulmonary surfactant. The aim of this study was to determine the lipid composition of pulmonary surfactant in patients receiving inhaled liposomal amphotericin B prophylaxis.

METHODS: A prospective, open, controlled multicenter study was conducted in 2 groups: 19 lung transplant recipients who received regular prophylaxis with inhaled amphotericin B (study group) and 19 recipients who did not receive inhaled prophylaxis (control group). From both groups, 15 ml of the third aliquot of bronchoalveolar lavage fluid was obtained and phospholipid content determined in the active fraction of surfactant (large aggregates) and in the inactive fraction (small aggregates). Large aggregate cholesterol content was also determined.

RESULTS: Patient demographic data and characteristics were similar in the 2 groups. No between-group differences in median phospholipid content were found for large aggregates (study group, 0.4 [range, 0.18–1.9] μmol vs controls, 0.36 [range 2.15–0.12] μmol; $p = 0.69$) or small aggregates (study group, 0.23 [range, 0.1–0.58] μmol vs controls, 0.29 [range, 0.18–0.65] μmol; $p = 0.33$). The small aggregate-to-large aggregate phospholipid ratio, commonly used as a marker of alveolar injury, showed no differences between the groups (study group, 0.56 vs controls, 0.69; $p = 0.28$). Nor were there differences in the cholesterol content of large aggregates (study group, 0.04 μmol [range 0.01–0.1] vs controls, 0.04 μmol [range 0.02–0.27); $p = 0.13$).

CONCLUSIONS: These results seem to indicate that prophylaxis with nebulized liposomal amphotericin B does not cause changes in the lipid content of pulmonary surfactant.
J Heart Lung Transplant 2013;32:313–319
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Inhaled amphotericin B is extensively used as prophylaxis for *Aspergillus* spp infection in lung transplant recipients.[1–3] Several types of amphotericin B preparations are available.[4–11] One such drug is liposomal amphotericin

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http://dx.doi.org/10.1016/j.healun.2012.11.013

B (LAB; AmBisome, Gilead Sciences SL, Madrid, Spain), which has proven to be safe and effective for this purpose.[12–14] We previously reported a low incidence of infection due to *Aspergillus* spp (7.7%) and invasive disease (1.9%) with this prophylaxis.[13] In patients who did not receive prophylaxis, the reported incidence of infection/colonization may be close to 40%,[15,16] and approximately 15% in invasive aspergillosis.[17–20] In another study in the 1990s, we reported an infection rate of 33% in our center.

Amphotericin B concentrations after nebulization remain high for 14 days in bronchioalveolar lavage (BAL) at adequate concentrations for prophylaxis of *Aspergillus* infection.[14] Hence, it is feasible to administer nebulized LAB (n-LAB) every 2 weeks, thereby improving treatment adherence and convenience for the patient. However, the portion of the respiratory tract studied ran from the segmental bronchus to the parenchyma, but concentrations achieved at the suture site were not determined. Because this area is particularly susceptible to infection, until more information becomes available, it seems reasonable to maintain a high frequency of n-LAB administration until the suture is completely healed.[14]

Surfactant has a vital part in reducing surface tension at the air–liquid interface of the alveolar wall, thus preventing alveolar collapse and transudation of capillary fluid into the alveolar lumen.[21] The inflammatory processes involved in lung transplantation can cause changes in surfactant function,[22–24] and this alteration may be theoretically implicated in a poorer transplant outcome, particularly in the development of primary graft dysfunction.[25–30]

The liposome composition of LAB is very similar to the lipid composition of pulmonary surfactant; therefore, inhalation of LAB during long periods could alter the composition of surfactant and cause graft dysfunction. Nebulization of a standard 25-mg dose of LAB entails nebulization of 106 mg of phosphatidylcholine.[31] It is plausible that inhalation of this amount of phosphatidylcholine might change the composition of pulmonary surfactant, which consists of proteins (10%) and lipids (90%), predominantly phospholipids, the most important of these being phosphatidylcholine (70%-80%). Cholesterol is also a component of the liposome, and therefore, inhalation of LAB could alter the lipid composition of surfactant. Surfactant concentrations in the alveolus are regulated by an equilibrium between production by type II pneumocytes and consumption and elimination by alveolar macrophages. Modification of this equilibrium has been implicated in the development of alveolar proteinosis.[21,32] In lung transplantation, the phagocytic function of macrophages can be impaired by immunosuppressive therapy, and this may contribute to alter the balance.[32] This study was initiated to investigate the repercussions of LAB inhalation on human pulmonary surfactant and designed to determine whether prolonged administration of inhaled LAB produces changes in the lipid content of the pulmonary surfactant in lung transplant patients.

Methods

This study was approved by the Ethics Committee of Hospital Universitari Vall d'Hebron and the Research Committee of Hospital Universitario Puerta de Hierro. Patients who took part in the study gave informed consent for participation.

Patients and study design

A prospective multicenter study was conducted of 38 consecutive lung transplant recipients aged older than 18 years who underwent bronchoscopy for graft follow-up or other clinical indications between December 2006 and February 2009. Patients with pneumonia, hemodynamic shock, or acute respiratory failure (oxygen pressure <60 mm Hg) were excluded, as were those with previous intolerance to inhaled amphotericin B, current treatment with some form of intravenous amphotericin, or an intellectual deficit or psychologic abnormality that would limit comprehension of the nature of the study. Two groups were studied: 19 patients who received standard prophylaxis with n-LAB at a minimum cumulative dose of 150 mg (study group) and 19 patients who did not receive inhaled prophylaxis (control group).

Immunosuppressive regimen

Depending on the local protocol, patients were treated with triple therapy based on cyclosporine or tacrolimus plus azathioprine, or mycophenolate mofetil plus corticosteroids. Cyclosporine was started on Day 1, with dose adjustments to maintain trough blood levels of 100 to 300 ng/ml, depending on the post-transplant period. When tacrolimus was used, the dose was adjusted to maintain trough levels of 5 to 15 ng/ml. Azathioprine dose was 1 to 3 mg/kg/day, depending on white cell count and avoiding a total leukocyte count of less than 4.0×10^9/liter. Initial mycophenolate mofetil dose was 1 to 3 g/day, with dose adjustment to maintain trough blood levels of 2 to 4 μg/ml and avoiding a total leukocyte count of less than 4.0×10^9/liter. Methylprednisolone was started in the operating room at a dose of 10 mg/kg before graft reperfusion, followed by 375 mg/day the first day and gradually tapering over the first year to reach a maintenance dose of approximately 0.1 to 0.2 mg/kg/day for life. Rapamycin or everolimus were used as rescue therapy in chronic and recurrent acute rejection or to substitute other immunosuppressive agents because of adverse effects. Induction therapy with basiliximab was used according to the local protocols. Acute rejection was treated with intravenous pulse administration of methylprednisolone at a dose of 5 to 10 mg/kg/day for 3 days or 1 mg/kg/day for 10 days, depending on the severity of the episode.

Antimicrobial prophylaxis

Patients without pre-operative septic disease received anti-biotic prophylaxis in the immediate post-operative period according to the protocols of the participating centers. In recipients with an underlying septic disease (mainly cystic fibrosis and bronchiectases), anti-biotic prophylaxis was modified according to the microorganisms isolated from the last cultures performed. Duration of prophylaxis was contingent on the results of recipient and donor intraoperative cultures. Isoniazid prophylaxis was prescribed in patients with tuberculosis infection (positive purified protein derivative test). Cytomegalovirus prophylaxis began with intravenous ganciclovir until resumption of oral intake, and then

valganciclovir up to Day 180 in cytomegalovirus-positive recipients, and Day 365 in cytomegalovirus donor+/recipient– mismatches. N-LAB was used as prophylaxis for *Aspergillus* infection in the study group, and itraconazole was used in the control group. The itraconazole dose was 200 mg/day for 6 months.

Preparation of n-LAB and administration

The n-LAB was prepared by dissolving 50 mg of LAB for injection (Ambisome, Gilead Sciences SL, Madrid, Spain) in 12 ml of sterile water. The solution remained stable for at least 7 days at 2°C to 8 °C. The prophylactic schedule was a 25-mg (6-ml) dose administered 3 times a week up to Day 60, a 25-mg dose once a week between Days 60 and 180, and a 25-mg dose once every 2 weeks thereafter for life. The technique consisted of amphotericin B nebulization by a one-jet nebulizer (Ventstream or Sidestream, Respironics, Murrysville, PA) with a CR60 compressor (air pressure, 27.2 psi and flow, 7.3 liters/min), equipped with a disposable bacterial exhale filter. This system produces aerosol droplets having a median mass diameter of 3 μm and a respirable fraction (percentage output contained in particles < 5 μm) of 80% of particles.

Patients were instructed by a trained staff nurse to inhale through a mouthpiece and exhale through the nose. The procedure took 10 to 15 minutes. To avoid contamination, the nebulizer was washed and brushed with soap and water after each administration; once rinsed, it was submerged in 1% sodium hypochlorite solution. We estimated the cumulative dose for each patient according to the time elapsed since transplantation, assuming that patients had followed the prophylactic schedule.

Bronchoscopic procedure and sample collection

Bronchoscopy was performed through the nose in most cases. Before sample collection, 2% lidocaine (10 ml) was administered as local anesthetic and immediately aspirated. BAL samples were obtained. The tip of the bronchoscope was wedged into a subdivision of a segmental bronchus of the right middle lobe or lingula. BAL was performed by instillation of a preliminary aliquot of 20 ml sterile isotonic saline solution, which was excluded from the analysis, and 3 separate 50-ml aliquots of saline. A 15-ml sample from the third aliquot was used for the surfactant assays. The third aliquot provides an optimum sample from the distal airway.[33] The instilled fluid was reaspirated by gentle manual suction. Dwell time of the instilled fluid in BAL averaged 20 seconds. Samples were frozen at –80°C until analysis.

Surfactant assay

Samples were all analyzed at the same time. The BAL sample was centrifuged at 400*g* for 10 minutes to eliminate cells. The cell-free sample was then centrifuged at 48,000*g* for 1 hour at 4°C to obtain a pellet of large aggregates (LA), which is the active fraction of pulmonary surfactant, and a supernatant containing small aggregates (SA) which is the inactive fraction.[22] Organic extraction in chloroform/methanol was performed to isolate the hydrophobic components of pulmonary surfactant,[34] and these underwent quantitative inorganic phosphorus assay to measure the phospholipid content.[35] The amount of cholesterol in the active fraction of surfactant and its proportion with respect to the amount of phospholipids were also determined. Quantitative determination of cholesterol content in LA was carried out using an enzymatic colorimetric assay (Cayman Chemical, Ann Arbor, MI).

Statistical analysis

Sample size was calculated for a 0.05 2-sided significance level, with a power level (1–β) of 80% to detect differences between the 2 groups. The demographic data and characteristics of the patients and the mean concentrations of the different molecules analyzed in surfactant were compared with the chi-square test or Fisher's exact test for discrete variables and the Mann Whitney *U*-test for continuous variables. Differences were considered significant at $p < 0.05$.

Results

Patient characteristics are reported in Table 1. The 2 study groups did not differ with respect to demographic variables or characteristics (Table 1). Patients received a median of 24 n-LAB doses (range, 6–128) before BAL was obtained, which represents a median cumulative amphotericin B dose of 600 mg (range, 150–3,200 mg). The median of timing of BAL in relationship with the LAB dose was 3 days (range, 0.5–15 days).

The median phospholipid content of LA was 0.40 μmol (range, 0.18–1.9 μmol) in the study group and 0.36 μmol (range, 2.15–0.12 μmol) in the controls, which was not significantly different ($p = 0.69$; Figure 1), nor was the median phospholipid content of SA, which was 0.23 μmol (range, 0.1–0.58 μmol) in study patients and 0.29 μmol (range, 0.18–0.65 μmol) in controls ($p = 0.33$; Figure 1). Likewise, no differences were observed in the SA-to-LA phospholipid ratio, which was 0.56 in study patients vs 0.69 in controls ($p = 0.28$). There were no differences in the LA cholesterol content between the study group (median, 0.04; range, 0.01–0.1 μmol) and the controls (median, 0.04; range, 0.02–0.27 μmol; $p = 0.13$). The cholesterol-to-phospholipid ratio in LA was also similar, 8.03 in the study group and 9.43 in the controls ($p = 0.38$). There were no significant differences in sub-analysis stratified by time since transplant (Table 2).

Discussion

The negative result obtained—no differences between groups—is a new piece of the puzzle providing additional information about safety in this type of prophylaxis. In this study evaluating the effect of inhaled LAB on human pulmonary surfactant, we found no differences in the lipid composition of surfactant between lung transplant recipients who received LAB and those who did not. The content of phospholipids in the LA (active fraction) and the SA (inactive fraction) was similar in the 2 groups. These results suggest that in the long-term, phospholipids present in the LAB formulation metabolize without disturbing the phospholipid content in the active and inactive surfactant fractions.

The SA-to-LA ratios obtained seem to indicate that pulmonary surfactant is not affected in patients treated with

Table 1 Characteristics of Lung Transplant Recipients in the 2 Groups

Variable [a]	Group with n-LAB ($n = 19$)	Group without n-LAB ($n = 19$)	p-value
Sex			0.72
Men	13 (68.4)	14 (73.7)	
Women	6 (31.6)	5 (26.3)	
Age, years	49.5 ±12.8	54.6 ±11.4	0.19
Underlying disease			
Chronic obstructive pulmonary disease	9 (47.4)	8 (42.1)	0.74
Pulmonary fibrosis	4 (21.1)	8 (42.1)	0.16
Others	6 (31.6)	3 (16.7)	0.16
Bronchiectasis	1 (5.3)	1 (5.3)	
Bronchiolitis	1 (5.3)	0 (0)	
Hypersensitivity pneumonitis	0 (0)	1 (5.3)	
Pulmonary hypertension	0 (0)	1 (5.3)	
Lymphangioleiomyomatosis	1 (5.3)	0 (0)	
Cystic fibrosis	3 (15.8)	0 (0)	
Transplant type			0.32
Single	9 (47.4)	6 (31.6	
Double	10 (52.6	13 (68.4)	
Time since transplant, days	364 (14–2,044)	336 (19–1,741)	0.85
Reason for bronchoscopy			0.74
Surveillance	9	8	
Deterioration of respiratory functional tests	7	9	
Bronchiolitis obliterans	3	2	
Bronchial stenosis	1 (5.3)	3 (15.8)	0.60
Isolation of bacteria and/or fungi in BAL	5 (26.3)	2 (10.5)	0.40
Bronchiolitis obliterans (chronic rejection)	4 (21.1)	7 (36.8)	0.28

BAL, bronchoalveolar lavage; n-LAB, nebulized liposomal amphotericin B.
[a]Continuous variables are shown as the mean ± standard deviation or mean (range), and categoric variable as number (%).

LAB. The SA-to-LA phospholipid ratio is commonly used as a marker of alveolar damage. In normal conditions, the amount of phospholipids in LA is double that of SA. The ratio increases in pathologic conditions owing to a decrease in surfactant secretion or to accelerated LA-to-SA conversion.[36] This ratio in our study was close to 0.5 in both groups.

Because cholesterol is one of the constituents of LAB, the large aggregate cholesterol content was also analyzed. Again, the results showed no differences between treated and non-treated patients. This finding could also indicate that LAB is rapidly metabolized by epithelial and alveolar fluid cells. It is also possible that the small size of the liposomes administered would facilitate their incorporation

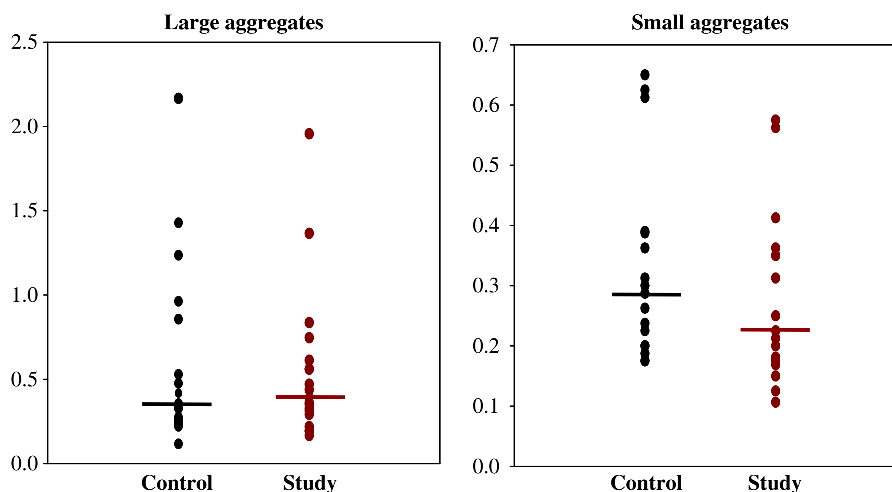

Figure 1 Phospholipid content is shown in large aggregates and small aggregates isolated from bronchoalveolar lavage fluid (μmol) obtained in 19 study patients who received inhaled prophylaxis compared with 19 control patients who did not. The solid line indicates the median.

Table 2 Phospholipid and Cholesterol Content in Large Aggregates and Small Aggregates Isolated From Bronchoalveolar Lavage Fluid From Patients Who Did and Did Not Receive Inhaled Prophylaxis: Sub-analysis Stratified by Time Since Transplant

Time since transplant	Group	No.	PL in LA (μmol)	PL in SA (μmol)	PL SA/LA (μmol)	Cholesterol LA (μmol)	Cholesterol/PL in LA (μmol)
14-180 days	Control	9	0.33 (1.43-0.22)	0.24 (0.65-0.18)	0.67 (1,19-0.17)	0.03 (0.28-0.02)	7.2 (22.33-5-98)
	Study	12	0.42 (1.37-0.17)	0.22 (0.58-0.15)	0.51 (1.17-0.26)	0.04 (0.1-0.01)	8.38 (17,32-3.44)
	p-value		0.80	0.63	0.63	0.77	0.74
181-365 days	Control	3	0.48 (0.86-0.28)	0.39 (0.39-0.2)	0.73 (0.82-0.46)	0.44 (0.1-0.03)	9.43 (11.88-9.32)
	Study	2	0.43 (0.56-0.29)	0.31 (0.36-0.25)	0.75 (0.86-0.65)	0.06 (0.07-0,04)	13.04 (13.23 -12.86)
	p-value		>0.99	0.56	0.56	>0.99	0.083
>365 days	Control	7	0.48 (2.17-0.12)	0.31 (0.63-0.18)	0.82 (1.50-0.29)	0.04 (0.22-0.01)	10.20 (24.39-5.13)
	Study	5	0.36 (1.96-0.31)	0.17 (0.56-0.11)	0.47 (1.07-0.05)	0.02 (0.08-0.02)	5.24 (25.05-0.81)
	p-value		0.69	0.17	0.37	0.22	0.17

LA, large aggregates; PL, phospholipid; SA, small aggregates.

in cells or extravasation to the blood or lymphatic fluid, thereby accelerating their metabolism.

Even though the mean cumulative amphotericin B dose was high (around 600 mg), only 10% of the dose is deposited in the lung.[37] That would represent a relatively small amount of drug and could explain the lack of differences between the groups. Only 66% of the dose nebulized is inhaled, and some of the particles inhaled are too large to reach the lungs and others are too small to be deposited.[37]

In contrast to these results, other forms of inhaled amphotericin B have been found to cause surfactant changes. Griese et al[38] reported that conventional amphotericin B alters bovine surfactant function, measured as the minimal surface tension and absorbance. The authors found that this effect is not caused by pure amphotericin B, but instead, results from the extremely lipophilic deoxycholic acid used as an excipient. In the same study, LAB inhibited surfactant function, but only mildly and at high doses. Similar results were reported by Ruijgrok et al[39] in an in vitro study with bovine surfactant. The authors found a dose-dependent inhibition of surfactant function with the use of amphotericin B desoxycholate, whereas no effect was seen with LAB.

Other aspects related to the safety of LAB have also been evaluated. It is well tolerated, inhalation does not alter lung function,[12,13] and there is no systemic absorbance.[14] These favorable characteristics and the fact that it does not induce changes in surfactant lipid content, as was shown in the present study, delineate an optimal safety profile for its long-term use as prophylaxis.

Surfactant stabilizes the alveoli at low lung volumes and prevents alveolar collapse and edema during physiologic conditions and in acute lung injury. In addition, an important component of the specific innate immune defense mechanism of the lung is provided by the phospholipids and proteins of pulmonary surfactant.[25,40] There is increasing awareness of the relevant role of surfactant in the evolution of lung grafts. The lung transplantation process involves an inflammatory component that is more or less severe in relation to graft ischemia, lung preservation, and reperfusion. This inflammatory process apparently involves, among other things, the action of resident macrophages, the release of multiple inflammatory mediators, the recruitment and activation of circulating platelets, and death of pulmonary cells. As a result, vascular endothelium and alveolar type I and II cells are injured and a subsequent alteration of alveolar surfactant may occur.[41]

Some data have suggested a persistent impairment of biophysical surfactant properties after lung transplantation, possibly due to poor functioning of type II pneumocytes. Hohlfeld et al[24] compared phospholipid concentrations, proteins, and surface activity in BAL between 60 lung transplant recipients and 10 healthy individuals and observed differences in the minimum surface tension and the ratio of SA to LA, suggesting an impairment in surfactant properties. A possible mechanism for such impaired surface activity is that changes in surfactant-specific protein alter surfactant function. Another possible explanation could be changes in the phospholipids composition (mainly dipalmitoyl-phosphatidylcholine).[24] This would have important implications in primary graft dysfunction.[23,41] In fact, exogenous surfactant has been used in the prevention and treatment of primary graft dysfunction, with promising results.[25–30] In experimental studies, Van Puten et al[29] observed a significant improvement in lung histologic characteristics, reduced apoptosis, and an increased anti-inflammatory marker in rat lung transplants that had received porcine surfactant compared with controls. Saez et al[30] noted a recovery of oxygen levels, a normalized alveolar–arterial oxygen tension difference, and a recovery of surfactant function when KL_4, a new synthetic surfactant, was used in an experimental model of ischemia–reperfusion injury.

Studies in humans regarding treatment of primary graft dysfunction with surfactant are very few, with scarce patients and without control groups, thus limiting their value. For example, in 5 patients with severe life-threatening primary graft dysfunction who failed to respond to conventional measures, Amital et al[28] observed a significant improvement within hours of treatment with bronchoscopic instillation of mammalian surfactant. There are, however, some randomized studies of prevention of primary graft dysfunction. In an open, randomized, prospective controlled study with 42 patients, Amital et al[27] demonstrated that surfactant instillation during lung transplantation improved

oxygenation, prevented primary graft dysfunction, shortened intubation times, and enhanced early post-transplant recovery. There is also some evidence relating surfactant functional status to chronic graft dysfunction. In this respect, d'Ovidio et al[40] observed an association between reflux effect, alteration of surfactant, and chronic dysfunction.

To summarize, there were no differences in the lipid content of LA and SA in lung surfactant of patients treated or not with LAB. Thus, prophylactic administration of this drug does not seem to have an effect on surfactant lipid composition. One limitation of this study may be the small number of patients enrolled and the wide range in time since transplant and therefore, in cumulative dose. Future studies including a larger number of patients, investigating surfactant protein content and activity would be desirable to further define the influence of inhaled LAB on pulmonary surfactant.

Disclosure statement

This study was supported by a Spanish Society of Pneumology and Thoracic Surgery grant (2005).

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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