

Original Article

Effects of azathioprine on mucociliary clearance after bronchial section and anastomosis in a rat experimental model*

Efeitos da azatioprina sobre a depuração mucociliar após secção e anastomose brônquica em um modelo experimental em ratos

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Abstract

Objective: To evaluate the effects of azathioprine on the mucociliary system in a model of bronchial section and anastomosis in rats. **Methods:** Thirty-six male Wistar-Furth rats were submitted to left bronchial section and anastomosis and divided into two groups to receive either saline solution or azathioprine. After 7, 15 and 30 days of treatment, six animals from each group were killed, after which *in situ* mucociliary transport velocity, *in vitro* mucus transportability, and contact angle of mucus in the right (intact) and left (sectioned) bronchi were measured. **Results:** *In situ* mucociliary transport velocity was significantly lower in the sectioned bronchi than in the intact bronchi ($p < 0.001$). *In situ* mucociliary transport velocity was lower in the intact bronchi of the animals treated with azathioprine for 7 days ($p < 0.05$), and those bronchi presented full recovery after 30 days of treatment. The contact angle was higher in the mucus samples collected from the sectioned bronchi of the animals treated with saline solution for 30 days ($p < 0.001$), which is in accordance with the decreased *in vitro* mucus transportability observed in the same animals ($p < 0.001$). **Conclusions:** We conclude that, in the sectioned bronchi of rats, treatment with azathioprine causes only transitory impairment of mucociliary transport, whereas administration of saline solution impairs mucociliary transport for up to 30 days. In addition, azathioprine protects against alterations in mucus surface properties.

Keywords: Mucociliary clearance; Anastomosis, surgical; Immunosuppression; Lung.

Resumo

Objetivo: Avaliar os efeitos da azatioprina sobre o sistema mucociliar em um modelo de secção e anastomose brônquica em ratos. **Métodos:** Trinta e seis ratos machos da raça Wistar-Furth foram submetidos à secção e anastomose brônquica esquerda e separados aleatoriamente em dois grupos para receberem solução salina ou azatioprina. Após 7, 15 e 30 dias de terapia, seis animais de cada grupo foram sacrificados, e foram realizadas as medidas da velocidade de transporte mucociliar *in situ*, da transportabilidade do muco *in vitro* e do ângulo de contato do muco nos brônquios direito (intacto) e esquerdo (seccionado). **Resultados:** A velocidade de transporte mucociliar *in situ* foi significativamente menor nos brônquios seccionados do que nos brônquios intactos ($p < 0,001$). Houve redução da velocidade de transporte mucociliar *in situ* nos brônquios intactos dos animais tratados com azatioprina por 7 dias ($p < 0,05$), havendo completa recuperação após 30 dias de terapia. O ângulo de contato do muco foi maior nos brônquios seccionados dos animais tratados com solução salina por 30 dias ($p < 0,001$), estando de acordo com a redução da transportabilidade do muco *in vitro* observada nos mesmos animais ($p < 0,001$). **Conclusões:** Concluímos que, nos brônquios seccionados de ratos, a terapia com azatioprina causa um prejuízo apenas transitório do transporte mucociliar, enquanto a administração de solução salina prejudica o transporte mucociliar por até 30 dias. Além disso, a azatioprina contribui para prevenir alterações nas propriedades da superfície do muco.

Descritores: Depuração mucociliar; Anastomose cirúrgica; Imunossupressão; Pulmão.

Introduction

Bronchial section during lung transplant interrupts blood flow through the bronchial artery and causes sensory denervation distal to the suture, affecting the cough reflex and consequently making patients more dependent on mucociliary transport (MCT). This allows us to speculate that bronchial section, in conjunction with the administration

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of immunosuppressants, leads to MCT and mucus alterations, contributing to the greater susceptibility of such patients to respiratory infection in the post-operative period following lung transplant.

As a mechanism to defend the organism against pathogenic agents present in inhaled air, MCT is fundamental. Adequate clearance of the airways occurs through the joint activity of ciliated and mucus-secreting cells present in the tracheobronchial epithelium.⁽¹⁾ For MCT to be effective, it is essential that its components be morphologically and physiologically integrated.⁽²⁾ An adequate mucociliary clearance is fundamental not only for the normal functioning of the lungs in a healthy state but also, and principally, in the presence of respiratory infections.⁽³⁾ Previous studies have shown significant impairment of MCT after lung transplant.⁽⁴⁾ This alteration, when present, can place graft recipients, who are already immunosuppressed as an effect of the anti-rejection drugs, at an even greater risk of acquiring respiratory tract infections, which constitute one of the principal limiting factors for their survival.⁽⁵⁾

The mechanisms involved in the impairment of mucociliary function after lung transplant are not well established, and could be related to a series of factors present in the peri-operative scenario, such as mechanical injury resulting from bronchial section, which leads to denervation and devascularization of the portion distal to the anastomosis,

as well as to the effects of anesthetics, dehydration, and mechanical ventilation.^(4,6) In addition, since the drugs used for immunosuppression present a series of side effects, such as nephrotoxicity and hepatotoxicity, we raised the hypothesis that such drugs have a deleterious effect on the airway epithelial tissue as well. Since azathioprine is one of the drugs most commonly used by patients after transplant, and since its direct effect on the mucociliary system is not clearly known, it was chosen as the drug to be tested in this study.

Therefore, the objective of the present study was to evaluate the joint effect of bronchial section/anastomosis and immunosuppressive therapy with azathioprine, two factors directly involved in lung transplant.

Methods

The present study was approved by the Ethics Committee for the Analysis of Research Projects of the University of São Paulo School of Medicine. We used 36 male Wistar-Furth rats, each weighing approximately 300 g, according to the international norms that regulate laboratory animal research.⁽⁷⁾ All animals were submitted to the same surgical procedure and then randomly distributed into two groups: the Aza group (n = 18), treated daily with azathioprine; and the Sal group (n = 18), treated daily with saline solution. Six animals from

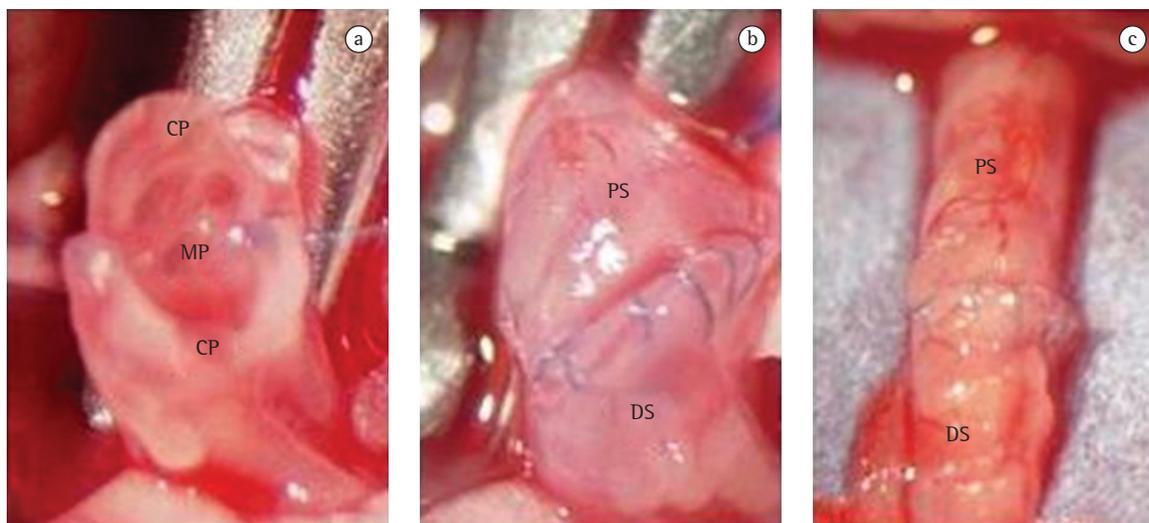


Figure 1 - Bronchial anastomosis technique: stereomicroscope view (x8) of the membrane (a) and cartilage (b) portions as well as of the final aspect (c). DS: distal stump; PS: proximal stump; CP: cartilage portion; and MP: membrane portion.

each group were killed after 7, 15, and 30 days of treatment, after which *in vitro* mucus transportability, *in situ* MCT velocity, and contact angle (between a mucus drop and a surface) were analyzed.

The animals were previously anesthetized in a chamber containing isoflurane (Isothane; Baxter Health Care Corporation, Guayama, Puerto Rico) and intubated orotracheally using a polyethylene catheter. Artificial ventilation (model 683; Harvard Apparatus, South Natick, MA, USA) was maintained at a tidal volume of 10 mL/kg of body weight and a respiratory rate of 70 cycles/min. General anesthesia was maintained with 0.5% isoflurane in pure oxygen administered by a nebulizer (model 1223; Takaoka Indústria e Comércio Ltda, São Paulo, SP, Brazil). Access to the thoracic cavity was achieved through a left thoracotomy performed in the fifth intercostal space. The main left bronchus was carefully dissected, being subsequently clamped near the carina and sectioned in its medial third. Bronchial anastomosis was performed using continuous 8-0 polypropylene sutures (Figure 1). Finally, airflow was restored, and the atelectasis of the left lung was resolved through hyperinflation. Prior to the closure of the incision, a chest tube was inserted. The chest tube was removed, together with the tracheal tube, immediately after spontaneous respiration had been restored. The surgical procedure was performed with the aid of a stereomicroscope, at a magnification of $\times 8$. All surgical procedures were performed by the same researcher using a clean, albeit non-sterile, technique.

From the day of surgery, azathioprine (Imuran, 50 mg; Merck, Darmstadt, Germany) was administered to the animals via an orogastric tube in a daily dose of 3 mg/kg, diluted in saline solution (1 mg/mL). The animals in the Sal group were submitted to the same therapeutic regimen, receiving only the vehicle in an equivalent volume.

After 7, 15, and 30 days of treatment, six animals from each group were anesthetized intraperitoneally with sodium pentobarbital (30 mg/kg), and killed by exsanguination through section of the abdominal aorta.⁽⁸⁾

Immediately after the animals were killed, their lungs were removed en bloc from the thoracic cavity and placed in Petri dishes. Only the bronchi that did not present any sign of stenosis, confirmed macroscopically, were included in the study. After dissection, an incision was made in each main

bronchus, and mucus collection was performed selectively by inserting a small hair paintbrush into the lumen of each bronchus. The mucus adhered to the paintbrush was then placed in an Eppendorf tube containing mineral oil (to prevent dehydration) and stored at -70°C .

The relative MCT velocity in the mucus samples was measured using an *in vitro* frog palate model. The mucus samples, previously defrosted at room temperature, were placed on the frog palate ciliated epithelium, and their movement was observed and timed with the aid of a stereomicroscope equipped with a reticulated eyepiece (Figure 2). The MCT velocity in the rat mucus samples was compared to that in the frog mucus itself, and the results were therefore expressed as relative velocity (rat/frog).

Contact angle was measured using an eyepiece ($\times 25$) equipped with a goniometer with a scale from 0 to 180 degrees. The samples were placed on slides treated with sulfochromic acid to eliminate electrical currents, after which the angle formed between the air-liquid interface and the slide surface was registered.⁽⁹⁾

The MCT velocity was monitored through direct observation of the carbon particles deposited on the mucus layer of the distal region of each bronchus using a stereomicroscope with a reticulated eyepiece.

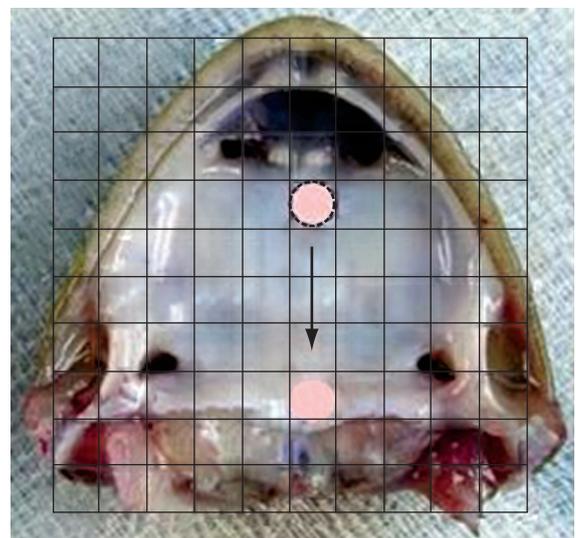


Figure 2 - Photograph of the frog palate with a diagram representing the technique for measuring mucus transportability by visualization using a stereomicroscope equipped with a reticulated eyepiece.

The movement of the particles was timed and registered as the distance covered in one minute.

The statistical analysis was performed using the program GraphPad Prism for Windows, version 4.0 (GraphPad Software Inc., San Diego, CA, USA). Analysis of variance was used in order to determine the interference of the factor analyzed, as well as any possible interaction between them. In addition, the Bonferroni test was used in order to determine the differences between the groups for each factor analyzed. The data are presented in the form of charts and expressed as mean \pm standard deviation. Values of $p < 0,05$ were considered significant.⁽¹⁰⁾

Results

The analysis of the measurements of the contact angle of the mucus samples revealed greater values in the animals evaluated after 30 days of treatment, with a significant difference in the sectioned bronchi of the animals treated with saline solution ($p < 0,001$; Figure 3). This increase in the contact angle demonstrated a lower capacity of the mucus for spreading over a flat surface, characterizing it as more rigid and, therefore, more difficult to transport. This was demonstrated in the overall evaluation of mucus transportability on frog palate, especially in relation to the samples from the sectioned bronchi of

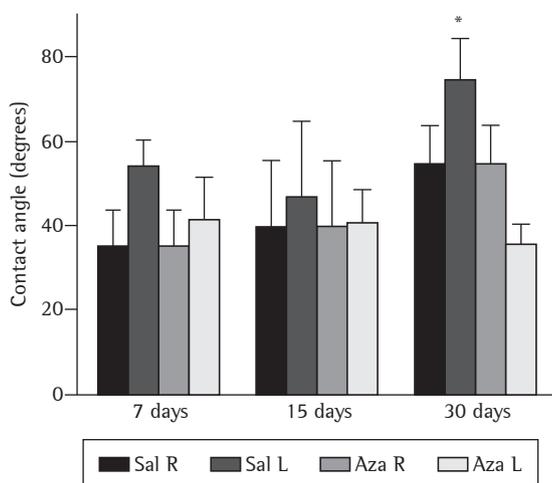


Figure 3 – Comparison of the contact angle of mucus samples from the right (R) and left (L) bronchi of the animals treated with saline solution (Sal) or azathioprine (Aza). The contact angle of mucus from the sectioned bronchi (L) treated with Sal for 30 days was significantly greater (* $p < 0.001$).

the animals treated with saline solution ($p < 0,001$; Figure 4).

The results show that, in the animals that received saline solution, MCT velocity was lower, at all three time points evaluated, in the left bronchi (submitted to sectioning and anastomosis) than in the right (intact) bronchi ($p < 0.05$). The analysis of the left (sectioned) bronchi from the animals that received azathioprine showed a decrease in MCT velocity (with a statistical difference) after 7 days of treatment in relation to those that received saline solution, showing an interaction between the factors analyzed. When the right and left bronchi of the animals that received azathioprine were compared, the left (sectioned) bronchi presented an evident decrease in MCT velocity after 7 days of treatment ($p < 0.001$). The analysis of the right (intact) bronchi of the animals that received azathioprine showed a decrease in MCT velocity after 7 days of treatment ($p < 0.05$) in relation to the right (intact) bronchi of those that received saline solution, and the bronchi of the animals that received azathioprine presented full recovery after 30 days of treatment (Figure 5).

Discussion

Many advances have been made in the areas of immunosuppression, antibiotic therapy, and post-operative intensive care. Nevertheless, in lung transplants, in which there is manipulation of the bronchi, the most frequent complication is infection resulting from malfunction of the mucociliary system.⁽¹¹⁾ The immunosuppressive effect of the anti-rejection drug is easily identified as a risk factor for complications and constitutes a major hindrance to the success of the lung transplant.⁽¹²⁾

We observed that various immunosuppressive regimens have been tested in clinical practice, with a retrospective evaluation of the results obtained.⁽¹³⁾ Such testing has become more common since the introduction of new immunosuppressive drugs aimed at replacing those, such as azathioprine, that have been used for more than 30 years. To date, this 'competition' between the new and the old has not produced sufficient scientific data to indicate which immunosuppressive drugs should or should not be used in lung transplant, since not all of their effects have been defined.

We sought to develop, in the present study, the idea of an experimental model that could clarify the

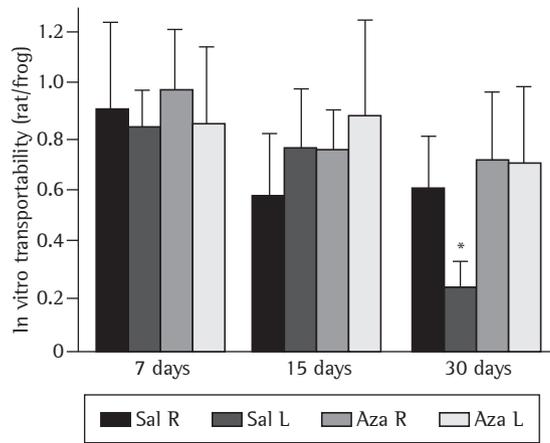


Figure 4 – Comparison of transportability of mucus samples from the right (R) and left (L) bronchi of the animals treated with saline solution (Sal) or azathioprine (Aza) using an *in vitro* frog palate model. The mucus from the sectioned bronchi (L) treated with Sal for 30 days was transported more slowly by the ciliated epithelium of the frog palate (* $p < 0.001$).

effects of immunosuppressive drugs as well as of bronchial section and anastomosis on the mucociliary system, and that could be extrapolated to clinical practice. In this model, we evaluated the effects of bronchial section in animals treated with saline solution or azathioprine. In our model, MCT velocity was determined by direct observation of the India ink solution deposited in the region distal to the anastomosis of the left (sectioned) and right (intact) bronchial epithelium.

Our results demonstrate that there is MCT impairment and altered mucus quality on the left (operated) side but not on the right (intact) side, reinforcing the concept that bronchial denervation is responsible for decreasing MCT velocity. The use of azathioprine minimized these effects by improving mucus quality, contradicting our initial hypothesis that, since azathioprine is a drug that reduces cellular metabolism, it would further impair MCT.

In the left bronchi of all animals studied, there was a reduction in MCT and a worsening of mucus quality. As the scarring process evolved, confirmed through macroscopic analysis, we observed that a tendency toward recovery of MCT after 30 days, although the mucus remained thick, viscous, and very sticky. This results from the fact that sectioning leads to MCT inefficiency in the acute post-operative

phase, which in turn results in the accumulation of mucus and consequent alterations in its properties. Therefore, sectioning has no direct effect on the formation of mucus or on its properties. However, it has an indirect effect: MCT inefficiency leads to a worsening of mucus quality. Sectioning did not affect MCT or mucus quality on the right (intact) side. This demonstrates that surgery has only a local, rather than a systemic, effect. Indirectly, altered mucus quality promotes the occurrence of infections, and, through this mechanism, systemic complications occur. Therefore, longer-term mucus treatment and the use of mucus-thinning procedures in the post-operative period should be emphasized. Broad-spectrum antibiotics with a protective effect on the mucosa will bring greater benefits than do those without such properties.

Some authors have found a correlation between lung transplant and impairment of pulmonary surfactant activity.⁽¹⁴⁾ Therefore, pulmonary surfactant might play an important role in normal mucociliary interaction if applied to the bronchial surface as a therapeutic option in transplant patients. Azathioprine has no direct effect on the production or quality of pulmonary surfactant.⁽¹⁵⁾

Other authors have studied, in other animal species, the effects of bronchial denervation on the mucociliary system and observed a decrease in MCT velocity.^(16,17) Bronchial devascularization might have the additional effect of severely impairing MCT velocity. The principal hypothesis is that the reversibility of MCT impairment is due to vascular neof ormation (revascularization of the bronchial tree), since bronchial anastomosis does not lead to recovery of the nerves.⁽¹⁸⁾ Therefore, our hypothesis was that this effect would add to the effect of azathioprine on the reduction of cell activity and inflammatory activity, thereby prolonging the time to bronchial revascularization.

In the animals that received azathioprine, we found that it reduced MCT velocity in the group killed after 7 days of treatment both on the right (intact) side and on the left (operated) side. Over time, there is evident recovery of MCT velocity on the right and left sides, indicating that MCT velocity is affected by azathioprine only in initial period of up to 7 days of treatment. This mechanism might result from the fact that azathioprine decreases cell activity and reaches the ciliated cells, altering their function. The recovery of ciliated cell function, over time, in

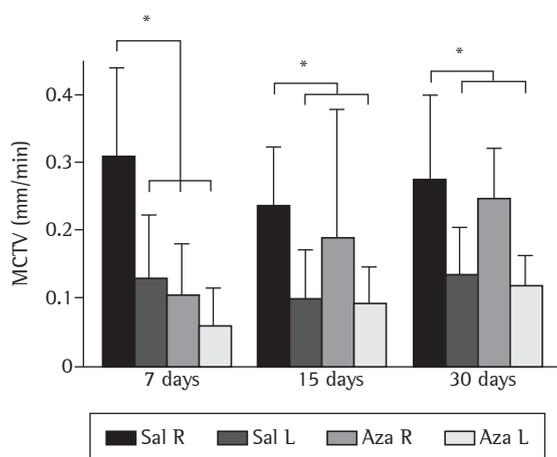


Figure 5 - Comparison of *in situ* mucociliary transport velocity (MCTV) in the right (R) and left (L) bronchi of the animals treated with saline solution (Sal) or azathioprine (Aza). MCTV was lower in the sectioned bronchi (L) than in the intact bronchi (R) in all animals treated with Sal or Aza at all time points evaluated (* $p < 0.05$). MCTV in the intact bronchi (R) decreased after 7 days of treatment with Aza (* $p < 0.05$), and those bronchi presented full recovery after 30 days of treatment.

the group that received azathioprine is probably due to two factors that lead to increased MCT velocity: bronchial scarring and improved mucus formation/properties. Many immunosuppressive protocols include azathioprine as a pre-operatively administered drug.⁽¹⁹⁾ Based on the results of the present study, we suggest that azathioprine administration, as well as the duration of azathioprine treatment in the post-transplant period, be the object of several other experimental studies.

In the Aza group, there was less worsening of mucus quality over time, which prevented changes in the properties of the mucus. The anti-inflammatory effect of azathioprine, as described by various authors,^(20,21) seems to be responsible for this fact. Acting on the mucus-secreting cells, it presented a regulatory effect on mucus and periciliary fluid production. Our hypothesis was that azathioprine leads to lower MCT velocity and poorer mucus quality. However, these parameters did not worsen after administration of the drug, and the effect of the drug was not diminished after sectioning. The MCT velocity was affected by sectioning, with azathioprine-related worsening, only in the animals killed after 7 days of treatment.

The quality of the mucus worsened over time in the animals submitted to bronchial section and treated with saline solution, and this worsening was greater in the animals killed after 30 days of treatment. We found, therefore, that bronchial section in isolation worsens mucus quality over time, and that this worsening is prevented by azathioprine administration. Further scientific evidence regarding drug effects is needed before immunosuppressive protocols for use in clinical practice can be defined.

In summary, MCT velocity was impaired in the bronchi submitted to sectioning, and azathioprine had a protective effect on the viscoelastic properties of mucus. The results obtained can add to the understanding of the MCT dysfunction observed after lung transplant, as well as increasing knowledge regarding the therapeutic interventions that more rapidly lead to the recovery of this system. Further studies are needed in order to evaluate the joint effect of combining azathioprine with other immunosuppressants.

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