Prevention of Tracheal Stenosis With Pirfenidone After Tracheotomy: An Experimental Study

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**Objectives:** In this study, pirfenidone's role about reducing tracheal stenosis by suppressing fibrosis and inflammation was examined.

**Methods:** Tracheotomy was performed on 14 rats, and their cannulas were fixed to tracheotomy area by stoma suture. Two working groups were established. Rats in the first group were given 15 mg/kg/day (1 mL pirfenidone solution) pirfenidone intraperitoneally for 10 days. In the second group as a control group, 1 mL saline solution was applied intraperitoneally. Ten days later, rats were decanulated and kept alive for 3 more weeks. Anesthetized rats were sacrificed on day 30. All rat tracheas were resected between the first and seventh rings. Epithelial damage, inflammation, and fibrosis were determined histopathologically; diameters of intratracheal lumen and their mucosal thickness parameters were determined histomorphometrically; and TGFβ-1 (the growth factor beta), TNFα (tumor necrosis factor alpha), and IL-1β (Interleukin-1 beta) values were determined immunohistochemically.

**Results:** According to the parameters of the control group, fibrosis; diameters of intratracheal lumen; and values of TGFβ-1, TNFα, and IL-1β were found to be statistically significant.

**Conclusion:** In our study, it was found that pirfenidone reduces fibrosis and narrowing of intratracheal lumen diameter significantly.

**Level Words:** Tracheal stenosis, tracheotomy, pirfenidone.

**Level of Evidence:** NA

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**INTRODUCTION**

Tracheal stenosis (TS) is the most common complication after tracheotomy and tracheal tube placement. Due to the pressure applied by the tracheal tube cuff, ischemia, edema, inflammation, and eventually fibrosis develops, playing an important role in the pathogenesis of tracheal stenosis, which occurs with an incidence of 0.6% to 21%.1,2

Current methods used in the treatment of tracheal stenosis include balloon dilatation, endoscopic laser surgery, tracheal resection, and end-to-end anastomoses.3 However, the increased risk of restenosis after these treatment methods paved the way to search for innovative pharmacologic agents inhibiting the fibrosis, which is the main leading cause in the pathogenesis of fibrosis.

Pirfenidone (5-methyl-1-phenyl-2-[1h]-pyridone) is a medicine approved by the Food and Drug Administration (FDA) for oral use in the treatment of idiopathic pulmonary fibrosis (IPF). Although its exact mechanism of action has not been established yet, it is suggested that it acts by decreasing the production rate of TGFβ-1 (the growth factor beta), TNFα (tumor necrosis factor alpha), and IL-1β (Interleukin-1 beta) in the in vitro and in vivo fibrotic disease models.4–6 Despite the use of pirfenidone in primarily pulmonary fibrosis, as well as the experimental studies using it in the disorders manifesting with fibrosis—including the cutaneous scars,4 vocal fold scars,7 abdominal adhesions,8 intestinal9 and esophageal strictures,10 and benign biliary strictures11—the number of studies on tracheal stenosis is limited.3,12,13

This study aimed to evaluate the effect of pirfenidone on the development of tracheal stenosis in the rats intubated with a tracheal tube.

**MATERIALS AND METHODS**

**Experimental Animals and Ethics Statement**

This study was conducted in Yeditepe University Faculty of Medicine, Center of Experimental Studies, with the approval of Yeditepe University Experimental Animals Ethics Committee, dated January 7, 2014, and registered with no. 405. All animal maintenance processes and procedures were performed by hand. In order to exclude the rats with any unobservable signs of any infection or diseases, 16 Sprague Dawley rats weighing 200 to 250 grams were observed over a period of 7 days. Rats were housed in an environment with a 12-hour light–dark cycle and at a constant temperature (approximately 22 °C).
Experimental Model and Application of Treatment

The rats were anesthetized by administering each rat a 40 mg/kg dose of ketamine base (Ketalar 10 mL flacon, Pfizer, New York, NY) and a 7 mg/kg dose of xylazine hydrochloride (Ksiiazol 50 mL flacon, Provet, Istanbul, TUR). After the surgery, the rats were left for spontaneous respiration. Rats were left in supine position after being anesthetized. The anterior part of the necks of the rats was shaved, cleaned by using povidone iodine, and draped with sterile covers. The area of incision was applied 0.2 mL lidocaine hydrochloride with adrenaline in order to alleviate pain during and after the operation, as well as to facilitate hemostasis. A vertical skin incision on the midline was performed, extending from the upper border of the thyroid cartilage to incisura jugularis. Passing through the skin and subcutaneous tissues, the submandibular gland was retracted superiorly. The strap muscles were retracted, exposing the laryngotracheal framework. After elevating the pretracheal fascia, a vertical tracheotomy was generated between the second and the fourth cervical tracheal rings and a 6 French (F) feeding tube was placed as a tracheotomy cannula. The cannula was fixed in the tracheotomy region by means of stoma sutures. The strap muscles, subcutaneous tissue, and skin were closed anatomically using 4.0 Vycril Rapide (Ethicon, Diegem, Belgium), and then the operation was terminated. Two rats died in the perioperative period. Because the number of rats was appropriate to conduct a statistical analysis, these rats were not replaced.

The rats were observed and followed up for any respiratory distress during the early postoperative period, and they were placed into their cages when they were fully awake. The animals were randomized into two groups. Pirfenidone (Santa Cruz Biotechnology, Santa Cruz, CA) prepared as a 3 mg/mL solution was applied intraperitoneally to the first group (experiment group, group P) at a dose of 1.5 mg/day over a period of 10 days (total dose 150 mg for each rat in group P). To the second group (control group, group C), 1 mL saline solution was applied intraperitoneally over a period of 10 days. The intraperitoneal route of administration was preferred because it was easy to administer, effective, and allows for correct dosing.8 The tracheotomized rats were decannulated after the period of 10 days. The animals were sacrificed by bleeding under intramuscularly administered 40 mg/kg ketamine base (Ketalar 10 mL flacon, Pfizer) anesthesia 3 weeks after the decannulation.

Microscopic Evaluation

The epithelial damage, inflammation, and fibrosis were evaluated as parameters by the histopathological examination. All parameters were qualified as none (−), mild (+), moderate (++), and high (+++). During the microscopic evaluation, the parameters qualified with “+” and “−” signs were converted into numerical values of 0, 1, 2, and 3 to conduct statistical analyses. The epithelial damage was assessed by the loss in the epithelial thickness of the tracheal mucosa. In order to evaluate the inflammation, the total number of inflammatory cells in the injured submucosal region were counted under 400x magnification, and the mean number of inflammatory cells per mm² was evaluated. In the course of this evaluation, the findings qualified as (−), (+), (++), and (+++) were scored as 0, 1, 2, and 3, respectively. The findings of fibrosis were graded in a range from 0 to 3 (Table I).

Immunohistochemical Evaluation

Sections of 5 microns were created to conduct an immunohistochemical examination. Then, they were stained by processing them with TGF BETA; (Santa Cruz Biotechnology, Santa Cruz, CA, Lot No: E1914, 1:50 dilution) using a Leica Bond Max immunohistochemical staining device (Leica Biosystems, Wetzlar, DEU). Depending on the degree of staining, the sections stained by immunohistochemical staining were scored in a range from 0 to 3 under an Olympus BX 51 microscope (Olympus Corporation, Tokyo, Japan).

Morphometric Evaluation

The tracheas collected from the rats were resected in the area between the superior border of the first tracheal ring and the inferior border of the seventh tracheal ring. Serial sections were created from each tracheal specimen, starting from the distal end and extending to the proximal one. The tracheal specimens of 0.5 cm were fixed in 10% neutral formaldehyde and put into tissue cassettes. The biopsy materials in the cassettes were followed up by using a Shandon Excelsior tissue processor (Thermo Fisher Scientific, Waltham, MA). After the follow-up period, they were embedded in paraffin and then inserted in a recipient paraffin block. Horizontal sections of 5 microns were created by means of a Shandon FINESSE-ME microtome (Thermo Fisher Scientific, Waltham, MA). After the sections were stained with hematoxylin and eosin (H&E) and Masson Trichrome (MT) stains, they were examined under a light microscope (Olympus BX 51) (Fig. 1) (Fig. 2). In addition, the sections were examined under a Leica light microscope integrated with Stereo Investigator software 7.0.5 software (Microbrightfield, Williston, VT), and the internal diameters of the trachea lumens and the thickness of the trachea mucosa were measured.

The sections stained with MT were examined with a stereology working station composed of the following devices, including a CCD digital camera (Optronics Microfire 1600 × 1200P, Goleta, CA); a graphic card (ATI FireGL Advance MicroDevice, Camberley, U.K.); a computer-controlled, motor-driven stage (Bioprecision, Hawthorne, NY); a histology microscope (Heidenhain, Traunreut, Germany); and a light microscope (Leica DM 4000B). The measurements were conducted using the Stereo Investigator 7.0.5 software and the microscope described above (Fig. 1) (Fig. 2). The internal diameter of the lumen and the thickness of mucosa were measured in micrometers by calculating the mean of values collected from four different areas by using a Leica C Plan ×4 objective (NA = 0.10) and quick measure line.

Statistical Analysis

The sample size and the post hoc power analysis of the study were calculated by the G*Power 3.1.9.2 program. Accepting Pata et al.14 study in the literature as a reference for the thickness of the fibrous tissue, the sample size for each group was defined to include at least seven rats to test a one-way hypothesis with a high effect size of 1.5, 80% power, and 5% margin of error.

The data collected in the study was analyzed with SPSS 22.0 (IBM Corp., Armonk, NY) program. The descriptive statistics of the continuous variables were expressed as mean ± standard deviation; for the categorical variables, frequencies and percentages were used. The t test for two independent samples was used to compare the means of two independent groups. The chi-square and Fisher exact tests were used as appropriate to analyze the differences between the categorical variables. The difference was accepted to be significant at a level of P < 0.05.

RESULTS

The following parameters were determined in each histology slide, including the epithelial damage; fibrosis; Turkmen and Pata: Effect of Pirfenidone After Tracheotomy; Francisco, CA, Lot No: A2142, 1:50 dilution), and IL-1BETA ([H-153]: SC 7884; Santa Cruz Biotechnology, Santa Cruz, CA, Lot No: E1914, 1:50 dilution] using a Leica Bond Max immunohistochemical staining device (Leica Biosystems, Wetzlar, DEU). Depending on the degree of staining, the sections stained by immunohistochemical staining were scored in a range from 0 to 3 under an Olympus BX 51 microscope (Olympus Corporation, Tokyo, Japan).
TABLE I.
Histopathological Evaluation of Epithelial Damage, Inflammation, and Fibrosis Parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(-) No</th>
<th>(+) Mild</th>
<th>(+++) Moderate</th>
<th>(+++) Severe</th>
</tr>
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<tbody>
<tr>
<td>Epithelial damage</td>
<td>&lt; 25%</td>
<td>26%–50%</td>
<td>51%–75%</td>
<td>&gt; 75%</td>
</tr>
<tr>
<td>Inflammation</td>
<td>There are 0–30 inflammatory cells in mm³ (×400 magnification)</td>
<td>There are 31–100 inflammatory cells in mm³ (×400 magnification)</td>
<td>There are 101–150 inflammatory cells in mm³ (×400 magnification)</td>
<td>There are more than 151 inflammatory cells in mm³ (×400 magnification)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>No fibrosis findings</td>
<td>Mild degree of fibrosis</td>
<td>Moderate degree of fibrosis</td>
<td>Severe degree of fibrosis</td>
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Fig. 1. (a, b) Histology of a rat trachea without signs of fibrosis (−) in group P and with (+++) fibrosis (black arrow) in group C (MT staining, 200 × -fold, 400 × -fold magnification). (c, d) Histology of a rat trachea with complete regeneration of damaged epithelium (black arrow) in group P and with damaged epithelium (black arrow) in control group C (H&E staining, 200 × -fold magnification). (e, f) Histology of a rat trachea without signs of inflammation in group P and with (+++) inflammation (black arrows) in group C (H&E staining, 200 × -fold magnification). H&E = hematoxylin and eosin; MT = Masson Trichrome.
inflammation; histomorphometric measurements of the internal diameters of tracheal lumens and mucosal thicknesses; and the levels of TGFβ-1, TNFα, and IL-1β evaluated by immunohistochemical methods. The investigators who performed the evaluations were blinded to the groups.

**Microscopic Findings**

The numerical values of the findings of inflammation, epithelial damage, and fibrosis; and the immunohistochemically defined expression levels of TGFβ-1, TNFα, and IL-1β were determined for each group.

**Fibrosis**

In the pirfenidone group, almost no fibrosis was observed in two subjects, and mild fibrosis was observed in five subjects. In the control group, the degrees of fibrosis were mild in two subjects, moderate in three, and severe in two.
When the groups were compared by the degrees of fibrosis, there was a statistically significant difference between the pirfenidone and control groups ($P = 0.04$; the statistical significance was accepted at the level of $P < 0.05$) (Fig. 3a).

**Epithelial Damage**

The epithelial damage was evaluated under a light microscope. In the pirfenidone group, mucosa epithelium was observed to be regenerated in the injured area in all rat subjects but one.

When the two groups were compared by the degree of epithelial damage, there was not a statistically significant difference between the pirfenidone and the control groups. ($p: 0.549$; the statistical significance was accepted at the level of as $P < 0.05$.) In the pirfenidone group, the epithelial regeneration was almost complete in all rat subjects excluding one subject (Fig. 3b).

**Inflammation**

In the pirfenidone group, the inflammation findings were mild in four subjects, moderate in one subject, and severe in two subjects; however, it was observed in the control group that they were mild in one subject, moderate in two subjects, and severe in four subjects (Fig. 3c).

Fig. 3. (a) Distribution of the rats in both groups regarding severity of fibrosis. (b) Distribution of the rats in both groups regarding severity of epithelial damage. (c) Distribution of the rats regarding severity of inflammation. (d) Distribution of the rats in both groups regarding TGFβ-1 expression. (e) Distribution of the rats in both groups regarding TNFα expression. (f) Distribution of the rats in both groups regarding IL-1β expression. IL-1β = Interleukin-1 beta; TGFβ-1 = growth factor beta; TNFα = tumor necrosis factor alpha.
Although the inflammation was inhibited to a higher extent in the pirfenidone group, there was not a statistical difference between the two groups ($P = 0.247$; the statistical significance was accepted at the level of $P < 0.05$).

**Immunohistochemical Findings**

**TGFβ-1.** The degrees of the expression of TGFβ-1 were mild in six subjects and moderate in one in the pirfenidone group; however, they were qualified as moderate in four and severe in three subjects in the control group (Fig. 3d). The comparison of the groups by the degree of TGFβ-1 expression revealed a statistically significant difference between the pirfenidone and control groups ($P = 0.005$; the statistical significance was accepted at the level of $P < 0.05$).

**TNFα.** In the pirfenidone group, the expression of TNFα was qualified to be severe in one subject and mild in five subjects, whereas it was almost not at all expressed in one subject. In the control group, the degrees of the expression of TNFα were mild in one subject, moderate in five, and severe in one (Fig. 3e).

In the comparison of groups by degree of TNFα expression, there was a statistically significant difference between the pirfenidone and control groups ($P = 0.034$; the statistical significance was accepted at the level of $P < 0.05$).

**IL-1β.** The degrees of the expression of IL-1β were qualified to be mild in six and moderate in one subject in the pirfenidone group; however, they were mild in one subject, moderate in four, and severe in two in the control group (Fig. 3f).

When the two groups were compared by the degrees of IL-1β expression, there was a statistically significant difference between the pirfenidone and control groups ($P = 0.025$; the statistical significance was accepted at the level of $P < 0.05$).

**Morphometric Findings**

The internal diameter of the lumen and the thickness of mucosa was measured in μm (micrometers) in the course of the histomorphometric evaluation of the slides (Fig. 4A, Fig. 4B, Fig. 4C, and Fig. 4D).

The comparison of the groups by the thicknesses of tracheal mucosa did not reveal a statistically significant difference between the pirfenidone and control groups ($P = 0.995$; the statistical significance was accepted at the level of $P < 0.05$).

The results of the histomorphometric evaluation showed that the internal diameters of the tracheal lumens were significantly wider in the pirfenidone group compared to the control group. There was a statistically significant difference between the two groups ($P = 0.01$; the statistical significance was accepted at the level of $P < 0.05$). The power of the study was found out to be 79.24% by post hoc analysis.

**DISCUSSION**

Tracheal stenosis is a pathological scar with a prolonged inflammatory phase of cicatrization leading to fibrosis in the area of the lesion by the increased production of TGFβ, collagen synthesis, and increased levels of accumulation. Although the surgical methods used in the treatment of TS are the treatment of choice, they are still not effective by 100%. Therefore, it is important to prevent the development of fibrosis before stenosis occurs.

As adjuncts to the surgical interventions, primarily mitomycin C has been used in the treatment of TS, followed by several other pharmacological agents including the steroids, antibiotics, anti-reflux medications, halofuginone, 5-FU (fluorouracil), and carnitine. These treatment options were used in order to be effective on one or more stages of wound healing, including inflammation, proliferation, maturation, and remodeling.

The corticosteroids and antibiotics are effective during the stages of inflammation and proliferation; mitomycin C, the combination of 5-FU and triamcinolone acetate, and carnitine are effective during the proliferation stage; and halofuginone, β-aminopropionitrile, colchicine, penicillamine, and N-acetyl-L-cysteine are effective during the maturation stage. Provided by its mechanism of action, pirfenidone is effective during all three stages of wound healing.

Hirshoren and Eliashar reviewed the PubMed and Ovid databases covering the years between 1960 and 2007. They have found out that the agents modulating the wound-healing process were used in the treatment of upper airway stenoses; however, they reported these were not investigated sufficiently. Furthermore, they found out controversial findings associated with their roles in the prevention and treatment of subglottic stenoses. Evaluating these data, we preferred to investigate the effects of pirfenidone as an effective agent on all three stages of wound healing and as an agent with anti-inflammatory, antioxidant effects in the literature, as investigated in three studies on the prevention of tracheal stenosis.

Pirfenidone is approved by the FDA to be used in the treatment of idiopathic pulmonary fibrosis. The data reported thus far by the studies conducted on pirfenidone inform that it has a minimum level of side effects, including diarrhea, nausea, photosensitivity, rash, and vomiting.

After it was discovered that pirfenidone had antifibrotic effects on the pulmonary fibrosis induced by bleomycin in hamsters, it was studied in several models of fibrotic diseases, demonstrating its antifibrotic, anti-inflammatory, and antioxidant effects. Liu et al. conducted a study on lung transplantation on two different species of rats and demonstrated that 0.5% pirfenidone administered in the food inhibited peribronchial and interstitial fibrosis. Zhou et al. studied a heterotopic tracheal allograft model on murines and demonstrated that the degrees of inflammation, intraluminal degranulation, and fibrosis were reduced in the transplanted tissues collected from the murine subjects, which were administered 0.5% pirfenidone in the food for prophylactic purposes.
Wigenstam et al.\textsuperscript{27} conducted a study in 2018 and observed that, with some mucosal damage occurring to a lesser degree, there was no pulmonary edema. In addition, the development of fibrosis was inhibited compared to the control group, as revealed by the histopathological examination of the tissues collected from the subjects treated with three doses of 200 mg/kg intraperitoneal pirfenidone after inducing iatrogenic pulmonary damage by sulfur dioxide.

Cardiac fibrosis has similarities with the fibrosis occurring in the other organs, as demonstrated both at the cellular and molecular levels. Mirkovic et al. demonstrated a reduction in the degree of hypertrophy in the ventricular myocytes and significantly prevented the development of cardiac fibrosis caused by hypertension\textsuperscript{28} in hypertensive rats, which were administered oral pirfenidone at approximately 250 to 300 mg/kg/day doses. Similarly, in an ischemic reperfusion model on rats, Nguyen et al.\textsuperscript{29} reported that the left ventricular fibrosis developing after myocardial infarction occurred at a 50% lesser degree in the subjects administered 1.2% pirfenidone in the food when compared to the results of the control group.

Several studies have been conducted to investigate the effect of pirfenidone on renal fibrosis. Shimizu et al.\textsuperscript{30} conducted a study with the aim of reducing renal fibrosis induced by unilateral ureteral obstruction, prophylactically administering an oral dose of 500 mg/kg/day pirfenidone in the food. They reported a reduction in the accumulation of collagen and the degree of fibrosis. In two different studies on a model of subtotal nephrectomy in rats as a good model for chronic kidney disease, Takakuta et al.\textsuperscript{31} and Shimizu et al.\textsuperscript{32} demonstrated the antifibrotic and anti-inflammatory effects of pirfenidone at a level of significance.

Another organ is the liver, on which fibrotic disease models have been studied commonly. In their study, Salazar-Montes et al.\textsuperscript{33} reported that pirfenidone administered at oral doses of 200 mg/kg prevented the development of liver fibrosis by 40% in a liver fibrosis model induced by carbon tetrachloride. Another common experimental model is the liver fibrosis induced by dimethylnitrosamine. Tada et al.\textsuperscript{34} conducted a study where an area of fibrosis was created iatrogenically by the chronic administration of dimethylnitrosamine and reported that pirfenidone administered at oral doses of 500 mg/kg reduced the area of fibrosis by 40%.

The efficacy of pirfenidone was investigated in cellular level models, too, besides the fibrotic disease models. Hall et al.\textsuperscript{4} conducted a study on myofibroblasts derived from the human skin covering the mammary tissue, stimulating the cell cultures with different doses of pirfenidone. They observed that pirfenidone reduced the profibrotic responses of the myofibroblasts in a dose-dependent manner.

Another recent experimental study at the cellular level has been conducted by Kodama et al.\textsuperscript{7} aiming to prevent the scar tissue formation in the vocal folds. After forming a scar tissue by electrosurgery in the vocal cords of ferrets, the animals were treated with pirfenidone. The authors observed that the collagen gel contraction and collagen expression was suppressed in the fibroblasts isolated from these vocal cords of the animals.
We employed an animal model in our study consisting of tracheotomized animals intubated by a tracheal tube, which has already been described as a model for TS,14,35,36 and investigated the effects of pirfenidone on the initial stages of the fibrotic process using this model. Our study has demonstrated that pirfenidone is effective in preventing TS developed due to the effects of tracheostomy and the pressure caused by the long-term indwelling of the cannula. The histopathological examination of the tracheal sections revealed almost normal findings in the pirfenidone group compared to the control group. The narrowing in the intraluminal diameter of the trachea has occurred to a significantly lesser degree compared to the control group. Immunohistochemical examinations have shown that the expression of important cytokines of inflammation, namely TGF-β, TNFα, and IL-1β, have been suppressed significantly compared to the control group.

There is only one study available in the literature conducted to examine the use of pirfenidone in tracheal stenosis similar to our study. Olmos-Zuniga et al.3 conducted a study on tracheal wound healing and administered two oral doses of 40 mg/kg/day pirfenidone to one group of animals who underwent tracheoplasty and an end-to-end anastomosis. The authors concluded that the subjects in that group developed a lesser amount of fibrous tissue in the region of anastomosis, the diameters of the tracheal lumens were close to those values measured preoperatively, and the expression of TGF-β1 occurred at a lesser degree compared to the other groups. Similarly, Orozco-Perez et al.10 conducted a study using an esophageal stricture model and demonstrated that the fibrosis was prevented in the pirfenidone group by the suppressed expression of the profibrotic genes, resulting in the development of narrowness in the esophageal lumen to a lesser degree.

This study has several limitations: The first is small group sizes. The reason why we create small groups is based on the principle of reduction of the animal experiments ethics, 3Rs rule (Replacement, Reduction, Refinement). However, the results of our study are convincing when the results of the post hoc power analysis considered. The second limitation is a short observation period. There will be need for further research with longer observation period in order to find the best results in tracheal mucosa and structure changes. The third limitation of our study is the intraperitoneal administration of pirfenidone. There is not enough data on dose optimization for intraperitoneal administration in previous animal experiments. Further studies are needed to determine the optimal parenteral dose of pirfenidone and use it in clinical trials.

CONCLUSION

Despite the several treatment options used, restenosis commonly occurs after tracheal stenosis as a result of developing fibrosis after treatment. Therefore, the recent studies on tracheal stenosis have focused on medications efficacious on the processes of wound healing. Our study demonstrates that pirfenidone can be used to prevent tracheal fibrosis due to its antifibrotic and anti-inflammatory effects. However, we are of the opinion that more extensive studies will be further required for pirfenidone to be used in clinical studies associated with tracheal stenosis in the human.

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