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Effectiveness of Curcumin on Tracheal Anastomosis Created in the Rat Model

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Abstract

Objective: We aimed to investigate the effect of curcumin on tracheal wound healing in the anastomosis model following tracheal incision in rats.

Methods: Twenty-one healthy male Wistar Albino rats were included in the study. A horizontal incision was made between the 2nd-3th rings of the rat trachea and damage was created in the inner tracheal mucosa. Tracheal incision area was sutured and anastomosis was performed. Rats were divided into three groups. Ringer was given to the first group, corticosteroids to the 2nd group, and curcumin to the 3rd group for 28 days. The rats were sacrificed on the 28th day and the tracheal anastomosis line samples were sent for histological examination. Wound healing parameters, tracheal lumen width, wall thickness, and stenosis index were evaluated.

Results: Statistically significant difference was detected in tracheal lumen width and wall thickness in the curcumin group ($p<0.05$). Statistically significant differences were observed in the curcumin group in parameters of inflammation, collagen production and fibrosis. ($p<0.05$).

Conclusion: It is important to prevent the formation of tracheal stenosis, which is difficult to treat in clinical practice. Desired results cannot be achieved in the side-effect profile of some medical drugs used in this regard. We found the positive effect of Curcumin in the prevention of stenosis following tracheal injury.

Key Words: Rat, Trachea, Stenosis

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INTRODUCTION

Tracheal stenosis is one of the difficult pathologies that usually develops as a result of prolonged endotracheal intubation or tracheotomy. Stenosis can also develop as a result of trauma, some autoimmune diseases and neoplasia. It has been reported that stenosis developed in 66% after intubation and 26% after tracheotomy (1). Factors affecting the development of tracheal stenosis include the timing and mode of mechanical ventilation, mucosal damage caused by endotracheal tube cuff pressure, radiotherapy and recurrent upper respiratory tract infections (2,3). Today, despite many studies, the pathological mechanism and risk factors of tracheal stenosis have not been fully understood (4). There are different treatment modalities such as endoscopic methods, open surgical procedures, mitomycin-c, steroid therapy in its treatment. Among surgical treatments, resection of the stenotic area and end-to-end anastomosis stand out as the best method (5). Restenosis can be seen even after the most appropriate intervention. The uncontrolled excess of granulation tissue seen during wound healing as a result of tracheal mucosal damage is accepted as a precursor lesion for contraction and stenosis in wound healing (6).

Curcumin is a natural polyphenol compound and has been used in traditional medicine for centuries in Far East Asian countries. It is known that curcumin has antioxidant, anti-inflammatory activity and positive effects on wound healing (7). Although there are many studies on the effectiveness of curcumin on many inflammatory diseases, studies on tracheal stenosis have not been conducted. The aim of our study was to determine the efficacy of curcumin in preventing stenosis secondary to experimentally induced tracheal injury. In order to evaluate the efficacy of curcumin, granulation tissue secondary to tracheal damage performed on rat models was formed and histological parameters were determined.

METHODS

The study was approved by the animal experiments ethics committee of Kahramanmaraş Sütçü İmam University Faculty of Medicine, with the date/ protocol number 27.02.2020/13 and the decision number 06, and was carried out at Kahramanmaraş Sütçü İmam University Experimental Animals Application and Research Center.

Animals

The minimum sample size was calculated as 7 for each group, with a 95% confidence interval and 5% tolerable error assumption, based on the study by Mirapoglu et al. (8). This study was carried out using 21 healthy and adult male Wistar-Albino type rats, weighing 250-350 grams, obtained from Kahramanmaraş Sütçü İmam University Medical Faculty Experimental Animal Laboratory. The rats were kept in groups of seven in 3 separate cages at 22 ± 2 °C, in a humidity-adjusted

environment suitable for a 12-hour light and dark cycle, and were fed ad libitum with standard feed and tap water.

Drugs

Curcumin preparation Sigma-Aldrich (Product number; C1386-10G, St Louis, MO, USA), Methylprednisolone drug (Prednol-L 40 mg. ampoule, Mustafa Nevzat) and Ringer's solution (Bioflex Ringer Lactate 500 ml solution, Osel) were used in the study.

Study Design

The rats were divided into three groups of seven as control group and two study groups. The 1st group was the control group, the 2nd group was the curcumin group, and the 3rd group was the steroid group.

Surgery

Anesthesia was achieved by administering 75-100 mg/kg ketamine hydrochloride (Ketalar 10 ml vial, E.Warner Lambert) and 0.5 mg/kg chlorpromazine (Rompun 50 ml 2% flacon, Bayer) intraperitoneally to the rats in all groups, and they were allowed to breathe spontaneously. A milliliter of epinephrine lidocaine hydrochloride (Jetokaine ampul, Adeka) was administered subcutaneously to the anterior neck of the rats in the supine position as a local anesthetic. The anterior neck of the rats was shaved and cleaned with povidone iodine (Poviiodeks ®,1000 ml). The operation area was covered sterile. Then, surgical procedures were performed under the operating microscope and with a microsurgery set. Strap muscles were reached by starting with a vertical two-centimeter incision made from the upper part of the thyroid cartilage to the incisura jugularis level in the midline. The submandibular gland was removed superiorly and the strep muscles were retracted laterally, the pretracheal fascia was passed, and the trachea was exposed. Inferior thyroid vein and recurrent laryngeal nerves were identified in the tracheoesophageal groove. The tracheal lumen was reached by making a horizontal incision with the help of the number 11 scalpel between the second and third rings of the trachea, based on the inferior thyroid veins laterally. Tracheal inner mucosa was damaged with the help of microcurette. Open tracheal ends were anastomosed end-to-end with 6/0 vicryl (Ethicon, Diegem, Belgium) starting from the lateral side. Approximately two sutures were placed, leaving the knots outside the trachea (Fig.1). After the bleeding control, the layers were duly closed, the operation was terminated, and the rats were placed in their cages after awakening. All rats in three groups were kept in the same environment and fed the same way. Peroperative intraperitoneal 50 mg/kg cefazolin sodium (Cefazol 250 mg, Mustafa Nevzat) was administered. The rats, who were followed up for respiratory distress after the operation, were taken to their cages when they woke up.

Treatment Modalities

All rats were followed for 28 days, which is known as sufficient time for stenosis

development after tracheal injury (9). Meanwhile, 1 mg/kg saline solution i.p. was administered to the control group, 100 mg/kg curcumin per oral to the 2nd group (curcumin group), and 1 mg/kg methylprednisolone i.m. to the 3rd group (steroid group) once a day. The administered drug doses were applied with reference to literature studies (10,11).

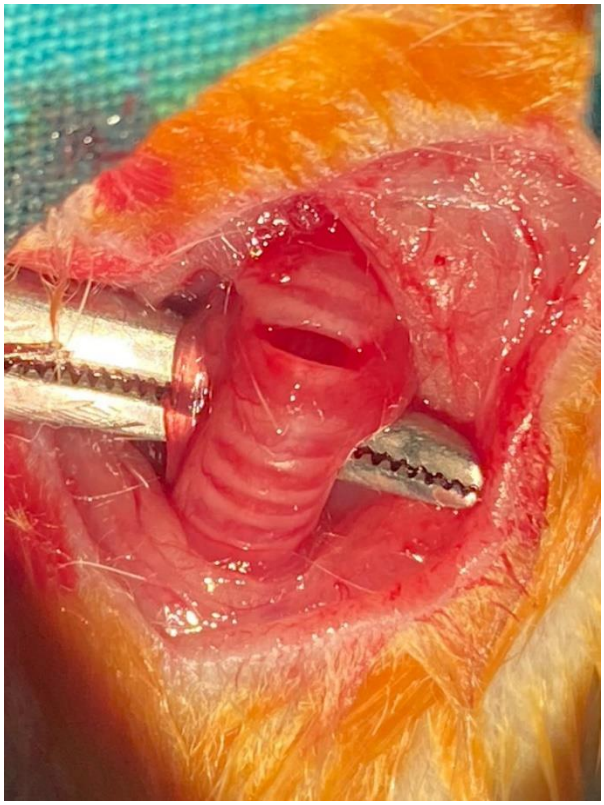


Figure 1. Incision and suturation of Trachea

Histological Evaluation

At the end of the follow-up periods, the rats in the study and control groups were sacrificed with high-dose intraperitoneal pentothal on the same day, and tracheal segments of five millimeters in length including the anastomosis line were removed.

The cross-sectional areas of the trachea were measured histometrically and compared. These excised tracheal segments were placed in 10% formaldehyde and turned into paraffin blocks, and then 4 micron thick sections were taken. Cross-sections of 4 microns were created using a Shandon FINESSE-ME microtome (Thermo Fisher Scientific, Waltham, MA). These sections were stained with hematoxylin and eosin and evaluated for inflammatory cell infiltration, epithelial degeneration and regeneration. The mean of these two diameters was determined by measuring the largest diameter and the diameter perpendicular to this diameter in the sample selected for the average lumen width. For the wall thickness, the mean value of the maximum and minimum distance between the luminal surface of the mucosal surface epithelium and the outer radial border of the wall was determined. Tracheal cartilage tissue was included in the wall thickness. The following formula was used to calculate the stenosis index; stenosis index = (wall thickness/2)/(lumen diameter). n addition, Masson trichrome staining was performed to evaluate collagen deposition by collagen fiber density. A semi-automated stereology workstation stage for stereological analysis consisting of a digital camera (Nicon Coolpix E 4500, Tokyo, Japan), image capture card (Flash Point 3D, Integral Technologies, Indianapolis, IN, USA), personal computer and computer-controlled motorized sample (Proscan II, Prior,

Rockland, MA, USA), a microcamera (Heidenhein, Traunreut, Germany) and a light microscope (Nicon, Eclips E 600, Tokyo, Japan) were used. In order to evaluate wound healing, the degrees of inflammation (polymorphonuclear leukocytes (PMNL), lymphocytes, histiocytes, macrophages), fibrosis, neovascularization and epithelialization were examined. In all parameters; mild, moderate and severe characterizations were made as follows: 0-10 inflammatory (PMNL, lymphocyte, histiocyte, macrophage) cells (-), 11-100 inflammatory cells mild or (+), 101-1000 inflammatory cells moderate or (++), more than 1000 inflammatory cells were evaluated as severe or (+++) inflammation. If the thickness of the collagen fibers was normal, it was evaluated as (-), minimal thickening as mild or (+), moderate thickening as moderate or (++), intense thickening as severe or (+++) fibrosis. In terms of neovascularization, if there is no neovascularization in the x 400 magnification field (-), less than 3 neovascularization is mild or (+), 4-10 neovascularization is moderate or (++), more than 10 neovascularization is severe or (+++). These parameters were digitized for statistical evaluation, and (-) evaluations were scored as 1, (+) evaluations as 2, (++) evaluations as 3, (+++) evaluations as 4 (12).

Statistical Analysis

In the evaluation of the data, the conformity of the variables to the normal distribution was examined with the Shapiro Wilk test. The comparisons of 3 groups in the variables conforming to the normal distribution were examined with the One Way Anova test. Tukey HSD test, Tamhane T2 test and Dunnett test were used for pairwise comparisons. Comparisons of 3 groups in variables that did not conform to normal distribution were analyzed with the Kruskal Wallis H test. Dunn's test, one of the post-hoc tests, was used for pairwise comparisons. The differences in frequency distributions according to the groups in qualitative variables were examined with the Chi-Square test and Exact test. Statistical significance was accepted as $p<0.05$. Statistical parameters were expressed as Mean±SD, Median (25% quartile-75% quartile) and n(%). IBM SPSS version 22 (IBM SPSS for Windows version 22, IBM Corporation, Armonk, New York, United States) and R.3.3.2 software were used to evaluate the data.

RESULTS

When evaluated in terms of inflammation parameters, it was found that inflammatory cell infiltration, fibrosis and collagenosis parameters in the curcumin group were statistically significantly lower than in the other groups (Fig. 2,3). There was no significant difference between the groups in neovascularization, epithelial degeneration and regeneration parameters. Histopathological findings of all groups are given in Table 1.

Table 1. Comparison of Histopathological Parameters Between Groups

		None		Mild		Moderate		Intensive		
		N	%	N	%	N	%	N	%	P
Inflammation	Curcumin	3	42,9	3	42,9	1	14,3	0	0,0	**0.032***
	Steroid	0	0,0	2	28,6	4	57,1	1	14,3	
	Control	0	0,0	2	28,6	2	28,6	3	42,9	
Fibrosis	Curcumin	2	28,6	3	42,9	2	28,6	0	0,0	**0.047***
	Steroid	1	14,3	3	42,9	2	28,6	1	14,3	
	Control	0	0,0	1	14,3	3	42,9	3	42,9	
Collagen	Curcumin	4	57,1	1	14,3	2	28,6	0	0,0	**0.028***
	Steroid	2	28,6	2	28,6	2	28,6	1	14,3	
	Control	1	12,5	3	37,5	1	14,3	2	28,6	
Neo vascularization	Curcumin	1	16,7	1	16,7	2	33,3	3	42,9	0.940
	Steroid	1	14,3	2	28,6	2	28,6	2	28,6	
	Control	0	0,0	3	42,9	2	28,6	2	28,6	
Epithelial degeneration	Curcumin	0	0,0	2	28,6	2	28,6	3	42,9	0.739
	Steroid	1	14,3	2	28,6	3	42,9	1	14,3	
	Control	2	28,6	2	28,6	2	28,6	1	14,3	
Epithelial regeneration	Curcumin	1	14,3	2	28,6	3	42,9	1	14,3	0.651
	Steroid	1	14,3	3	42,9	2	28,6	1	14,3	
	Control	2	28,6	2	28,6	2	28,6	1	14,3	

*Exact test; p< 0.05 difference statistically significant

When the histomorphometric results are examined, it is seen that the lumen width is greater in the curcumin group than in the other groups (Graph 1, p<0.001). Statistically significant wall thickness was lower in the curcumin group and steroid group compared to the control group (Graph 2, p=0.011, p=0.002). There was no significant difference between the curcumin group and the other groups in the stenosis index (Graph 3, p=0.151). There was no significant bleeding during or after the operations in any of the groups during the study period. We did not observe any wound infection in the groups. No cutaneous or esophageal fistula developed in any of the rats, and there was no rat death in any group.

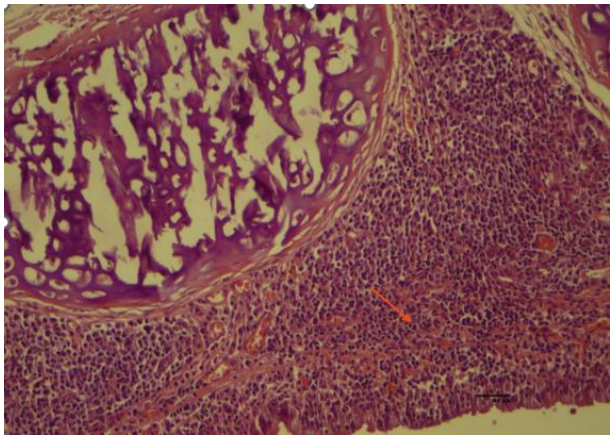


Figure 2a. Section from the tracheal injury site of a rat in the control group. Severe lymphoplasmocytic inflammation in the lamina propria (marked with an arrow) (H/EX200)

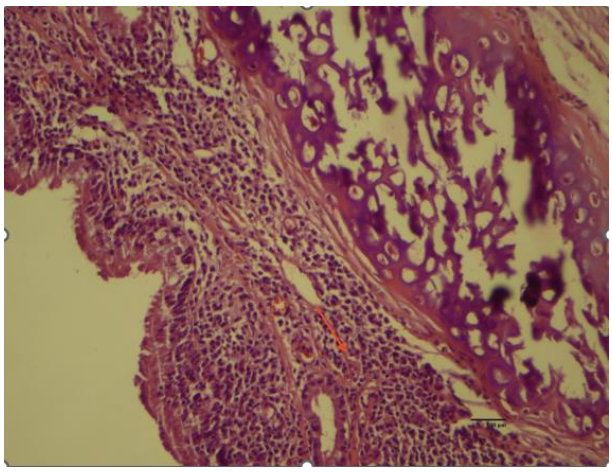


Figure 2b. Section from the tracheal injury site of a rat in the steroid group, moderate lymphoplasmocytic inflammation in the lamina propria (marked with arrow) (H/EX200)

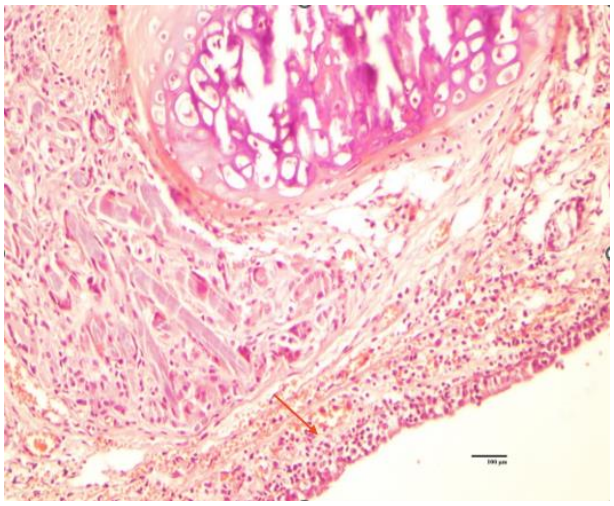


Figure 2c. Section from the tracheal injury site of a rat in the curcumin group, mild lymphoplasmocytic inflammation in the lamina propria (marked with arrow) (H/EX200)

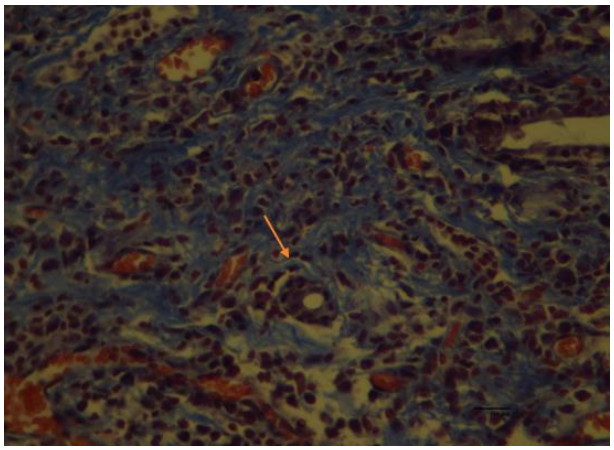


Figure 3a. Moderate fibrosis (Asterisk) with a condensed and bundled bluish appearance in the control group (MTX400)

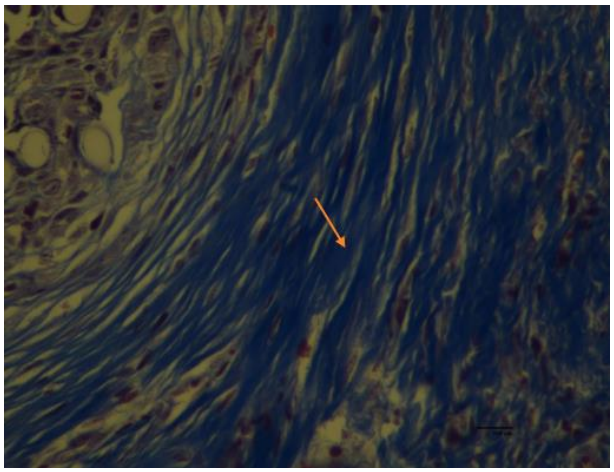


Figure 3b. Moderate fibrosis with a bluish appearance concentrated in the steroid group (marked with an asterisk) (MTX400)

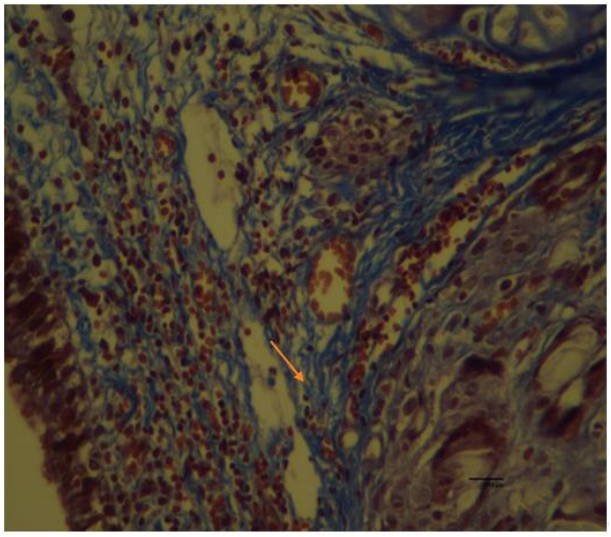
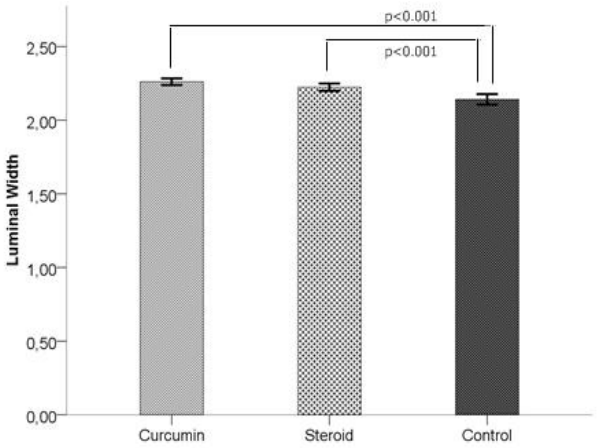
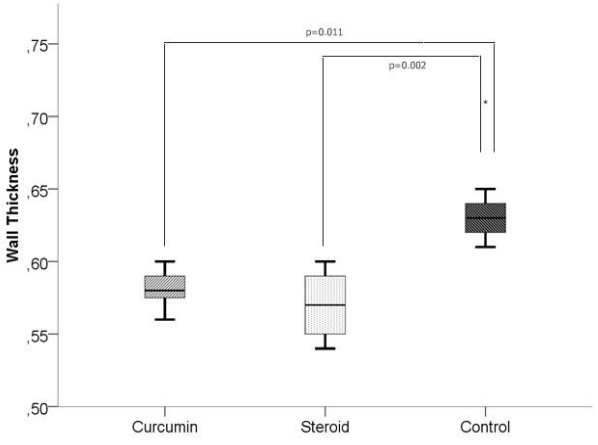


Figure 3c. Mild fibrosis (Asterisked) stained bluish in the curcumin group (MTX400)



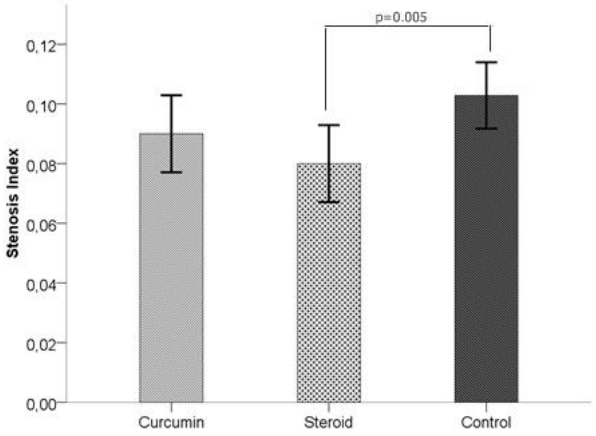
* lumen width measurement unit is mm

Graph 1. Comparison of Tracheal Lumen Width between Groups



*Tracheal Wall Thickness measurement unit is mm

Graph 2. Evaluation of tracheal wall thickness between groups



Graph 3. Evaluation of Intergroup Stenosis Index

DISCUSSION

Tracheal stenosis is narrowing of the airway caused by hypertrophic scar tissue that develops as a result of abnormal wound healing in the tracheal lumen. Damage to the mucosal tissue on the cartilage ring of the trachea leads to ischemic necrosis (13). In the acute phase of wound healing secondary to tracheal injury, polymorphonuclear leukocytes and macrophages are abundant in injured tissue. Macrophages increase the release of chemical mediators such as interleukins (IL) 1 and 6, tumor necrosis factor - α (TNF -α) and transforming growth factor - β (TGF - β), which play an important role in wound healing. These chemical mediators activate endothelial cells. With the effect of factors such as Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF) and TGF-β, fibroblasts and keratinocytes migrate to the wound to repair wound healing and initiate collagen synthesis (14). TGF-β1 overexpression, which plays a key role here, causes overproduction of matrix metalloproteinase enzyme. This situation continues with the overexpression and contraction of collagen synthesis. This pathological condition, which is observed in the tracheal mucosa, then progresses to granulation tissue and fibrosis with the involvement of deep tissues, resulting in thickening of the submucosal and mucosal layer and thus stenosis in the tracheal lumen(15). Considering this situation, reducing fibrosis by regulating wound healing in treatment will be an effective application in preventing the formation of tracheal stenosis (16). For this purpose, we used curcumin, which has a strong immunomodulatory activity in the prevention of experimental tracheal stenosis. Our histopathological and histomorphometric data showed that curcumin may be effective in preventing tracheal stenosis.

The treatment applied in tracheal stenosis varies according to the patient's clinic and classification of stenosis. While endoscopic methods are applied to cases with Grade I-II stenosis according to the Cotton-Myer classification, surgical treatments are prominent in cases with grade III-IV stenosis (17). Along with surgical treatment, bronchoscopic dilatation, laser ablation, electrocautery, stent application, medical treatment is also performed (18). It has been used to prevent restenosis after medical treatment, surgical or bronchoscopic intervention and to reduce the need for additional intervention. Corticosteroids are one

of the first drugs used for this purpose (19). Corticosteroids inhibit the expression of injury-induced growth factors and cytokines released by macrophages at the wound site with their immunosuppressive effects, delay wound contraction and impair the healing of epithelialization (20). In some studies, on steroids, which seem to be an effective drug in preventing stenosis, it has been reported that systemic corticosteroid administration is insufficient to prevent stenosis formation. In addition, it is seen that there is no accepted approach in the literature in terms of effective dose, application form and duration of corticosteroids in preventing tracheal stenosis (21-23). On the other hand, it has been stated that intralesional steroid administration can be an effective treatment in preventing the formation of restenosis (24). In the treatment of tracheal stenosis, drugs such as 5-Fluorouracil, carnitine, mitomycin-C are also used in addition to steroids (25-27). However, the effectiveness of these drugs is also limited. To the best of our knowledge, there is no study with curcumin in the prevention of tracheal stenosis. Our work on this issue can be considered as a pioneering work. In our study, it is noteworthy that the tracheal lumen width was wider and the wall thickness was less in the curcumin group compared to the control group. Considering that the results were similar with the group given steroid treatment, we think that curcumin may be as effective as steroids.

Curcumin (diferuloylmethane) is a natural polyphenol compound obtained from the root of turmeric (curcuma longa) in the ginger family in the South Asian region. It is used in the prevention and treatment of various diseases such as pain treatment, digestive system, skin, liver diseases, menstrual diseases and chronic wounds in Far East Asian traditional medicine practices (28). Curcumin's healing effect is due to its beneficial effects such as antioxidant, anti-inflammatory, antiproliferative, anti-infectious. In recent studies, the anti-inflammatory activity of curcumin is influenced by cytokines such as PDGF, VEDF, TGF-β, TNF – α, IL – 1,6,8, nuclear factor – κB (NF- κB), cyclooxygenase, protein kinase, Matrix Metalloproteinase (MMP). It has been shown to be associated with the regulation of the activity of mediators and enzymes that are effective in the inflammatory process (28,29). Effects such as reepithelialization, acceleration of wound healing and prevention of fibrosis formation were observed in biopsies taken from wounds treated with curcumin. This effect of curcumin is associated with its regulation of TGF-β and inducible Nitric Oxide synthetase (iNOS). While it induces TGF-β in normal wound healing, it prevents overexpression(30). At the same time, it has been observed that curcumin inhibits the overexpression of proangiogenetic growth factors VEGF, FGF, and Epidermal Growth Factor (EGF)(31). This indicates that

curcumin can prevent hypertrophic scarring and advanced fibrosis, which are extreme forms of wound healing. In our study, it was found statistically significant that the parameters of inflammation, collagenization and fibrosis were lower in the curcumin group compared to the other groups. We think that it can be an effective treatment agent in the prevention of acquired tracheal stenosis when evaluated together with factors such as tracheal lumen width and wall thickness.

The main limitations of our study are performing the tracheal intervention by making an incision on the anterior surface of the trachea and not removing the tracheal ring. Since it is a surgical procedure with a high risk of mortality and morbidity, the surgical procedure was performed on the tracheal anterior surface in order to minimize rat death and complications. In addition, the inability to perform immunohistochemical staining and electron microscopic examination are among the other limitations of the study.

CONCLUSION

It is important to prevent the formation of tracheal stenosis, which is difficult to treat in clinical practice. Desired results cannot be achieved in the side-effect profile of some medical drugs used in this regard. There is a need for preparations that have less adverse effects and are at least as effective as existing drugs in preventing the formation of stenosis. In our study, we determined that curcumin may be as effective as corticosteroids. We believe that our study will shed light on advanced studies on tracheal stenosis.

Ethics Committee Approval: The study was approved by the animal experiments ethics committee of Kahramanmaraş Sütçü İmam University Faculty of Medicine, with the date/ protocol number 27.02.2020/13 and the decision number 06 and was carried out at Kahramanmaraş Sütçü İmam University Experimental Animals Application and Research Center.

Author Contributions:

Concept: M.G.Y, E.K, N.Y Design: M.G.Y, E.K, N.Y; Literature search: D.C, A.D, Data Collection and Processing: I.O, A.Y, Analysis or Interpretation A.D, Writing: M.G.Y, E.K, D.C

Conflict of Interest: There are no conflicts of interests related to this study.

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