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Evaluation of the Efficacy of Colchicine, Pirfenidone and Prednisolone in Preventing Stricture Due to Inflammation as a Result of Urethral Mucosal Damage in Rats

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What's known on the subject? and What does the study add?

Urethral stricture is a disease characterized by fibrotic scar formation causing narrowing of the urethra, decreased urethral volume, and failure of normal voiding function, and can frequently recur despite endoscopic or open surgical treatment. We administered pirfenidone, colchicine, and corticosteroids orally to prevent proliferation, excessive fibrosis, and scarring during the healing process of urethral stricture. This is the only study in the literature comparing three oral pharmacological agents with known anti-inflammatory and antifibrotic properties to prevent recurrence of urethral stricture. As a result of clinical studies, we believe that the use of these agents before and after all interventions for urethral stricture should be included in clinical practice.

Abstract

Objective: To evaluate the effectiveness of oral use of drugs with anti-inflammatory and antifibrotic properties that are used in the treatment of many diseases in clinical practice in rats with stenosis caused by urethral damage.

Materials and Methods: Forty male rats were equally divided into 5 groups. After anesthesia, penectomy was performed in group 1 without any other procedure. Urethral stenosis was created in rats in the groups 2, 3, 4, and 5. The rats in group 2 underwent penectomy at the end of 6 weeks. Groups 3, 4, and 5 were treated with colchicine, prednisolone, and pirfenidone, respectively by gavage for 6 weeks and underwent penectomy. Inflammation, fibrosis, and urethral lumen area were evaluated histopathologically in urethral tissue or all animals.

Results: The urethral lumen area increased in group 4, although this incease was not statistically significant compared to group 2. In addition, prednisolone lead to a significant decrease in the inflammation and fibrosis scores compared to group 2. We also found that there was a significant decrease in inflammation and fibrosis scores and a significant increase in the urethral lumen area in groups 3,5 compared to group 2. Moreover, the group 3 had a significant increase in matrix metalloproteinase-9 expression compared to group 2. Bone morphogenetic protein-2 expression was increased especially in groups 3,5.

Conclusion: We concluded that oral administration of colchicine or pirfenidone prevented the formation of urethral stenosis in rats to a large extent. The oral steroid treatment also reduced the formation of urethral stenosis, although not as effectively as colchicine or pirfenidone.

Keywords: Functional urology, pathology, reconstructive urology

Introduction

The urethra is a tube-shaped canal with two open ends that allows urine, which is stored in the bladder, to be excreted from the body. The typical length of the urethra is 18-20 cm in men and 3-4 cm for women. Since the urethra is much longer in men than in women, urethral stricture is more commonly seen in men. It is a disease characterized by narrowing in the urethra, decreased urethral volume, and abnormal voiding function due to fibrotic wound formation. Etiology of the disease usually

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includes trauma, infections, and previous surgical interventions. Cellular changes in the extracellular matrix of the urethral spongiosis tissue are involved in the pathogenesis of urethral stricture (1). Here, the normal connective tissue is replaced by dense fibers lined with fibroblasts. Histopathological studies have shown a decrease in the ratio of collagen type III to collagen type I (1). This change is accompanied by a decrease in the smooth muscle to collagen ratio and a marked increase in nitric oxide synthesis in the constricted fibrotic region (2).

Male patients with symptomatic urethral stricture often present to the outpatient clinic due to symptoms such as difficulty urinating, inability to empty the bladder completely, and weak urine flow. The location, size, and length of the urethral stricture are important factors when considering various treatment options. Treatment methods include endoscopic treatments (urethral dilation, internal urethrotomy, injectable materials, laser urethrotomy, and urethral stents) and open surgical reconstructions (urethroplasties, end-to-end anastomotic urethroplasty, onlay free graft, and pedicle flap) (3). However, in some cases, effective results cannot be obtained with these methods, and the disease can recur. It is often emphasized that healing the damaged tissue in urethral stricture would require stopping the bleeding and properly balanced modelling that will not cause infection, proliferation, excessive fibrosis, and scarring (1-3). Therefore, anti-inflammatory agents or antifibrotics can be used for the treatment of urethral stricture. The final feature is progressive fibrosis resulting from extracellular matrix control in which matrix metalloproteinase (MMP) is believed to play an important role. In the evaluation of the antifibrotic and anti-inflammatory effect, MMP-9, which is effective in inhibiting granulation tissue formation, and bone morphogenetic protein (BMP-2), which has an anti-fibrogenic function in multiple organs, are also known to be active in this mechanism (4,5). However, there are a limited number of studies about this subject.

In this study, we aimed to evaluate the use, efficacy, and advantages of drugs such as colchicine, corticosteroids, and pirfenidone. These drugs are used in the treatment of many diseases in clinical practice, due to their anti-inflammatory and antifibrotic properties, in terms of non-recurrence of urethral stricture.

Materials and Methods

Forty male Albino-Wistar rats (220-450 g) were used in this study. All procedures were carried out in a room with a constant temperature (22±2 °C) with a 12-hour light-dark cycle. Institutional guidelines and the Guide for Care and Use of Laboratory Animals of the National Research Council were followed while handling the rats. All procedures were performed in compliance with the provisions of the 1986 Strasbourg Universal Declaration on Animal Welfare and by the approval of the local ethics committee (approval number: HADYEK 23, date: 15.01.2018 - Gaziosmanpaşa University Rectorate Animal Experiments Ethics Committee). The animals were divided into 5 groups with 8 animals in each group. A sharp tip device is made by twisting the 21-gauge syringe needle approximately 1 mm backward. The urethral stricture model was created by rotating this device 360 degrees and back and forth in an approximately 2 cm section starting from about 0.5 cm proximal to the urethral meatus towards the anterior urethral mucosa (Figure 1). The animals were anesthetized with 50 mg/kg IP ketamine hydrochloride and 10 mg/kg IP xylazine. After the anesthesia injection, group 1 underwent penectomy without any other procedure. Urethral stricture was created in rats in groups 2, 3, 4, and 5. The rats in group 2 underwent penectomy at the end of 6 weeks. Groups 3, 4, and 5 were treated with 1 mg/kg colchicine, 0.5 mg/kg prednisolone, and 50 mg/kg/day pirfenidone, respectively by gavage for 6 weeks and underwent penectomy at the end of 6 weeks (6-8). After all the procedures, the animals were sacrificed by cervical dislocation under ketamine, and xylazine anesthesia.

Histopathological Evaluation

Rats' penises were immediately put into a 4% buffered neutral (pH: 7.2) formalin solution for 48 hours after being resected to be fixed. Since it is known that there are bone and cartilage pieces in some areas of the rat penis, the penises were postfixed in decalcification solution with EDTA.

After the fixation process, the penises were divided into two right from the midpoint in one move of a sharp scalpel. Afterwards, the tissue was rinsed under running water, dehydrated with

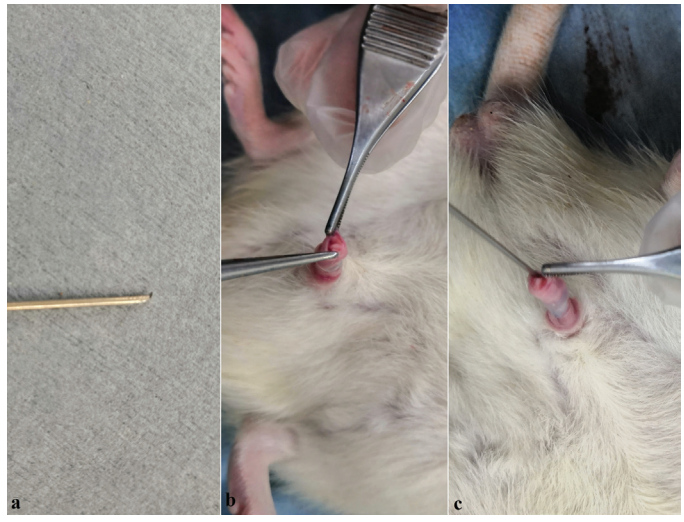

Figure 1. a. Sharp tip tool created by twisting the 21 gauge syringe needle approximately 1 mm back, b. Urethral meatus of rat, c. Creation of urethral mucosal damage

increasing alcohol concentrations (70%, 80%, 90%, 96%, and 100%) for 5 minutes each. Then the samples were cleared in increasing xylene concentrations and were incubated in 3 different paraffin series at 60 °C. The samples were then buried upright in a clean paraffin block. Serial consecutive sections of 5 µm thickness were taken from the blocked tissue with a rotary microtome (Leica RM2135, Germany). The penis tissue sections were placed in poly-L-lysine slides and used for hematoxylin-eosin staining, triple staining, and immunohistochemical analysis.

Hematoxylin-Eosin Staining

The tissue sections of rat penile tissue fixed with formalin and embedded in paraffin blocks were cut into 5 µm thick sections, stained with hematoxylin for 10 minutes after deparaffinization and rehydration procedures. Slides were rinsed in water for 5 minutes, and then immersed in acid alcohol and rinsed under running water. After incubating for 3 minutes in eosin dye solution, the slides were immersed in distilled water, which was changed several times to remove excess dye. The sections were then passed through the increasing concentrations of alcohol (80%, 90%, 95%, and 100%). After incubating in xylene (3x15 min), the sections were sealed with entellan drops and covered with coverslips. Histological analyses of the prepared hematoxylin, eosin-stained slides were done using a light microscope (Nikon Eclipse 200; Nikon).

Triple Staining (Modified Masson Trichrome)

The paraffin from the penile tissue sections of 5 µm thickness was melted by incubating the sections in a 60°C oven, then they were deparaffinized in a xylene series, and rehydrated with decreasing concentrations of alcohol followed by distilled water. The slides were incubated in Weigert's hematoxylin solution for 10 minutes. Then, the slides were rinsed under running tap water for 5 minutes and immersed in distilled water. Next, the slides were dipped into acid fuchsin solution for 1 minute and then immersed in distilled water two times and incubated in phosphotungstic acid solution for 10-15 minutes. Soaked in phosphotungaustic acid solution. Tissue sections were dipped in distilled water twice, soaked in aniline blue solution for 1 minute, and then immersed in distilled water. Then, after dipping through increasing concentrations of alcohol (80, 90, 96, and 100%) and 3 separate xylene series, the slides were closed with entellan and covered with a coverslip, in preparation for microscopic analysis.

Histopathologic Analysis

Histopathological evaluations were performed by a histologist blinded to the study. Hematoxylin-eosin-stained preparations were scored by semi-quantitative inflammation scoring criteria after microscopic analyses (9). Modified Masson Trichrome-stained preparations were scored according to semi-quantitative fibrosis scoring criteria (9). For both scoring procedures, the weighted averages of the 8 sections from each animal and the 5 different areas in each section were scored under a microscope with a 40x objective. The calculated mean inflammation and fibrosis score values of each group were compared statistically.

Immunohistochemical Analysis

Five-µm-thick tissue sections were immunohistochemically stained with BMP and MMP primary antibodies to detect expression of BMP and MMP molecules. Immunohistochemical staining procedures were carried out as follows: 5 µm thin tissue sections were incubated in a 60 °C oven, to melt the paraffin, and sequentially passed through three different xylene series for deparaffinization, followed by alcohol washes of decreasing concentration (100%, 90%, 80%, and 70%) and rehydrated with distilled water. After the microwave antigen retrieval treatment in 10 mM citric acid, endogenous peroxidase blockade was performed by incubating the slides in 3% hydrogen peroxide (H_2O_2) solution for 10 minutes. The slides were washed with phosphate buffer solution (PBS) three times for 5 minutes each, and the perimeter was outlined with a hydrophobic pen (PAP pen). The non-immune blocking serum was applied and the slides were kept in a humid dark environment for 15 minutes. After the blocking agent was removed, BMP and MMP primary antibodies (1:100; Abcam, Cambridge, UK) were added to the slides. The slides were incubated overnight in a closed humid box at 4 °C. After 3 washes with PBS (5 min each), the slides were incubated with biotinylated secondary antibody (goat immunoglobulin G) in a humid, dark environment for 45 minutes at room temperature. This was followed by three PBS washes (5 min each), and secondary antibody (streptavidin-horseradish peroxidase conjugated reagent) was applied, after which the slides were incubated in a dark and humid environment for 30 minutes. After 3 PBS washes (5 min each), coloring was achieved using aminoethyl carbazole chromogen solution. Following contrast with hematoxylin, the sections were passed through distilled water and were covered with a coverslip with a water-based (aqueous mounting reagent) closure solution. For negative control slides, PBS was used instead of primary antibody.

The immune staining intensities were analyzed based on the staining intensity scoring criteria using a light microscope (Nikon Eclipse 200; Nikon) at 40x magnification in the NIS-Element program (Hasp ID: 6648AA61; Nikon) (10). For this purpose, the immunostained and unstained areas of the urethra were categorized based on the intensity of the staining reaction in five sections for each protein, using immunohistochemistry. The obtained weighted group average results were converted to H-score values with the formula [∑Pi (i + 1)]. In the formula, represents the staining intensity score, while Pi is the percentage of stained cells.

Statistical Analysis

IBM SPSS 22 Windows statistical software was used for statistical comparison of the results. The Kruskal-Wallis test was used in the comparison of the groups' mean urethral lumen area. The comparison of groups' means values of inflammation scores, fibrosis scores, and immunohistochemical H scores was conducted using one-way ANOVA, and multiple comparisons were made with Tukey's HSD. A p-value of less than 0.05 was considered significant.

Results

Histopathological Results

Histomorphometric (Urethral Lumen Area) Findings

The result of the analysis indicated that the urethral lumen area of group 2 (G2) decreased significantly compared to control (G1) and other groups (p=0.001). The results of the treatment groups were calculated to be similar to the control (p=0.1420) (Figure 2).

Inflammation Findings

The analyses of hematoxylin-eosin-stained preparations based on inflammation scale criteria showed that the inflammation score was significantly increased in the stricture-only group (G2), while in the control group it was normal (p=0.001). The inflammation scores of the treated groups were similar (p=0.19) and higher than the control (p=0.01), but lower than the stricture only group (G2) (Figure 3).

Fibrosis Findings

The histopathological analysis of triple stained slides showed normal urethrae in G1. It was found that fibrosis score increased significantly in G2 compared to the control (G1) (p=0.001). In treatment groups (G3, G4, G5), the score was significantly lower compared to G2 (p=0.01), but significantly higher than the control group (G1) (p<0.001) (Figure 4).

Immunohistochemical Findings

The immunohistochemical analyses showed that expression of BMP-2 was significantly decreased in G2 compared to G1 (p=0.007). In treatment groups, especially in G3 and G5, there was a significant increase in BMP-2 expression compared to G2 (p=0.03).

MMP-9 expression was similarly found to be decreased in G2 and increased in treatment groups (p=0.01). However, this increase was not statistically different from G1 and G2 (p=0.11) (Tables 1 and 2) (Figure 5).

Discussion

Urethral stricture is a complex pathology characterized by the narrowing of the urethral lumen with varying depths and lengths. Histopathologically, it manifests as spongiofibrous tissue and scarring and can be seen in male patients at any age (1). The prevalence of urethral stricture is between 0.6-1.4%, and 15-20% of adult males have been reported to be affected by this pathology at some time in their lives (11). Urethral stricture occurs mostly as idiopathic, iatrogenic, inflammatory, and, to a lesser extent, secondary to trauma (12). There are many invasive treatment approaches for urethral stricture. Although the success rates with treatment methods such as urethroplasty are around 90% in the first year, it decreases to 60-70% by the 5th year (13). Failure of dilatations due to urethral stricture is up to 80% (14). Yi et al. (15) evaluated 80 patients who had undergone balloon dilatation for bulbomembranous stricture

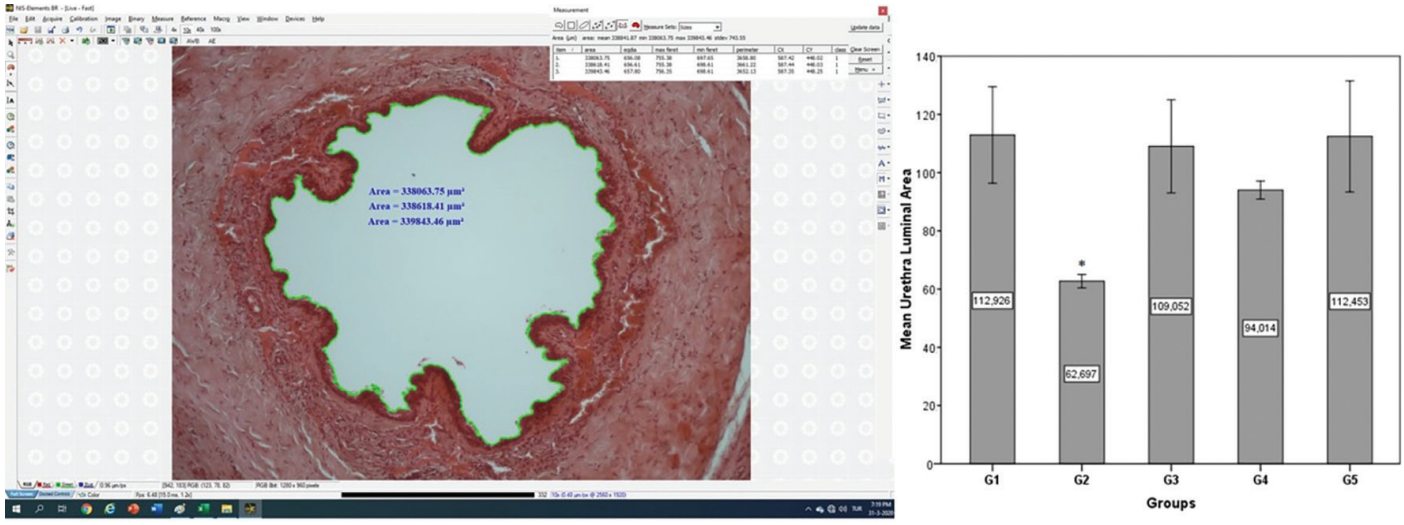

Figure 2. Screen shot from an example of urethral lumen area measurements and graphical view of the mean values of urethral lumen areas of the groups. *: p<0.05 vs. others (Kruskal-Wallis Mann-Whitney test)

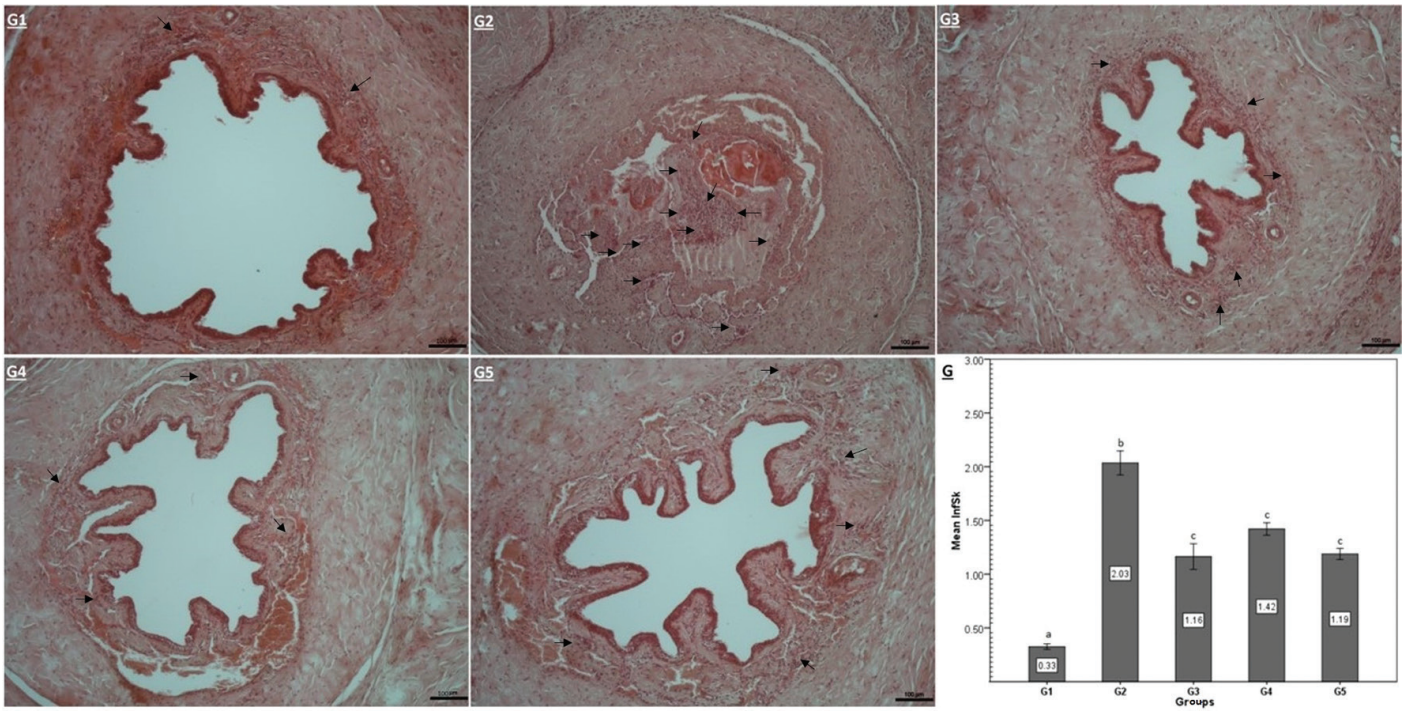

Figure 3. Representative hematoxylin eosin-stained urethra images from each group. Arrows indicate areas with inflammatory cell density. G1-G5, working groups (Bar: 100 µm); G is the values of inflammation scores group environment. Letters on the bars show statistical similarities and differences (b: $p<0.05$ vs. G1, c: $p<0.05$ vs. G1 and G2 One-Way ANOVA, Tukey HSD)

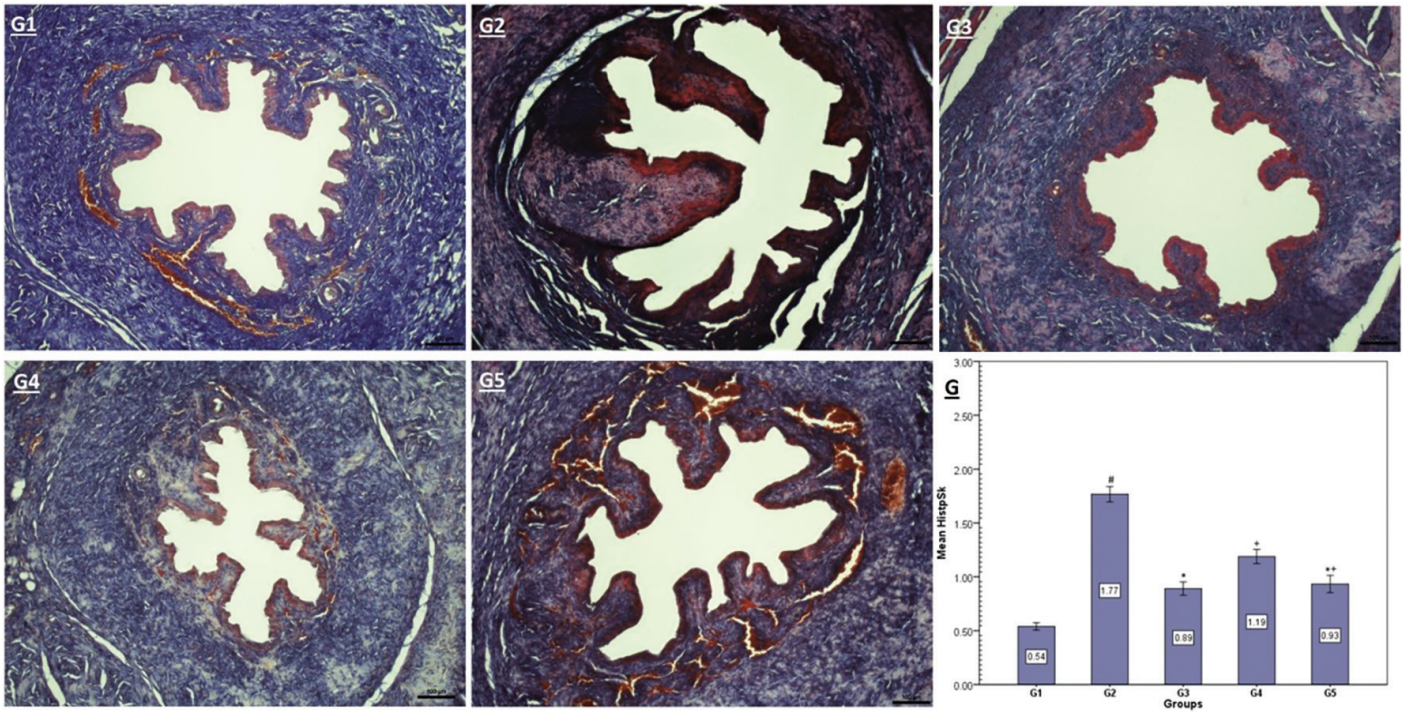

Figure 4. Representative modified masson trichrome painted urethra images (Bar: 100 µm) from each group. G1-G5, working groups; G fibrosis scores are group mean values. The icons on the graphic bars show statistical similarities and differences (#: $p<0.05$ vs. G1, *: $p<0.05$ vs. G1 and G2 One-Way ANOVA, Tukey HSD)

Table 1. The mean Hsc values (±: SEM) of the immunohistochemical staining intensities of the BMP and MMP expressions of the groups

	G1	G2	G3	G4	G5
BMP Hsc	117.71±9.3^{A}	72.71±8.4^{B}	82.42±8.0^{AC}	77.84±10.2^{BC}	89.58±7.1^{AC}
MMP Hsc	99.77±12.3^{a}	74.65±11.5^{b}	81.72±3.2^{ab}	67.22±13.3^{ab}	72.59±8.6^{ab}

G1-G5 are working groups. Capital letters on values represent statistical similarities and differences for BMP, and small letters for MMP. BMP: Bone morphogenetic protein, MMP: Matrix metalloproteinase, Hsc: H-score, SEM: Standard error of mean

Table 2. Mean values of urethra lumen area, inflammation score and fibrosis score of the study groups

	G1	G2	G3	G4	G5
Urethra luminal area (μm²)	112925,51±16575.8	62697,44±2310.7	109051,64±16019	94014,38±3120.1	112452,67±19090.1
Inflammation grade	0.33±0.02	2.03±0.11	1.16±0.11	1.42±0.05	1.19±0.05
Fibrosis grade	0.54±0.03	1.77±0.07	0.89±0.06	1.19±0.06	0.93±0.08

A. Inflammation grading scale (6)
Grade - Amount of inflammation
0 - None
1 - Giant cells, occasional lymphocytes, and plasma cells
2 - Giant cells, plasma cells, eosinophils, neutrophils
3 - Many inflammatory cells, microabscesses

B. Fibrosis grading scale (7)
Grade - Amount of fibrosis
0 - None
1 - Minimal, loose
2 - Moderate
3 - Florid, dense

C. Criteria for grading immunohistochemical staining intensities (10)
Score - Immune reactivity
0+ / None staining
1+ / Minimal staining
2+ / Moderate staining
3+ / Strong staining

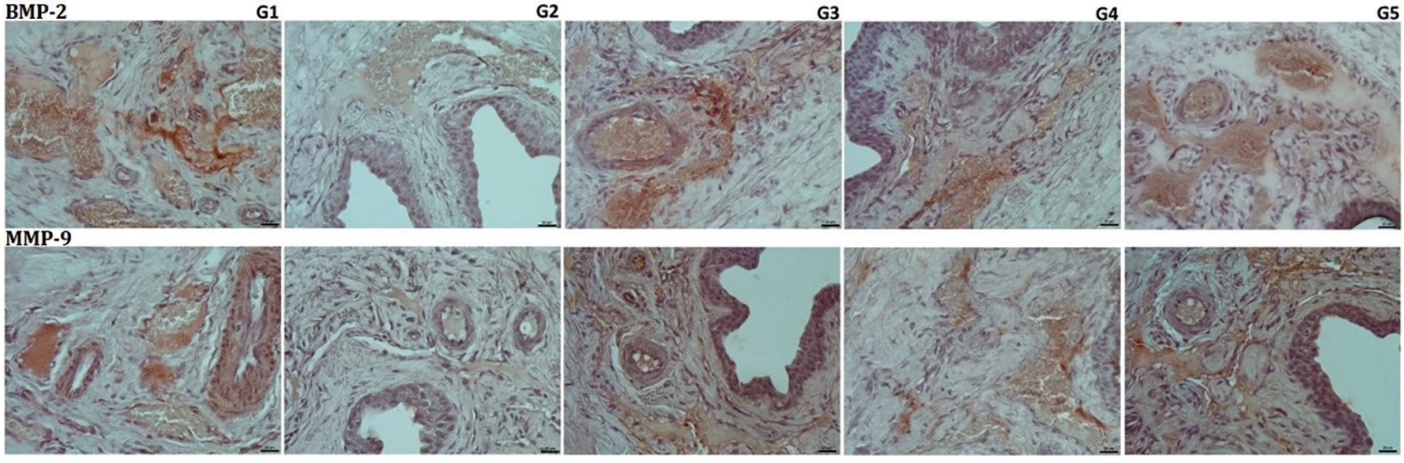

Figure 5. Representative images for top-line BMP-2 and low-order MMP-9 expressions from immunohistochemically stained preparations from each group (Bar: 20 μm, IHC-AEC)

BMP-2: Bone morphogenetic protein, MMP-9: Matrix Metalloproteinase, IHC: Immunohistochemistry, AEC: Amino ethyl carbochol

and observed that 33.8% of the patients redeveloped stricture during the mean follow-up period of 8.4 months. Long-term balloon dilatation success varies between 35 and 70%. Success rates of a single session of optic urethrotomy are only 8% (16). Other urethral surgeries are based on highly complicated techniques and/or can result in higher rates of complications (13,14). Urethral stricture is a disease that significantly impairs quality of life due to lower urinary tract complaints, recurrent infections, sexual dysfunction, and hematuria. Since it may require many urological interventions, it is important to treat urethral stricture with as minimally invasive methods as possible.

Studies on alternative methods, such as medical treatment approaches or tissue engineering, aiming to increase success in the treatment of urethral stricture are increasing. Recently, some studies have suggested using steroids. It is known that corticosteroid therapy, which can be used in the treatment of urethral stricture, reduces collagen production (17). Yıldırım et al. (18) treated half of the 83-patient cohort, whose mean age was 56.4 years, with 40 mg suburethral methyl prednisolone during internal urethrotomy, and observed that stricture recurred in 19 (46%) cases. They reported that the rate of recurrence was significantly lower in the steroid group than the control group. In a meta-analysis in which Zhang et al. (19) examined 8 studies, the time until relapse was longer in 203 cases who underwent internal urethrotomy and steroid injection than in patients that did not receive steroids. In the case-control study involving 72 cases with bulbar urethral stricture, patients were divided into two groups: those who received oral steroid treatment (n=36, deflazocort 6 mg tablets) after urethrotomy and those who did not (n=36) (20). At the end of the six months on average, the maximum flow rates in the first and second groups were determined as 18.2 mL/sec and 13.7 mL/sec, respectively, and recurrence of urethral stricture was lower in those receiving steroid therapy (20). In many studies, except for one, various steroids were given locally by invasive methods (17-19). In our study, the urethral lumen area increased, although not statistically significant, in the rats given oral prednisolone treatment compared to the stricture-only group (G2). Moreover, prednisolone treatment significantly decreased the inflammation and fibrosis scores compared to the G2 group.

In urology, pirfenidone has been used for urethral stricture, for reducing the effect of kidney damage, and in prostate cancer cell cultures (21-23). Transforming growth factor (TGF)-beta (β)1 is known to stimulate fibroblast differentiation and increase extracellular matrix production. Pirfenidone prevents fibrosis by reducing the effect of TGF-β1, and therefore is widely used in the treatment of pulmonary fibrosis. In addition, pirfenidone has been shown to inhibit cell proliferation and collagen I synthesis in intestinal cells, and is effective in reducing fibrosis in many organs (24). In a study conducted in rabbits with urethral stricture, it was reported that catheters coated with nanoparticle/pirfenidone complexes reduced urethral stricture and fibrosis (21). In our study, we also found that there was a significant decrease in inflammation and fibrosis scores, and a significant increase in the urethral lumen area in rats treated with pirfenidone compared to the stenosis-only group.

The use of antifibrotic agents appears to be a rational approach in the treatment of urethral stricture. Colchicine is an alkaloid chemically known as colchicinum-N-(5,5,7,-tetrahydro-1,2,3,-tetramethoxy-9-oxobenzo [alpha] heptalen-7-yl) acetamide. It has been used as an antifibrotic agent because it inhibits procollagen secretion and prevents its conversion into collagen (25). It has also been used to reduce fibrosis in the liver, lung, and kidney as well as serosal adhesions due to its antifibrotic and anti-inflammatory effects (26,27). Colchicine disrupts microtubule formation and binds to tubulin to inhibit microtubule polymerization (28). In a retrospective study evaluating 84 patients who received 1 g of oral colchicine per day and underwent internal urethrotomy due to urethral stricture, it was reported that the recurrence rate of urethral stricture was significantly reduced (29). In our study, we also found that there was a significant decrease in inflammation and fibrosis scores and a significant increase in the urethral lumen area in rats treated with oral colchicine compared to the stricture-only group (G2).

The main features of urethral stricture include epithelial damage, fibroblast proliferation, inflammation, and production of increased extracellular matrix. The ultimate feature is progressive fibrosis due to extracellular matrix deposition, in which MMP is believed to play an important role (1). MMPs are a group of proteinases known to regulate the remodeling of the extracellular matrix and are therefore important in the process of fibrosis and scarring that cause urethral stricture. A drug called verapamil has been reported to prevent excessive formation of urethral scars by inhibiting proliferation of urethral scar fibroblasts and increasing MMP activity in human cell culture (30). MMP-9 has been reported to be effective in inhibiting granulation tissue formation caused by metallic stent placement in the rat urethral model (4). In our study, MMP-9 expression was significantly increased in the group treated with colchicine compared to the stricture-only group (G2).

TGF-β1 is a protein with a wide range of biological functions in cell growth, differentiation, and extracellular matrix production. TGF-β1 plays a supportive role in the development of fibrosis in the kidney, lung, liver, and pancreas (31). Meanwhile, BMP-2 has an anti-fibrogenic function in multiple organs. It antagonizes TGF-β1-induced fibrogenic signals in renal fibroblasts and is effective in the treatment of rat renal fibrosis caused by unilateral ureteral obstruction (32). Wound healing in human skin has been reported, to be partly through induction of BMP-2 (33). Similarly, in a study with mice, mutual regulation between BMP-2 and TGF-β1 signal axes, has been reported to elucidate the anti-fibrogenic mechanism of BMP-2 in the pathogenesis of liver fibrosis (5). In our study, the increase of BMP-2 expression, especially in G3 and G5, supports the notion of the antifibrotic effect of colchicine and pirfenidone in immunohistochemistry.

Study Limitations

Since this study is an animal experiment, we cannot use methods such as endoscopy, radiological imaging, or uroflowmetry in the creation of urethral stricture or in the evaluation of healing.

The sample size of the experimental groups is also one of the limitations of the study. In addition, we cannot use the medical treatments applied in the study before and after opening the urethral stricture with the endoscopic methods in use at our current urology clinic.

Conclusion

We concluded that oral administration of colchicine or pirfenidone prevents the formation of urethral stricture to a large extent, and that oral steroid treatment reduces this formation, albeit not as effectively as colchicine or pirfenidone. Colchicine reduced fibrosis via BMP and contributed to partial urethral remodeling via MMP. Based on these findings, we believe that using these agents before and after endoscopic surgery or dilatation of the urethra in clinical practice following necessary clinical studies.

Ethics

Ethics Committee Approval: All procedures were performed in compliance with the provisions of the 1986 Strasbourg Universal Declaration on Animal Welfare and by the approval of the local ethics committee (approval number: HADYEK 23, date: 15.01.2018 - Gaziosmanpaşa University Rectorate Animal Experiments Ethics Committee).

Informed Consent: Not necessary.

Footnotes

Authorship Contributions

Surgical and Medical Practices: F.F., Concept: F.F., Design: F.F., K.Y., F.E., F.G., Data Collection or Processing: F.F., K.Y., F.E., F.G., Analysis or Interpretation: F.F., K.Y., F.E., F.G., Literature Search: F.F., K.Y., F.E., F.G., Writing: F.F., K.Y., F.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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